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PROPERTIES OF THREE DIFFERENT FORMS OF ANGIOTENSIN I-CONVERTING ENZYME FROM HUMAN LUNG

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Summary

We compared some properties of three different forms of angiotensin I-converting enzyme from human lung obtained with trypsin treatment. The inhibition studies were performed using bradykinin potentiator C, Arg-Pro-Pro and EDTA. The I_{50} values for these inhibitors on the enzymes of peak I (mol. wt. 290 000) and peak II (mol. wt. 180 000) were identical (bradykinin potentiator C, $5 \cdot 10^{-6}$ M; Arg-Pro-Pro, $1.2 \cdot 10^{-4}$ M; EDTA, $5 \cdot 10^{-5}$ M). But the enzymic activity of peak III (mol. wt. 98 000) was not inhibited by bradykinin potentiator C and Arg-Pro-Pro. The I_{50} value for EDTA on the enzyme of peak III was $2 \cdot 10^{-3}$ M. The enzymic activities of peak I and peak II were reduced to 10% of the initial level of the enzymic activity by the preincubation for 10 min at 50°C, but the enzymic activity of peak III to 55%. The pH optimums for three different forms of the enzyme were identical and pH 8.3 in potassium phosphate buffer, but the pH optimums were changed and pH 7.3—8.0 in borate/sodium carbonate buffer. K_m values of three different forms of the enzyme for Hippuryl-His-Leu-OH were identical and 0.6 mM in borate/sodium carbonate buffer, pH 7.8. The enzymic activities of peak I and peak II were dependent of Cl^- ion, but the enzymic activity of peak III was independent of Cl^- ion. Moreover, the enzymes of peak I and peak II were adsorbed on a column of concanavalin A-Sepharose, but the enzyme of peak III was not adsorbed on the column. It was suggested that the enzymes of peak I and peak II were glycoprotein, but the enzyme of peak III did not have a mannose in a suitable position to react with concanavalin A.

Introduction

Angiotensin I-converting enzyme which converts angiotensin I to angiotensin II by releasing the C-terminal dipeptide was first isolated from horse plasma [1]. We reported previously that the enzyme purified from human lung

was capable of inactivating bradykinin [2]. This means that the enzyme has a significant role on the metabolism of angiotensin I and bradykinin in the lung, and may contribute the regulation of the systemic blood pressure. On the purification of this enzyme from human lung using trypsin treatment, we found that there were three different forms of the enzyme (molecular weight: 290 000, 180 000 and 98 000). In this report, we compare some kinetic properties of three different forms of the enzyme.

Materials and Methods

Human cadaveric lung was obtained from the victim of a traffic accident. Hippuryl-His-Leu-OH, angiotensin I, angiotensin II, bradykinin, Arg-Pro-Pro and bradykinin potentiator c were purchased from the Institute for Protein Research, Osaka Univ., Osaka, Japan. Trypsin was from Difco Lab., Detroit, Michigan, U.S.A. The molecular weight marker kit was obtained from Boehringer Mannheim GmbH, West Germany and Sephadex G-200, hydroxyapatite, Dextran Blue 2000 and Con A-Sepharose from Pharmacia Fine Chemicals AB, Uppsala, Sweden. DE-52 was from Whatman, Maidstone, Kent, England.

Enzyme assays

Angiotensin I-converting enzyme assay was performed by the spectrophotometric method of Cushman and Cheung [3]. 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. Another assay of the enzyme was performed by using angiotensin 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].

Protein concentration was determined by the method of Lowry et al., using bovine serum albumin as a standard [5].

Preparation of the three different forms of the enzyme from human lung

The purification procedure has been previously reported in detail [2].

Human cadaveric lung (40 g) was chopped into small pieces and suspended in 20 mM potassium phosphate buffer, pH 7.8, containing 0.25 M sucrose. The suspension was homogenized in a Waring blendor 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) which was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.8, was incubated with trypsin (1 mg/200 mg protein) for 2 h at 37° C in the presence of 1 mM $CaCl_2$. The solution was readjusted to pH 5.2 and centrifuged for 30 min at 15 000 × *g*. The supernatant (Step 3) was adjusted 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) in the same buffer. The first peak of the enzyme (peak A) (Step $4a_1$) was eluted at the concentration of 0.09 M NaCl and the second peak (peak B) (Step $4b_2$) at the concentration of 0.2 M NaCl. 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 (Step $4a_2$), concentrated with Amicon PM 10 filter, was applied to a column of Sephadex G-200 (2.6 × 89 cm). Fractions (3.25 ml) were collected at a flow rate of 20 ml per h. The enzymic activity separated into two peaks. The first peak appeared in fractions 54—62 (peak I) and the second peak in fractions 72—84 (peak II) (Step $4a_3$). Next, the peak B fraction (Step $4b_1$) which was eluted at the concentration of 0.2 M NaCl on a column of DE 52-cellulose was also purified using columns of hydroxyapatite (Step $4b_2$) and Sephadex G-200 (Step $4b_3$) in the same methods described above. The enzymic activity appeared in fractions 98—110 by gel filtration (peak III).

