Cathepsin S

The cysteine proteinase from bovine lymphoid tissue is distinct from cathepsin L (EC 3.4.22.15)

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Cathepsin S was purified from bovine spleen by acid autolysis, (NH₄)₂SO₄ fractionation and chromatography on CM-Sephadex C-50, CM-cellulose and activated-thiol-Sepharose. Cathepsin L was isolated from lysosomal fractions of rat liver, rat kidney and bovine liver. Generally, cathepsin L was bound tightly to CM-Sephadex C-50. Preparations of cathepsin L from rat liver, rat kidney and bovine liver were shown to have kinetic constants for the substrate benzyloxycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide in the same range (Kₘ 2–3 μM). Benzyloxycarbonyl-Phe-Phe-diazomethane proved to be a sensitive irreversible inhibitor of cathepsin L from different species. Cathepsin S differed in all these characteristics from cathepsin L. A polyclonal antibody to cathepsin L from rat reacted with bovine cathepsin L but not with bovine cathepsin S.

INTRODUCTION

'Cathepsin S' was the name given to a cysteine proteinase purified from bovine lymph nodes (Turnšek et al., 1975) and from bovine spleen (Turk et al., 1980). Later this enzyme was renamed 'cathepsin L', because of its similarity (high proteolytic activity and negligible activity against Bz-Arg-NHNap) to cathepsin L (Turk et al., 1983). We have since compared some properties of cathepsin L from rat liver with those of cathepsin S from bovine spleen and concluded that the enzymes differ from each other more than cathepsin L preparations from rat, rabbit and human (Kirschke et al., 1984). We therefore suggested retaining the name cathepsin S for the enzyme from bovine spleen.

In the present paper we compare the two bovine enzymes, cathepsin S from spleen and cathepsin L from liver, with each other and with cathepsin L from rat liver and rat kidney, by biochemical and immunological criteria.

EXPERIMENTAL

Materials

Z-Phe-Phe-CHN₄ was kindly provided by Dr. E. Shaw (Friedrich Miescher Institute, Basel, Switzerland). Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, Z-Arg-NHMec and Arg-NHMec were obtained from Bachem (Torrance, CA, U.S.A.). Sephadex and Sepharose were from Pharmacia (Uppsala, Sweden) and microgranular CM-cellulose CM-52 was from Whatman (Maidstone, Kent, U.K.). Soluble complex of horseradish peroxidase with rabbit anti-peroxidase antibody was obtained from Dakopatts (Copenhagen, Denmark). Serva Blau G and papain were from Serva (Heidelberg, Federal Republic of Germany) and 3,3′-diaminobenzidine tetrahydrochloride was from Chemapol (Prague, Czechoslovakia). Nitrocellulose paper was from FMC Corp. Marine Colloids Division Bio Products (Rockland, ME, U.S.A.). Samples of human liver removed at autopsy were kindly provided by Professor Dr. Langkopf (Martin-Luther-University, Halle, German Democratic Republic).

Enzyme preparation

Cathepsin L was prepared from lysosomal fractions from rat liver, rat kidney and bovine liver as described previously for rat liver (Kirschke et al., 1977). Cathepsin B and cathepsin H were purified from a lysosomal extract of rat liver (Barrett & Kirschke, 1981). Cathepsin L from human liver was a preparation (not completely purified) from a lysosomal extract of a piece of human liver.

Cathepsin S was purified from bovine spleen. The first steps of the purification procedure were the same as described for cathepsin L from human liver (Mason et al., 1985). A 800 g batch of beef spleen was homogenized in 2 vol. of 1% NaCl/0.1% EDTA. The extract was subjected to autolysis at pH 4.2 and 4°C overnight and then to (NH₄)₂SO₄ fractionation. The 20–80% satn.-(NH₄)₂SO₄ fraction was applied to a column (4.9 cm x 110 cm) of Sephadex G-75 equilibrated with 100 mM-sodium acetate buffer, pH 5.8, containing 200 mM-NaCl and 1 mM-EDTA. The pooled active fractions (Mₑ in the range 20000–35000) were applied, after dialysis against 20 mM-sodium acetate buffer, pH 5.8, containing 1 mM-EDTA, to a column (1.25 cm x 55 cm) of CM-Sephadex C-50 equilibrated in the same buffer. The column was washed with 2 bed volumes of the buffer and then eluted with a linear gradient (0–600 mM) of NaCl in the buffer (2 x 250 ml).

