NATURE OFLYSOSOMAL ANGIOTENSINASE ACTIVITY

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Acid angiotensinase activity is present in lysosomes of rat kidney and liver. The nature of this activity was investigated by bioassay and paper chromatography, using various angiotensin analogues as substrates. The main part of the lysosomal angiotensinase activity, especially on [Ile\(^{8}\)]-angiotensin II, is due to a carboxypeptidase which releases C-terminal phenylalanine. This enzyme is independent of sulphydryl groups and is inhibited by DFP. It is differentiated from cathepsin A or catheptic carboxypeptidase by its heat stability. The remaining part of the angiotensinase activity is sulphydryl-dependent and not inhibited by DFP. This is attributed to a chymotrypsin-like or a trypsin-like action or both. Rat plasma after dialysis against EDTA had a slight acid angiotensinase activity and released phenylalanine.

In spite of numerous studies on the renin-angiotensin system, relatively little is known about the process of removal of circulating angiotensin II. Some peptidases capable of inactivating angiotensin in tissues or body fluids have been termed "angiotensinases" although none has been found so far to be specific for angiotensin. It was first believed that destruction of angiotensin was mainly due to plasma angiotensinases. However, recent observations have shown that from 60 to 90% of injected angiotensin was removed from plasma after one passage through the hepatoporal, renal or femoral vascular beds. These results, together with those obtained from metabolic and immunologic studies with radioactive angiotensin II, underline the importance of an enzymatic inactivation in tissues, hence of tissue angiotensinases.

A variety of angiotensinases have been identified in tissues. Angiotensinases with aminopeptidase activity near neutral pH have been found in kidney microsomes. Another group of angiotensinases active at lower pH, including a carboxypeptidase called angiotensinase C, has also been found in extracts of various tissues.

Studies on the subcellular distribution in renal and hepatic cells have shown that "acid angiotensin" activity is localized in lysosomes. The purpose of the present paper was to investigate further the nature of this angiotensinase activity of lysosomes.

METHODS

Tissue Fractionation

Sprague-Dawley female rats, weighing 200–250 g, were anesthetized with ether and killed by bleeding. Kidneys and livers were removed immediately and placed into a cold sucrose solution at a concentration of 0.45 M for kidneys and 0.25 M for liver. Tissues were homogenized and fractionated by the method of Shibko and Tappel for kidney and of Ragab et al. for liver. The lysosome II fraction was used as liver lysosome. The purity of the fractions was examined enzymatically with acid phosphatase, glucose-6-phosphatase and the succinoxidase system as marker enzymes. Each fraction was frozen and thawed ten times prior to enzyme assay. The kidney and liver lysosomal fractions

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were diluted with 0.9% NaCl to contain 1 mg and 2 mg of protein per milliliter respectively. For paper chromatography, 3 to 5 ml of the diluted sample were dialyzed at 4°C against an ethylenediaminetetraacetate solution (2.2 g EDTA-Na₂ per liter) and then against distilled water.

Lysosomal Angiotensinase Activity

The following substrates were used: [Asn¹ Val⁵]-angiotensin II (0.5 µg), [α-L-Asp¹ Ile⁵]-angiotensin II (0.5 µg), [Arg¹ Ile⁵]-angiotensin II (1 µg), and [α-L-Asp¹ Ile⁵]-angiotensin I (1 µg). A mixture containing 0.125 M tris-acetic acid buffer (pH 5.5), 0.1 ml of the diluted lysosomal fraction and the substrate for a final volume of 4 ml was incubated at 37°C for 15 minutes. Then tubes were placed in boiling water for 10 minutes and centrifuged. Aliquots of 0.05 ml of the supernatant were then assayed in an amobarbital-anesthetized pentolinium-treated rat, using [Asn¹ Val⁵]-angiotensin II (Hypertensin-Ciba) as standard. The pressor activity of angiotensin was not modified by the nature of the incubation mixture nor by heating. Samples without substrate showed neither pressor nor depressor activity. Angiotensinase activity was expressed as percentage of substrate inactivated during incubation.

Cathepsin A Activity

A mixture containing 1 ml of 0.2 M citrate buffer (pH 5.0), 0.2 ml of 0.05 M N-carbobenzyloxy-α-glutamyl-L-tyrosine (Cbz-Glu-Tyr), 0.1 ml of sample and 0.7 ml of distilled water was incubated at 37°C for 15 minutes. After removal of proteins as above, free tyrosine was measured by the ninhydrin reaction. This activity was expressed in millimicromoles of released tyrosine.

