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SOLUBILIZATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM RABBIT LUNG USING TRYPSIN TREATMENT

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Summary

The solubilization of angiotensin I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) from rabbit lung was carried out using trypsin treatment. A good recovery of 76% was obtained. The enzyme from solubilized fraction was purified using columns of Sephadex G-200, hydroxyapatite and DEAE-cellulose. The purified enzyme was shown to convert angiotensin I to angiotensin II and also to inactivate bradykinin. The specific activity of the enzyme was 24.3 units/mg protein for hippurylhistidylleucyl hydroxide and 0.182 μmol/min per mg protein for angiotensin I. The enzymic activity obtained after trypsin treatment for 5 h could be divided into two components: (i) an enzyme of molecular weight 300 000 (peak II) and (ii) an enzyme of molecular weight 145 000 (peak III), by Sephadex G-200 gel filtration. The molecular weight of the denatured enzyme was found to be 155 000 by disc gel electrophoresis in the presence of sodium dodecyl sulfate. K_m values of peak II and peak III fraction for Hippuryl-His-Leu-OH were 2.6 mM.

Introduction

Angiotensin I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) which converts angiotensin I to angiotensin II by releasing the terminal dipeptide was first isolated from horse plasma [1]. Ng and Vane pointed out the significance of the lung in metabolising angiotensin I in vivo [2] and Cushman and Cheung reported that a high activity of the enzyme existed in the lung [3]. The enzyme is also thought to be capable of inactivating bradykinin and to be identical with kininase II [4—6]. Purification of the enzyme has been attempted from rabbit, calf, hog, rat and human lung [7—13], from bovine, rat and hog kidney [14—16], and from hog, rabbit and human plasma [6,17,18]. Since this enzyme is thought to exist as a membrane-bound protein [12,19,20,21] there

are some problems in solubilizing it from a pulmonary particulate fraction. Cheung and Cushman tried to extract the enzyme from acetone powders of rabbit lung [22]. Soffer et al. [23] and Oshima et al. [16] attempted to solubilize the enzyme by using detergents.

We investigated the effect of trypsin treatment on solubilization of the enzyme from rabbit lung. In this report, we describe the details of trypsin treatment and a purification procedure and also 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 Biochemical Co., New Jersey, 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

Angiotensin I-converting enzyme assay was performed by the spectrophotometric method of Cushman and Cheung [7]. One unit of the enzyme activity was defined as that amount of the enzyme which hydrolysed 1 μmol of Hippuryl-His-Leu-OH per min at 37° C under the conditions described by them. The other assay of the enzyme was performed 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 reaction was stopped by boiling for 5 min. After appropriate dilution, the angiotensin II formed was assayed in isolated rat uterus [6].

Angiotensinase and kininase activities were determined by using either angiotensin II or bradykinin as substrates. The reaction mixture, containg 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 had been stopped by boiling for 5 min, the peptides were assayed in the isolated rat uterus [6].

Disc gel electrophoresis

Analytical disc gel electrophoresis was performed on 5% acrylamide gel at pH 8.6 with a current of 2 mA per tube for 2 h [24]. 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 electrophoresis on 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 [25,26]. 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 [27].

Results

The effect of trypsin treatment on solubilization of angiotensin I-converting enzyme from rabbit lung

Fresh rabbit lungs (10 g) were chopped into small pieces and suspended in 50 ml of 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 (Fraction A) was filtered through two layers of gauze, adjusted to pH 5.2 with acetic acid and centrifuged for 20 min at 15 000 × *g*. The pellet was dissolved in 10 mM potassium phosphate buffer pH 7.8 and adjusted to pH 7.8 with 1 M NaOH. The acid-precipitated fraction (Fraction B) was divided into five parts and each of them was incubated with trypsin (1 mg/10 mg protein) in the presence of 1 mM $CaCl_2$ for 1, 2, 3, 4 and 5 h at 37°C, respectively. After the reaction had been stopped with soybean trypsin inhibitor (1 mg/mg protein), each sample was centrifuged for 90 min at 78 000 × *g*. The pellet contained about 5% of the enzyme activity after trypsin treatment for 2 h. Recovery of each enzyme activity as compared with the total activity of Fraction B was 40.4, 76.0, 57.7, 55.5, and 59.0% respectively. These values are the means of the experiments repeated three times.