Results

Preparation of the three different forms of angiotensin I-converting enzyme from human lung

The purification of angiotension I-converting enzyme was performed three times on three different materials from human lungs, and all the same data were obtained. This shows one of these experiments.

Purification steps of angiotensin I-converting enzyme from human lung using trypsin (1 mg/200 mg protein) are summarized in Tables I, II and III. The total enzymic activity was increased 3-fold as compared with the enzymic activity present in the initial lung extract on the steps of trypsin treatment and DE 52-cellulose column chromatography. This result was similar to that obtained with trypsin treatment (1 mg/500 mg protein) in the previous paper. The enzymic activity was separated into two peaks on a column of DE 52-cellulose. The first peak of the enzyme (peak A) was eluted at the concentration of 0.09 M NaCl and the second peak of the enzyme (peak B) at the concentration of 0.2 M NaCl. The ratio of the total enzymic activity of peak A and peak B was about 7 : 3. The peak A and peak B fraction were further purified separately using columns of hydroxyapatite and Sephadex G-200. Purification steps of the peak A and peak B fraction are summarized in Table II and III respectively. The peak A fraction was separated into two peaks (peak I and peak II) and the peak B fraction showed only one peak on a column of Sepha-

TABLE I

PURIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM HUMAN LUNG USING TRYPSIN (1 mg/200 mg PROTEIN)

Purification step	Volume (ml)	Total protein	Total activity (units)	Specific activity Hippuryl-His-Leu-HO (units/mg)
1 The supernatant of homogenate centrifuged at 700 X *g*	95	2551.3	3.572	0. 0014
2 Sediment from the pH 5.2 precipitation, resuspended in 10 mM phophate buffer, pH 7.8	45	1184.9	2.014	0.0017
3 After trypsin treatment and acidification to pH 5.2, the supernatant centrifuged at 15 000 X *g*	135	498.1	5.477	0.011
4 DE 52-cellulose eluate				
$4a_1$ Eluate at the concentration of 0.09 M (peak A)	65	11.9	8.169	0.684
$4a_2$ Eluate at the concentration of 0.2 M NaCl (peak B)	49	6.23	3.501	0.640

TABLE II

PURIFICATION OF THE PEAK A FRACTION OBTAINED BY A COLUMN OF DE 52-CELLULOSE

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity	
				Hippuryl-His-Leu-OH (units/mg)	Angiotensin I (nmol/min per mg protein)
$4a_1$ Eluate at the concentration of 0.09 M NaCl (peak A)	65	11.9	8.169	0.684	47.9
$4a_2$ Hydroxyapatite eluate (concentrated)	3	0.78	6.323	8.13	568.6
$4a_3$ Sephadex G-200 filtrate (concentrated) peak I	5	0.36	3.415	9.5	665.0
peak II	5	0.17	2.276	13.8	964.0

TABLE III

PURIFICATION OF THE PEAK B FRACTION OBTAINED BY A COLUMN OF DE 52-CELLULOSE

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity	
				Hippuryl-His-Leu-OH (units/mg)	Angiotension I (nmol/min per mg protein)
$4b_1$ Eluate at the concentration of 0.2 M NaCl (peak B)	49	6.23	3.501	0.562	
$4b_2$ Hydroxyapatite eluate (concentrated)	3	4.21	2.696	0.640	
$4b_3$ Sephadex G-200 filtrate (concentrated) peak III	5	4.00	2.561	0.640	18.0

dex G-200. 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).

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 and 0.665, 0.966 and 0.018 μmol/min per mg protein for angiotensin I respectively. The enzymes of peak I and peak II did not inactivate angiotensin II, however inactivated bradykinin. The enzyme of peak III had slight angiotensinase activity.

