Assay procedures for cathepsin B, H and L activities in rat tissue homogenates.

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Abstract

Cathepsin B, H and L activities in small amounts of rat tissue homogenates corresponding to 10 micrograms protein were determined with 7-amino-4-methyl-coumarin conjugates as substrates. A new procedure for serum cathepsin H activity was also developed. High cathepsin B and H activities were found in kidney, spleen and liver. Liver cathepsin B, H and L activities in D-galactosamine-injured rats were decreased concomitantly with an increase in serum cathepsin H activity.


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Assay Procedures for Cathepsin B, H and L Activities in Rat Tissue Homogenates

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Cathepsin B, H and L activities in small amounts of rat tissue homogenates corresponding to 10 µg protein were determined with 7-amino-4-methyl-coumarin conjugates as substrates. A new procedure for serum cathepsin H activity was also developed. High cathepsin B and H activities were found in kidney, spleen and liver. Liver cathepsin B, H and L activities in D-galactosamine-injured rats were decreased concomitantly with an increase in serum cathepsin H activity.

Key words: cathepsin B, H and L, serum cathepsin H, liver injury.

Purification procedures and biochemical characteristics of thiol proteases have been described in the literature (1, 2). The proteases are generally classified into cathepsin B, H and L by means of their susceptibility to a specific substrate and inhibitor (3). However, the assay methods have been described with the purified enzyme corresponding to 3 to 25 and 40 to 400 ng for cathepsin L and H, respectively, in a system using 7-amino-4-methyl-coumarin (NMec) conjugate as the specific substrates (4). It is, therefore, difficult to determine how much crude tissue preparation should be applied to an assay system. An easy and simple assay procedure should be developed in order to measure unknown activities of crude cathepsins in tissue homogenates.

In the present paper, we describe a simple assay system for determining cathepsin B, H and L activities in various tissue homogenates of rats. Furthermore, a method for serum cathepsin H activity, which has not been reported before, is also presented here. These procedures can be applied to the investigation of changes in the enzyme activities in rats with severe liver injury.

Materials and Methods

Three synthetic NMec substrates for cathepsin B, H and L, N-calbombenzoxyl-L-arginyl-L-arginine-NMec (Z-Arg-Arg-NMec), L-arginyl-NMec (Arg-NMec) and N-calbombenzoxyl-L-phenylalanyl-L-arginine-NMec (Z-Phe-Arg-NMec), were purchased from Peptide Institute, Inc. (Osaka). Leupeptin was obtained from Sigma Chemical Co. (St. Louis), and Brij-35 from Nakarai Chemical Co. (Kyoto).

Male Sprague-Dawley rats, weighing 200-300 g, were fasted overnight. D-galactosamine (GalN) was intraperitoneally injected at a dose of 100 mg/100 g body weight, and the rats were sacrificed by exsanguination 24 h following the treatment. Tissues were homogenized with a polytron homogenizer (Type PT 10/35, Kinematica, Switzerland)
at maximum speed for 20 sec in 9 volumes of physiological saline, and centrifuged for 10 min at 1000 × g. The supernatant fractions (tissue homogenate) were diluted before use.

The assay of cathepsin B, H and L activities were performed according to the method previously described by Barrett (5). The incubation mixture contained 250 μl of buffer (0.2 M phosphate buffer, pH 6.8, for cathepsin B; 0.4 M phosphate buffer, pH 6.8, for cathepsin H, and 0.4 M acetate buffer, pH 5.5, for cathepsin L), 1.33 mM disodium EDTA for cathepsin B or 4.0 mM for cathepsin H and L, and 2.7 mM cysteine (free base) for cathepsin B or 40 mM cysteine (monobase) for both cathepsin H and L. Each enzyme solution (tissue homogenate) was diluted to 500 μl with 0.1% Brij-35, and the mixture was preincubated for 1 min at 30°C, followed by adding 250 μl of the respective substrate (20 μM). After incubating at 30°C for exactly 10 min, the reaction was terminated by adding 1 ml of the stopping reagent containing 100 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid. A fluorometric spectrophotometer (Hitachi 650-10 type, Tokyo) was used to read the fluorescent intensity of the free NMec by excitation at 370 nm and emission at 460 nm. A fluorescent intensity of 0.05 M NMec was defined as 10 units of enzyme activity. Protein concentrations were determined according to Lowry et al. (6). Specific enzyme activity in tissue homogenates was expressed as 10−7 mU/mg protein.

The data obtained were expressed as the mean ± SD and compared statistically by Student’s t-test.

