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PURIFICATION OF THE ANGIOTENSIN I-CONVERTING ENZYME OF THE LUNG

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SUMMARY

The angiotensin I-converting enzyme (peptidyl dipeptide hydrolase) was purified from hog lung. The molecular weight of the enzyme in sodium dodecyl sulfate polyacrylamide gel electrophoresis was established as 206 000. The pI in isoelectrofocusing was found to be 4.3. The purified enzyme cleaved Bz-Gly-Gly-Gly and bradykinin. It was inhibited by a high concentration of urea and by the nonapeptide SQ 20881. Both compounds inhibited reversibly as shown in studies done with the water-insoluble Sepharose-converting enzyme complex. After gel filtration on a Sephadex G-200 column two additional forms of converting enzyme were detected.

INTRODUCTION

Although the angiotensin I-converting enzyme was discovered in horse plasma in 1956 (ref. 1), systematic investigations of this protein were undertaken much later. Research was stimulated by the synthesis of angiotensin I in appreciable quantities[2] and by the new availability of various chemical techniques to assay converting enzyme[3–6]. During studies of the metabolism of bradykinin[7–9], we found that, in addition to angiotensin I, the converting enzyme cleaves C-terminal dipeptides from a variety of peptide substrates. These included bradykinin, the B-chain of insulin and other peptides that could be used as substrates in spectrophotometric, fluorimetric, or radiometric assays[4,6,10,11]. Because of this broad substrate specificity we call the converting enzyme peptidyl dipeptide hydrolase (or dipeptide hydrolase)[6].

Initially we concentrated and purified an enzyme (first named peptidase P or kinase II) from homogenized kidney cortex[7] and from plasma[8] that cleaved the C-terminal dipeptide of bradykinin. Then converting enzyme was purified from swine plasma[4,6] and homogenized lung as reported briefly elsewhere[10–13]. In this communication we describe the details of our purification techniques and some other investigations of the enzyme.

Abbreviations: Boc-, *tert*-butyloxycarbonyl; Bz-, benzoyl; Z-, benzyloxycarbonyl; Bz-Gly-Gly-Gly, hippurylglycylglycine; DNS-, 1-dimethylaminonaphthalene-5-sulfonyl.

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MATERIALS AND METHODS

200 g of swine lung obtained fresh from the slaughterhouse were homogenized in a Waring blender for 4 min in 2 l of 0.25 M sucrose solution buffered with 0.05 M Tris to pH 7.4. The homogenate was centrifuged for 30 min in a Sorvall refrigerated centrifuge at 5000 × *g* and for 2 h in a Spinco L-2 65 ultracentrifuge at 50 000 × *g*. The final supernatant was the source of enzyme. This was concentrated under N_2 pressure to approximately 10% of the initial volume over an Amicon XM 100A membrane filter. The same membrane filter was used to concentrate the enzyme preparation before each subsequent column chromatography step.

The enzyme was purified first on a Sephadex G-200 column (80 cm × 10 cm). Gel filtration was done at the rate of 20 ml/h in an 0.05 M Tris buffer of pH 7.4, containing 0.1 M NaCl. Fractions of 18 ml were collected. After concentration with the membrane filter, the sample was dialyzed overnight against 5 l of 0.005 M Tris, pH 7.4. The active material was then adsorbed on a DEAE-Sephadex A-50 column (40 cm × 2.5 cm) and eluted with a linearly increasing gradient of NaCl ranging from zero to 0.3 M in 0.005 M Tris buffer, pH 7.4. The ionic strength was determined in a Radiometer conductivity meter. 50 ml/h were collected in 13-ml fractions.

Final purification was achieved by means of a hydroxylapatite (BioRad) column (18 cm × 3 cm). The active fraction after DEAE-Sephadex column chromatography was concentrated to about 6 ml with the Amicon membrane filter and dialyzed against 1 l of 0.001 M phosphate buffer, pH 6.8, containing 0.005 M NaCl. The hydroxylapatite column was equilibrated with the same buffer, and the converting enzyme adsorbed on the column was eluted with a linear gradient of phosphate buffer increasing in molarity from 10^{-3} to 10^{-1} and containing 0.005 M NaCl. 30 ml/h was collected in 10-ml fractions. The active peak containing the enzyme was dialyzed against the Tris buffer used in the spectrophotometric assay.

