Acid Angiotensinase as a Lysosomal Enzyme

MASATO MATSUNAGA*, NOBORU SAITO, JUN KIRA,
KOICHI OGINO, AND MASAO TAKAYASU

THE MAIN enzymes in the rat kidney which degrade angiotensin are located in lysosomes and microsomes.¹ Lysosomal angiotensinase is active at an acid pH and inhibited by di-isopropylfluorophosphate (DFP), while microsomal angiotensinase is active near neutral pH and requires calcium ions. Some of the modalities of the action of acid angiotensinase contained in kidney lysosomes have already been described.^{1,2} The present paper reports on further properties of this enzyme and its differentiation from other lysosomal peptidases. Distribution of the acid angiotensinase is also surveyed, since it is reasonable to expect that this enzyme would be contained in lysosomes not only of the kidney but also of other tissues.

MATERIALS AND METHODS

Tissue Fractionation and Extraction

Closely inbred Wistar rats (Animal Center of Kyoto University) of either sex weighing 200–300 g were killed by bleeding from the aorta. Tissues were removed and cooled immediately.

a) Subcellular Fractions

Lysosomal, mitochondrial, microsomal and soluble (final supernatant) fractions were prepared from the kidney and liver as described previously.³ Each fraction was frozen and thawed ten-times prior to enzyme assay.

b) Tissue Extract

Fresh tissues were kept frozen overnight. After thawing at room temperature, 10 ml of distilled water was added to 1 g of wet weight and homogenized with a Potter-Elvehjem homogenizer. The homogenates were then frozen and thawed, and centrifuged at 6,700 g for 30 minutes. Presipitates were discarded.

Enzyme Assay

Angiotensinase activity was determined according to the method previously reported.¹ 0.25

–0.50 μ g of asparaginy¹-valyl⁵-angiotensin II (Hypertensin-Ciba), 1.0 ml of 0.5 M tris-acetic acid buffer and a sample, in a total volume of 4.0 ml were incubated at 37°C for 15–30 minutes. The amount of inactivated angiotensin was bioassayed in the rat.

Arylamidase activity was measured at pH 7.0 in the presence of 2×10^{-3} M dithiothreitol (DTT) by the method of MAHADEVAN and TAPPEL.⁴

For the assay of cathepsin A activity, 0.3 ml of a sample was incubated with 0.2 ml of 0.1 M carbobenzoxy-L-glutamyl-L-tyrosine (cbz-glu-tyr) as substrate and 1.5 ml of 0.2 M acetate buffer, pH 5.0, at 37°C for 60 minutes. Reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid. After filtration through a double layer of Toyo filter paper No.7, released tyrosine in 0.2 ml of the filtrate was estimated by the ninhydrin reaction.⁵

Hemoglobin-splitting cathepsin activity was determined as follows, modifying Anson's method.^{6,7} To 0.3 ml of a sample were added 1.2 ml of 0.1 M acetate buffer, pH 3.6, and 0.3 ml of 6% (w/v) solution of denatured hemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio). After incubation at 37°C for 30 minutes, reaction was stopped by the addition of 5 ml of 5% (w/v) trichloroacetic acid. The mixture was filtered through a double layer of Toyo filter paper No.7. Optical density of the filtrate was measured at 280 m μ with a spectrophotometer (Shimadzu QV-50).

Gel-Filtration

Lysosomal fraction of the kidney after ten-time freezing and thawing was dialyzed against 0.9 per cent saline overnight, and centrifuged at 100,000 g for 60 minutes. The supernatant fluid was placed on the column of Sephadex G-100 or G-200 (Pharmacia, Uppsala, Sweden). The methods for gel-filtration were the same as those

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3rd Division of Internal Medicine, School of Medicine, Kyoto University, Kyoto

* Present address: Research Division, Cleveland Clinic Foundation, 2020 East 93rd Street, Cleveland, Ohio 44106, U.S.A.

reported earlier.² Protein was estimated by absorption at 280 m μ . All the procedure for gel-filtration and column chromatography were carried out at 4°C.