Results

Assay of cathepsin B and L activities in tissue homogenates. Ten to 100 μl volumes of a liver homogenate were diluted to 500 μl with 0.1% Brij-35 and used as the enzyme source for the cathepsin L assay. The fluorescent intensity of NMec after 10 min incubation curvilinearly increased up to 25 μl of liver homogenate (Fig. 1), and then decreased even as the sample volume increased. The activities were inhibited to approximately 80% by adding 0.5 μM leupeptin, which is a specific inhibitor of cathepsin B and L (Fig. 1A). Therefore, less than 10 μl of the homogenate (the actual sample used in the assay was the homogenate diluted 50 times) was applied to the assay system, in which leupeptin was included as
the blank (Fig. 1B). Linear increases in fluorescent intensity were observed up to 2.5 μl of homogenate in both samples with low or high enzyme activity (Fig. 2). A proportional increase in the activity was recognized depending upon the length of incubation time (inset of Fig. 2). The activities were not changed by treating the samples with Triton X-100 or extensive freezing and thawing (data not shown). From these results, the assay system of cathepsin L in tissue homogenates was set as follows: a) the enzyme sample was 1 μl of tissue homogenate (actual volume in the assay was 500 μl of the homogenate diluted 500 times), b) the incubation time was 10 min, and c) 0.5 μM leupeptin was included as the blank.

Have the same property as cathepsin L with respect to leupeptin inhibition. A proportional increase in the activity was observed up to 5 μl of homogenate (data not shown), and, therefore, 2.5 μl of the sample (actual volume was 500 μl of 200-fold-diluted homogenate) was used in the following assay.

Assay of serum and tissue cathepsin H activities. A linear increase in cathepsin H activities was observed up to 50 μl of serum and 5 μl of tissue homogenate prepared in the same way as described above (Fig. 3).

![Graph A](image-url)

**Fig. 2** Effects of various sample volumes of liver homogenates on cathepsin L activity. Small volumes (0.5–2.5 μl of liver homogenate: actual sample volumes were 100 to 500 μl of 200-times-diluted liver homogenate) were used as samples. Two samples with low (O—O) or high (●—●) cathepsin activity were tested. Leupeptin was used as described in Fig. 1. The slit width for excitation and emission was 8 nm, and sensitivity 0.3. Effect of incubation time on the activity of 1 μl of liver homogenate is shown in the inset.

Cathepsin B has already been shown to

![Graph B](image-url)

**Fig. 3** Effects of sample volumes of serum (A) and liver homogenate (B) on cathepsin H activity. The reaction was terminated after 10 min incubation. Various sample volumes of 10-times-diluted serum or 100-times-diluted liver homogenate were tested, and proportional increases in the activity were recognized both in serum and liver homogenate (undiluted sample volumes are shown in this figure). The assay conditions were the same as described in Fig. 2.

An inhibitory effect of leupeptin on cathepsin H activities was not recognized. Therefore, a blank was prepared for each sample by withholding the substrate until addition of
the stopper. The assay was conducted with 25 μl of serum (actual volume for assay was 500 μl of 20-times-diluted serum) and 2.5 μl of tissue homogenate (500 μl of 200-times-diluted sample), and both with 10 min incubation.

**Cathepsin B and H activities in the various organs.** Distribution of cathepsin B and H activities in liver, spleen, kidney, lung, heart and skeletal muscle from overnight-starved rats was investigated (Table 1). The highest levels of cathepsin B and H activities were seen in kidney. Considerably high cathepsin B activity was observed in liver and spleen, but low activity was observed in other tissues. Cathepsin H activities were similar in heart, spleen and lung, and low in liver and skeletal muscle. A high ratio of cathepsin B/cathepsin H activity (B/H in the table) was observed in liver, and a low ratio was observed in heart, lung and skeletal muscle, suggesting that the different tissues showed specific distribution patterns of those cathepsins.

**Liver cathepsin B, H and L activities and serum cathepsin H activity in GalN-treated rats.** Liver cathepsin L and H activities 24 h following GalN administration were significantly diminished, and cathepsin B activity also tended to decrease. A marked elevation of serum cathepsin H activities was recognized in GalN-treated rats concomitantly with a decrease in the tissue activities, indicating release of this enzyme from the injured hepatocytes to the circulating blood due to extensive hepatic necrosis (Table 2).