Paper Chromatography

Solutions and their concentrations used in this experiment arc as follows:

- [Asn¹ Val⁵]-angiotensin II (Ciba), 5 mg/ml
- Chz-glutamylytyrosine, 0.05 M
- p-chloromercuriphenyl-sulfonic acid (PCMS), 4 × 10⁻³ M
- dithiothreitol (DTT), 0.01 M
- α-chymotrypsin (bovine pancreas, Sigma type II), 0.1 mg/ml
- trypsin (bovine, Sigma type XI), 0.1 mg/ml
- tris-acetic acid buffer (pH 5.5 and pH 7.5), 0.05 M
- di-isopropylfluorophosphate (DFP), 5% (w/v) in isopropanol.

Aliquots of 0.05 ml of buffer (pH 5.5), substrate and enzyme were mixed; 0.05 ml of DTT or PCMS, or 0.015 ml of DFP was added when necessary. The final volume was made up to 0.2 ml with distilled water. Incubation was carried out at 37°C for 15 to 180 minutes. The reaction was stopped by heating in boiling water and precipitates were removed by centrifugation. A volume of 0.07 ml of the supernatant was applied as a spot on Whatman No. 1 filter paper. Ascending chromatography was carried out with n-butanol-acetic acid-water (5:1:4) system. The chromatogram was stained with ninhydrin reagent then with Pauly's reagent. Isatin (2% in acetone) was used for detection of proline.

Plasma Acid Angiotensinase

Blood from normal or nephrectomized rats was withdrawn in the presence of EDTA as anticoagulant. About 5 ml of plasma were dialyzed against EDTA and then against distilled water in the same way as the lysosomal fraction. A mixture containing 1 ml of the dialyzed plasma, 0.5 ml of 0.5 M tris-acetic acid buffer (pH 5.5), 0.125 µg of [Asn¹ Val⁵]-angiotensin II and enough distilled water to a total volume of 2 ml was incubated at 37°C for 24 hours. The same mixture was also incubated in the presence of DFP (10⁻³ M). The reaction was stopped by cooling in iced water and the remaining angiotensin bioassayed in the rat. Since DFP inhibits acid angiotensinase⁻²⁰ the difference in amounts of angiotensin in the incubation mixtures with and without DFP is referred to as plasma acid angiotensinase activity.

A mixture of 2 ml of the dialyzed plasma containing 0.5 mg of [Asn¹ Val⁵]-angiotensin II was incubated at pH 5.5, at 37°C for 24 to 48 hours. After heating in boiling water for 10 minutes and centrifugation, the supernatant was evaporated under reduced pressure. The residue was dissolved in 0.5 ml of distilled water. Redistilled ethanol was added in a concentration of 85% and the precipitate was removed by centrifugation. The supernatant was evaporated to dryness and redissolved in 0.2 ml of distilled water. One half of this solution was used for paper chromatography.

Results

Angiotensinase Activity of the Kidney and Liver Lysosomes (Tables I and II)

In the absence of cofactor, lysosomal angiotensinase(s) was active on all angiotensin II analogues, including Arg¹-angiotensin II which is resistant to aminopeptidase-angiotensinase. It was inhibited by DFP, but scarcely affected by
Nature of lysosomal angiotensinase activity

Table I. Angiotensinase Activity (at pH 5.5) of Kidney Lysosomes

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Substrate</th>
<th>[Asn₁ Val₅]-angiotensin II</th>
<th>[Asp₁ Ile₅]-angiotensin II</th>
<th>[Arg₁ Ile₅]-angiotensin II</th>
<th>[Asp₁ Ile₅]-angiotensin I</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>69</td>
<td>70</td>
<td>91</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>DTT + DFP</td>
<td>34</td>
<td>8</td>
<td>40</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>PCMS</td>
<td>56</td>
<td>69</td>
<td>84</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PCMS + DFP</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>56</td>
<td>68</td>
<td>82</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>DFP</td>
<td>6</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are percentages of the substrate inactivated by 0.1 mg of protein at 37°C for 15 minutes.

