Characterization of New Fluorogenic Substrates for the Rapid and Sensitive Assay of Cathepsin E and Cathepsin D

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Received February 17, 1999; accepted March 18, 1999

Cathepsin E and cathepsin D are two major intracellular aspartic proteinases implicated in the physiological and pathological degradation of intra- and extracellular proteins. In this study, we designed and constructed highly sensitive synthetic decapeptide substrates for assays of cathepsins E and D based on the known sequence specificities of their cleavage sites. These substrates contain a highly fluorescent (7-methoxycoumarin-4-yl)acetyl (MOCAc) moiety and a quenching 2,4-dinitrophenyl (Dnp) group. When the Phe-Phe bond is cleaved, the fluorescence at an excitation wavelength of 328 nm and emission wavelength of 393 increases due to diminished quenching resulting from the separation of the fluorescent and quenching moieties. The first substrate, MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-NH₂, in which the Lys-Pro combination at positions P5 and P4 was designed for specific interaction with cathepsin E, is hydrolyzed equally well by cathepsins E and D (kcat/Km = 10.9 μM⁻¹·s⁻¹ for cathepsin E and 15.6 μM⁻¹·s⁻¹ for cathepsin D). A very acidic pH optimum of 4.0 was obtained for both enzymes. The second substrate, MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-NH₂, in which the isoleucine residue at position P2 was meant to increase the specificity for cathepsin E, is also hydrolyzed equally by both enzymes (kcat/Km = 12.2 μM⁻¹·s⁻¹ for cathepsin E and 16.3 μM⁻¹·s⁻¹ for cathepsin D). The kcat/Km values for both substrates are greater than those for the best substrates for cathepsins E and D described so far. Unfortunately, each substrate shows little discrimination between cathepsin E and cathepsin D, suggesting that amino acids at positions far from the cleavage site are important for discrimination between the two enzymes. However, in combination with aspartic proteinase inhibitors, such as pepstatin A and Ascaris pepsin inhibitor, these substrates enable a rapid and sensitive determination of the precise levels of cathepsins E and D in crude cell extracts of various tissues and cells. Thus these substrates represent a potentially valuable tool for routine assays and for mechanistic studies on cathepsins E and D.

Key words: aspartic proteinase, cathepsin D, cathepsin E, fluorogenic substrate.
activities in crude cellular lysates. A very common method employs bovine hemoglobin as a substrate (35-37) from which the trichloroacetic acid (TCA)-soluble products liberated by enzyme reaction are measured by their absorbance at 280 nm and the Folin reaction. This method, however, is time consuming. Meanwhile, a number of synthetic substrates possessing more extended peptide chains designed to be cleaved by the endopeptidase mechanism have been developed to monitor these protease activities (38-40). The use of synthetic substrates appears to have advantages over assay with protein substrates because it provides a simple and fast method for the measuring enzymatic activities of large numbers of samples. Recently, fluorogenic substrates containing fluorophor and quencher groups within the same molecule have been developed for kinetic studies of aspartic proteinases (41, 42). The fluorescent signal in these uncleaved substrates is quenched by resonance energy transfer between the fluorophor and quencher groups. After cleavage of the peptide chain, the quenching efficiency decreases substantially resulting in an increase in fluorescence. Early substrates designed along this line had such fluorophors as 5-(2-aminoethyl)aminonaphthalene-1-sulfonic acid, o-aminobenzoinic acid, or p-nitroanilide, and such quenchers as γ-hydroxypropyl-N-methylpyridium or 4-(4-dimethylaminophenylazo)benzoic acid. The fluorophor MOCAc and the quencher Dnp pair has been recently described as an excellent fluorogenic substrate for matrix metalloproteinases (43, 44) and caspases (45).

In this paper, we describe the design, construction, catalytic properties, and kinetic evaluation of two new fluorogenic decapeptide substrates suitable for assessing the activities of cathepsins E and D from various sources.

