BBA 65988

PURIFICATION AND PROPERTIES OF ENDOPEPTIDASE FROM RABBIT RED CELLS AND ITS PROCESS OF DEGRADATION OF ANGIOTENSIN

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SUMMARY

Endopeptidase, showing angiotensinase activity in rabbit red cells, was purified by fractionation with $(NH_4)_2SO_4$, DEAE-cellulose column chromatography and Sephadex G-200 gel filtration. The specific activity of angiotensinase on the purified preparation was increased about 8000-fold compared with that of the crude hemolysate.

The peptide bonds cleaved by the enzyme preparation in [α-L-Asp[1], Ile[5]]-angiotensin II were Arg–Val, Tyr–Ile and Ile–His. It was demonstrated that hydrolysis of the Tyr–Ile bond with the enzyme was the first step in the inactivation process of angiotensin.

The results indicated that the purified so-called angiotensinase might be an endopeptidase without hydrolytic activity on casein, *p*-toluenesulfonyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester.

INTRODUCTION

Angiotensin II which has pressor activity is inactivated by an angiotensin-destroying substance, so-called angiotensinase. It has not been decided whether the inactivation of angiotensin *in vivo* is due to nonspecific peptidases or to a specific enzyme. Most studies on angiotensinase have been conducted using a crude preparation such as plasma and tissue extract or homogenate without any attempt to purify it.

Our previous paper[1] has already reported the presence of much higher angiotensinase activity in red cells in comparison with that in plasma from rabbits. The present experiments were designed to purify the enzyme showing angiotensinase activity from rabbit red cells and to determine the peptide bond cleaved by the enzyme in synthetic angiotensin II.

Abbreviations: ATEE, acetyl-L-tyrosine ethyl ester; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

METHODS AND MATERIALS

Bioassay of angiotensinase activity

The material was incubated with a constant amount of [α-L-Asp[1], Val[5]]-angiotensin II amide (CIBA) or [α-L-Asp[1], Ile[5]]-angiotensin II (Institute for Protein Res., Osaka University) *in vitro* under the conditions mentioned below. Following incubation, the enzyme reaction was stopped by placing the sample in a boiling-water bath for 10 min. Pressor activity of the supernatant was tested on the blood pressure in rat. The rat weighing 170–180 g was vagotomized, anesthetized by injecting pentobarbitone sodium, 50 mg/kg, intraperitoneally and given pentolinium tartrate, 10 mg/kg, subcutaneously. The pressor response of unknown solution was bracketed between known amounts of synthetic angiotensin II used as substrate. The amount of angiotensin inactivated (ng) was used as an indicator for the angiotensinase activity.

Enzyme material was usually incubated with 0.1–1.0 μg of synthetic angiotensin II, 0.2 ml of 0.1 M phosphate buffer (pH 6.8), added saline solution in a total volume of 2.0 ml for 5–30 min at 37°.

Assay of arylamidase activity

The extent of hydrolysis of arylamides was determined by a modified method of GOLDBARG and RUTENBURG[2]. The substrates were L-leucyl-β-naphthylamide, L-aspartyl-β-naphthylamide and L-glutamyl-β-naphthylamide. Arylamidase assay with amino acid β-naphthylamide substrate was carried out by assaying the β-naphthylamine liberated from the substrate in a 2.0-ml reaction mixture containing 0.5 ml of substrate solution (400 μg/ml), 0.5 ml of 0.2 M phosphate buffer (pH 7.2) and enzyme preparation. The incubation was done at 37° for 15–30 min, and the reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (25%). The amount of β-naphthylamine liberated, expressed as μg, was calculated from the standard curve.

Assay of hydrolytic activity on leucinamide

The hydrolysis assay with L-leucinamide as substrate was carried out by the method of FURIYA *et al.*[3]. The amount of enzymatically released ammonia was estimated by phenolhypochloride–nitroprusside method.

