Biochimica et Biophysica Acta, 483 (1977) 398—408

BBA 68164

PURIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM HUMAN LUNG

KAZUTAKA NISHIMURA, NORIKO YOSHIDA, KUNIO HIWADA, EINOSUKE UEDA and TATSUO KOKUBU

The 2nd Department of Internal Medicine, Ehime University School of Medicine, Shigenobu, Onsen-gun, 791-02 Ehime (Japan)

(Received December 8th, 1976)

Summary

Angiotensin I-converting enzyme (peptidyl dipeptide hydrolase, EC 3.4.15.1) was solubilized from the membrane fraction of human lung using trypsin treatment and purified using columns of DE 52-cellulose, hydroxyapatite and Sephadex G-200. The purified enzyme was shown to convert angiotensin I to angiotensin II and also to inactivate bradykinin. The specific activity of the enzyme was 9.5 units/mg protein for Hippuryl-His-Leu-OH and 0.665 μmol/min per mg protein for angiotensin I. The enzymic activity obtained after trypsin treatment (1 mg/200 mg protein) for 2 h could be divided into three components: (i) an enzyme of molecular weight 290 000 (peak I), (ii) an enzyme of molecular weight 180 000 (peak II) and (iii) an enzyme of molecular weight 98 000 (peak III), by columns of DE 52-cellulose and Sephadex G-200. K_m values of peak I, II and III fraction for Hippuryl-His-Leu-OH were identical at 1.1 mM. pH optimum of the enzyme was 8.3 for Hippuryl-His-Leu-OH.

Introduction

Angiotensin I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) which converts angiotensin I to angiotensin II by releasing the C-terminal dipeptide was first isolated from horse plasma [1]. The enzyme is also thought to be capable of inactivating bradykinin and to be identical with kininase II [2—4]. Purification of the enzyme has been attempted from plasma, kidney and lung of a few sorts of animals [4—11]. Moreover, purification works of the enzyme from human materials were also reported, although details have not been given [12,13]. Since this enzyme is thought to exist as a membrane-bound protein [10,14], there are some problems in solubilizing it from membrane fraction. We attempted to solubilize the enzyme from rabbit lung using trypsin and

obtained good result [15]. So we tried to purify the enzyme from human lung using trypsin.

In this report, we describe the details of purification procedure and some properties of the enzyme.

Materials and Methods

Hippuryl-His-Leu-OH, angiotensin I, angiotensin II, bradykinin were purchased from the Institute for Protein Research, Osaka Univ., Osaka, Japan. Trypsin was from Difco Lab., Detroit, Michigan, U.S.A. and soybean trypsin inhibitor from Worthington Biochem. Co., New Yersey, U.S.A. The molecular weight marker kit was obtained from Boeringer Mannheim GmbH, West Germany and Sephadex G-200, hydroxyapatite and Dextran Blue 2000 from Pharmacia, Uppsala, Sweden. DE-52 was from Whatman, Maidstone, Kent, England. Chemicals used for acrylamide gel electrophoresis were from Wako Pure Chemicals, Osaka, Japan.

Enzyme assays

Angiotension I-converting enzyme assay was performed by the spectrophotometric method of Cushman and Cheung [5]. One unit of the enzyme activity was defined as that amount of the enzyme which hydrolysed 1 μmol of Hippuryl-His-Leu-OH/min at 37°C under the conditions described by them. The another assay of the enzyme was performed by using angitotensin I as substrate. The reaction mixture, containing 0.4 ml of 100 mM potassium phosphate buffer, pH 7.8, 0.5 ml of 10 μM angiotensin I in saline, 0.1 ml of the enzyme solution and a few drops of 0.27 M diisopropylfluorophosphate, was incubated at 37°C for 10 min and stopped by boiling for 5 min. After appropriate dilution, the angiotensin II formed was assayed in the isolated rat uterus [4].