### Table 1 Cathepsin B and H activities in various rat organs

<table>
<thead>
<tr>
<th></th>
<th>Cathepsin B (4)</th>
<th>Cathepsin H (4)</th>
<th>B/H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>23.8 ± 2.5</td>
<td>11.8 ± 3.3</td>
<td>2.15</td>
</tr>
<tr>
<td>Kidney</td>
<td>52.5 ± 9.5</td>
<td>54.3 ± 2.5</td>
<td>0.97</td>
</tr>
<tr>
<td>Heart</td>
<td>5.0 ± 2.2</td>
<td>25.8 ± 5.6</td>
<td>0.19</td>
</tr>
<tr>
<td>Spleen</td>
<td>22.8 ± 5.1</td>
<td>26.5 ± 7.4</td>
<td>0.87</td>
</tr>
<tr>
<td>Lung</td>
<td>5.5 ± 1.0</td>
<td>21.8 ± 4.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.8 ± 0.5</td>
<td>12.8 ± 3.6</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values (×10⁻² mU/mg protein) are expressed as the mean ± SD.

( ) = No. of rats.

### Table 2 Cathepsin L, B and H activities in GalN-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control (3)</th>
<th>GalN (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>69.3 ± 15.8</td>
<td>31.4 ± 5.6</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>11.0 ± 4.4</td>
<td>4.6 ± 1.5</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>3.7 ± 0.6</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>124 ± 20</td>
<td>218 ± 63</td>
</tr>
</tbody>
</table>

The activities were determined 24 h after GalN administration. Data are expressed as the mean ± SD (×10⁻² mU/mg protein for liver and mU/l for serum).

*: p < 0.05. **: p < 0.01. NS: Not significant.

( ) = No. of rats.

**Discussion**

The assay procedures of cathepsin activities in tissues have been established and are generally used for analyzing the biochemical properties of the enzymes (7, 8). However, it is difficult to measure the activities due to lack of details in these reports, i.e., the volume of enzyme solutions with unknown activity that should be applied to the assay...
system was not indicated. The present study clearly showed that the volume of tissue homogenate (1:10) in Barrett's system should be less than 5 μl. Diminution of the activities by using more than this volume may be due to strong inhibition by endogeneous inhibitors (8) in tissue homogenates. As previously suggested, Z-Phe-Arg-NMec is hydrolyzed by plasma and tissue kallikreins and other trypsin-like enzymes (5). Therefore, it seems likely that complete inhibition of cathepsin B and L activities is not obtained by leupeptin, and, thus, its addition to blank tubes is needed in order to measure the true activities.

Measurement of serum cathepsin activities is generally believed to be impossible because of severe inhibition by endogeneous inhibitors in serum (10). However, Pietras (11) has reported elevated serum cathepsin B activity in patients with gynecological malignancy, and increased serum cathepsin B-like activity in patients with hepatocellular carcinoma was recently described by Dufek (12). However, we found in the present study serum cathepsin B measurement using Z-Arg-Arg-NMec as the substrate was impossible and serum cathepsin H activity could be determined easily. Since leupeptin does not strongly affect this activity, it would be better to designate it as cathepsin H-like activity at the present until a specific inhibitor is identified. Recent advances in histochemical analysis have revealed that cathepsin H exists in the plasma membrane (13), suggesting the possibility of easy release of this enzyme into the circulating blood.

The tissue distribution pattern of cathepsin B and H analyzed in our assay system is nearly identical with that of other assay systems (14) in which the activities of cathepsin B and H in kidney are 3-times higher than those in liver and spleen. Alterations in cathepsin activities in injured livers have not been reported yet. Diminished levels of cathepsin activity in GalN-treated rats were confirmed by determining the activity in purified soluble lysosomes obtained by Garab's method (15) (data not shown). Furthermore, we observed a marked increase in serum cathepsin H activity in patients with fulminant hepatitis. Therefore, increases in serum cathepsin H activities and decreases in liver cathepsin activities directly reflect the degree of hepatic cell necrosis. Serum cathepsin H measurement may be a useful diagnostic tool for liver diseases and also possibly for myocardial infarction and myodystrophic disorders.

Thiol proteases have been documented to be involved in malignant cell proliferation (16, 17), and cathepsin D, an acid protease, may be important in hepatic regeneration after partial hepatectomy (18, 19). Further investigation of the relation between cathepsin activities and cell proliferation is necessary to find a way to protect against malignant cell metastasis and promote hepatocyte regeneration in patients with fulminant hepatitis. Changes in cathepsin activities in hepatocellular carcinoma using autopsy and operation materials are under investigation in our laboratory.

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References


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