Assays of dipeptidase, tripeptidase and carboxypeptidase activities

L-Leu–Gly, DL-Glu–Gly, L-Arg–Gly, carbobenzoxy (Z)-Gly–L-Phe, Z-Gly–L-Arg and L-Leu–Gly–Gly, as substrates were obtained from the Institute for Protein Res., Osaka University. These peptidase activities were estimated by the ninhydrin method of Yemm and Cocking, as modified by MATHESSON AND TATTRIE[4]. The enzyme material was incubated for 30–60 min at 37° with 0.5 μmole of each substrate, except for DL-Glu–Gly (1.0 μmole), 0.1 ml of 0.1 M phosphate buffer (pH 6.8) and added distilled water in a total volume of 1.0 ml. The reaction was stopped by heating the incubate in a boiling-water bath for 10 min. The peptidase activity was expressed as L-leucine equivalent.

Assay of caseinolytic activity

Hydrolytic activity of the enzyme material on casein was determined by the casein–Folin A method[5]. The incubation solution for the assay was composed of

0.2 ml of 2% casein solution denaturated by heating and enzyme material made up to a total of 1.0 ml with 0.01 M phosphate buffer (pH 6.8). After incubation for 60 min at 37°, the reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (7%). Amount of peptide in the supernatant was estimated by the Folin method.

Assay of esterase activity

The assay was performed by the method of ROBERTS[6], using as substrates *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and acetyl-L-tyrosine ethyl ester (ATEE), obtained from the Institute for Protein Res., Osaka University. The incubation solution for the assay was composed of 0.1 ml of 0.1 M substrate solution, 0.5 ml of 0.4 M Tris–HCl buffer (pH 8.5) and enzyme material, made up to a total of 1.0 ml with distilled water. The incubation was carried out for 60 min at 37°.

Thin-layer chromatography on silica gel G

The peptides cleaved in synthetic angiotensin by the enzyme material were separated by means of thin-layer chromatography on silica gel G, using a solvent mixture of *n*-butanol–acetic acid–water (4:1:5, by vol.).

High-voltage paper electrophoresis

In order to characterize the degradation products demonstrable after incubating angiotensin II with the enzyme material, high-voltage paper electrophoresis (Toyo Roshi, paper No. 51, 30 cm × 60 cm) was run in a buffer solution (pH 3.5) of pyridine–HCl–water (1:10:289, by vol.). A current density of 0.5 mA/cm at a potential of 4000 V was applied for 90 min.

Amino acid analysis

Analysis of amino acid composition of the peptide developed by paper electrophoresis was carried out using a Nippon Denshi amino acid autoanalyzer (JEOL-3BC) The peptides from a paper were eluted with 10% acetic acid and hydrolyzed with 5.7 M HCl at 105° for 24 h.

RESULTS

Purification of the enzyme showing angiotensinase activity from normal rabbit red cells

Hemolysate preparation. Normal rabbit red cells, washed 3 times with saline, were hemolyzed by freezing and diluted 1:10 with distilled water. The preparation was centrifuged for 15 min at 15 000 × *g*, and the supernatant was employed for the experiment (Fraction 1).

All steps of the purification were carried out at 0–4°.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to Fraction 1 to 30% saturation. The enzyme was allowed to stand for several hours before being centrifuged at 15 000 × *g* for 15 min. The supernatant was fractionated with solid $(NH_4)_2SO_4$ to 70% saturation, followed by centrifugation at 15 000 × *g* for 20 min. The precipitate was dissolved in a small volume of distilled water and was dialyzed for 5 h against 0.01 M phosphate buffer (pH 6.8) (Fraction 2).

