A Comparison of the Substrate Specificities of Cathepsin D and Pseudorenin*

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Cathepsin D, purified from hog spleen, releases angiotensin I from tetradecapeptide renin substrate and from protein renin substrates purified from hog and human plasma. However, the enzyme does not act on the naturally occurring renin substrate as it exists in plasma nor on purified substrate in the presence of plasma. Cathepsin D releases angiotensin I quantitatively from tetradecapeptide renin substrate and does not further degrade the angiotensin I on prolonged incubation. The pH optimum for cathepsin D acting on tetradecapeptide renin substrate is 4.5, and there is very low activity above pH 7. These properties are very similar to those of pseudorenin, an angiotensin-forming enzyme originally isolated from human kidney, indicating that cathepsin D and pseudorenin may be identical.

In 1969, Skeggs et al. (1) described some of the properties of a new angiotensin-forming enzyme which was named pseudorenin. This enzyme, partially purified from human kidney, was distinguished from renin by (a) its lower pH optimum with TDP, (b) its inability to release angiotensin I from the naturally occurring renin substrate in plasma, and (c) its wide distribution in mammalian tissues.

Cathepsin D is a widely distributed acid protease described by Press et al. (2) and Lapresle and Webb (3) in 1960. It is a major intracellular proteolytic enzyme of spleen and kidney but does not hydrolyze the synthetic peptide substrates used to characterize cathepsins A, B, and C.

In the present investigation we have shown that cathepsin D, purified from hog spleen, can release angiotensin I from TDP as well as from protein renin substrates purified from hog and human plasma. A study of the substrate specificities shows that cathepsin D is similar to pseudorenin but clearly different from renin.

**EXPERIMENTAL PROCEDURES**

**Preparation of Enzymes**—Hog spleen pseudorenin was prepared as follows. Hog spleen (3 kg) was defatted and homogenized with 3 volumes of water in a Waring Blender. The pH was adjusted to 2.95, the mixture was centrifuged, and the supernatant solution was readjusted to pH 6.0 and fractionated with (NH₄)₂SO₄. The protein precipitating between the limits 2.8 and 3.8 M (NH₄)₂SO₄ was dissolved in and dialyzed against distilled water. After centrifugation to remove insoluble protein, the supernatant solution was dialyzed against 0.005 M sodium phosphate buffer, pH 7.0, and pumped onto a column (2.5 x 100 cm) of DEAE-cellulose (Whatman DE32) which had been equilibrated with the same buffer. Elution was carried out at 1 ml/min with a gradient made by pumping 0.025 M sodium phosphate buffer, pH 5.0, at 1 ml/min, into the gradient mixing chamber which initially contained 500 ml of 0.005 M phosphate buffer, pH 7.0. Fractions containing pseudorenin activity were pooled, yielding 1980 pseudorenin units (1) with a specific activity of 4.6 pseudorenin units/mg of protein. The preparation was free of renin (assay with crude hog renin substrate at pH 7.5) and angiotensinase activities.

Human kidney pseudorenin was prepared by batch treatment with and chromatography on DEAE-cellulose as described previously (1). The final preparation had a specific activity of 0.6 pseudorenin unit/mg of protein and was free of renin (assay with crude hog renin substrate at pH 7.5) and angiotensinase activities.

Human kidney renin was prepared by batch treatment with and chromatography on DEAE-cellulose as described previously (1). The final preparation had a specific activity of 35 nmol of angiotensin I/h/mg of protein (assay with human renin substrate at pH 6.0) was free of angiotensinase activity, but contained traces of pseudorenin activity.

Hog kidney renin, prepared as described previously (4), was free of angiotensinase and pseudorenin activities and had a specific activity of 1.0 nmol of angiotensin I/h/mg of protein (assay with hog renin substrate at pH 7.5).

Cathepsin D, purified from hog spleen, was a gift from Dr. Jordon Tang.

**Preparation of Substrates**—TDP was synthesized and purified as described previously (1). Hog renin substrate, form A, was purified by solvent extraction using polyethylene glycol (see Fig. 2, Ref. 6) and had a specific activity of 14 nmol of angiotensin I/h/mg of protein (assay with hog renin substrate at pH 7.5). Human kidney renin substrate was fraction E, procedure I (7) with a specific activity of 18 nmol of angiotensin I/h/mg of protein. The renin substrate at pH 7.5.

