Cathepsin S

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

Heidrun KIRSCHKE, Ingeburg SCHMIDT and Bernd WIEDERANDERS

Institute of Biochemistry, Medical Faculty, Martin-Luther-University, DDR-4020 Halle (Saale), German Democratic Republic

Cathepsin S was purified from bovine spleen by acid autolysis, $(\text{NH}_4)_2\text{SO}_4$ 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 (K_m 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-CHN₂ 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). Nitro-cellulose 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 $(\text{NH}_4)_2\text{SO}_4$ fractionation. The 20–80% -satn. $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a column (4.9 cm $\times$ 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 (M_r 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 $\times$ 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 $\times$ 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 thiopropyl-Sepharose. The adsorbed active fractions were then chromatographed on CM-cellulose CM-52 as described

Abbreviations used: Z-, benzyloxycarbonyl-; Bz-, benzyl-; -NHmec, 4-methyl-7-coumarylamide; -NHnap, 2-naphthylamide; -CHN₂, -diazomethane; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane.

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 methylenebisacrylamide) 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 M-urea and 0.5 μ M-pepstatin 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-NH₂ and cathepsin H with Bz-Arg-NH₂ and Lys-NH₂ (Barrett & Kirschke, 1981). Papain was assayed with Bz-Arg-NHMec at pH 6.8.

K_m 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 μ M with cathepsin L and 16–200 μ M 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-CHN₂ was measured by the following procedure. Cathepsin S was preincubated with 2 mM-dithiothreitol, 2 mM-EDTA and Z-Phe-Phe-CHN₂ (1–5 μ M final concentration). At various times, samples were withdrawn and diluted for assay with 10 μ M-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 $k_{obs.} = 0.693/t_{0.5}$. The apparent second-order rate constant for inactivation was $k_{obs.}/[I]$.

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 together with cathepsins B and H. The enzyme responsible for this activity against azocasein (in the presence of urea and pepstatin) 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 μ M-Z-Phe-Phe-CHN₂ (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

Fig. 1. SDS/polyacrylamide-gel electrophoresis of cathepsin L and cathepsin S

SDS/12.5%-(w/v)-polyacrylamide-gel electrophoresis was used for the separation of: lane 1, standard; lane 2, cathepsin L, unreduced; lane 3, cathepsin L, reduced; lane 4, cathepsin S, unreduced; lane 5, cathepsin S, reduced; lane 6, standard. Approx. 6 μ g of each enzyme was applied.

approx. 2 mg from 1 kg of rat liver, 3–4 mg from 1 kg of rat kidney and 0.8 mg from 1 kg of bovine liver.

M_r values

Cathepsin S appeared as a single protein with M_r in the same range as the heavy chain of cathepsin L on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions (M_r 24000) (Fig. 1).

Data previously reported on the M_r of cathepsin S as 24000 and the single-chain structure of the enzyme (Turk *et al.*, 1980) were thus confirmed. The M_r of purified preparations of cathepsin L from bovine liver (Fig. 1), rat liver (Kirschke *et al.*, 1984), rabbit liver (Mason *et al.*, 1984) and human liver (Mason *et al.*, 1985) was approx.

29000 and it was shown that the molecule consisted of two chains (M_r 24000–25000 and 5000) linked by disulphide bonds.

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 μ 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 μ 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 μ 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-CHN₂

Z-Phe-Phe-CHN₂, 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 μ M-Z-Phe-Phe-CHN₂ and 5.0 μ M-Z-Phe-Phe-CHN₂ respectively in the presence of 1 mM-dithiothreitol and 1 mM-EDTA for 10 min at 30 °C before the assay with 5 μ M-Z-Phe-Arg-NHMec. Inhibition after 10 min by 0.5 μ M-Z-Phe-Phe-CHN₂ was 96–99% for cathepsin L, 51–58% for cathepsin S and 1–4% for cathepsin B. After the same incubation time, 5.0 μ 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 1. Activities of cathepsin L and cathepsin S

One unit corresponds to 1 μ mol of substrate degraded in 1 min at 30 °C. The concentration of the enzymes was determined by titration with E-64. Substrate concentration in the assays was 10 μ M, or 100 μ M where indicated by the asterisk. Abbreviation: N.D., not determined.

		Activity (unit/ μ mol)		
		Z-Phe-Arg-NHMec	Z-Arg-Arg-NHMec	Arg-NHMec
Cathepsin L, rat liver	1180		1.06	< 0.1
Cathepsin L, rat kidney	1120		0.22	0.3
Cathepsin L, bovine liver	1080		0.11	0.1
Cathepsin S, bovine spleen	78		0.50	< 0.1
Cathepsin S, bovine spleen*	285		N.D.	N.D.