DEAE-cellulose column chromatography. Fraction 2 was chromatographed on a DEAE-cellulose column (2 cm × 43 cm), equilibrated with 0.01 M phosphate buffer

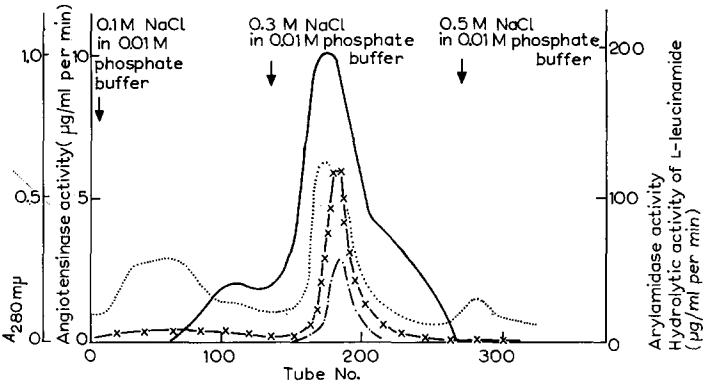


Fig. 1. Chromatographic pattern of enzymic activities on DEAE-cellulose. The column, 2 cm × 43 cm, equilibrated with 0.01 M phosphate buffer (pH 6.8) was subsequently eluted with a stepwise NaCl gradient, consisting of two chambers. The initial volume in the closed mixing vessel was 250 ml of 0.01 M phosphate buffer (pH 6.8), followed by stepwise changes in the reservoir with 500 ml each of 0.1, 0.3 and 0.5 M NaCl in the starting buffer. · · · · ·, protein; ————, angiotensinase activity; ×—×, arylamidase activity; — · —, hydrolytic activity on L-leucinamide.

(pH 6.8), followed by elution with a NaCl stepwise gradient. Hemoglobin was not adsorbed onto the column. For elution with a NaCl stepwise gradient, the initial volume in the closed mixing vessel was 250 ml of 0.01 M phosphate buffer (pH 6.8), followed by stepwise changes in the reservoir with 500 ml each of 0.1, 0.3 and 0.5 M NaCl in the starting buffer. 10 ml of the eluate were collected in each tube. Fig. 1 shows chromatographic pattern of enzymic activities on a DEAE-cellulose column.

The bulk of the enzyme showing angiotensinase activity was eluted in the 0.1–0.3 M NaCl fraction. In this step, this fraction also had hydrolytic activity on L-leucyl-β-naphthylamide and L-leucinamide. The precipitate of the 80% $(NH_4)_2SO_4$ saturation in this active fraction was dialyzed against 0.01 M phosphate buffer (pH 6.8) (Fraction 3).

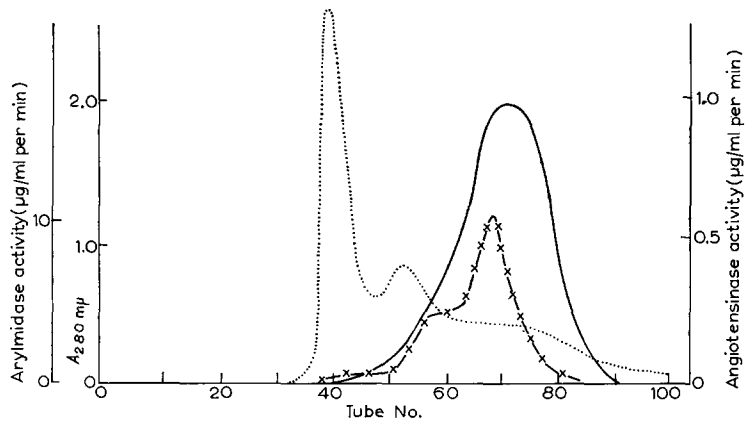


Fig. 2. Distribution of angiotensinase activity after passage through Sephadex G-200. Column size was 4 cm × 100 cm; flow rate was 20 ml/h. Elution was with 0.01 M phosphate buffer (pH 6.8). The volume of each tube was 8 ml. · · · · ·, protein; ————, angiotensinase activity; ×—×, arylamidase activity.