In our laboratory, renin preparations are judged to be free of pseudorenin by examination of the pH profile of TDP hydrolysates. A second maximum between pH 4 and 5 indicates the presence of pseudorenin.

The cathepsin D used in this study was purified by a modification (3, S. Huang, S. Huang, and J. Tang, unpublished results) of the method given in Ref. 5.

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1 The abbreviation used is TDP, tetradecapeptide renin substrate (Asp-Arg-Val-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser).

2 In our laboratory, renin preparations are judged to be free of pseudorenin by examination of the pH profile of TDP hydrolysates. A second maximum between pH 4 and 5 indicates the presence of pseudorenin.

3 The cathepsin D used in this study was purified by a modification (S. Huang, S. Huang, and J. Tang, unpublished results) of the method given in Ref. 5.
Substrate specific activity is expressed as nanomoles of renin substrate/mg of protein.

**Reaction of Enzymes with TDP**—Incubation tubes contained 500 pmol of TDP, 5 mg of lysozyme (to prevent adsorption of peptides on glassware), and enzyme in 0.05 m sodium citrate buffer, pH 4.5, at a final volume of 1.0 ml. In a few additional experiments the buffer used was 0.05 m sodium phosphate, pH 7.0. Tubes were incubated for 15 min at 37°C, 0.5 ml of 0.15 m NaCl was added, and the tubes were heated for 10 min at 100°C. After centrifugation, the supernatant solutions were assayed for angiotensin I by a modification of the radioimmunoassay method of Haber et al. (8) using [Asp₁, Ile₉]angiotensin I (Beckman) as standard. In selected samples, angiotensin I was also assayed by bioassay in the rat (9). In both the radioimmunoassay and the bioassay, appropriate corrections were made for the TDP "blank" (incubation of TDP in the absence of enzyme). Enzyme activity is expressed as picomoles of angiotensin I formed per 15 min and specific activity as nanomoles/min/mg of enzyme protein. Protein concentrations were measured by an automated modification of the method of Lowry et al. (10).

**Reaction of Enzymes with Protein Renin Substrates**—Incubation tubes contained 1 nmol of substrate (except 0.20 and 0.09 nmol in the case of hog and human plasma, respectively) and enzyme in 0.05 m sodium citrate buffer, pH 5.0, at a final volume of 1.0 ml. Tubes were incubated for 30 min at 37°C, 0.5 ml of 0.15 m NaCl was added, and the tubes were heated for 10 min at 100°C. Assays were carried out as described above. Enzyme activity is expressed as picomoles of angiotensin I formed per 30 min and specific activity as nanomoles/min/mg of enzyme protein.

**Reaction of Enzymes with Hemoglobin**—Incubation tubes contained 2.0 ml of 2.5% denatured hemoglobin (Worthington) and enzyme in 0.07 m sodium citrate buffer, pH 3.4, at a final volume of 0.5 ml. Tubes were incubated at 37°C for 30 min, and the reaction was stopped by adding 4.5 ml 5% trichloroacetic acid (11). After 10 min, the tubes were centrifuged, and the clear supernatant solutions were assayed for hemoglobin by an automated modification of the method of Lowry et al. (10) using bovine serum albumin as standard. Enzyme specific activities are expressed as milligrams bovine serum albumin equivalents/min/mg of enzyme protein.

**RESULTS AND DISCUSSION**

We have determined the rates of angiotensin release from several renin substrates and the rate of hydrolysis of hemoglobin. In Table I are shown the specific activity values for hog spleen cathepsin D, hog spleen pseudorenin, and human kidney pseudorenin. For comparison, the values for hog kidney renin and human kidney renin have been included.

Cathepsin D, like pseudorenin, hydrolyzed TDP very rapidly at pH 4.5, but much more slowly at 7.0. Also, cathepsin D hydrolyzed hog and human renin substrates in the purified states, but not as they exist in plasma. Hog kidney renin did not hydrolyze hemoglobin and had much less activity toward TDP at pH 4.5 than at 7.0. Human kidney renin was the only enzyme tested which released angiotensin I from human spleen cathepsin D, hog spleen pseudorenin, and human kidney pseudorenin. For comparison, the values for hog kidney renin and human kidney renin have been included.