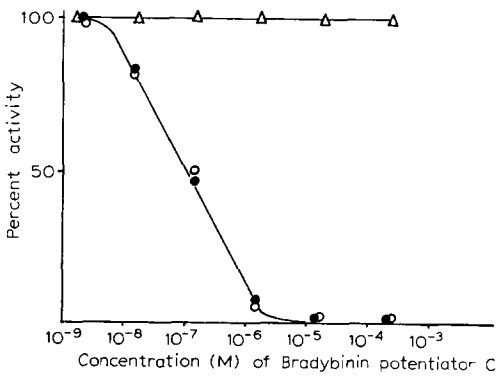
Inhibition by bradykinin potentiator c, Arg-Pro-Pro or EDTA

Inhibition assays were performed using 5 mM Hippuryl-His-Leu-OH as substrate and bradykinin potentiator c, Arg-Pro-Pro or EDTA as inhibitor.

(i) The inhibitory effect of bradykinin potentiator c on the three different forms of angiotensin I-converting enzyme from human lung is shown in Fig. 1. The I_{50} values for bradykinin potentiator c on the enzymes of peak I and peak II were identical and $5 \cdot 10^{-6}$ M, but the enzymic activity of peak III could not be inhibited even at the concentration of $5 \cdot 10^{-3}$ M.

(ii) The inhibitory effect of Arg-Pro-Pro on the three different forms of the enzyme is shown in Fig. 2. The I_{50} values for Arg-Pro-Pro on the enzymes of peak I and peak II were identical and $1.2 \cdot 10^{-4}$ M, but the enzymic activity of peak III was not inhibited at the concentration of $5 \cdot 10^{-2}$ M.

(iii) The inhibitory effect of EDTA on the three different forms of the enzyme was shown in Fig. 3. The I_{50} values for EDTA on the enzymes of peak I and peak II were identical and $5 \cdot 10^{-5}$ M, and that on the enzyme of peak III was $2 \cdot 10^{-3}$ M.



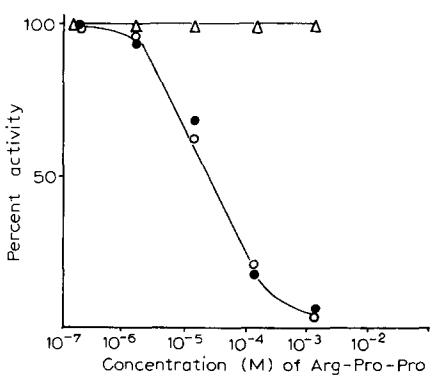


Fig. 1. The inhibitory effect of bradykinin potentiator C on the enzymic activities of peak I, peak II and peak III. Inhibition assays were performed using 5 mM Hippuryl-His-Leu-OH as substrate and bradykinin potentiator C as inhibitor. ○———○: the enzymic activity of peak I, ●———●: the enzymic activity of peak II; △———△: the enzymic activity of peak III.

Fig. 2. The inhibitory effect of Arg-Pro-Pro on the enzymic activities of peak I, peak II and peak III. Inhibition assays were performed using 5 mM Hippuryl-His-Leu-OH as substrate and Arg-Pro-Pro as inhibitor. ○———○: the enzymic activity of peak I; ●———●: the enzymic activity of peak II; △———△: the enzymic activity of peak III.