Disc gel electrophoresis was done in a Canalco apparatus for 2 h in a Tris–glycine buffer, pH 8.3. A current of 2 mA per tube was applied at 4 °C. The proteins in the polyacrylamide gel were stained with Coomassie blue.

The molecular weight of the enzyme was determined by disc gel electrophoresis in the presence of sodium dodecyl sulfate[14], and confirmed by gel filtration on a Sephadex G-200 column. Before electrophoresis in 5% polyacrylamide gel, the proteins were incubated with sodium dodecyl sulfate and 2-mercaptoethanol[14] for 60 min at 37 °C. A current of 6 mA per tube was used for 6 h. For comparison, the molecular weight was also estimated by gel filtration on a Sephadex G-200 (2.5 cm × 90 cm) column equilibrated with 0.05 M Tris buffer and 0.1 M NaCl according to the instructions of the manufacturer (Pharmacia). The flow rate was 15 ml/h and 3.6-ml fractions were collected.

Isoelectric focusing was done in an LKB electrofocusing column in ampholyte buffers ranging from pH 3 to 5 (ref. 15). The experiment was carried out for 70 h at the starting current of 9 mA and 700 V.

The amount of protein was measured according to Lowry *et al.*[16] or estimated in the ultraviolet-recording spectrophotometer at 280 nm.

Converting enzyme obtained after DEAE-Sephadex column chromatography was rendered water-insoluble by coupling it covalently to Sepharose-4B (ref. 17), as described previously[10,13].

The activity of the soluble enzyme was determined in a Cary Model 15 recording ultraviolet spectrophotometer at 254 nm with 10^{-3} M Bz-Gly–Gly–Gly substrate[6]. The method is based on measuring the increase in absorbance when glycylglycine is cleaved from the substrate and hippuric acid is liberated in 0.1 M Tris, pH 7.4, containing 0.1 M NaCl at 37 °C.

The action of converting enzyme was also measured in bio-assay with bradykinin substrate[9]. We assayed kininase activity on the isolated rat uterus by inactivation of added synthetic bradykinin, the concentration of which was 3.3 μg/ml. The enzyme and the peptide were incubated in Tris buffer (pH 7.4, 0.1 M). Samples were withdrawn from the incubation mixture every 5 min, immediately diluted with saline (1:200), and added to an isolated tissue bath containing the rat uterus in estrus constantly bubbled through with O_2. The decrease in the activity of bradykinin was then determined by using a converted density balance. The uterus in the bath was fastened to the balance arm. The mechanical movements of the balance arm were converted by differential transformers and by the necessary circuits to electrical signals which were recorded automatically on a recorder.

RESULTS

Purification

Converting enzyme from the final supernatant of the lung homogenate obtained after centrifugation for 2 h at 50 000 × *g* was purified as shown in Table I.

TABLE I

PURIFICATION OF THE ANGIOTENSIN I-CONVERTING ENZYME OF THE LUNG

Purification step	*Volume (ml)*	*Total units**	*Protein (mg/ml)*	*Units per mg protein*	*Yield (%)*	*Purification*
Final supernatant of homogenate	1380	135	9.5	0.01	100	1
Amicon XM 100A membrane filtration	118	137	61	0.02	101	2.0
Sephadex G 200 gel filtration**	823	77	1.3	0.07	57	7.0
DEAE-Sephadex A-50 column chromatography**	25	65	5.8	0.45	48	45
Hydroxylapatite column chromatography	9	28	0.2	13.8	20.7	1380

* 1 unit = 1 μmole of Bz-Gly–Gly–Gly cleaved per min.