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*Comparison of Cathepsin D and Pseudorenin*

**Table I**

Specific activities with various substrates

Assays were carried out and specific activities are reported as described under "Experimental Procedures." Hb, hemoglobin. pH 3.4: TDP (4.5), tetracapeptide renin substrate, pH 4.5; TDP (7.0), tetracapeptide renin substrate, pH 7.0; Hog A, purified hog renin substrate, form A, pH 5.0; Human, purified human renin substrate, pH 5.0.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hb</th>
<th>TDP (4.5)</th>
<th>TDP (7.0)</th>
<th>Hog A Human</th>
<th>Hog plasma Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hog spleen cathepsin D</td>
<td>11</td>
<td>200</td>
<td>1.3</td>
<td>2.2</td>
<td>0.57</td>
</tr>
<tr>
<td>Hog spleen pseudorenin</td>
<td>1.2</td>
<td>21</td>
<td>0.13</td>
<td>0.24</td>
<td>0.065</td>
</tr>
<tr>
<td>Human kidney pseudorenin</td>
<td>0.80</td>
<td>19</td>
<td>0.12</td>
<td>0.15</td>
<td>0.039</td>
</tr>
<tr>
<td>Hog kidney renin</td>
<td>0</td>
<td>0.57</td>
<td>12</td>
<td>4.1</td>
<td>0</td>
</tr>
<tr>
<td>Human kidney renin</td>
<td>0.13</td>
<td>1.9</td>
<td>0.13</td>
<td>0.12</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*The cathepsin D assay is much less sensitive than the assay for angiotensin I. The specific activity of hog kidney renin at the lower limit of detection would be 0.003 mg of bovine serum albumin/min/mg of enzyme protein.*
Comparison of Cathepsin D and Pseudorenin

TABLE II

Inhibitory effect of plasma on the enzymatic release of angiotensin I from purified hog renin substrate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Angiotensin I formed pmol/30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>Hog plasma</td>
</tr>
<tr>
<td>Hog spleen cathepsin D</td>
<td>104</td>
</tr>
<tr>
<td>Human kidney pseudorenin</td>
<td>109</td>
</tr>
<tr>
<td>Hog kidney renin</td>
<td>100</td>
</tr>
</tbody>
</table>

Assays were carried out as described under "Experimental Procedures" in the absence or presence of 0.5 ml of plasma.

FIG. 2. Total hydrolysis of TDP (160 pmol) by excess enzyme. Assay conditions are described under "Experimental Procedures." ●, 0.5 μg of cathepsin D at pH 4.5; ○, 18 μg of hog kidney renin at pH 7.0.

as when a similar incubation was carried out with excess hog kidney renin at pH 7.0 (Fig. 2). When the incubations were continued for up to 4 h, there was no change in the quantity of angiotensin I measured. These results indicate that the leucylleucine bond of TDP is hydrolyzed quantitatively by cathepsin D and that the angiotensin I formed is not further degraded. In a separate experiment, cathepsin D was incubated at 37°C with 200 pmol of angiotensin I at pH 4.5 for time intervals up to 2 h. There was no loss of angiotensin I as measured by either radioimmunoassay or bioassay. These results conflict with a report by Reinharz and Roth (12) that cathepsin D from bovine anterior pituitary hydrolyzed benzoyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamide at both the phenylalanylhistidine and leucylleucine bonds. However, their enzyme had only been purified by gel filtration on Sephadex G-200 and could have been contaminated by other peptidases.

Work of other investigators has suggested that cathepsin D and pseudorenin could be the same enzyme. Both are inhibited by pepstatin (13, 14) and both are bound to hemoglobin-Sepharose affinity columns (15, 16). Day and Reid (17) have suggested that dog brain renin activity may be due to cathepsin D since these two enzyme activities could not be separated by gel chromatography or isoelectric focusing. On the other hand, Corvol et al. (18) found no cathepsin-like activity in highly purified hog kidney renin.

Our present results clearly distinguish cathepsin D from hog and human kidney renin. However, the finding that cathepsin D and pseudorenin have similar substrate specificities indicates that these two enzymes may be identical.

Acknowledgment—We thank Dr. Jordon Tang for the gift of cathepsin D.

REFERENCES

A comparison of the substrate specificities of cathepsin D and pseudorenin.
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