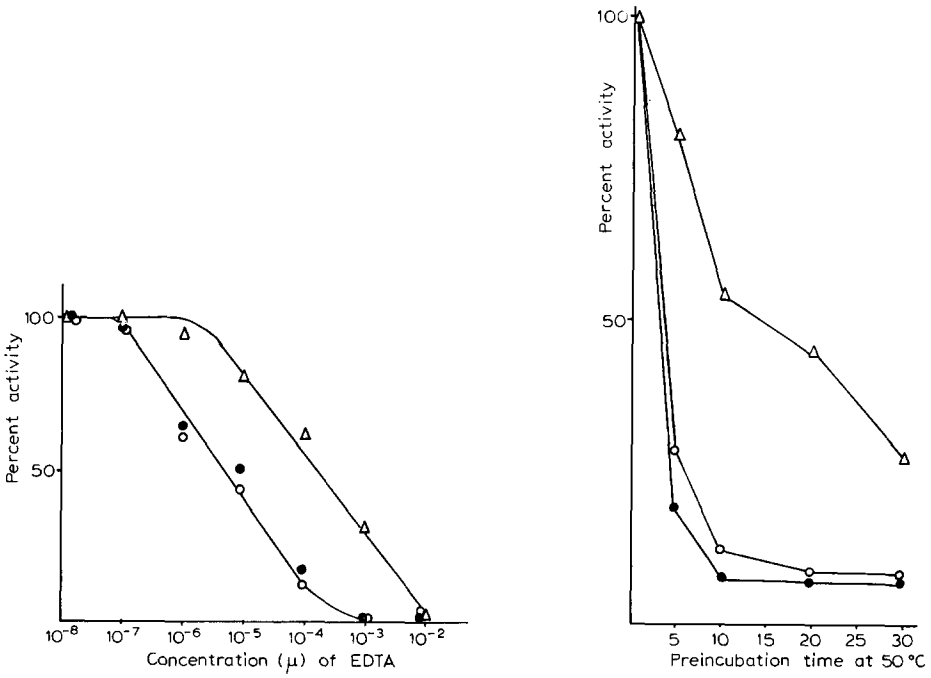


Fig. 3. The inhibitory effect of EDTA on the enzymic activities of peak I, peak II and peak III. Inhibition assays were performed using 5 mM Hippuryl-His-Leu-OH as substrate and EDTA as inhibitor. ○———○: the enzymic activity of peak I; ●———●: the enzymic activity of peak II; △———△: the enzymic activity of peak III.

Fig. 4. Heat stabilities of peak I, peak II and peak III. The enzymes of peak I, peak II and peak III were preincubated for 5, 10, 20 and 30 min at 50°C and assayed using 5 mM Hippuryl-His-Leu-OH. ○———○: the enzymic activity of peak I; ●———●: the enzymic activity of peak II; △———△: the enzymic activity of peak III.

Heat stability of angiotensin I-converting enzyme

The enzymes of peak I, peak II and peak III were preincubated for 5, 10, 20 and 30 min at 50°C and assayed using 5 mM Hippuryl-His-Leu-OH described above (Fig. 4). The enzymic activities of peak I and peak II were reduced to 10% of the initial level of the enzymic activity by the preincubation for 10 min, but the enzymic activity of peak III to 55%.

Cl^- ion dependency

The enzymic assays of peak I, peak II and peak III were performed with 5 mM Hippuryl-His-Leu-OH, in 100 mM potassium phosphate buffer, pH 8.3, both in the presence of Cl^- ion (0.3 M NaCl) and in the absence of Cl^- ion. The enzymic activities of peak I and peak II were dependent of Cl^- ion, but the enzymic activity of peak III was independent of Cl^- ion.

pH optimum

The pH optimums for the enzymes of peak I, peak II and peak III were identical and pH 8.3 for Hippuryl-His-Leu-OH when the enzyme which was dialyzed against 1 mM potassium phosphate buffer, pH 7.8 was incubated with

the substrate in 0.1 M potassium phosphate buffer, containing 0.3 M NaCl. But, when the enzyme and substrate were incubated in 0.1 M borate/sodium carbonate buffer, containing 0.3 M NaCl, the pH optimums were changed and pH 7.3—8.0 shown in Fig. 5. The maximal enzymic activity of peak III, obtained in phosphate buffer, was only 60% of that obtained in borate/sodium carbonate buffer.

K_m value

K_m values of peak I, peak II and peak III for Hippuryl-His-Leu-OH in 100 mM potassium phosphate buffer, pH 8.3, were identical and 1.1 mM. And, K_m values of peak I, peak II and peak III for Hippuryl-His-Leu-OH in 100 mM borate/sodium carbonate buffer, pH 7.8, were identical and 0.6 mM.

Con A-Sepharose column

The enzymes of peak I, peak II and peak III which was dialyzed against 50 mM Tris · HCl buffer, pH 7.0, containing 0.5 NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ were applied to a column of Con A-Sepharose which was equilibrated with the same buffer. The enzymes of peak I and peak II was adsorbed on the column, but the enzyme of peak III was eluted in the starting buffer. It was suggested that the enzymes of peak I and peak II were glycoprotein, but the enzyme of peak III did not have a mannose in a suitable position to react with concanavalin A.