Angiotensinase and kininase activities were determined by using either angiotensin II or bradykinin as substrates. The reaction mixture, containing 0.4 ml of 100 mM potassium phosphate buffer, pH 7.8, 0.5 ml of 0.8 μM angiotensin II (or of 10 μM bradykinin), and 0.1 ml of the enzyme solution, was incubated for 2 h at 37°C (or for 5 min at 37°C for the measurement of kininase activity). After the reaction was stopped by boiling for 5 min, the peptides were assayed in the isolated rat uterus [4].

Disc gel electrophoresis

Analytical disc gel electrophoresis was performed on 7.5% acrylamide gel at pH 8.6 with a current of 2.5 mA per tube for 4 h [16]. For the determination of the molecular weight of the enzyme which was incubated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 h at 50°C, disc gel electrophoreis on 7.5% acrylamide gel was performed with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulfate. A current of 8 mA per tube was used for 5 h [17,18]. Gels were stained 0.05% Coomassie Blue in 12.5% trichloroacetic acid.

Protein concentration was determined by the method of Lowry et al., using bovine serum albumin as a standard [19].

Results

Purification of angiotensin I-converting enzyme from human lung

Purification steps of angiotensin I-converting enzyme from human lung using trypsin (1 mg/500 mg protein) are summarized in Table I. Human cadaveric lung (36 g), which was obtained from a patient who had died from a traffic accident, was chopped into small pieces and suspended in 90 ml of 20 mM potassium phosphate buffer, pH 7.8, containing 0.25 M sucrose. The suspension was homogenized in a Waring blender for 4 min and centrifuged for 20 min at 700 × *g*. The supernatant (Step 1) was filtered with two layers of gauze and adjusted to pH 5.2 with acetic acid and centrifuged for 30 min at 15 000 × *g*. The pellet (Step 2) was suspended in 10 mM potassium phosphate buffer, pH 7.8 and adjusted to pH 7.8 with 1 M NaOH. The blood component was almost excluded at this step. The acid precipitated fraction (Step 2) was incubated with trypsin (1 mg/500 mg protein), containing 1 mM $CaCl_2$ for 120 min at 37°C. The solution was readjusted to pH 5.2 with acetic acid and centrifuged for 30 min at 15 000 × *g*. The supernatant (Step 3) was dialyzed overnight against 3000 ml of 10 mM potassium phosphate buffer, pH 7.8 and applied to a column of DE 52-cellulose (2.6 × 30 cm) which was equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0—0.5 M) in the same buffer (initial volume of buffer in gradient mixing chamber was 500 ml), indicated as peak A in Fig. 1. The enzyme emerged at the concentration of 0.09 M NaCl. At the stages of Steps 1, 2 and 3, the enzymic activities were abnormally low levels, which may be due to the presence of inhibitors in the lung tissue. Also, the total activity of the enzyme was increased 2.7-fold by

TABLE I

PURIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM HUMAN LUNG USING TRYPSIN (1 mg/500 mg PROTEIN)

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity	
				Hippuryl-His-Leu-OH (units/mg)	Angiotensin I (nmol/min per mg)
1 The supernatant of homogenate centrifuged at 700 × *g*	81.4	2296.4	3.215	0.0014	
2 Sediment from the pH 5.2 precipitation, resuspended in 10 mM phosphate buffer, pH 7.8	40.4	1066.5	1.813	0.0017	
3 After trypsin treatment and acidification to pH 5.2, the supernatant centrifuged at 15000 × *g*	149.0	448.3	4.93	0.011	
4 DE 52-cellulose eluate	48.0	15.4	10.50	0.684	47.9
5 Hydroxyapatite eluate (concentrated)	3.0	1.0	8.13	8.13	568.6
6 Sephadex G-200 filtrate (concentrated)	5.0	0.7	6.75	9.50	665.0