DTT: dithiothreitol, 2.5 x 10⁻³ M  DFP: di-isopropylfluorophosphate, 10⁻³ M
PCMS: p-chloromercuriphenylsulfonic acid, 5 x 10⁻⁴ M

Table II. Angiotensinase Activity (at pH 5.5) of Liver Lysosomes

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Substrate</th>
<th>[Asn₁ Val₅]-angiotensin II</th>
<th>[Asp₁ Ile₅]-angiotensin II</th>
<th>[Arg₁ Ile₅]-angiotensin II</th>
<th>[Asp₁ Ile₅]-angiotensin I</th>
</tr>
</thead>
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<tr>
<td>DTT</td>
<td>78</td>
<td>82</td>
<td>91</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>DTT + DFP</td>
<td>60</td>
<td>25</td>
<td>45</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>PCMS</td>
<td>59</td>
<td>76</td>
<td>86</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PCMS + DFP</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>61</td>
<td>73</td>
<td>88</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>DFP</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are percentages of the substrate inactivated by 0.2 mg of protein at 37°C for 15 minutes.

Abbreviations as in Table I.

PCMS. In the presence of DTT, angiotensinase activity had a general tendency to increase; addition of DFP reduced but did not completely inhibit this activity. Lysosomal angiotensinase had little effect on angiotensin I in the absence of DTT.

These results indicate two types of lysosomal angiotensinase activity. One type is inhibited by DFP and is independent of sulphydryl groups. The fact that it is active on all angiotensin II analogues but not on angiotensin I suggests the requirement of C-terminal phenylalanine in the substrate. The other type which is not inhibited by DFP and is activated by sulphydryl compounds is represented by the activity shown in the presence of both DTT and DFP. Under these conditions, activity on [Asp₁ Ile₅]-angiotensin II was much less than that on [Asn₁ Val₅]-angiotensin II. The sulphydryl-dependent activity on the latter was observed between pH 4.5 and 6.5 without dominant peak.

Kidney and liver lysosomes showed the same pattern of angiotensinase activity except that the latter seemed to have more sulphydryl-dependent activity.

Products of Lysosomal Angiotensinases

Paper chromatograms of the reaction products of [Asn₁ Val₅]-angiotensin II with kidney lysosomes are illustrated in Fig. 1. Phenylalanine was liberated at an early stage. One isatin-sensitive spot was detected next. Although other amino acids or small peptides could give a positive reaction with isatin, this spot was considered as proline from its Rf, yellow color with ninhydrin and negative Pauly reaction. Later on, a spot corresponding to a tetrapeptide, Asn-Arg-Val-Tyr, appeared together with other amino acids. This process is in accordance with the amino acid analysis by Saito et al. Later changes were accelerated by DTT (2.5 x 10⁻³ M). PCMS (10⁻³ M) did not affect the liberation of phenylalanine, but completely inhibited further degradation. Therefore, it is apparent that an enzyme which is not dependent on sulphydryl groups, releases the C-terminal phenylalanine of angiotensin II but does not participate in the other
Fig. 1. Paper Chromatogram of Reaction Products of [Asn\textsuperscript{1}, Val\textsuperscript{5}]-angiotensin II with Kidney Lysosomes.
* incubated in the presence of PCMS
(1) phenylalanine, (2) valine, (3) tyrosine,
(4) angiotensin, (5) Asn-Arg-Val-Tyr, (6)
proline, (7) unidentified peptide, (8) unidentified.
The position of spot (6) is higher than that shown in this figure and partially overlaps spot (5).

changes.
Chromatograms of the reaction products in the presence of both DTT and DFP (Fig. 2) showed two spots with the same \( R_F \) and colors as those resulting from the action of chymotrypsin, i.e. Asn-Arg-Val-Tyr and Val-His-Pro-Phe. Two more spots similar to those produced by trypsin were detected, usually after longer incubation. These reactions are sulphydryl-dependent and not inhibited by DFP, whereas release of phenylalanine and other amino acids is inhibited by DFP.

- The same effects were observed with the liver lysosomes. Samples without angiotensin showed no detectable spots.
- Effect of Heating on Lysosomal Enzymes
The diluted lysosomal fraction of the kidney was kept at 60°C for 10 minutes. The acid angiotensinase activity remained intact even after heating, whereas cathepsin A activity disappeared (Table III). Paper chromatography showed release of phenylalanine from angiotensin, but no release of tyrosine from Cbz-glutamyl-tyrosine after heating.

Plasma Acid Angiotensinase
Although rat plasma after dialysis against EDTA had only a slight angiotensinase activity, 30 to 70 ng of angiotensin were inactivated when incubated during 24 hours at 37°C with 1 ml of plasma. Release of phenylalanine was observed by paper chromatography. No other changes.