The following three samples were applied to a column of Sephadex G-200 (2.6 × 90.5 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 hour. Sample 1 was the 78 000 × *g* supernatant obtained from Fraction B which was treated with trypsin for 2 h. The enzyme activity was obtained as a single peak of Fractions 56—66, indicated as peak II in Fig. 1a. Sample 2 was the 78 000 × *g* supernatant obtained from Fraction B which was treated with trypsin for 5 h. The enzyme activity separated into two peaks. The first peak appeared in the same fraction as peak II and the another peak in Fractions 68—80 indicated as peak III in Fig. 1b. Sample 3 was the Nonidet-P40 extract which was obtained according to the method of Soffer et al. [23]. The enzyme activity separated into two peaks. The first peak appeared in the void volume of the column (peak I) and the second peak in the same fraction as peak II (Fig. 1c).

Purification of angiotensin I-converting enzyme from peak II fraction obtained by trypsin treatment and gel filtration

Purification steps of peak II fraction are summarized in Table I. Fresh rabbit lungs (27.8 g) were chopped into small pieces and suspended in 160 ml of 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 (Fraction A) was filtered with two layers of gauze and adjusted to pH 5.2 with acetic acid and centrifuged for 20 min at 15 000 × *g*. The pellet was suspended in 10 mM potassium phosphate buffer, pH 7.8 and adjusted to pH 7.8 with 1 M NaOH (Fraction B). The enzyme suspension was incubated with trypsin (1 mg/10 mg protein) containing 1 mM $CaCl_2$ for 120 min at 37°C. After the reaction was stopped with soybean tryp-

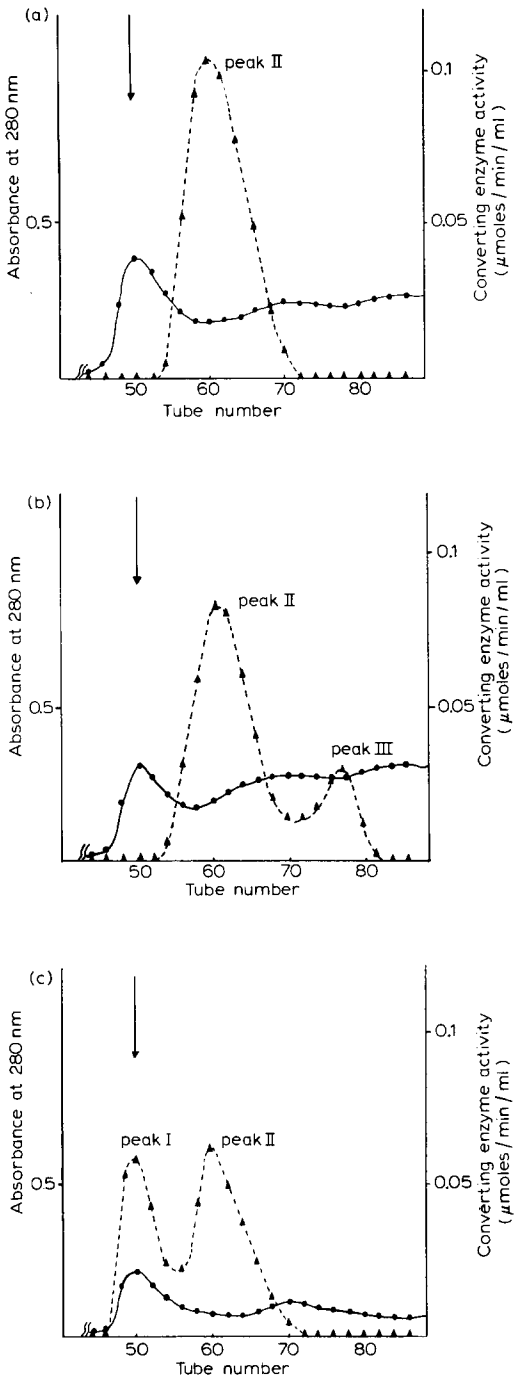

Fig. 1. Gel filtration on Sephadex G-200 column (2.6 X 90.5 cm). Fraction volume: 3.25 ml. Enzyme activity was measured using Hippuryl-His-Leu-OH as substrate. The arrow indicates the void volume of the column as determined with Blue Dextran 2000. (●———●): absorbance at 280 nm; (▲- - - - -▲): enzyme activity. a, applied sample; the 78 000 X *g* supernatant ontained from Fraction B which was treated with trypsin for 2 h. b, applied sample; the 78 000 X *g* supernatant obtained from Fraction B which was treated with trypsin for 5 h. c, applied sample; the Nonidet-P 40 extract.