** The enzyme was concentrated before the next purification step over the Amicon membrane filter.

Gel filtration on a Sephadex G-200 column yielded three active peaks (Fig. 1). Peak I moved with the void volume, but Peak II entered the gel. We assumed that Peak I contained a higher molecular weight aggregate of the converting enzyme and that Peak II contained the dimer of the enzyme. Peak III containing the enzyme, was purified further on a DEAE-Sephadex A-50 column. The enzyme adsorbed on the column was eluted with 0.15 M NaCl. This step of purification was followed by chro-

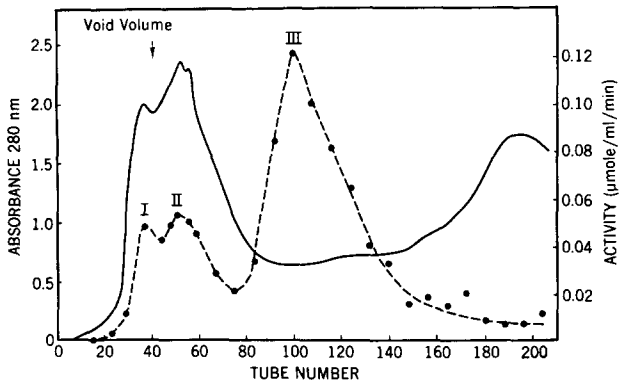

Fig. 1. Gel filtration of converting enzyme on a Sephadex G-200 column. Three peaks were obtained. Presumably peak I contains higher molecular weight aggregate of enzyme, and Peak II contains the dimeric form. The converting enzyme in Peak III was purified further. ———, absorbance at 280 nm; ●— —●, activity with Bz-Gly–Gly–Gly substrate.

matography on a hydroxylapatite column. Here converting enzyme was eluted with a 0.05 M phosphate buffer.

Because the activity of converting enzyme is lower in phosphate than in Tris buffer, after hydroxylapatite column chromatography it was dialyzed against Tris–NaCl used in the spectrophotometric assay. The cleavage of Bz-Gly–Gly–Gly substrate was observed to establish activity after each step of purification.

The purified enzyme also cleaved bradykinin as determined in bio-assay of bradykinin[6,9]. Using our standard conditions, 3 μg of bradykinin was inactivated within 30 min by 0.3 μg of converting enzyme protein at 37 °C in the Tris–NaCl buffer used.

The protein obtained after hydroxylapatite column chromatography was essentially homogeneous. In disc gel electrophoresis, it showed a single band after staining the gel (Fig. 2).

Molecular weight determination

The molecular weight of converting enzyme was determined in polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate[14]. The migration of converting enzyme against protein standards is shown in Fig. 3. Plotting the log of the molecular weight of standards against the ratios of distance of migration yielded a straight line. Using this plot, we estimated the enzyme to have a mol. wt of 206 000. After incubation with sodium dodecyl sulfate and mercaptoethanol[14] for 60 min a protein band of about 70 000 mol. wt was detected. This indicates that the converting enzyme contains three subunits of 70 000 mol. wt each. An almost identical mol. wt (210 000) was found after Sephadex G-200 gel filtration on a column calibrated with apoferritin, γ-globulin, bovine serum albumin, and cytochrome *c* as marker proteins.

Isoelectric focusing

The pI value of the enzyme was established by isoelectric focusing when 31 mg of protein obtained after DEAE-Sephadex column chromatography was applied in an electrofocusing column. After a 70-h run a pI of 4.3 was found.