TABLE I

PURIFICATION OF ENZYME SHOWING ANGIOTENSINASE ACTIVITY IN RED CELLS

Purification step	*Total protein (g)*	*Specific activity (μg/mg protein per h)*	*Total activity (mg/h)*
Hemolysate	546	0.20	110
Fractionation with $(NH_4)_2SO_4$ (20–70%)	26.2	5.71	150
DEAE-cellulose column chromatography	0.870	80.00	69.6
Sephadex G-200 gel filtration	0.010	1720.00	17.

Sephadex G-200 gel filtration. Fraction 3 was applied to a 4 cm × 100 cm column of Sephadex G-200, equilibrated with 0.01 M phosphate buffer pH (6.8). The column was eluted with the same buffer solution at a flow rate of 20 ml/h. The volume of each tube was 8 ml. The elution pattern of the enzyme showing angiotensinase activity is shown in Fig. 2. Angiotensinase activity appeared in Tubes 60–80 with L-leucyl-β-naphthylamidase activity. Hydrolytic activity on L-leucinamide, however, was not found in the peak.

The purification procedures of the enzyme showing angiotensinase activity and its specific activity in each step are shown in Table I. The specific activity of the enzyme on the purified preparation was increased 8000-fold compared with that of the crude hemolysate.

The enzyme preparation in the last step of purification was employed for the following studies on angiotensinase activity.

TABLE II

SUBSTRATE SPECIFICITY

Substrate	*Angiotensinase activity (μg per mg protein per h)*
α-Angiotensin II	265.5
α-Angiotensin II amide	515.5
L-Leu-β-naphthylamide	1400
DL-Glu-β-naphthylamide	0
L-Asp-β-naphthylamide	0
L-Leu-Gly	1.7
DL-Glu-Gly	0
Arg-Ala	8.7
L-Leucinamide	2.8
L-Leu-Gly-Gly	363.0
z-Gly-Phe	2.3
z-Gly-Arg	0
Casein	0
TAME	0
ATEE	0

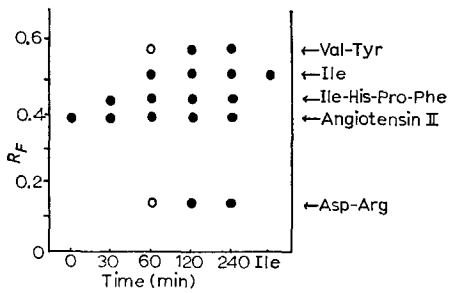
Properties of the purified enzyme showing angiotensinase activity.

pH optimum for the activity using [α-L-Asp^1]-Val^5-angiotensin II as substrate was 6.8. The angiotensinase activity was completely inhibited by the addition of EDTA (1 mM). The activity could not be restored by the addition of Co^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} or Zn^{2+} (1 mM). In addition, its activity was completely inhibited by DFP (1 mM) and by heating at 65° for 10 min.

Table II shows the specificity of the enzyme towards various substrates. The enzyme showed hydrolytic activity on [α-L-Asp^1, Val^5]-angiotensin II, [α-L-Asp(NH$_2$)^1, Val^5]-angiotensin II, L-leucyl-β-naphthylamide and L-Leu–Gly–Gly; however, it had no activity on L-leucinamide, L-aspartyl-β-naphthylamide, L-Leu–Gly, D,L-Glu–Gly, L-Arg–Ala, Z-Gly–Phe, Z-Gly–Arg, casein, TAME and ATEE.

Characterization of the peptide bond cleaved in angiotensin by the purified enzyme

[α-L-Asp^1, Ile^5]-angiotensin II was used as substrate. After incubating 2 mg of angiotensin II with the enzyme preparation at 37°, aliquots were taken periodically (30, 60, 120 and 240 min) for thin-layer chromatography on silica gel G. Two ninhydrin-positive spots in the mixture incubated for 30 min and five spots in the mixture incubated for 120 or 240 min were demonstrated (Fig. 3). No ninhydrin-positive spot was observed in the enzyme material incubated alone for 240 min at 37°.