Column Chromatography

Frozen-thawed lysosomal fraction of the kidney was dialyzed overnight against 0.005 M tris-phosphate buffer, pH 8.0, and centrifuged as described above. The supernatant was applied to the column. Diethylaminoethyl-cellulose (DEAE-cellulose, Nakarai Chemicals, LTD, Kyoto, Japan) with a capacity of 0.90 mEq/g was washed and packed according to PETERSON & SOBER.⁸ DEAE-cellulose column (26 X 2 cm.) was equilibrated with 0.005 M tris-phosphate buffer (pH 8.0). Elution was carried out with a NaCl gradient using a 2-chamber device. The limit buffer was 1 N NaCl-0.01 M tris-phosphate (pH 8.0). The concentration of chloride in the eluate was determined by the method of SCHALES & SCHALES.⁹

RESULTS

1. Effect of pH on Lysosomal Angiotensinase

Angiotensinase activity of kidney lysosomal fraction was measured at pH between 3.0 and 7.5 (Fig. 1). Activity was expressed by reciprocal of an amount of the sample which inactivates approximately one half of the substrate in 15 minutes. Maximum activity was found at pH 5.6–5.7.

2. Effect of Thiol Reagent on Lysosomal Angiotensinase

Acid angiotensinase activity of kidney lysosomal fraction was slightly activated by the addition of 2×10^{-3} M DTT and partially inhibited by 2×10^{-4} M p-chloromercuribenzoate (PCMB) (Table I).

Fig. 1. Effect of pH on Lysosomal Angiotensinase Activity.

TABLE I EFFECT OF THIOL REAGENT ON LYSOSOMAL ANGIOTENSINASE

Confactor	Activity
-	100
PCMB (2×10^{-4} M)	62
DTT (2×10^{-3} M)	127
PCMB + DTT	126

The activity was determined at pH 5.0.

PCMB; p-chloromercuribenzoate

DTT: dithiothreitol

PCMB: p-chloroner

TABLE II ANGIOTENSINASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF THE LIVER

Fraction	Activity	
	pH 5.0	pH 7.5
Lysosomal	178.4	42.0
Mitochondrial	93.2	16.0
Microsomal	0	24.0
Soluble	0	187.2

Activity is expressed in nanograms of angiotensin inactivated by 0.46 mg of protein at 37°C for 15 minutes.

3. Angiotensinases in Subcellular Fractions of the Liver

Angiotensinase activity of liver subcellular fractions was determined at pH 5.0 and pH 7.5 (Table II). At pH 5.0 most of the activity was found in the lysosomal fraction, and at pH 7.5 the soluble fraction showed the highest activity. Acid angiotensinase activity of lysosomal fraction was inhibited by the addition of one drop of 1 : 20 solution of DFP in isopropylalcohol to the incubation mixture. The activity of the soluble fraction was inhibited by dialysis against ethylenediamine tetraacetic acid (EDTA) solution (2.2 g EDTA- Na_2 and 10 g NaCl per liter, adjusted to pH 7.0 with 1 N NaOH), and restored by the addition of 5×10^{-3} M CaCl_2 (Table III).

4. Angiotensinases in Various Tissues (Table IV)

Activities of acid and neutral angiotensinases in extracts of various tissues were measured. To avoid interference by each other, acid angiotensinase was determined at pH 5.0 after dialysis against EDTA, while the neutral enzyme was measured at pH 7.5 following addition of one drop of the DFP solution. Activity of the neutral angiotensinase was found mostly in the kidney, liver, spleen and lung; there was a little activity in the brain, heart and skeletal muscle.

TABLE III INHIBITION AND ACTIVATION OF LIVER ANGIOTENSINASES

Treatment	Activity	
	Lysosomal	Soluble
-	178.4	187.2
DFP	42.0	-
Dialysis	-	0
against EDTA	-	145.6
Dialysis + Ca ⁺⁺	-	-

Activity is expressed in nanograms of angiotensin inactivated by 0.46 mg of protein at 37°C for 15 minutes.