EXPERIMENTAL PROCEDURES

Peptide Substrates and Enzymes—The two fluorogenic decapeptide substrates MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)γ-NH2 (substrate I) and MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)γ-NH2 (substrate II) were designed and custom-synthesized at the Peptide Institute, Osaka. They were dissolved in dimethylsulfoxide (DMSO) at 1 mM and stored at -80°C until use. The recombinant Ascaris pepsin inhibitor protein was purified from the culture medium of Saccharomyces cerevisiae transfected with its cDNA (46). Cathepsin E was purified from human erythrocytes (37) and rat erythrocytes (47), spleen (35), and gastric mucosa (unpublished) according to the previously described methods. Cathepsin D was purified from rat spleen (36) and gastric mucosa (unpublished) as described. All other chemicals were of reagent grade and were purchased from various commercial sources.

Assays—The hydrolysis of the two fluorogenic substrates at their Phe-Phe bonds was spectrofluorometrically determined at a variety of pH values. Reaction mixtures contained 80 μl of buffer (for routine assays, 50 mM sodium acetate buffer, pH 4.0, was used), 10 μl of 200 μM substrate solution, and 10 μl of sample solution in a total volume of 100 μl. The final substrate concentration range was 10-40 μM; the final DMSO concentration was 0.2% for all assays. Ionic strength was maintained constant at 0.1 by the addition of sodium chloride as necessary. Reaction mixtures were incubated at 40°C for 10 min and the reaction was terminated by adding 2 ml of 5% TCA. The increase in fluorescence intensity produced by substrate cleavage during incubation was measured at an emission wavelength of 393 nm with excitation at 328 nm using a fluorescence spectrophotometer. Kinetic parameters were obtained with appropriate concentrations of substrates. In all cases the kinetics of cleavage followed the Michaelis-Menten scheme. The Kₘ and Vₘₐₓ values were estimated from the intercepts and slopes of double-reciprocal plots of rate versus substrate concentration. The kₐₜ values (s⁻¹) were determined from the equation

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[S] + [E]} \]

where JA, [S], and [E] are the increase in absorbance, substrate concentration (μM), and enzyme concentration (molar), respectively. The active enzyme concentration was determined by titration against the competitive inhibitor pepstatin A.

In some experiments, the aspartic proteinase activity was determined at pH 3.5 using 1.5% acid-denatured hemoglobin substrate as described previously (37).

In order to quantify the amounts of cathepsins E and D in various rat tissues, the Ascaris pepsin inhibitor was used for the selective inhibition of cathepsin E. Enzyme solution containing 70 μl of 50 mM sodium acetate buffer (pH 4.0) and 10 μl of 20 μM Ascaris pepsin inhibitor in a total volume of 90 μl was preincubated at 40°C for 10 min. Then 10 μl of 200 μM substrate solution was added and the reaction mixture was incubated at 40°C for 10 min. The reaction was terminated by adding 2 ml of 5% TCA. After centrifugation, the fluorescence in the supernatant was measured at an emission wavelength of 393 nm with excitation at 328 nm.

RESULTS AND DISCUSSION

It has been demonstrated that, in addition to bulky hydrophobic amino acid residues at the P1 and P1' positions, residues at more peripheral subsites of substrates are important for the enzyme-substrate interaction with and efficient cleavage by aspartic proteinases (42, 48, 49). For example, Lys at the P5 position and Pro at the P4 position are preferred by cathepsin E rather than cathepsin D (14, 49, 50). In this study, new fluorogenic synthetic decapeptide substrates, MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)γ-NH₂ (substrate I), and MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)γ-NH₂ (substrate II), were synthesized and used for assays of cathepsins E and D. The major peptide parts of these substrates were derived from the sequences of the chromogenic peptide substrates Lys-Pro-Ile-Leu-Phe-Nphγ-Arg-Leu and Lys-Pro-Ile-Leu-Phe-Nphγ-Arg-Leu as described for the assay of cathepsin E by Rao-Naik et al. (50) (Nphγ: p-nitrophenylalanine). The present peptide substrates were labeled at their amino termini with the highly fluorescent MOCAc group. The quenching Dnp group was added at the carboxyl terminal. The unquenched peptide MOCAc-Pro-Leu-Gly showed a fluorescence maximum at an excitation wavelength of 328 nm with emission centered around 393 nm (not shown). From the known specificities of cathepsin E and cathepsin D towards substrates similar to...
those used in the present study, it is clear that only the Phe-Phe bond is susceptible to cleavage by cathepsin E and cathepsin D.