Fractions eluted with 100–200 mM-NaCl (containing cathepsins B, H and S) were further purified by covalent chromatography as described by Willenbrock & Brocklehurst (1985) with the exception that activated-thiol-Sepharose was used instead of thio propioyl-Sepharose. The adsorbed active fractions were then chromatographed on CM-cellulose CM-52 as described.
by Ločnikar et al. (1981). The ion-exchange chromatography on CM-cellulose was repeated to remove traces of cathepsin B from the preparation of cathepsin S.

Fractions eluted with 300–400 mm-NaCl from the CM-Sephadex C-50 column exhibited activity against azocasein in the presence of urea. They were pooled and concentrated by ultrafiltration on a YM-10 membrane (Amicon).

The enzyme preparations were stored in 100 mm-sodium acetate buffer, pH 5.0, containing 0.5 mm-HgCl₂ and 1 mm-EDTA at −20 °C.

**Gel electrophoresis**

SDS/polyacrylamide-gel electrophoresis was performed with separating gels of 12.5% polyacrylamide (2.6% of this total concentration being methylenebis-acrylamide) in a buffer system described by Barrett et al. (1979). Reference proteins were: serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor, myoglobin and insulin A chain. Proteins were stained with Serva Blau G.

**Enzyme assays**

All assays were generally as described by Barrett & Kirschke (1981). The reaction mixtures were buffered at pH 5.5 or 5.0 as stated with 100 mm-sodium acetate buffer and activated for 5 min at 30 °C with 2.5 mm-dithiothreitol and 2.5 mm-EDTA before incubation with the substrate.

Cathepsins L and S were routinely assayed with azocasein (1%, w/v) as substrate in the presence of 3 mm-urea and 0.5 mm-pepsatin at pH 5.0. The reactions (in 0.2 ml) were stopped after 10 or 15 min at 40 °C with 0.2 ml of 10% (w/v) trichloroacetic acid.

Cathepsin B was routinely assayed with Bz-Arg-NHNap and cathepsin H with Bz-Arg-NHNap and Lys-NHNap (Barrett & Kirschke, 1981). Papain was assayed with Bz-Arg-NHMec at pH 6.8.

Kₘ values were determined by the direct linear plot (Eisenthal & Cornish-Bowden, 1974) and the method of Wilkinson (1961). Enzymes, dithiothreitol and EDTA in 100 mm-sodium acetate buffer, pH 5.5, and 0.005% Brij-35 were preincubated for 5 min at 30 °C before the start of the reaction by addition of Z-Phe-Arg-NHMec as substrate in a concentration range of 0.8–40 μM with cathepsin L and 16–200 μM with cathepsin S. The reaction velocity was recorded by an Eppendorf fluorimeter with excitation at 313–366 nm and emission at 430–470 nm.

**Protein and enzyme concentration**

Protein was determined by a micro modification of the Lowry method (Langner et al., 1971), with bovine serum albumin as standard.

The concentration of active molecules of cathepsins L, S and B was determined by active-site titration with E-64, with Z-Phe-Arg-NHMec as substrate (Barrett & Kirschke, 1981).

**Inhibition studies**

The rate constant for the inhibition of cathepsin S by Z-Phe-Phe-CHN₄ was measured by the following procedure. Cathepsin S was preincubated with 2 mm-dithiothreitol, 2 mm-EDTA and Z-Phe-Phe-CHN₄ (1–5 μM final concentration). At various times, samples were withdrawn and diluted for assay with 10 μM-Z-Phe-Arg-NHMec as substrate to determine the extent of enzyme inactivation. The half-time of inactivation was used to calculate the pseudo-first-order rate of inactivation kₐbs. = 0.693/τₛ. The apparent second-order rate constant for inactivation was kₐbs./[I].