TABLE IV ANGIOTENSINASE ACTIVITIES IN EXTRACTS OF VARIOUS TISSUES

Tissue	Activity per 10 mg of tissue		Protein (mg)
	Acid	Neutral	
Kidney	192	343	0.55
Liver	127	309	0.76
Spleen	138	175	0.70
Adrenal	50	-	0.35
Lung	37	137	0.30
Heart	7	28	0.24
Brain	6	35	0.16
Muscle	5	17	0.17

Activity is expressed in nanograms of angiotensin inactivated by 0.46 mg of protein at 37°C for 15 minutes.

TABLE V EFFECTS OF DFP ON CATHEPSINS

Treatment	Hemoglobin-splitting Cathepsin	Cathepsin A
-	0.17	5.0
DFP	0.17	2.8

Activity is expressed in cathepsin units.

Since adrenal extract had to undergo prolonged dialysis to eliminate catecholamines, the calcium-dependent neutral angiotensinase was not determined. Acid angiotensinase activity was high in the kidney, spleen and liver, moderate in the adrenal and lung, and low in the heart, brain and skeletal muscle.

5. Effects of DFP on Cathepsins in Kidney Lysosomes

Addition of 0.04 ml of the DFP solution to the incubation mixture containing 2.2 mg protein of kidney lysosomal fraction caused a 43 per cent inhibition of cathepsin A activity. The hemoglobin-splitting cathepsin was not inhibited at all by DFP (Table V).

6. Isolation and Molecular Weight of Lysosomal Angiotensinase

Figures 2, 3 and 4 show elution diagrams of lysosomal enzymes by Sephadex gel-filtration

Fig.2. Sephadex G-100 Gel-Filtration of Lysosomal Enzymes.

Fig.3. Sephadex G-200 Gel-Filtration of Lysosomal Enzymes.

Fig.4. DEAE-cellulose Column Chromatography of Lysosomal Enzymes.

and DEAE-cellulose column chromatography. The acid angiotensinase was evidently separated from arylamidase and hemoglobin-splitting cathepsin by gel-filtration. However, cathepsin A could not be eliminated from the angiotensin-

ase by either Sephadex or DEAE-cellulose. Molecular Weight of acid angiotensinase fell within a range between 70,000 and 90,000, calculated according to ANDREWS¹⁰ while those of arylamidase and hemoglobin-splitting cathepsin

were less than 50,000.

DISCUSSION

It was revealed that the liver contains two kinds of angiotensinases similar to those observed in the kidney; the one is active at an acid pH and inhibited by DFP, the other is active near neutral pH and requires calcium ions. Because of difficulty in obtaining purer subcellular fractions of the liver, separation was not as evident as in the kidney. Nevertheless, it is obvious, considering the probability of contamination of one fraction by the other, that the acid angiotensinase is concentrated in lysosomes and the neutral angiotensinase is in the soluble fraction. The location of the former is the same as in the kidney, but that of calcium-dependent neutral angiotensinase is different. The latter observation is compatible with that reported by KOKUBU et al.¹¹ in rabbit liver. Rapid turnover of injected angiotensin in the liver¹² might result from the presence of this enzyme in the soluble fraction.

A survey showed a wide and similar distribution of both kinds of angiotensinases among various tissues: activities were high in parenchymatous tissues such as kidney and liver, and low in muscular or nervous tissues. But the proportion of the two was different from one tissue to another, e.g. the spleen had much more of the acid enzyme than the lung. The localization of acid angiotensinase in lysosomes is suggested by higher activity of this enzyme in such lysosome-rich tissues as kidney, spleen and liver, as well as by the studies on subcellular fractions of the kidney and liver. Similar effects of pH and inhibitors would indicate identity of acid angiotensinase.