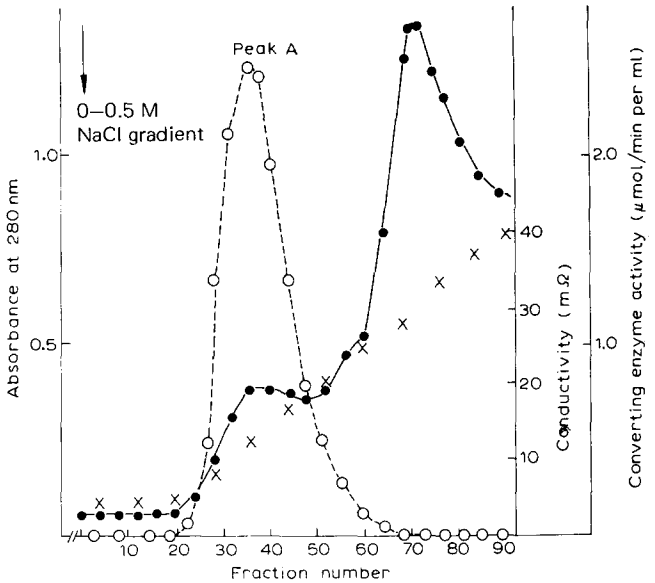


Fig. 1. DE 52-cellulose column chromatography of converting enzyme from human lung. Enzymic activity was measured using Hippuryl-His-Leu-OH as substrate. The arrow indicates the starting point of NaCl gradient (0—0.5 M). Applied sample: the supernatant (Step 3) which was treated with trypsin (1 mg/500 mg protein) for 2 h. ●———●: absorbance at 280 nm; ○- - - - -○: enzymic activity; X———X: conductivity.

trypsin treatment. The enzyme fraction (Step 4) was dialyzed for 48 h against 10 l of 1 mM potassium phosphate buffer, pH 6.8 and applied to a column of hydroxyapatite (2.6 × 8.5 cm) which was equilibrated with the same buffer. The enzyme adsorbed on the column was eluted with a linear gradient of phosphate buffer increasing in molarity from 1 to 30 mM (initial volume of buffer in gradient mixing chamber was 250 ml) (Fig. 2).

The active fraction was concentrated to 3 ml with an Amicon PM 10 filter (Step 5) and applied to a column of Sephadex G-200 (2.6 × 89 cm) which was equilibrated with 10 mM potassium phosphate buffer, pH 7.8. Fractions (3.25 ml) were collected at a flow rate of 20 ml per h. The enzymic activity was obtained as a single peak of Fractions 54—62, indicated as peak I in Fig. 3. The active fraction was concentrated to 5 ml using Amicon PM 10 filter (Step 6).

Disc gel electrophoresis

The sample of Step 6 showed only a single protein band after disc gel electrophoresis (Fig. 4).

The activity of the enzyme

The activity of the enzyme for Hippuryl-His-Leu-OH and for angiotensin I increased with increasing purification. The final preparation had a specific activity of 9.5 units/mg protein for Hippuryl-His-Leu-OH and 0.665 μmol/min per mg protein for angiotensin I. The purified enzyme did not inactivate angiotensin II, however inactivated bradykinin. The specific activity of the enzyme for inactivation of bradykinin was 9.5 μmol/min per mg protein.

