Amino Acid Analysis after Degradation of Angiotensin II by Rat Kidney Lysosomes

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MANY studies about degradation of angiotensin II have been carried out in plasma, erythrocytes or organ homogenates at near neutral pH¹⁻¹¹. However, there have been only a few reports about acid angiotensinase^{8, 12-14}. MATSUNAGA et al.¹³ reported that acid angiotensinase activity was demonstrated in rat kidney lysosomes. It has been known that lysosome particles have acid hydrolases including proteolytic enzymes^{15, 16}; so, the authors investigated how angiotensin II was split by rat kidney lysosomal fraction using automatic amino acid analyzer.

MATERIALS AND METHODS

Kidney lysosomal fraction of Wistar rats, weighing 200-300g, was prepared by the method of SHIBKO and TAPPEL¹⁷ with a slight modification as described previously¹⁸. Lysosomal fraction after ten-time freezing and thawing was dialyzed in the cold against 0.9 per cent saline overnight. After centrifugation at 100,000g for 30 minutes, the supernatant was applied to the column of Sephadex G-100 or G-200 (Pharmacia, Uppsala, Sweden). The suspension of Sephadex G-100 or G-200 with 0.9 per cent saline, after decantation, was packed into a column by gravity flow (3.1 cm diam. × 30.5 cm or 1.5 × 48 respectively). The supernatant of kidney lysosomes was pipetted on the top of the column and eluted at a constant rate with 0.9 per cent saline. Protein concentration was determined

by the method of LOWRY et al.¹⁹ The eluates, in which acid angiotensinase activities were shown by bioassay, were used as material.

1. Reaction mixtures

α -L-aspartic acid (NH₂)¹-valine⁵-angiotensin II (Hypertensin, Ciba) or α -L-aspartic acid¹-isoleucine⁵-angiotensin II (Protein Institute, Osaka, Japan) was used as substrate and dissolved in distilled water (1 mg/ml). 0.5 ml of the substrate solution, 1 ml of 0.2 M acetate buffer (pH 5.0) and 1 ml of material (containing 35 or 97 μ g of protein per ml) in a total volume of 2.5 ml were incubated at 37°C for various periods up to 24 hours. At the end of incubation, the reaction mixtures were transferred to an ice stocker and frozen at minus 20°C until the time when amino acid analysis of reaction products could be performed (Result 1). In another case, the reaction was stopped by boiling in a water bath for 5 minutes and then frozen (Results 2, 3).

2. Amino acid analysis of reaction products

After thawing, the sample was adjusted to pH 2.2 with 1N HCl and then to 5 ml with pH 2.2, 0.2N citrate buffer. This solution was filtered through a glass filter, and 2 ml of the filtrates were applied to the column of amino acid analyzer using Aminex A Resin (Hitachi KLA-3B), and analyzed at 55°C by the standard method of analysis. A 10 × 0.6 cm column was used for the basic amino acids and a 50 × 0.9 cm column for the acidic and neutral ones. Free amino acids of reaction products were recorded automatically on the recording paper at 440-, 570-, and 640-m μ with ninhydrin reagent proposed by SPACKMAN et al.²⁰ In the calculation, the position

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(a)

(b)

(c)

Fig. 1. Recording of free amino acids with automatic amino acid analyzer during various incubation periods.

- (a) Valyl¹⁵-angiotensin II amide was incubated with material at 37°C for 3 hours. Material was partially purified with Sephadex G-100.
- (b) The same as (a), but for 24 hours.
- (c) The same as (a), but for 15 minutes.

of the half height of the peak was determined and the peak area was computed by the $H \times W$ method with dot counting. The results were expressed as a percentage of free amino acids released (μmol) to angiotensin II (μmol). The procedure to obtain the distinction between serine and asparagine was carried out with a lithium form of Resin²¹.

RESULTS

1. Degradation of valyl⁵-angiotensin II amide by the eluates of Sephadex G-100

(a) Phenylalanine, proline, histidine and valine were released much more than the others during incubation time of 3, 6 or 24 hours (Figs. 1a, 1b, 2). Valine reached 118 per cent up to 24 hours, since two valines were involved in angiotensin II amide. No free amino acid was detected with the analyzer in the material incubated without angiotensin, or in angiotensin without material.

Fig. 2. The rate of amino acids released during various incubations until 24 hours to Valyl⁵-angiotensin II amide is expressed as a percentage.

(b) Phenylalanine was most released (85%) and the next was proline (50%) during incubation for 30 minutes, while valine and histidine were released slightly (Figs. 1c, 3).

Fig. 3. The rate of amino acids released during short periods up to 30 minutes to Valyl⁵-angiotensin II amide is expressed as a percentage.

2. Degradation of valyl⁵-angiotensin II amide by the eluates of Sephadex G-200

Phenylalanine was completely liberated (100%) and the next was proline (41%) during 10 minutes, while histidine was released to a lesser extent (Figs. 4a, 5). In 20 minutes, the release of proline reached 69 per cent, and valine, asparagine and arginine began to be liberated slightly (Fig. 5). Because of the presence of various amino acids, although very slight, the peak areas in the material were subtracted from the results (Fig. 4c).