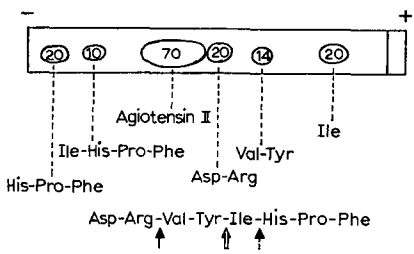


Fig. 3. Diagram showing degradation products demonstrable by chromatography after incubating [α-L-Asp^1, Ile^5]-angiotensin II with angiotensinase preparation for varying periods, at 37°. For further details, see METHODS AND MATERIALS.

Fig. 4. High-voltage paper electrophoretic pattern of the degradation products after incubating [α-L-Asp^1, Ile^5]-angiotensin II with angiotensinase preparation for 120 min at 37°. A current density of 0.5 mA/cm. at a potential of 4000 V was applied for 90 min. The figures listed represent percentages (calculated on a molar basis) of the angiotensin II originally used.

[α-L-Asp^1, Ile^5]-Angiotensin II (10 mg) was incubated with 2 ml of the enzyme material (protein amount: 165 μg/ml) for 120 min. The reaction was stopped by placing the sample in a boiling-water bath for 10 min. When an aliquot of the incubation mixture was analyzed using amino acid analyzer without hydrolysis, about 15% isoleucine was found. However, no other free amino acid was demonstrated except for only a trace of valine and tyrosine. The remaining incubation mixture was subjected to high-voltage paper electrophoresis in order to separate degradation products. After electrophoresis, the paper was cut off perpendicularly by a line 1 cm from the margin. Color reaction utilized spraying with a 0.2% ninhydrin butanol

solution. As shown in Fig. 4, six ninhydrin-positive spots resulted. The strips from the remaining paper which corresponded to the migrated sites of the six spots were eluted with 10% acetic acid, followed by hydrolysis with 5.7 M HCl. Using an amino acid autoanalyzer, these spots indicated Ile, Val–Tyr, Asp–Arg, angiotensin II, Ile–His–Pro–Phe and His–Pro–Phe (Fig. 4). Furthermore, using the mixture incubated for 30 min, thin-layer chromatography demonstrated two spots as angiotensin II and Ile–His–Pro–Phe (Fig. 3).

DISCUSSION

It is obvious from the experimental results (Fig. 4) that the enzyme preparation purified from rabbit red cells might attack three peptide bonds in [α-L-Asp[1], Ile[5]]-angiotensin II, that is, Arg–Val, Tyr–Ile and Ile–His bonds. The following discussion is based on the assumption that hydrolysis in the three peptide bonds could be caused by different enzymes.

Our attention was focused upon the elucidation of a peptide bond hydrolyzed with the purified enzyme as first step in the inactivation process of angiotensin. It could be considered that an endopeptidase that hydrolyzes the Tyr–Ile bond or the Arg–Val bond might be important for the inactivation process of angiotensin on the basis of minimum structural requirements of angiotensin II for biological activity[7]. It is possible that the enzyme that hydrolyzes the Ile–His bond is not essential in the angiotensin inactivation process and that the enzyme might attack, the Ile–His bond of the tetrapeptide, Ile–His–Pro–Phe, resulting from the hydrolysis of the Tyr–Ile bond in advance, because the existence of both Ile–His–Pro–Phe and the His–Pro–Phe was confirmed in the degradation products of angiotensin II, while the Asp–Arg–Val–Tyr–Ile and Val–Tyr–Ile were not observed. The enzyme that hydrolyzed the Ile–His bond might be exopeptidase, indicating hydrolysis on L-leucyl-β-naphthylamide and L-Leu–Gly–Gly. These results suggest that the enzyme showing angiotensinase activity purified from rabbit red cells might be a trypsin-like enzyme that hydrolyzes the Arg–Val bond or a chymotrypsin-like enzyme that hydrolyzes the Tyr–Ile bond.