**Immunological methods**

An antiserum against rat cathepsin L was raised in rabbits. The IgG fraction of the antiserum was prepared by the procedure of Steinbuch & Audran (1969). The titre of the IgG fraction was determined by measurement of the activity of cathepsin L not precipitated with the antibody. The IgG fraction did not inhibit papain. We therefore concluded that it did not contain traces of cysteine proteinase inhibitors present in the plasma. The reaction of the antibody (to rat cathepsin L) with enzymes of different species was detected by use of dot immunoblots, in which the antigen in a fixed concentration was adsorbed on nitrocellulose paper and treated with the following proteins: (1) skim milk (12%, w/v) for blocking the nitrocellulose; (2) rabbit IgG containing the antibody to rat cathepsin L (overnight); (3) pig anti-(rabbit IgG) antibody (30 min); (4) soluble complex of horseradish peroxidase and rabbit anti-peroxidase antibody (30 min); (5) 3.3'-diaminobenzidine (0.05%) and H₂O₂ (0.015%) (30 s). The paper was washed (five times) between the steps with buffered 0.1% Triton X-100.

**RESULTS AND DISCUSSION**

**Behaviour of the enzymes on CM-Sephadex C-50**

In the preparation from bovine spleen, the main azocasein-hydrolysing activity was eluted at only 100–200 mm-NaCl from CM-Sephadex C-50 together with cathepsins B and H. The enzyme responsible for this activity against azocasein (in the presence of urea and pepsatin) was subsequently purified by chromatography on thiol-Sepharose and CM-cellulose, and characterized as cathepsin S by the ratio of the activities with Z-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec as substrates (cathepsin S, 1:1.6; cathepsin L, 1:0.05; Kirschke et al., 1984), by the inhibition with 0.5 μM-Z-Phe-Phe-CHN₄ (after 10 min incubation cathepsin S about 50% and cathepsin L about 100% inhibition), and by the negligible reaction with an antibody to cathepsin L.

Only traces of the azocasein-hydrolysing activity could be eluted from the ion-exchanger at 300–400 mm-NaCl, and this was identified by immunological methods as cathepsin L from bovine spleen.

In contrast, the main proteolytic activity due to cathepsin L of a fraction from bovine liver was eluted at 300–400 mm-NaCl, and only traces could be detected in fractions containing cathepsins B and H.

Preparations of cathepsin L from different organs and species such as rat liver and kidney, rabbit liver, pig spleen and human liver have already been characterized by their tight binding to CM-Sephadex C-50 (Kirschke et al., 1977; Mason et al., 1984; Lynn & Labow, 1984; Mason et al., 1985). Cathepsin S differed in this property from cathepsin L.

**Yields of the enzymes**

Only 0.2 mg of cathepsin S could be obtained from 1 kg of bovine spleen. The amount of cathepsin L was
Action of the enzymes on synthetic substrates

Table 1 shows that the preparations of cathepsins S and L were contaminated neither by cathepsin B (substrate Z-Arg-Arg-NHMec) nor by cathepsin H (substrate Arg-NHMec). It can be seen that Z-Phe-Arg-NHMec is a very sensitive substrate of cathepsin L, but not of cathepsin S.

Cathepsin S differed clearly from all the preparations of cathepsin L in its $K_m$ of 42 $\mu M$ with Z-Phe-Arg-NHMec as substrate (Table 2). For this substrate, the $K_m$ of cathepsin L from rat liver and kidney and bovine liver was in the range of 2–3 $\mu M$ (Table 2).

The same low $K_m$ has been reported for cathepsin L from human and rabbit liver (Mason et al., 1985).

Z-Lys-4-nitrophenyl ester was hydrolysed by both cathepsin S and cathepsin L with a $K_m$ in the range 9–12 $\mu M$. The hydrolysis of this substrate is a property that is shared by many other proteinases and therefore cannot be regarded as a distinctive characteristic for cathepsins S and L.

Further similarities in catalytic properties of the enzymes are negligible activity against Bz-Arg-NHnap and similar proteolytic activity against azocasein in the presence and in the absence of urea (Kirschke et al., 1984).

