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Action of rat liver cathepsin L on collagen and other substrates

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1. It has been found that cathepsin L is very susceptible to loss of activity through autolysis. When this is prevented by purification and storage of the enzyme as its mercury derivative, preparations are obtained with higher specific activity than previously. 2. Active-site titration shows, however, that even the new purification method does not give preparations in which the enzyme is 100% active. 3. Benzyloxycarbonylphenylalanylarginine 7-(4-methyl)coumarylamide has been discovered to be a very sensitive substrate for cathepsin L. Like all other known substrates for cathepsin L, however, it is also cleaved by cathepsin B. 4. Cathepsin L degrades insoluble collagen at pH 3.5 over 5-fold faster than at pH 6.0. The specific activity at pH 3.5 is 5–10-fold higher than that of cathepsin B (rat or human) or bovine spleen cathepsin N ('collagenolytic cathepsin'). 5. Qualitatively, the action of cathepsin L on collagen is similar to that of cathepsins B and N, i.e. selective cleavage of terminal peptides leads to conversion of β- and higher components mainly to α-chains.

It has long been known that rat liver lysosomes contain a cysteine proteinase that has little or no action on the synthetic substrates usually used for such enzymes (Bohley *et al.*, 1971; Kirschke *et al.*, 1972). The enzyme has been named 'cathepsin L' (EC 3.4.22.15) (Kirschke *et al.*, 1976), and subsequently purified and further characterized (Kirschke *et al.*, 1977*a*; DeMartino *et al.*, 1977; Towatari *et al.*, 1978). We have now found that cathepsin L prepared as described previously tends to be contaminated with autolysis products, and we have devised a method to prevent this.

Cathepsin L purified by the improved method and standardized by active-site titration has been used to re-examine the action of the enzyme on synthetic substrates and proteins. A useful new synthetic substrate has been discovered, and the relationship of cathepsin L to cathepsin N has been somewhat clarified.

Abbreviations used: The abbreviations used for amino acid derivatives and *N*-terminal groups are based on the standard conventions [*Biochem. J.* (1972) **126**, 773–780]. The *C*-terminal groups are: CHN_2, diazomethane; NH_2, amide; NMec, 7-(4-methyl)coumarylamide; NNap, 2-naphthylamide; $NPhNO_2$, nitroanilide; $OPhNO_2$, nitrophenyl ester. E-64, L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Ep-475, L-*trans*-epoxysuccinyl-L-leucylamido(3-methyl)butane; SDS, sodium dodecyl sulphate.

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Experimental

Materials

Ultrogel AcA 54 was obtained from LKB Instruments Ltd. 2-Amino-2-methylpropane-1,3-diol (Ammediol) was from Cambrian Chemicals, Croydon CR0 4XB, Surrey, U.K. Collagen from bovine achilles tendon was obtained from Worthington. Acid-soluble collagen from rat tail tendon was the gift of Dr. M. Iwig, and azocasein the gift of Dr. J. Langner, both of the Physiologisch-chemisches Institut, Halle, Germany. Z-Arg-Arg-2-NNap was given by Dr. C. G. Knight, Strangeways Laboratory, Cambridge, U.K., and Z-Lys-$OPhNO_2$ and Z-Phe-Phe-CHN_2 were the gifts of Dr. E. N. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A. Z-Phe-Arg-NMec and Z-Arg-Arg-NMec were from Cambridge Research Biochemicals, Huntingdon, Cambridgeshire PE18 6DP, U.K. Z-Arg-Arg-NMec was also synthesized and given by Dr. D. C. Sunter, Strangeways Laboratory, Cambridge, U.K. E-64 and Ep-475 were kindly given by Dr. K. Hanada, Taisho Pharmaceutical Co., Saitama, Japan. Sephadex and Sepharose were from Pharmacia.

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Methods

Preparation of cathepsin L. A light mitochondrial/lysosomal fraction was prepared from livers of 100 male Wistar rats exactly as described previously (Bohley *et al.*, 1969). The fraction (50ml) was homogenized with 2.5vol. of water by ten passes of a Teflon/glass homogenizer (40μm clearance) at 0°C, and centrifuged at 360000$g_{max.}$ for 45min. (These conditions favour solubilization of lysosomal rather than mitochondrial proteins.) The supernatant was concentrated in an Amicon Diaflo apparatus over a UM-10 membrane to 15–20ml.

The enzyme was then run on a column of Sephadex G-75 (Kirschke *et al.*, 1977*a*) and the pool of active fractions was dialysed against 0.01M-potassium phosphate buffer, pH5.8, containing 0.1mM-$HgCl_2$ and 0.2mM-EDTA. Further chromatography on CM-Sephadex C-50 was also as described by Kirschke *et al.* (1977*a*). (Much better separations of cathepsins B and L have been obtained with CM-Sephadex than CM-cellulose in this step.) The next step was chromatography on concanavalin A–Sepharose; the active fractions from CM-Sephadex were concentrated and equilibrated with 20mM-sodium phosphate buffer, pH6.0, containing 0.2M-NaCl, 1mM-$CaCl_2$ and 1mM-$MnCl_2$, by ultrafiltration, and run on a column (15cm × 0.9cm, 9.5cm^3) of concanavalin A–Sepharose equilibrated with the same buffer. The column was eluted with 5 bed vol. of starting buffer and then with a gradient (10 bed vol.) of 0–100mM-methyl D-mannoside. Most (70–90%) of the applied activity of cathepsin L was adsorbed, and eluted with 25mM-methyl mannoside. The active fractions were concentrated and then equilibrated with 0.1M-sodium acetate, pH5.0, containing 0.5mM-$HgCl_2$ and 1mM-EDTA, by ultrafiltration.