When substrate II (20 μM) was incubated with cathepsin E (0.75 ng) or cathepsin D (0.86 ng) from different sources in a wide range of buffers (pH 3.0-6.5) at 40°C for 10 min, single peaks were observed for each enzyme (Fig. 1). The pH optima for the hydrolysis of this substrate by human erythrocyte cathepsin E, rat gastric cathepsin E, rat gastric cathepsin D, and rat spleen cathepsin D all were around pH 4.0, in agreement with the literature data (35-37, 47). At pH 3.0, cathepsin E showed less than 60% and cathepsin D less than 40% of their maximal activities at pH 4.0. While at pH 5.5 cathepsins E retained more than 55% and cathepsin D more than 60% of their maximal activities. Similar results were obtained with substrate I (20 μM) (not shown). Therefore, a constant pH of 4.0 was maintained throughout this work. The temperature for the optimal hydrolysis of substrate II by human erythrocyte cathepsin E and rat gastric cathepsin D was close to 45°C (Fig. 2). However, since longer incubation periods caused both enzymes to lose more of their activities at 45°C than at 40°C, the temperature for hydrolysis of the two peptides was set at 40°C in this study. Both enzymes were unstable following 10 min incubation at 60°C.

The cleavage of the substrates by cathepsins E and D is linear with enzyme concentration. Figure 3 shows the effect of substrate concentration on the activities of cathepsin E and cathepsin D. Both enzymes (0.75 ng of cathepsin E, 0.86 ng of cathepsin D) appear to be fully saturated for about 10 min when less than 10 μM of substrate II is present in the reaction mixture. A linear relationship, however, is observed with 20-40 μM of substrate. The results obtained when the standard assay was performed with varying concentrations of enzyme are described in Fig. 4. A linear relationship was observed up to a concentration of 1.8 ng/ml of cathepsin E or 2.2 ng/ml of cathepsin D. The use of fluorescent MOCAc labeling brought the assays for cathepsins E and D into the sensitivity range of 0.2-2.5 ng of each enzyme. This assay, therefore, is at least 30 and 100 times as sensitive as the measurement of cleaved hemoglobin peptides by cathepsins E and D, respectively. The useful substrate concentration range of the assay for both enzymes is 10 to 40 μM with a 10-min incubation time at 40°C. Both substrates were hydrolyzed only slightly by other lysosomal cysteine proteinases, such as cathepsins B, H, or L (data not shown).

The kinetic parameters determined for the hydrolysis of both substrates by human erythrocyte cathepsin E and rat gastric D are summarized in Table I. Lineweaver-Burk plots for the hydrolysis of substrate I by cathepsin E gave a $k_{cat}/K_m = 10.9 \mu M^{-1} \cdot s^{-1}$. Similarly, the $k_{cat}$ value for cathepsin D was determined to be $15.6 \mu M^{-1} \cdot s^{-1}$. The value obtained for substrate II with cathepsin E was $k_{cat}/K_m = 12.2 \mu M^{-1} \cdot s^{-1}$. Substrate II was also found to be an excellent substrate for cathepsin D with $k_{cat}/K_m = 16.3 \mu M^{-1} \cdot s^{-1}$. Similar kinetic parameters for the hydrolysis of both substrates were obtained for each enzyme from other sources (not shown). The data in Table I show the two substrates to have greater $k_{cat}/K_m$ values than the best substrates for cathepsins E or D described so far. There is a nearly 1.5-fold increase in the $k_{cat}/K_m$ values of cathepsin E with these substrates in comparison with the best known cathepsin E substrates (50), although the data were obtained under different assay conditions.