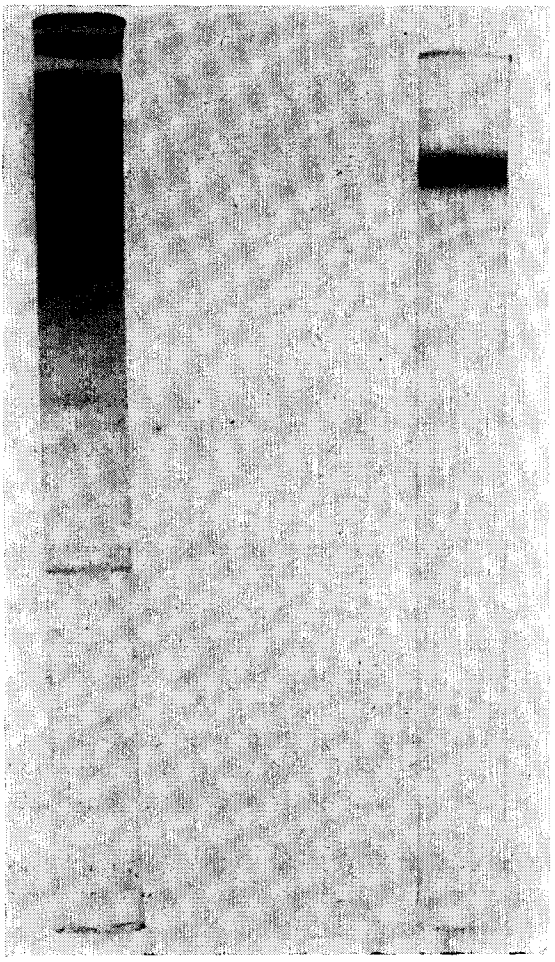

Fig. 2. Polyacrylamide disc gel electrophoresis of the enzyme. Left gel: proteins after DEAE-Sephadex column chromatography. Right gel: protein after hydroxylapatite column chromatography. The line on the lower portion of the gel indicates migration of the marker dye.

Inhibition

The nonapeptide SQ 20881 is a specific inhibitor of converting enzyme[6,10,18,24], inhibiting it completely at a concentration $1 \cdot 10^{-4}$ M (the I_{50} of this inhibitor with Bz-Gly-Gly-Gly as substrate is $3 \cdot 10^{-8}$ M (ref. 10)). SQ 20881 at a $1 \cdot 10^{-4}$ M concentration also completely blocked inactivation of bradykinin. This inhibition was reversible, as shown by experiments with insoluble Sepharose–converting enzyme complex. SQ 20881 (10^{-4} M) inhibited the hydrolysis of bradykinin by 0.10–0.15 ml settled volume of the insoluble complex. After the Sepharose–converting enzyme complex had been sedimented from the incubation mixture containing bradykinin (3 μg) and the inhibitor by brief centrifugation, and Sepharose–converting enzyme had been washed with 2–5 ml of Tris three times, the activity of converting enzyme was recovered. Bradykinin was inactivated again within 15 min by Sepharose–converting enzyme.

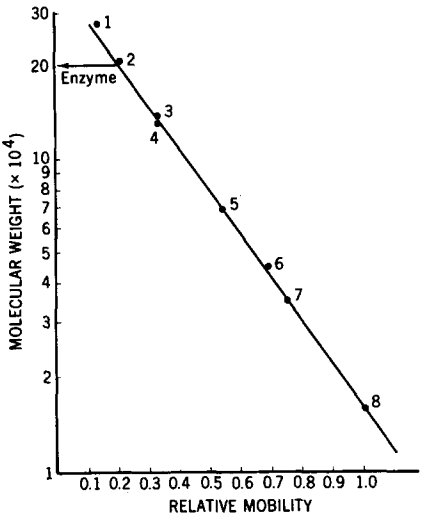

Fig. 3. Determination of the molecular weight of angiotensin I-converting enzyme. The mol. wt of 206 000 was obtained from the plot of the logarithm of the molecular weights of several proteins treated with sodium dodecyl sulfate against their mobilities relative to the migration of α and β chain of hemoglobin in disc gel electrophoresis on polyacrylamide gel. The proteins were stained with Coomassie blue. 50 μg of converting enzyme was used. 1, Bovine serum albumin tetramer (mol. wt 276 000); 2, bovine serum albumin trimer; 3, bovine serum albumin dimer; 4, β-galactosidase (mol. wt. 130 000); 5, bovine serum albumin monomer mol. wt 69 000); 6, ovalbumin (mol. wt 45 000); 7, pepsin (mol. wt 35 000); 8, $\alpha + \beta$ chains of hemoglobin (mol. wt 16 000).

The enzyme was also reversibly inhibited by urea. This was studied with both Bz-Gly–Gly–Gly (Table II) and bradykinin substrates. High concentration of urea (4 to 1 M) inhibited converting enzyme 100–74% after 20 min of preincubation. When converting enzyme was only preincubated with a high concentration of urea, but the final concentration in the spectrophotometric assay was reduced to 0.2 M by dilution, 70% of the activity was recovered, indicating that the blocking of the action of the enzyme by urea was reversible.

TABLE II

INHIBITION OF THE ANGIOTENSIN I-CONVERTING ENZYME BY UREA

Final concentration of urea (M)	*Inhibition (%)*
4	100
2	89
1	74
0.2	21
0.1	0
*4 → 0.2	30
*2 → 0.2	30

* Converting enzyme was preincubated with high concentrations of urea but the final concentration during the ultraviolet spectrophotometric assay was 0.2 M.