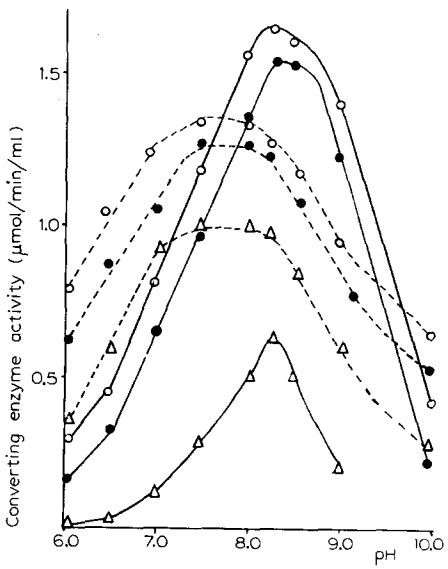


Fig. 5. The optimums for the enzymes of peak I, peak II and peak III. (———): the enzyme which was dialyzed against 1 mM potassium phosphate buffer, pH 7.8 and 5 mM Hippuryl-His-Leu-OH as substrate were incubated in 0.1 M potassium phosphate buffer, containing 0.3 M NaCl. (— — —): the enzyme which was dialyzed against 1 mM potassium phosphate buffer, pH 7.8 and 5 mM Hippuryl-His-Leu-OH as substrate were incubated in 0.1 M borate/sodium carbonate buffer, containing 0.3 M NaCl. ○———○: the enzymic activity of peak I; ●———●: the enzymic activity of peak II; △———△: the enzymic activity of peak III.

Discussion

The enzymic properties of peak I from human lung were identical with those of peak II with regard to inhibitory effect (bradykinin potentiator c, Arg-Pro-Pro and EDTA), heat stability, Cl^- ion dependence, pH optimum, K_m value and adsorbtion on a Con A-Sepharose column. These enzymic properties of peak I and peak II were similar to those reported by others [3,6,7]. However, the enzymic properties of peak III were different from those of peak I and peak II.

Cushman and Cheung reported that pH optimums of the enzyme from rabbit lung for Hippuryl-His-Leu-OH measured in the different buffers (sodium acetate buffer, potassium phosphate buffer, sodium borate buffer and Tris acetate buffer) were identical (pH 8.3) and sharp shaped, and the maximal activity obtained in Tris acetate buffer was 60% of that obtained in potassium phosphate or sodium borate buffer [3]. Eliseeva et al. reported that pH optimum of the enzyme from bovine lung for CBz-Phe-His-Leu-OH in Tris · HCl buffer was at 7.2—7.4 and in phosphate buffer, at pH 7.6—7.8, and maximal activity in Tris · HCl buffer was 1.5—2 times higher than in phosphate/borate buffer [8]. pH optima of peak I, peak II and peak III from human lung for Hippuryl-His-Leu-OH in potassium phosphate buffer were sharp-shaped (pH 8.3), but pH optima of those in borate/sodium carbonate buffer were broad (pH 7.3—8.0). K_m values of the enzymes in potassium phosphate buffer, pH 8.3 were 1.1 mM, but in borate/sodium carbonate buffer, pH 7.8 were 0.6 mM. Therefore, the enzymic activity in the serum and other samples will be safely assayed without distinct check of pH when borate/sodium carbonate buffer is used. The enzymic activities of peak I and peak II in 100 mM potassium phosphate buffer, pH 8.3 were 15% higher than those in 100 mM borate/sodium carbonate buffer, pH 7.8. But the enzymic activity in peak III in 100 mM potassium phosphate buffer, pH 8.3 was only 60% of that in 100 mM borate/sodium carbonate buffer, pH 7.8.

The enzymic activity of peak III for Hippuryl-His-Leu-OH as substrate was not inhibited by bradykinin potentiator c and Arg-Pro-Pro. And the enzyme hydrolyzed Hippuryl-His-Leu-OH in the abscence of Cl^- ion. These properties of the enzyme of peak III were similar to those of β-converting enzyme from rat submaxillary glands reported by Boucher et al. [9]. The enzymic activity of β-converting enzyme was not inhibited by EDTA but inhibited by plasma. However, the enzymic activity of peak III was inhibited by EDTA and not inhibited by plasma. The difference between the properties of the enzyme of peak III and those of the enzymes of peak I and peak II may be partly due to the difference of carbohydrate content because the enzyme of peak III was not adsorbed on a column of Con A-Sepharose. But we failed to purify the enzyme of peak III completely. Moreover, since the enzyme had slight angiotensinase activity, we could not clarify that the enzyme of peak III could inactivate bradykinin. Nakajima et al. reported that angiotensin I-converting enzyme, which was purified from hog lung, coupled to Sepharose 4 B could cleavage angiotensin I in the absence of Cl^- ion [10]. And Oshima et al. reported that Cl^- ion induced a shift in the conformation of the enzyme [11]. Our results suggest that the conformation of the smallest unit of angiotensin I-converting enzyme (peak III) which was obtained with trypsin treatment might not be

influenced by Cl^- ion and thus it acts both angiotensin I and bradykinin in the absence of Cl^- ion.

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