TABLE I

PURIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME FROM RABBIT LUNG

Fraction	Purification step	Volume (ml)	Protein (mg)	Specific activity		Yield (%)
				Hippuryl-His-Leu-OH (units/mg)	Angiotensin I (nmol/min per mg)	
A	The supernatant of homogenate centrifuged at 700 X *g*	123.8	4.640	0.0092		100
B	Sediment from the pH 5.2 precipitation, resuspended in 10 mM potassium phosphate buffer, pH 7.8	61.3	1.480	0.0197		68.6
C	After trypsin treatment. The supernatant centrifuged at 78 000 X *g*	53.5	491	0.043	0.32	50.1
D	Sephadex G-200 filtrate (conc.)	30.7	3.5	8.77	64.3	47.6
E	Hydroxyapatite eluate (conc.)	17.6	0.878	19.6		26.7
F	DE-52 cellulose eluate (conc.)	4.0	0.288	21.9	163.9	9.8

sin inhibitor (148 mg), the solution was centrifuged for 90 min at 78 000 × *g*. The supernatant (Fraction C) was concentrated to 23.6 ml with Amicon PM 10 filter and applied to a column of Sephadex G-200 (2.6 × 90.5 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 peak II fraction containing the enzyme was pooled and concentrated to 30.7 ml using an Amicon PM 10 filter (Fraction D). It was dialyzed for 48 h against 3000 ml of 1 mM potassium phosphate buffer pH 6.8 and applied to a column of hydroxyapatite (2.6 × 10 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 500 ml). The active fraction was concentrated to 17.6 ml with an Amicon PM 10 filter (Fraction E) and applied to a column of DE-52 cellulose (2.6 × 12 cm) which was equilibrated with 5 mM potassium phosphate buffer, pH 7.8. The enzyme was eluted with a linear gradient of NaCl (0—0.5 M) (initial volume of buffer in gradient mixing chamber was 250 ml). The enzyme fraction was concentrated to 4.0 ml with an Amicon PM 10 filter (Fraction F).

Disc gel electrophoresis. Fraction F showed only a single major protein band and two faint bands after disc gel electrophoresis. The proportions of major protein band and two faint bands were 94 and 6%, densitometrically. The gel was cut into 5-mm slices and incubated with 1 ml of 10 mM potassium phosphate buffer, pH 7.8 at 37°C for 24 h. The enzyme activity was detected in the same place as the major protein band. Recovery of the activity was approximately 30%.

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 obtained from the polyacrylamide disc gel fraction had a specific activity of 24.3 units/mg protein for Hippuryl-His-Leu-OH and 0.182 μmol/min per mg protein for angiotensin I. Also, it was confirmed that the purified angiotensin I-converting enzyme did not inactivate angiotensin II, but did inactivate bradykinin. The specific activity of the enzyme for inactivation of bradykinin was 2.6 μmol/min per mg protein.

Molecular weight determinations. Molecular weight determinations were carried out by gel filtration and by disc gel electrophoresis. The molecular weights of peak II and peak III were estimated to be approximately 300 000 and 145 000 respectively. The molecular weight of the denatured enzyme was determined in disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and estimated to be 155 000.

K_m value. K_m value of peak II fraction for Hippuryl-His-Leu-OH was 2.6 mM and identical with the peak III fraction.

Discussion

The specific activity (24.3 units/mg protein) of the purified enzyme for Hippuryl-His-Leu-OH approximates the value of 22 units/mg protein reported by Cushman and Cheung [3] and is lower than the value of 89 units/mg protein reported by Das and Soffer [8]. The specific activity for angiotensin I was 10

times lower than that reported by Soffer et al. [23]. This discrepancy may be due to the difference in the concentration of angiotensin I used as substrate and the method of the enzyme assay.

The molecular weight and Km value for Hippuryl-His-Leu-OH of the enzyme were consistent with those reported by Soffer et al. [23]. In respect to the difference of molecular weight of the enzyme calculated by gel filtration and by disc gel electrophoresis in the presence of sodium dodecyl sulfate, there are two possibilities: (i) peak II may contain the dimer of the enzyme, or (ii) the difference may be only due to the presence of carbohydrate, which was reported by Soffer et al. [23]. It is necessary to investigate this problem furthermore.

Using our method, the enzyme was solubilized by trypsin treatment for 2 h from a particulate fraction of rabbit lung and a good recovery of 76% was obtained. Thus trypsin treatment enables us to carry out a large scale preparation of the angiotensin I-converting enzyme from a particulate fraction.

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