Similar results were obtained in bio-assay using bradykinin as substrate. The activity of Sepharose–converting enzyme was also inhibited 100% by 4 M urea, but it was recovered after resedimenting and washing the insoluble complex of the enzyme.

When the source of the enzyme was Peak II obtained after Sephadex G-200 gel filtration (Fig. 1), 4 M urea inhibited it completely. Preincubation of converting enzyme for 20 min at 37 °C in 4 M urea and dilution of the incubation mixture before assay to yield 0.2 M urea, abolished the inhibition; 109% of the uninhibited control activity was recovered.

DISCUSSION

The peptidyl dipeptide hydrolase studied releases dipeptides from the C-terminal end of a variety of peptide substrates, including bradykinin[7,8] angiotensin I[1], B-chain of insulin[10], Bz-Gly–His–Leu[3], Bz-Gly–Gly–Gly[6,19], Boc-Phe–(NO_2)Phe–Gly[6], Z-Phe–His–Leu[20] and others[21]. Our most sensitive assay used the radioactive and fluorescent substrate [^{14}C]DNS-Gly–Gly–Gly[10]. The converting enzyme has a divalent metal ion cofactor and needs Cl^-. The Cl^- requirement, however, depends on the structure of the substrate. In absence of Cl^-, the rate can drop to zero with Bz-Gly–Gly–Gly, or nearly to zero with angiotensin I substrate, but only to about 53% with bradykinin substrate[13]. Other substrates produce values between these two extremes. When the converting enzyme is coupled to Sepharose-4B to form a water-insoluble complex, the Cl^- requirement is reduced and the enzyme converts angiotensin I to angiotensin II in a medium free of Cl^- at 24% of the control rate[13].

The lung regulates the metabolism of various vasoactive materials[22,23]. We purified converting enzyme from lung[6,10,11,13], but also from plasma[8] and kidney cortex[7]. Possibly converting enzyme coming from various sources is still a single enzyme.

Others report various degrees of purification of the enzyme from bovine kidney[20] and lungs of rabbit[24,25], calf[21], and man[26]. After completing our experiments we noticed an additional paper on the purification of converting enzyme from hog lung[19]. During purification of the lung converting enzyme the ratio of the rate of hydrolysis of angiotensin I to that of Bz-Gly–Gly–Gly[19] or Bz-Gly–His–Leu[24] stayed constant, thus all of these actions can be attributed to a single enzyme. The protein we obtained after hydroxylapatite chromatography appears to be homogeneous with a mol. wt of 206 000. We detected three subunits of the converting enzyme of a mol. wt of 70 000 each in sodium dodecyl sulfate polyacrylamide electrophoresis. The previously published estimates for the molecular weight of converting enzyme vary from 150 000 (ref. 27) to 480 000 (ref. 26). This discrepancy may be partially due to differences in the enzyme sources since the converting enzyme came from the lungs of various animals and man; but it may also be due to the fact that estimates of molecular weight were based upon using only partially purified enzyme preparations.

After gel filtration on a Sephadex G-200 column two additional forms of the enzyme are detectable. The activity in the first peak (Fig. 1) may represent the large molecular weight aggregate of converting enzyme, since it moves with the void volume. The second peak might contain the dimer of the enzyme. After incubation in urea, the activity of the latter increases upon dilution of the incubating mixture,

while the final urea concentration we used inhibits the activity of the other peak by about 30%. We attribute these results to the dissociation of the dimer form of converting enzyme to the monomer form at the high urea concentration (4 M) and the subsequent partial inhibition of the enzyme in the final concentration of 0.2 M. Urea at a concentration of 4 M inhibited reversibly the inactivation of bradykinin by a Sepharose–converting enzyme complex[28]. Binding of converting enzyme to Sepharose provides us with a useful tool to study the reversibility of enzyme inhibition. The enzyme complex can be separated in the centrifuge within minutes, the inhibitor can be removed with the supernatant and then the enzyme can be reequilibrated with the buffer. The inhibition by the potent specific nonapeptide inhibitor SQ 20881 (refs 6, 10, 18) is also reversed by sedimenting the Sepharose–converting enzyme complex and reequilibrating it with fresh buffer.

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