Irreversible inhibition by Z-Phe-Phe-CHN$_2$

Z-Phe-Phe-CHN$_2$, a rapid irreversible inhibitor of cathepsin L, proved not to be very effective on cathepsin S. Cathepsins L, S and B were preincubated with pH 5.0 buffer alone, 0.5 $\mu M$-Z-Phe-Phe-CHN$_2$ and 5.0 $\mu M$-Z-Phe-Phe-CHN$_2$ respectively in the presence of 1 mM-dithiothreitol and 1 mM-EDTA for 10 min at 30°C before the assay with 5 $\mu M$-Z-Phe-Arg-NHMec. Inhibition after 10 min by 0.5 $\mu M$-Z-Phe-Phe-CHN$_2$ was 96–99% for cathepsin L, 51–58% for cathepsin S and 1–4% for cathepsin B. After the same incubation time, 5 $\mu M$ inhibitor had caused an inhibition of 100% of cathepsin L, 86–90% of cathepsin S and 28–30% of cathepsin B. This made it impossible to assay both cathepsin S and cathepsin B in mixtures, as has been
Table 2. Kinetic constants with Z-Phe-Arg-NHMec as substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin L, rat liver</td>
<td>23</td>
<td>2.0</td>
<td>11.50</td>
</tr>
<tr>
<td>Cathepsin L, rat kidney</td>
<td>17</td>
<td>2.5</td>
<td>6.80</td>
</tr>
<tr>
<td>Cathepsin L, bovine liver</td>
<td>18</td>
<td>3.0</td>
<td>6.00</td>
</tr>
<tr>
<td>Cathepsin S, bovine spleen</td>
<td>7</td>
<td>42.0</td>
<td>0.17</td>
</tr>
</tbody>
</table>

The reaction velocity was recorded at 30 °C and pH 5.5 with Z-Phe-Arg-NHMec as substrate in a concentration range 0.8–40 µM for cathepsin L and 16–200 µM for cathepsin S.

The apparent second-order rate constant for Z-Phe-Phe-CHN$_2$ was measured to be approx. 900 m$^{-1}$s$^{-1}$ for cathepsin S from bovine liver.

The values were determined to be 136,000 m$^{-1}$s$^{-1}$ for human cathepsin L (Mason et al., 1985) and 160,000 m$^{-1}$s$^{-1}$ for rat cathepsin L (Barrett et al., 1982). The apparent second-order rate constant of inhibition by Z-Phe-Phe-CHN$_2$ was initially reported to be as high as 660,000 m$^{-1}$s$^{-1}$ for rat liver cathepsin L (Kirschke & Shaw, 1981), but this erroneous value possibly included an additional inactivation of the enzyme by the assay temperature of 40 °C. The value of approx. 900 m$^{-1}$s$^{-1}$ for cathepsin S is more like the value of 193 m$^{-1}$s$^{-1}$ determined for bovine cathepsin B by Leary & Shaw (1977).

**Reactions with an antibody against cathepsin L**

The IgG fraction containing an antibody against cathepsin L from rat liver had a concentration of 10 mg of protein/ml and a titre of 0.98 µg of precipitated cathepsin L/mg of IgG. This antibody was used for all experiments described here. In Ouchterlony double-diffusion tests the antibody precipitated cathepsin L from rat liver and rat kidney, but no precipitation could be detected with cathepsin L (bovine), cathepsin S (bovine), cathepsin H (rat), cathepsin B (rat) or papain.

By use of the very sensitive method of immuno-dotting on nitrocellulose, a reaction of the antibody also with preparations of cathepsin L from different species was shown (Fig. 2).

In Fig. 2 a positive reaction of the antibody can be seen with cathepsin L from rat and bovine in amounts of 50–100 ng and with cathepsin L from human liver, thus showing the close relationship of cathepsin L from different species. In contrast, 2 µg of cathepsin S did not react with the antibody against cathepsin L under the given conditions, which shows that cathepsin S is different from cathepsin L.

**CONCLUSIONS**

Cathepsin L and cathepsin S are similar proteinases with regard to their high activity against azocasein and their stability to 3 M-urea in the presence of this substrate. They hydrolyse Z-Lys 4-nitrophenyl ester, but exhibit no activity against Bz-Arg-NHNap. The two enzymes differ, however, in some properties that are characteristic for cathepsin L from several species: high activity against Z-Phe-Arg-NHMec ($K_m$ approx. 2 µM), sensitivity to inhibition by Z-Phe-Phe-CHN$_2$ and reaction with an antibody to cathepsin L in a certain concentration range. Therefore it can be concluded that cathepsin S is a separate enzyme from cathepsin L, and should continue to have a different name.

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**REFERENCES**


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