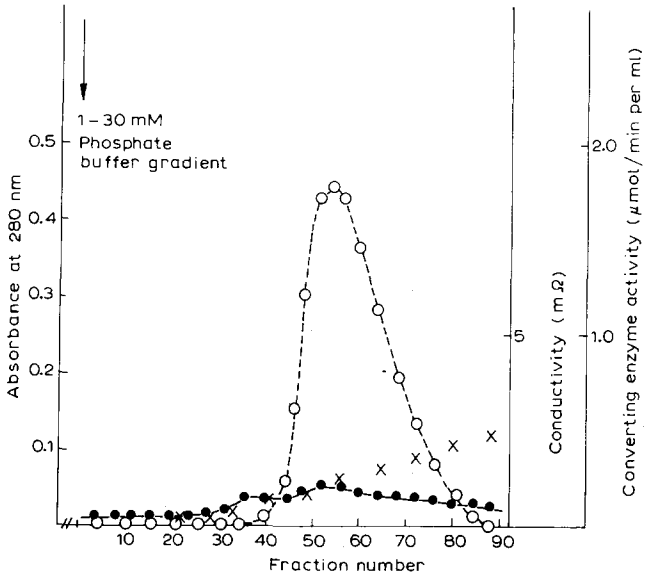
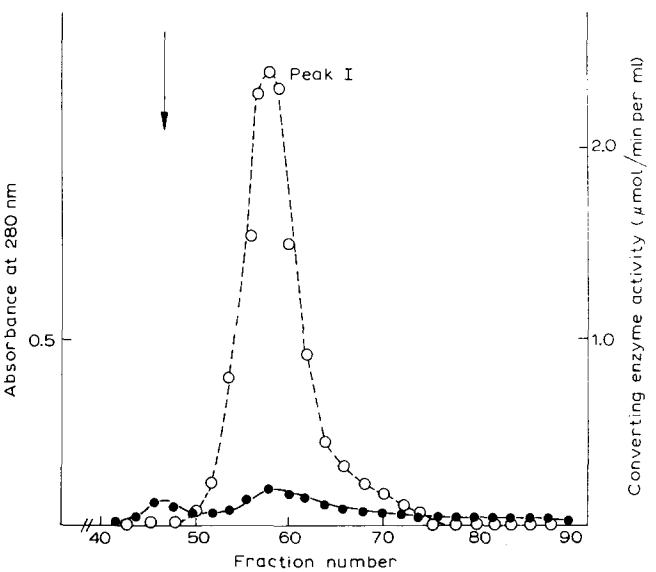


Fig. 2. Hydroxyapatite column chromatography of converting enzyme from human lung. The arrow indicates the starting point of phosphate buffer gradient (1—30 mM). Applied sample: DE 52-cellulose eluate (Step 4) ●———●: absorbance at 280 nm; ○- - - - -○: enzymic activity; X———X: conductivity.



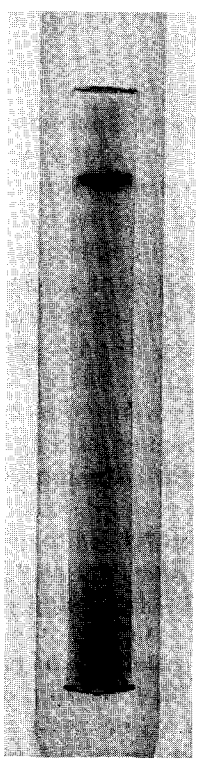


Fig. 3. Sephadex G-200 column chromatography of converting enzyme from human lung. The arrow indicates the void volume of the column as determined with Blue Dextran 2000. Applied sample: hydroxyapatite eluate (Step 5). ●———●: absorbance at 280 nm; ○- - - - -○: enzymic activity.

Fig. 4. 7.5% polyacrylamice disc gel electrophoresis. Applied sample: 10 μg of the purified enzyme (Step 6). The line on the lower portion of the gel indicates migration of the marker dye.

Molecular weight determination

Molecular weight determination was carried out by gel filtration, calibrated with apoferritin, catalase, aldolase and bovine serum albumin and by disc gel electrophoresis. The apparent molecular weight of this enzyme was estimated to be 290 000 by gel filtration of Sephadex G-200 (2.6 × 89 cm). After the enzyme was incubated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 h at 50° C, the denatured enzyme failed to enter the separating gel by dis gel electrophoresis in the presence of sodium dodecyl sulfate, suggesting that the enzyme had a molecular weight of more than 200 000.

pH optimum

The pH optimum for the enzyme was 8.3 for Hippuryl-His-Leu-OH when the enzyme which was dialyzed against 1 mM potassium phosphate buffer, pH 7.8 and substrate were incubated in 0.1 M potassium phosphate buffer, containing 0.3 M NaCl.

The effect of trypsin treatment on solubilization of the enzyme from human lung

The acid precipitated fraction (Step 2) which was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.8, was divided into two parts and incubated with two different concentrations of trypsin (1 mg/200 mg protein and 1 mg/10 mg protein) in the presence of 1 mM $CaCl_2$ for 2 h at 37° C.