It has already been demonstrated that hydrophobic and aromatic amino acid residues in the P1 and P1' positions of peptide substrates are preferred by cathepsins E and D (14, 50). Positions other than P1 and P1' are also known to be
important for the cleavage specificity by these enzymes. A Pro residue in the P4 position appears to be preferred by cathepsin E. Further, the Lys-Pro combination in the P4-P5 position is expected to be preferred by cathepsin E (14, 50).

Rao-Naik et al. (50) synthesized a series of chromogenic substrates based on the parent peptide Lys-Pro-Ile-Glu-Phe-Nph*-Arg-Leu, and showed the replacement of Glu by Ile in the P2 position to result in a slight increase in the overall cleavage efficiency by cathepsin E. They also demonstrated that the replacement of Leu by Ile in the P2 position leads to a 4-fold increase in the cleavage efficiency by cathepsin E. However, the present results show that the replacement of Leu by Ile in the P2 position of substrate I results in no significant improvement in the overall cleavage efficiency by either cathepsin E or cathepsin D. Moreover, there is no significant difference in the cleavage efficiency by cathepsins E or D between substrates I and II. This suggests that amino acid residues at positions far from the sites of cleavage are important for the discriminative interaction between the substrates and each enzyme. In this context, Kageyama (14) reported the importance of the amino acid residues at the P6 and P7 positions for the full interaction between substrate and cathepsin E, whereas cathepsin D hydrolyzes the peptides very slowly even though the cleavage sites are identical for the two enzymes.

The availability of these convenient fluorogenic substrates facilitates the examination of the interaction of aspartic proteinase inhibitors with the two enzymes. Pepstatin is known to be a potent inhibitor of both enzymes. Ascaris pepsin inhibitor, originally isolated from the round worm Ascaris lumbricoides (51), inhibits pepsins (57) and cathepsin E (52), but does not affect other types of aspartic proteinases, including cathepsin D (46, 53). The $K_i$ values for the hydrolysis of substrate II were measured at pH 4.0.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_i$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_i$ (µM⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin E</td>
<td>Substrate I</td>
<td>3.3</td>
<td>35.9</td>
<td>10.9</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Substrate I</td>
<td>3.7</td>
<td>57.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Cathepsin E</td>
<td>Substrate II</td>
<td>3.2</td>
<td>39.1</td>
<td>12.2</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Substrate II</td>
<td>3.7</td>
<td>59.6</td>
<td>16.3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human erythrocyte cathepsin E</td>
<td>7.6</td>
</tr>
<tr>
<td>Rat erythrocyte cathepsin E</td>
<td>3.8</td>
</tr>
<tr>
<td>Rat gastric cathepsin E</td>
<td>10.1</td>
</tr>
<tr>
<td>Rat spleen cathepsin E</td>
<td>9.6</td>
</tr>
<tr>
<td>Rat gastric cathepsin D</td>
<td>1.4</td>
</tr>
<tr>
<td>Rat spleen cathepsin D</td>
<td>4.6</td>
</tr>
</tbody>
</table>

TABLE II. Inhibition constants ($K_i$) for the interaction of cathepsin E and cathepsin D from various sources with pepstatin A and Ascaris pepsin inhibitor. All measurements were carried out at pH 4.0 in 50 mM sodium acetate buffer at 40°C for 10 min. The substrate used was MOCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-γ-NH$_2$. All inhibitions were competitive and the estimated precision of the values obtained was in the range of ±5-10%.
for cathepsins E and D from different sources (Table II). Pepstatin A strongly inhibits both cathepsins E and D from different sources with nanomolar K values. On the other hand, cathepsins E from different sources are all inhibited strongly by the Ascaris pepsin inhibitor with K values similar to those obtained using hemoglobin as a substrate (52-54). There was no significant difference seen in the K values for either enzyme between substrates I and II (not shown). Altogether, the present study shows the two substrates to be excellent substrates for the assay of cathepsins E and D.