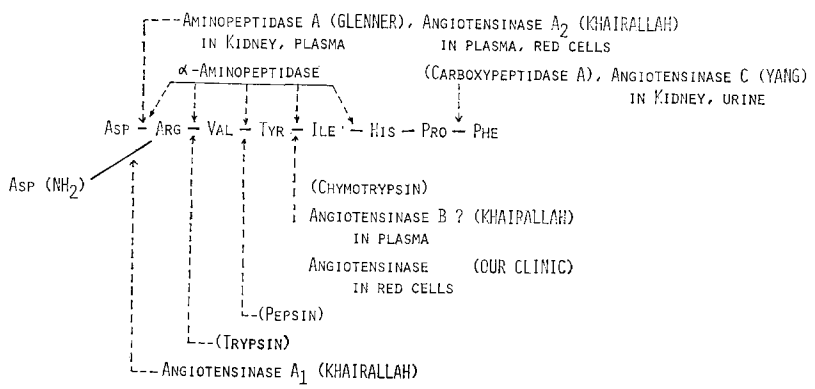


Fig. 5. Enzymatic cleavage of angiotensin.

However, further purification could not be carried out because the activity of enzyme preparation was unstable after gel filtration on Sephadex G-200 column.

Furthermore, based on the following three experimental results, it was supposed that hydrolysis of the Tyr–Ile bond in angiotensin with the endopeptidase, suggesting a chymotrypsin-like enzyme, might be the first step in the inactivation process of angiotensin. First, in the test for the demonstration of fragments by thin-layer chromatography on silica gel G (Fig. 3), only the tetrapeptide Ile–His–Pro–Phe was already found when angiotensin was incubated with the enzyme for 30 min, whereas, both Asp–Arg and Val–Tyr were demonstrated after the incubation for at least 60 min or more. Secondly, most of the free amino acid liberated from angiotensin incubated with the enzyme preparation was isoleucine, whereas, valine or tyrosine were present only in trace amounts. This finding indicates that hydrolysis of the Tyr–Ile bond might proceed at a faster rate than of the Arg–Val bond. Thirdly, in the test for degradation products, using high-voltage paper electrophoresis, after incubating angiotensin II with the enzyme preparation, (Fig. 4), the total amount of Ile–His–Pro–Phe and His–Pro–Phe was 30% of angiotensin II used, the amount of Asp–Arg was 20% and the total amount of Val–Tyr and Val (or Tyr) was also 20%. This finding indicates that the amount of the Tyr–Ile bond broken down is about 10% greater than that of the Arg–Val bond.

Fig. 5 shows a scheme of enzymatic degradation of angiotensin by various so-called angiotensinases according to the theories known for their substrate specificities. Although the peptide bonds hydrolyzed by trypsin or chymotrypsin were identical with those hydrolyzed by our purified angiotensinase preparation, it was not identical with trypsin or chymotrypsin because our enzyme preparation had no hydrolytic activities on TAME, ATEE and casein. Angiotensinase B in plasma reported by KHAIRALLA AND PAGE[8] was supposed to be endopeptidase, based only on the evidence that it inactivated angiotensin analogues such as β-angiotensin II and succinic acid-angiotensin II. Consequently, it is difficult to compare both angiotensinase B and our angiotensinase isolated from rabbit red cells.

It is clear that angiotensinase purified from red cells is different from angiotensinase A reported by KHAIRALLA *et al.*[9] or angiotensinase C by YANG *et al.*[10] which were exopeptidases.

Our experimental results suggested that the enzyme showing angiotensinase activity purified from rabbit red cells might be one of the endopeptidases without hydrolytic activities on casein, TAME and ATEE. Furthermore, it was demonstrated that peptide bonds cleaved by the enzyme in angiotensin were the Arg–Val and Tyr–Ile bonds. It seemed that the hydrolysis of the Tyr–Ile bond was the first step in the inactivation process of angiotensin.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Bloch (Ciba Pharmaceutical Institute) for his kind supply of synthetic angiotensin II. The authors' grateful thanks are also expressed to the members of the Institute for Protein Research, Osaka University, for their collaboration.

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