3. Degradation of isoleucyl⁵-angiotensin II by the eluates from Sephadex G-200

Phenylalanine was released most predominantly up to 20 minutes (79%).

The next was proline (56%). Isoleucine and histidine were liberated slightly (Figs. 4b, 6). No free amino acids were found after heating in angiotensin II.

DISCUSSION

It was found that four amino acids from the carboxyl terminal end of octapeptide was released during short reaction periods, that phe-

(a)

(b)

(c)

Fig. 4. Recording of free amino acids with automatic amino acid analyzer during short incubation periods. This incubation mixture was stopped by boiling in a water bath.

- (a) Valyl⁵-angiotensin II amide was incubated with material at 37°C for 10 minutes. Material was partially purified with Sephadex G-200.
- (b) Isoleucyl⁵-angiotensin II was incubated with material at 37°C for 20 minutes. Material was the same as (a).
- (c) Material employed above was incubated without angiotensin under the same condition.

Fig. 5. The rate of amino acids released during incubations up to 20 minutes to Valyl⁵-angiotensin II amide is expressed as a percentage.

Fig. 6. The rate of amino acids released during incubations up to 20 minutes to Isoleucyl⁵-angiotensin II is expressed as a percentage.

nylalanine was released most predominantly, followed by proline, and that histidine and valine or isoleucine were liberated slightly. Therefore, it was suggested that the attack sites of rat kidney lysosomes against synthetic angiotensin II were at least three bonds such as Pro-7-Phe-8, His-6-Pro-7 and Tyr-4-Val-5 or Tyr-4-Isoleu-5. (Fig. 7). But this suggestion is not completely correct because the peptides of reaction products may not be recorded by the amino acid analyzer, and because of incomplete purification of material.

Fig. 7. The probable attack sites.

YANG et al.¹⁴ recently reported that "angiotensinase C" active at pH 5.6–5.7 from swine kidney hydrolyzed angiotensin II amide to phenylalanine and heptapeptide, and that it was not identical with carboxypeptidases from pH optimum and inhibitors. MATSUNAGA et al.^{13,22} observed that lysosomal angiotensinase activity having optimum pH 5.5 in rat kidney was markedly inhibited by DFP, but scarcely by EDTA. PICKENS et al.¹² used DFP to inhibit angiotensinase activity in the acid region in the assay of plasma renin activity. Angiotensinases are certainly present in various organ homogenates besides plasma^{4,6,7,9,10}. MATSUNAGA et al.²² found the presence of acid angiotensinase in rat liver lysosomes.

It is also suspected that angiotensinase may not be a single enzyme, but a multiple of enzymes^{2,4}. The present data supports this suspicion. It has been shown that such proteolytic enzymes as carboxypeptidase A, chymotrypsin, trypsin, pepsin, papain, subtilisin and leucine aminopeptidase inactivate angiotensin II amide^{8,23–27}. Both endopeptidase and exopeptidase may play a role in degradation of angiotensin. REGOLI et al.⁴ reported that rat kidney homogenate showed both aminopeptidasic degradation (60%) and chymotrypsin-like attack (40%) at pH 7.4. But they obtained no evidence of carboxypeptidase, although one of two tetrapeptides induced probably by chymotrypsin-like attack, Val-His-Pro-Phe,

was rapidly split up into free amino acids⁴. JOHNSON et al.²⁸ reported that phenylalanine was only liberated by EDTA-treated liver extracts at pH 7.4.

It is generally considered that the constituent amino acids of angiotensin II are released more predominantly from N-terminal by various organ homogenates, erythrocyte, serum or plasma at near neutral pH than from C-terminal^{1, 2, 4, 11, 29}. It remains to be studied whether the amino acids of angiotensin II can be liberated more predominantly from C-terminal in the acid region, such as YANG et al.¹⁴ and the present data suggest. Carboxypeptidase A can release only phenylalanine from angiotensin II because proline resists this enzyme. It is even said that the action of carboxypeptidase A may decrease when the penultimate residue is proline³⁰. But carboxypeptidase C in plants can release proline from polypeptides including angiotensin II amide³¹. ZUBER et al.³² reported recently that plant tissues contained acid carboxypeptidase especially in the lysosomes. The contributions of carboxypeptidase C and catheptic carboxypeptidase, having acid pH optimum, may be assumed in the splitting of angiotensin II in the acid region^{31, 33}, while YANG et al.¹⁴ thought these enzymes unlikely. Prolidase should also be examined in detail³⁴. It requires further clarification as to how endopeptidase and exopeptidase participate in degradation of angiotensin II.

SUMMARY

Phenylalanine and proline in this order were liberated more predominantly at pH 5.0 during short incubation periods by rat kidney lysosomal fraction, followed by slight releasing of histidine, valine or isoleucine. It was observed that the amino acids released were limited to four successive ones from COOH-terminal during short incubation up to 15 minutes in degradation of angiotensin II. Therefore, the possibility of endopeptidasic (chymotrypsin-like) and exopeptidasic actions was suggested in the present investigation.

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