Among the peptidases contained in kidney lysosomes, arylamidase and hemoglobin-splitting cathepsin were separated from acid angiotensinase by Sephadex gel-filtration. Cathepsin B, C, D and E are also distinguished from the angiotensinase by their insusceptibility to DFP. However, further study is required to determine whether cathepsin A is responsible for inactivation of angiotensin, since this enzyme which splits cbz-glu-tyr was inhibited by DFP and not separable from acid angiotensinase by the methods used in this study. As to the nature of the enzyme action, SAITO et al.² analyzed the amino acids resulting from the reaction of semipurified lysosomal acid angiotensinase on angiotensin II (or its amide). They found that phenylalanine was released first, and proline next. Histidine and iso-

leucine (or valine) were liberated later on, but other part of angiotensin seemed to remain intact during short incubations. Similar results were obtained from paperchromatographic studies by MATSUNAGA.¹³ REGOLI et al.¹⁴ observed that valyl⁵-angiotensin II derivatives whose N-terminal group was protected against aminopeptidases were splitted to N-terminal tetrapeptide and four amino acids, valine, histidine, proline and phenylalanine, by rat kidney homogenate or plasma. They attributed this result to an action of a chymotrypsin-like endopeptidase followed by rapid degradation of C-terminal tetrapeptide by aminopeptidases and prolidase. However, involvement of aminopeptidases and consequently of prolidase would be slightest under the present experimental conditions with lysosomal angiotensinase. Therefore, it can be possible that the whole process was completed by the action of a carboxypeptidase (of carboxypeptidases), though release of phenylalanine is enough to inactivate angiotensin II.¹⁵

The effects of dithiothreitol and p-chloromercuribenzoate imply that a part of lysosomal acid angiotensinase activity depend on sulfhydryl group. In this respect, the possible involvement of catheptic carboxypeptidase could be taken into account, since it is a sulfhydryl-dependent enzyme which cleaves Cbz-glu-tyr.^{16,17} Whether lysosomes contain this enzyme remained to be studied. However, it would play a minor part, if any, in the lysosomal angiotensinase activity, considering its pH optimum (pH 3.0–4.0) and the degree of sulfhydryl-dependence of lysosomal angiotensinase. YANG et al.¹⁸ reported the existence of a carboxypeptidase (angiotensinase C) in swine kidney extract and human urine, which cleaved the prolyl-phenylalanine bond of angiotensin II. The effects of pH and DFP on this enzyme coincide with those on lysosomal angiotensinase. JOHNSON and RYAN¹⁹ showed that an aqueous extract of rabbit liver contained an angiotensinase which released C-terminal phenylalanine. This also can be considered as the same enzyme which is derived from lysosomes, though their experimental condition, incubating at pH 7.4, seems inappropriate. It could be said as a conclusion that a carboxypeptidase (angiotensinase C) is one of the lysosomal enzymes and the main contributor to the lysosomal angiotensinase activity, although partial dependence of the latter on sulfhydryl group suggests involvement of more than one enzyme.

It remains to be investigated how far the

lysosomal angiotensinase participates in metabolism of angiotensin *in vivo* and whether it contributes to plasma angiotensinase activity in normal and/or pathological states. Considerably rapid degradation or disappearance of angiotensin II derivatives in peripheral vascular bed, which are protected against aminopeptidases²⁰⁻²² may speak for an important role of lysosomes in angiotensin metabolism *in vivo*. KHAIRALLAH and PAGE²³ distinguished plasma angiotensinases into three groups. Angiotensinase A₁ and A₂, calcium-dependent aminopeptidases with near neutral pH optima, would correspond to microsomal angiotensinases in the kidney.²⁴ Their assumption that angiotensinase B with an acid optimum pH was the chymotrypsin-like endopeptidase postulated by REGOLI et al.¹⁴ requires direct evidence. They found an increase in angiotensinase activity different from aminopeptidases in the renal vein of a patient with renal artery disease. ITSKOVITS et al.²⁵ showed an increased angiotensin degradation in plasma of dogs with hemorrhagic or endotoxin shock, which was distinguished from the action of normally observed aminopeptidases. These observations would indicate the liberation of lysosomal angiotensinase(s) into blood stream at least under such diseased states, since it is known that ischemia, anoxia, shock etc. are associated with labilization of the membranes of lysosomes and release of some lysosomal enzymes into circulation.²⁶⁻³⁰