(i) The concentration of 1 mg trypsin per 200 mg protein in the acid-precipitated fraction. The acid-precipitated fraction which was incubated with trypsin (1 mg/200 mg protein) was adjusted to pH 5.2 with acetic acid and centrifuged for 30 min at 15 000 × *g*. The supernatant was readjusted to pH 7.8 with 1 M NaOH and applied to a column of DE 52-cellulose which was equilibrated with 10 mM potassium phosphate buffer, pH 7.8. The enzyme was eluted as two peaks with a linear gradient of NaCl (0—0.5 M) in the same buffer. The first peak of the enzyme was eluted at a concentration of 0.09 M NaCl (peak A) and the second peak at a concentration of 0.2 M NaCl (peak B), indicated in Fig. 5. The first peak of the enzyme (peak A), dialyzed against 1 mM potassium phosphate buffer, pH 6.8, was applied to a column of hydroxyapatite which was equilibrated with the same buffer and eluted with a linear gradient of phosphate buffer increasing in molarity from 1 to 30 mM. The active fraction, concentrated with an AMICON PM-10 filter, was applied to the column of Sephadex G-200. The enzymic activity separated into two peaks. The first peak appeared in the same fractions as peak I, showed in Fig. 3, and the second peak in Fractions 72—84, indicated as peak II in Fig. 6. Next, the peak B fraction which was eluted at a concentration of 0.2 M NaCl on a column of DE 52-cellulose was also purified using columns of hydroxyapatite and Sephadex G-200 in the same methods described above. The enzymic activity appeared in Fractions 98—110 by gel filtration, indicated as peak III in Fig. 7.

(ii) The concentration of 1 mg trypsin per 10 mg protein in the acid-precipitated fraction. The acid precipitated fraction which was incubated with trypsin (1 mg/10 mg protein) was adjusted to pH 5.2 with acetic acid and centrifuged for 30 min at 15 000 × *g*. The supernatant was readjusted to pH 7.8 with 1 M NaOH and applied to a column of DE 52-cellulose and showed two peaks

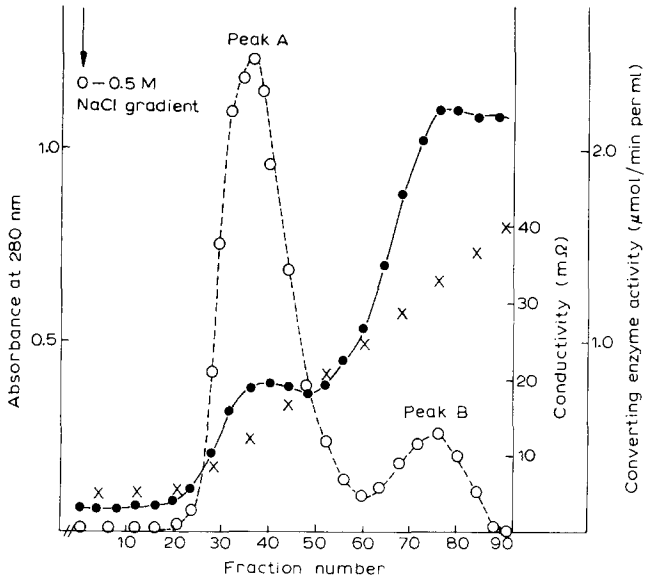


Fig. 5. DE 52-cellulose column chromatography of the converting enzyme from human lung. Applied sample: the supernatant which was treated with trypsin (1 mg/200 mg protein) for 2 h. ●———●: absorbance at 280 nm; ○- - - - -○: enzymic activity; X———X: conductivity.

which appeared in the same concentrations as peak A (eluted at the concentration of 0.09 M NaCl) and peak B (eluted at the concentration of 0.2 M NaCl), indicated in Fig. 8. But the ratio of the total enzymic activity of peak A and peak B was about 1 : 8. The peak A fraction was purified using columns of