Table 2. Kinetic constants with Z-Phe-Arg-NHMec as substrate

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.

	$k_{\text{cat.}}$ (s^{-1})	K_{m} (μM)	$k_{\text{cat.}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)
Cathepsin L, rat liver	23	2.0	11.50
Cathepsin L, rat kidney	17	2.5	6.80
Cathepsin L, bovine liver	18	3.0	6.00
Cathepsin S, bovine spleen	7	42.0	0.17

done successfully with cathepsin L and cathepsin B (Mason *et al.*, 1985).

The apparent second-order rate constant for Z-Phe-Phe-CHN₂ was measured to be approx. 900 $\text{M}^{-1} \cdot \text{s}^{-1}$ for cathepsin S from bovine spleen.

The values were determined to be 136000 $\text{M}^{-1} \cdot \text{s}^{-1}$ for human cathepsin L (Mason *et al.*, 1985) and 160000 $\text{M}^{-1} \cdot \text{s}^{-1}$ for rat cathepsin L (Barrett *et al.*, 1982). The apparent second-order rate constant of inhibition by Z-Phe-Phe-CHN₂ was initially reported to be as high as 660000 $\text{M}^{-1} \cdot \text{s}^{-1}$ 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 $\text{M}^{-1} \cdot \text{s}^{-1}$ for cathepsin S is more like the value of 193 $\text{M}^{-1} \cdot \text{s}^{-1}$ 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

Fig. 2. Reaction of an antibody to cathepsin L with different antigens

The antigens were bound to nitrocellulose: 1, 50 ng of rat cathepsin L; 2, 100 ng of bovine cathepsin L; 3, 2 μg of bovine cathepsin S; 4, human cathepsin L (not completely purified); 5, blank. The antibody to rat cathepsin L was diluted 1:100. The reaction was performed as described in the Experimental section.

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-CHN₂ 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.

We are very grateful to Dr A. J. Barrett (Strangeways Research Laboratory, Cambridge, U.K.) for his help in the preparation of this manuscript, and we thank Mrs. U. Broghammer and Mrs. C. Rupf for their expert technical assistance.

REFERENCES

- Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561
- Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418
- Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. & Hanada, K. (1982) *Biochem. J.* **201**, 189–198
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
- Kirschke, H. & Shaw, E. (1981) *Biochem. Biophys. Res. Commun.* **101**, 454–458
- Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. & Bohley, P. (1977) *Eur. J. Biochem.* **74**, 293–301
- Kirschke, H., Ločnikar, P. & Turk, V. (1984) *FEBS Lett.* **174**, 123–127
- Langner, J., Ansorge, S., Bohley, P., Kirschke, H. & Hanson, H. (1971) *Acta Biol. Med. Germ.* **26**, 935–951
- Leary, R. & Shaw, E. (1977) *Biochem. Biophys. Res. Commun.* **79**, 926–931
- Ločnikar, P., Popovič, T., Lah, T., Kregar, I., Babnik, J., Kopitar, M. & Turk, V. (1981) in *Proteinases and their Inhibitors: Structure, Function and Applied Aspects* (Turk, V. & Vitale, L., eds.), pp. 109–116, Mladinska Knjiga, Ljubljana
- Lynn, K. R. & Labow, R. S. (1984) *Can. J. Biochem. Cell Biol.* **62**, 1301–1308
- Mason, R. W., Taylor, M. A. J. & Etherington, D. J. (1984) *Biochem. J.* **217**, 209–217
- Mason, R. W., Green, G. D. J. & Barrett, A. J. (1985) *Biochem. J.* **226**, 233–241
- Steinbuch, M. & Audran, R. (1969) *Arch. Biochem. Biophys.* **134**, 279–284
- Turk, V., Kregar, I., Popovič, T., Ločnikar, P., Kopitar, M. & Brzin, J. (1980) *Period. Biol.* **82**, 363–368

- Turk, V., Brzin, J., Kopitar, M., Kregar, I., Ločnikar, P., Longer, M., Popovič, T., Ritonja, A., Vitale, L., Machleidt, W., Giraldi, T. & Sava, G. (1983) in *Proteinase Inhibitors, Medical and Biological Aspects* (Katunuma, N., Umezawa, H. & Holzer, H., eds.), pp. 125–134, Japan Scientific Societies Press, Tokyo
- Turnšek, T., Kregar, I. & Lebez, D. (1975) *Biochim. Biophys. Acta* **403**, 514–520
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Willenbrock, F. & Brocklehurst, K. (1985) *Biochem. J.* **227**, 511–519

Received 24 April 1986/30 June 1986; accepted 13 August 1986