The amounts of cathepsins E and D in various rat tissues were determined using a combination of substrate II and the aspartic proteinase inhibitors pepstatin A and Ascaris pepsin inhibitor (Table III). Almost all the activity in the tissue extracts, except the kidney and jejunum extracts, can be completely inhibited by pepstatin A. Part of the activity in the stomach is known to be attributable to other aspartic proteinases such as pepsin and gastricsin (4). The distributions of the two enzymes are apparently different. Cathepsin D is distributed ubiquitously in all tissues examined, although the levels vary among tissues. Of the tissues tested, stomach, spleen, and adrenal, in that order, have the highest levels of cathepsin D, whereas cecellebllum, hippocampus, jejunum, and heart show low levels of activity. In contrast, cathepsin E has a relatively limited distribution. Stomach, lymphoid tissues, and urinary bladder contain high levels of cathepsin E, but the enzyme is barely detectable in brain tissues, liver, and heart. The activity ratios of cathepsins E and D, therefore, vary among tissues. The data for the quantitation of cathepsins E and D are in good agreement with the results obtained using a combination of the hemoglobin assay and immunoprecipitation with discriminatory antibodies specific for each enzyme (4).

In conclusion, the two synthetic decapeptide substrates described here are the most sensitive substrates for cathepsins E and D described so far. Not only are the kcat/Km values for each enzyme higher than those reported previously, but also the cleavage products are more readily detectable. The simple assay method described here is suitable for the assessment of cathepsin E or cathepsin D activity in crude preparations. Further work to develop peptides that are cleaved specifically by the individual enzymes will permit their measurement under these conditions.

The authors wish to thank Dr. Hitoshi Maegawa (Discovery Research Laboratory, Ono Pharmaceutical Co., Ltd., Osaka, Japan) for his valuable suggestions concerning the construction of the peptide substrates.

REFERENCES


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TABLE III. Levels of cathepsins E and D in various rat tissues. Soluble extracts of various tissues from perfused rats were assayed with substrate II at pH 4.0 and 40 C for 10 min in the presence or absence of pepstatin A or Ascaris pepsin inhibitor. The contents of cathepsins E and D were calculated from the amounts of the Ascaris pepsin inhibitor sensitive and insensitive activity, respectively, for the pepstatin-sensitive proteinase activity found in each tissue extract. Relative enzyme activities are expressed as percent of the total activity in each tissue extract.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration (µg/mg)</th>
<th>Relative enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cathepsin E</td>
<td>Cathepsin D</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.002</td>
<td>0.32</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>not detectable</td>
<td>0.10</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.001</td>
<td>0.13</td>
</tr>
<tr>
<td>Heart</td>
<td>0.008</td>
<td>0.18</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.05</td>
<td>0.22</td>
</tr>
<tr>
<td>Liver</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.01</td>
<td>0.23</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.02</td>
<td>0.29</td>
</tr>
<tr>
<td>Lung</td>
<td>0.03</td>
<td>0.31</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.04</td>
<td>0.17</td>
</tr>
<tr>
<td>Colon</td>
<td>0.06</td>
<td>0.28</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>0.16</td>
<td>0.34</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.26</td>
<td>0.71</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.27</td>
<td>0.89</td>
</tr>
</tbody>
</table>

The value indicated by the asterisk (*) includes activities due to Ascaris pepsin inhibitor sensitive enzymes other than cathepsin E, such as pepsins.
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