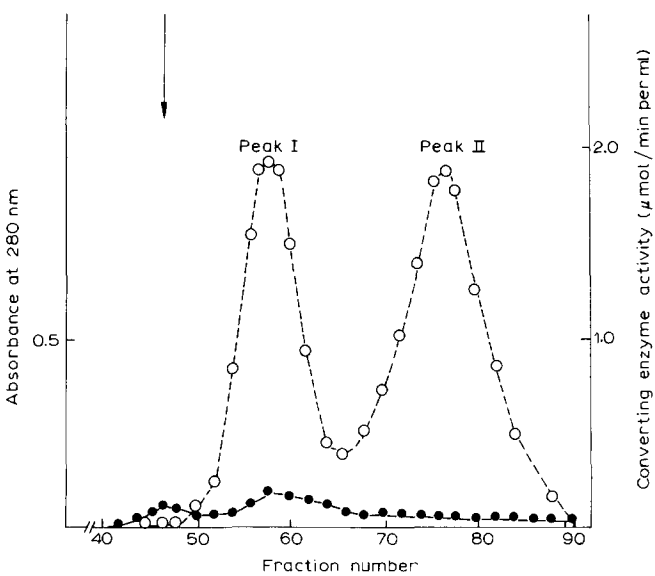


Fig. 6. Sephadex G-200 column chromatography of converting enzyme from human lung. The arrow indicates the void volume of the column as determined with Dextran Blue 2000. Applied sample: the peak A fraction obtained by trypsin treatment (1 mg/200 mg protein) for 2 h and DE 52-cellulose column chromatography. ●———●: absorbance at 280 nm; ○- - - - -○: enzymic activity.

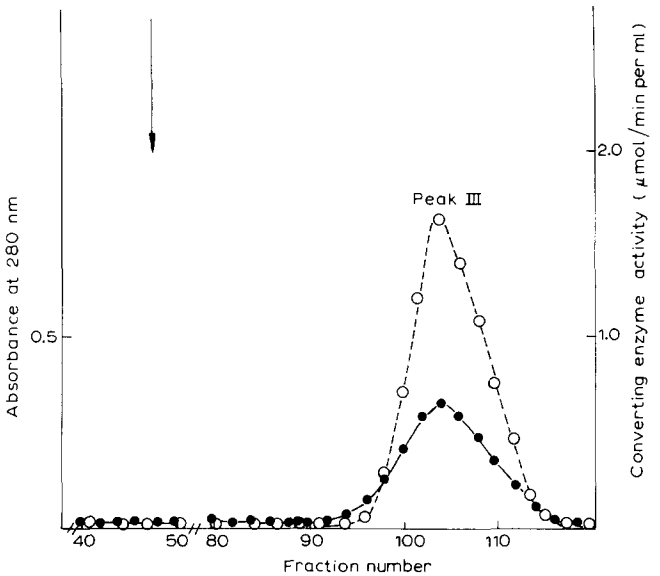


Fig. 7. Sephadex G-200 column chromatography of converting enzyme from human lung. The arrow indicates the void volume of the column as determined with Dextran Blue 2000. Applied sample: the peak B fraction. ●——●: absorbance at 280 nm; ○- - - - -○: enzymic activity.

hydroxyapatite and Sephadex G-200 (2.6 × 89 cm) in the same methods described above. The enzymic activity showed only one peak by gel filtration which appeared in the same fractions as peak II, indicated in Fig. 6. The peak B fraction was also purified using columns of hydroxyapatite and Sephadex