SUMMARY

1. Both acid angiotensinase and calcium-dependent neutral angiotensinase are widely distributed among various tissues of the rat. High activity of acid angiotensinase in such lysosome-rich tissues as kidney, spleen and liver suggests localization of this enzyme in lysosomes.
2. Main angiotensinases in rat liver are concentrated in lysosomal and soluble fractions. The lysosomal enzyme which is inhibited by DFP is acid angiotensinase, while the other is calcium-dependent neutral angiotensinase.
3. The pH optimum of lysosomal angiotensinase of the kidney is 5.6-5.7. Properties of acid angiotensinase indicate that a carboxypeptidase, so-called angiotensinase C, is a lysosomal enzyme and plays a main role in lysosomal angiotensinase activity. On the other hand, partial inhibition and activation by p-chloromercuribenzoate and dithiothreitol imply that some other enzyme(s) may take part in this activity.

4. Acid angiotensinase of kidney lysosomes was separated from arylamidase and hemoglobin-splitting cathepsin by Sephadex gel-filtration. Cathepsin A activity, however, could not be eliminated from the acid angiotensinase by either Sephadex gel-filtration or DEAE-cellulose column chromatography. DFP showed an inhibitory effect on cathepsin A activity of kidney lysosomes.
5. Molecular weight of lysosomal acid angiotensinase of rat kidney is between 70,000 and 90,000, calculated by Sephadex gel-filtration.
6. Possible role of the lysosomal enzyme in angiotensin metabolism *in vivo* is discussed.

REFERENCES

1. MATSUNAGA, M., KIRA, J., SAITO, N., OGINO, K. & TAKAYASU, M.: Acid Angiotensinase, Renin, and Acid Adenosine Triphosphatase in Rat Kidney Lysosomes, *Jap. Circul. J.* **32**: 137, 1968.
2. SAITO, N., SANADA, K., MATSUNAGA, M., KIRA, J., MUKAINO, S., OGINO, K., & TAKAYASU, M.: Amino Acid Analysis after Degradation of Angiotensin II by Rat Kidney Lysosomes, *Jap. Circul. J.* **33**: 87, 1969.
3. OGINO, K., MATSUNAGA, M., SAITO, N., KIRA, J., TAKAYASU, M., & ONO, K.: Renin and Acid Adenosine Triphosphatase as Lysosomal Enzymes, *Jap. Circul. J.* **31**: 1, 1967.
4. MAHADEVAN, S. & TAPPEL, A.L.: Arylamidases of Rat Liver and Kidney, *J. Biol. Chem.* **242**: 2369, 1967.
5. COCKING, E.C. & YEMM, E.W.: Estimation of Aminoacids by Ninhydrin, *Biochem. J.* **58**: XII, 1954.
6. ANSON, M.L.: The Estimation of Cathepsin with Hemoglobin and the Partial Purification of Cathepsin, *Gen. Physiol.* **20**: 565, 1937.
7. TALLAN, H.H., JONES, M.E., & FRUTON, J.S.: On the Proteolytic Enzymes of Animal Tissues: X. Beef Spleen Cathepsin C, *J. Biol. Chem.* **194**: 793, 1952.
8. PETERSON, E.A. & SOBER, H.A.: Column Chromatography of Proteins: Substituted Celluloses, in "Method in Enzymology", vol. 5, edited by Colowick, S.P. and Kaplan, N.O., Academic Press, New York and London, p. 3, 1962.
9. SCHALES, O. & SCHALES, S.S.: A Simple and Accurate Method for the Determination of Chloride in Biological Fluids, *J. Biol. Chem.* **140**: 879, 1941.
10. ANDREWS, P.: Estimation of the Molecular Weights of Proteins by Sephadex Gel-Filtration, *Biochem. J.* **91**: 222, 1964.
11. KOKUBU, T., FUJIMOTO, S., HIWADA, K., AKUTSU, H., KATO, A., & YAMAMURA, Y.: Inactivation of Angiotensin II in Liver, *Jap. Circul. J.* **31**: 879, 1967.
12. BUMPUS, F.M., SMEBY, R.R., PAGE, I.H., & KHAIRALLAH, P.A.: Distribution and Metabolic Fate of Angiotensin II and Various Derivatives, *Canad. Med. Assoc. J.* **90**: 190, 1964.