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TABLE III EFFECTS OF HEATING ON KIDNEY LYPOSOMAL ENZYMES*

<table>
<thead>
<tr>
<th></th>
<th>Angiotensinase Activity**</th>
<th>Cathepsin A Activity***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>186</td>
<td>810</td>
</tr>
<tr>
<td>After</td>
<td>181</td>
<td>0</td>
</tr>
</tbody>
</table>

* Heated at 60°C for 10 minutes in 0.9% NaCl (pH 6.5)
** Nanograms of [Asn₁ Val⁵] -angiotensin II inactivated during incubation.
*** Nanomoles of tyrosine released from Cbz-Glu-Tyr.

were detected even in the presence of DTT.

DISCUSSION

Previous studies have already demonstrated the carboxypeptidase nature and the partial sulfhydryl-dependence of the lysosomal angiotensinase activity of the kidney.¹³,²² This is confirmed in the present paper which also reveals that these two properties belong to different kinds of enzymes.

One enzyme, independent of sulfhydryl groups, inactivates angiotensin II by releasing C-terminal phenylalanine, and is inhibited by DFP. It is not responsible for further degradation of angiotensin II. Biological inactivation of angiotensin II following removal of C-terminal phenylalanine is already known.²³ Lysosomal angiotensinase activity on various angiotensin analogues shows that this enzyme represents the main portion of the activity, particularly the one related to inactivation of [Asp¹ Ile⁵] -angiotensin II. Among the peptidases contained in lysosomes, cathepsin A or catheptic carboxypeptidase might exert such a carboxypeptidase action.²⁴,²⁵ The fact that the lysosomal angiotensinase could not be separated from cathepsin A by either sephadex gel-filtration or DEAE-cellulose column chromatography suggested that the two are identical.¹³ However, this possibility seems to be excluded because of differences in heat stability between angiotensinase and cathepsin A activity as revealed in the present study. Independence of sulfhydryl group also excludes involvement of catheptic carboxypeptidase. The properties mentioned above—release of C-terminal phenylalanine of angiotensin II, independence of sulfhydryl group, and heat stability—, together with pH dependence shown in the previous report,¹³ indicate the identity of this angiotensinase with angiotensinase C described by Yang et al.¹⁴

A lysosomal angiotensinase activity which is not inhibited by DFP was observed in the presence of DTT. Failure in finding a dominant peak of pH dependence in the presence of DFP and DTT may speak for the involvement of more than one sulfhydryl enzyme. Chymotrypsin-like and trypsin-like actions were observed in the presence of these two cofactors. One or both of these actions should be responsible for the sulfhydryl-dependent angiotensinase activity of lysosomes, because only slight biological activity remained after cleavage of 4–5 bond, or removal of two N-terminal amino acids of angiotensin.²³ Production of N-terminal tetrapeptide would imply the predominance of chymotrypsin-like action. The chymotrypsin-like endopeptidase found by Regoli et al.²⁶ in rat kidney homogenate may be identical to the sulfhydryl-dependent lysosomal enzyme mentioned above, since most angiotensinase activity in rat kidney is localized in microsomes and lysosomes.²² and microsomal angiotensinases have been identified as aminopeptidases.¹⁰,¹¹ The trypsin-like activity might be attributable to cathepsin B or cathepsin C, or both, which are sulfhydryl-dependent lysosomal peptidases and could cleave Arg-Val bond of angiotensin.²⁷,²⁸ For the precise identification of sulfhydryl-dependent lysosomal angiotensinases, further study including isolation of each enzyme is needed. At least a part of sulfhydryl-dependent angiotensinase activity found in various tissues and blood could be attributed to the lysosomal enzyme(s), whereas another kind of sulfhydryl-dependent angiotensinase exists in liver cell sap and hemolysate.³¹

Liberation of amino acids such as proline, valine, etc. in later stages of incubation was also sulfhydryl-dependent and may be due to the action of various peptidases in the lysosomes. However, these secondary reactions are not essential for lysosomal angiotensinase activity, since cleavage of the 7–8 bond or the 4–5 bond is enough for complete inactivation of angiotensin.²³

Pickens et al.²⁹ found an acid angiotensinase
activity in human plasma which was inhibited by DFP but not EDTA. *Khairallah* and *Page* attributed this action to the chymotrypsin-like endopeptidase proposed by *Regoli* et al. In our present experiments, rat plasma after dialysis against EDTA showed a slight acid angiotensinase activity by releasing C-terminal phenylalanine. This angiotensinase C-like action in plasma suggests a release of the lysosomal enzyme into the blood stream, but we could not confirm the existence of a chymotrypsin-like enzyme which is not inhibited by EDTA.

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**REFERENCES**