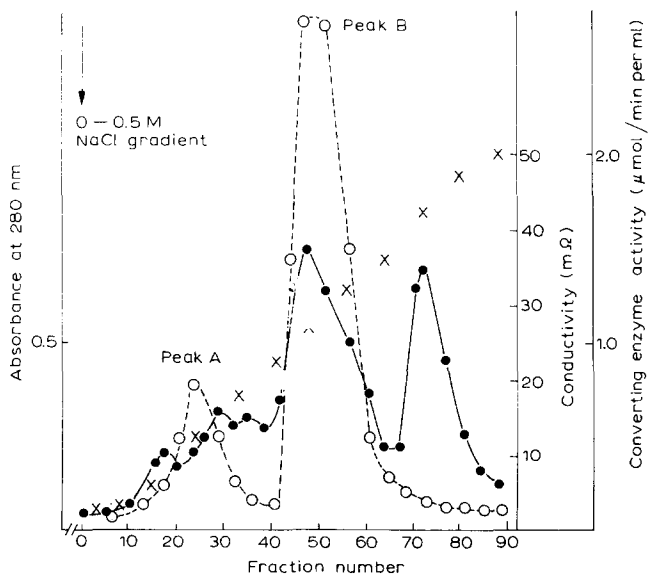


Fig. 8. DE 52-cellulose column chromatography of converting enzyme from human lung. Applied sample: the supernatant which was treated with trypsin (1 mg/10 mg protein) for 2 h. ●——●: absorbance at 280 nm; ○- - - - -○: enzymic activity; X——X: conductivity.

TABLE II

THE EFFECT OF TRYPSIN TREATMENT ON SOLUBILIZATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM HUMAN LUNG

Trypsin treatment	Eluted concentration of NaCl on a column of DE 52-cellulose	Molecular weight by gel filtration on Sephadex G-200	Specific activity of the enzyme (units/mg protein for Hippuryl-His-Leu-OH)
Trypsin 1 mg/500 mg protein	0.09 M NaCl (peak A)	290 000 (peak I)	9.5
Trypsin 1 mg/200 mg protein	0.09 M NaCl (peak A)	290 000 (peak I)	9.5
		180 000 (peak II)	13.8
	0.2 M NaCl (peak B)	98 000 (peak III)	0.64
Trypsin 1 mg/10 mg protein	0.09 M NaCl (peak A)	180 000 (peak II)	13.8
	0.2 M NaCl (peak B)	98 000 (peak III)	0.64

G-200. The enzymic activity showed only one peak by gel filtration which appeared in the same fractions as peak III, indicated in Fig. 7.

Molecular weight determinations

The apparent molecular weights of peak I, peak II and peak III were estimated to be 290 000, 180 000 and 98 000 respectively by gel filtration of Sephadex G-200 (2.6 × 89 cm).

The activities of the enzymes

The specific activities of peak I, peak II and peak III were 9.5, 13.8 and 0.64 units/mg protein for Hippuryl-His-Leu-OH respectively. The turnover number of the enzyme of peak I was very consistent with that of the enzyme of peak II.

K_m values

K_m values of peak I, peak II and peak III for Hippuryl-His-Leu-OH were identical and 1.1 mM. Results of these experiments are summarized in Table II.

Discussion

The specific activity (9.5 units/mg protein) of the purified enzyme for Hippuryl-His-Leu-OH is lower than the values of 24.3 units/mg protein from rabbit lung reported by Nishimura et al. [15] and of 17.6 units/mg protein from rat lung reported by Lanzillo and Fanburg [20] and approximates the value of 10.0 units/mg protein from hog lung reported by Dorer et al. [7]. But the specific activity (0.665 μmol/min per mg protein) for angiotensin I was higher than the value of 0.182 μmol/min per mg protein from rabbit lung reported by us [15]. The specific activities of the initial samples were very low, but on the steps of trypsin treatment and DE 52-cellulose column chromatography, the total enzymic activity was increased 3-fold as compared with the enzymic activ-