13. MATSUNAGA, M.: unpublished results
14. REGOLI, D., RINIKER, B., & BRUNNER, H.: The Enzymatic Degradation of Various Angiotensin II Derivatives by Serum, Plasma or Kidney Homogenate, *Biochem. Pharmacol.* **12**: 637, 1963.
15. BUMPUS, F.M. & SMEBY, R.R.: Importance of Amino Acid Side Groups for Biologic Activity of Angiotensin II, *Circulation* **25**: 183, 1962.
16. GREENBAUM, L.M. & SHERMAN, R.: Studies on Catheptic Carboxypeptidase, *J. Biol. Chem.* **237**: 1082, 1962.
17. GREENBAUM, L.M. & YAMAFUJI, K.: Catheptic Degradation of Bradykinin, *Life Sci.* **4**: 657, 1965.
18. YANG, H.Y.T., ERDÖS, E.G. & CHIANG, T.S.: New Enzymatic Route for the Inactivation of Angiotensin, *Nature* **218**: 1224, 1968.
19. JOHNSON, D.C. & RYAN, J.W.: Degradation of Angiotensin II by a Carboxypeptidase of Rabbit Liver, *Biochim. Biophys. Acta* **160**: 196, 1968.
20. DOYLE, A.E.: Studies on the Metabolism of Angiotensin, *Postgrad. Med. J.* **44**: 31, 1968.
21. DOYLE, A.E., LOUIS, W.J., JERUMS, G., & OSBORN, E.C.: Metabolism and Blood Levels Following Infusion of a Radioactive Analogue of Angiotensin, *Am. J. Physiol.* **215**: 164, 1968.
22. NG, K.K.F. & VANE J.R.: Fate of Angiotensin I in the Circulation, *Nature* **218**: 144, 1968.
23. KHAIRALLAH, P.A. & PAGE, I.H.: Plasma Angiotensinases, *Biochem. Med.* **1**: 1, 1967.
24. HESS, R.: Arylamidase Activity Related to Angiotensinase, *Biochim. Biophys. Acta* **99**: 316, 1965.
25. ITSKOVITZ, H.D., MILLER, L., URAL, W., ZAPP, J., & WHITE, R.: Inactivation of Angiotensin in Shock, *Am. J. Physiol.* **216**: 5, 1969.
26. DE DUVE, C.: Lysosomes, A New Group of Cytoplasmic Particles, in *Subcellular Particles*, edited by Hayashi, T., Ronald Press, New York, p. 128, 1959.
27. JANOFF, A., WEISSMANN, G., ZEIFACH, B.W., & THOMAS, L.: Pathogenesis of Experimental Shock. IV. Studies of Lysosomes in Normal and Tolerant Animals Subjected to Lethal Trauma and Endotoxemia, *J. Exp. Med.* **116**: 451, 1962.
28. BITENSKY, L., CHAYEN, J., CUNNINGHAM, G.J., & FINE, J.: Behavior of Lysosomes in Hemorrhagic Shock, *Nature* **199**: 493, 1963.
29. DUMONT, A.E. & WEISSMANN, G.: Lymphatic Transport of Beta-Glucuronidase during Hemorrhagic Shock, *Nature* **201**: 1231, 1964.
30. REICH, T., BRECKNELL, M.D., & REYNOLDS, B.M.: Plasma Cathepsin-like Acid Proteinase Activity during Hemorrhagic Shock, *J. Surg. Research* **5**: 116, 1965.