ity present in the initial lung extract. This may be due to the difference of the enzymic activity between membrane-bound and solubilized form, to the presence of the unknown inhibitors contained in the lung [3] and to the change of the enzymic activity by trypsin digestion [20]. Such a phenomenon was not seen on the purification procedure of angiotensin I-converting enzyme from rabbit lung. By varying concentrations of trypsin, three different enzymes of molecular weight were obtained by gel filtration. Though the purified enzyme from rabbit lung after the denaturation showed molecular weight 155 000 by disc gel electrophoresis [15], the denatured enzyme of peak I fraction from human lung failed to enter the separating gel under the standard condition. Thus this enzyme from human lung was suggested to contain a single polypeptide and its molecular weight changed regularly by trypsin digestion. The smallest unit of the enzyme (molecular weight 98 000) was very resistant to trypsin. Molecular weight of peak II fraction (180 000), K_m value (1.1 mM), and pH optimum (8.3) for Hippuryl-His-Leu-OH are consistent with those reported by Lieberman and Beutler from human plasma [21] and by Oshima et al. from human lung [22]. Lanzillo and Fanburg [23] recently reported that the lung served as a sourse for the serum angiotensin I-converting enzyme. According to their report, our data strongly suggested that the released form of the enzyme from human lung in plasma was peak II fraction (molecular weight 180 000). Overturf et al. [24] recently reported that two protein fractions (molecular weights 450 000 and 600 000) were separated by Sepharose 6-B gel filtration, and agiotensin I-converting enzyme and bradykininase were different enzymes. But we confirmed that the purified enzyme did convert angiotensin I to angiotensin II and also inactivate bradykinin, of which the specific activity for bradykinin was 10 times that for angiotensin I.

Acknowledgement

We thank Dr. C. Wakasugi, Department of Legal Medicine, Osaka University School of Medicine, for the supply of human lung materials. This work was supported a grant from Research Foundation for Cancer and Cardiovascular Diseases for 1976.

References

1 Skeggs, Jr., L.T., Kahn, J.R. and Shumway, N.P. (1956) J. Exp. Med. 103, 295—299
2 Yang, H.Y.T., Erdös, E.G. and Levin, Y. (1970) Biochim. Biophys. Acta 214, 374—376
3 Yang, H.Y.T., Erdös, E.G. and Levin, Y. (1971) J. Pharmacol. Exp. Ther. 117, 291—300
4 Ueda, E., Akutsu, H., Kokubu, T. and Yamamura, Y. (1971) Jap. Circul. J. 35, 801—806
5 Cushman, D.W. and Cheung, H.S. (1971) Biochem. Pharmacol. 20, 1637—1648
6 Stevens, R.L., Micalizzi, E.R., Fessler, D.C. and Pals, D.T. (1972) Biochemistry 11, 2999—3007
7 Dorer, F.E., Kahn, J.R., Lents, K.E., Levine, M. and Skeggs, L.T. (1972) Circ. Res. 31, 356—366
8 Fitz, A. and Overturf, M. (1972) J. Biol. Chem. 247, 581—584
9 Nakajima, T., Oshima, G., Yeh, H.S.J., Igic, R. and Erdös, E.G. (1973) Biochim. Biophys. Acta 315, 430—438
10 Lanzillo, J.J. and Fanburg, B.L. (1974) J. Biol. Chem. 249, 2312—2318
11 Das, M. and Soffer, R.L. (1975) J. Biol. Chem. 250, 6762—6768
12 Oshima, G., Gecse, A. and Erdös, E.G. (1974) Biochim. Biophys. Acta 350, 26—37
13 Erdös, E.G. and Oshima, G. (1974) Acta Physiol. Latinoam. 24, 507—511
14 Bakhle, Y.S. (1968) Nature 220, 919—921
15 Nishimura, K., Hiwada, K., Ueda, E. and Kokubu, T. (1976) Biochim. Biophys. Acta 452, 144—150

16 Davis, B.J. (1974) Ann. N.Y. Acad. Sci. 121, 404—427
17 Shapiro, A.L., Vin Uela, E. and Maizel, Jr., J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815—820
18 Neville, Jr., D.M. (1971) J. Biol. Chem. 246, 6328—6334
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Oshima, G., Nagasawa, K. and Kato, J. (1976) J. Biochem. 80, 477—483
21 Lieberman, J. and Beutler, E. (1976) N. E. J. Med. 294 (26), 1442—1444
22 Oshima, G., Gecse, A. and Erdös, E.G. (1974) Circ. Res. 34, 5: I-210-212.
23 Lanzillo, J.J. and Fanburg, B.L. (1976) Biochim. Biophys. Acta 445, 161—168
24 Overturf, M., Wyatt, S., Boaz, D. and Fitz, A. (1976) Life Sc. 16, 1669—1682