Preparation of cathepsins B and H. Cathepsins B and H were purified from rat liver lysosomes (Kirschke *et al.*, 1972, 1976, 1977*b*) and human liver (Barrett, 1973; Schwartz & Barrett, 1980) as previously described, and were stored in the acetate/EDTA/$HgCl_2$ buffer used for cathepsin L.

Gel electrophoresis. Samples were made 1% (w/v) with respect to SDS and 0.5% with respect to 2-mercaptoethanol, heated at 100°C for 5min and run in slab gels containing 7 or 12.5% (w/v) polyacrylamide (2.6% of this total concentration being cross-linker) in the buffer system described by Barrett *et al.* (1979) at a current of 20mA/gel for about 2h.

Enzyme assays. The final concentrations of substrates were: Z-Arg-Arg-NNap, 1mM; Z-Lys-$OPhNO_2$, 0.1mM; azocasein, 1%, and insoluble collagen, 0.33%. Reaction mixtures were buffered at pH6.0 with 40mM-sodium phosphate buffer, at pH5.4 with 40mM-sodium acetate buffer, and at pH3.5 with 0.2M-sodium formate buffer, and contained 2mM-dithiothreitol and 1mM-EDTA. Incubation was generally for 30min at 30° or 40°C (as stated), but for Z-Lys-$OPhNO_2$ the time was 1–2min, for insoluble collagen the temperature was 37°C (see below), and for soluble collagen 24°C. Free 2-naphthylamine was determined by coupling with diazotized 4-amino-2',3-dimethylazobenzene (Barrett, 1976). The liberation of 4-nitrophenol was measured directly at 430nm. The trichloroacetic acid-soluble reaction products of azocasein were measured at 366nm (Langner *et al.*, 1973).

The assay with Z-Phe-Arg-NMec was generally as described by Barrett (1980) for cathepsin B, but the stock buffer/activator was 0.4M-sodium acetate, pH5.5, containing 8mM-dithiothreitol and 4mM-EDTA, and the incubation temperature was 30°C.

Collagenolytic activity was determined essentially as described by Etherington (1972). Bovine tendon collagen (100mg) was dispersed in 10ml of 25% glycerol/0.35% acetic acid with an Ultra-Turrax homogenizer while surrounded by ice/water, and diluted with 10ml of 0.2M-sodium formate buffer, pH3.5, containing 2mM-EDTA. Each assay tube contained 200μl of the resulting collagen suspension, 50μl of the formate buffer containing 6mM-EDTA and 6mM-dithiothreitol, and 50μl of enzyme diluted as necessary with the formate buffer. Incubation was at 37°C for 20min–4h. The mixtures were centrifuged and 100μl of each clear supernatant was transferred to a glass capillary with 100μl of conc. HCl and hydrolysed for 18h at 110°C. The hydrolysate was mixed with 1.8ml of 40mM-citrate buffer, pH6.0, which contained NaOH equivalent to the HCl. In a 200μl sample of this solution, hydroxyproline was determined by a micro-modification of the method of Woessner (1961). The A_{546} of the reaction product was read, and the standard curve was found to be linear from 0.1 to 1μg of hydroxyproline/tube.

Active-site titration of cathepsin L. The method was generally as described elsewhere (Barrett *et al.*, 1981; Barrett & Kirschke, 1982). To each of a series of tubes containing 25μl of enzyme solution (about 0.3mg/ml) and 50μl of 0.4M-sodium acetate buffer, pH5.5, was added 25μl of 1, 2, 3...10μM-E-64. After standing for 30min at 30°C, the mixtures were diluted by addition of 4.9ml of 0.1% Brij 35, and 10μl samples were assayed for activity against Z-Phe-Arg-NMec (see above). The plot of residual activity against inhibitor molarity was linear to at least 95% inactivation, and showed the molarity of the enzyme solution (intercept on the abcissa) and the specific activity of cathepsin L in the assay on a molar basis (slope).

Determination of protein. Protein concentrations were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and discussion

Purification of cathepsin L

Cathepsin L was previously purified in its active form, and stored in the presence of glutathione and EDTA at −70°C (Kirschke *et al.*, 1977*a*). The present method of isolation and storage as the inactive mercury derivative has given more stable preparations of higher specific activity. This is attributable to the prevention of autolysis (detectable by SDS/polyacrylamide-gel electrophoresis; see Fig. 1) and to the fact that affinity chromatography on concanavalin A–Sepharose became possible (yields were only 10% when this step was tried with the free-thiol form of the enzyme). Better results were obtained with Hg^{2+} as inhibitor than with tetrathionate, which has been used to stabilize cathepsin H (Schwartz & Barrett, 1980); Hg^{2+} has now been found to give excellent results with cathepsin H too.

The specific activity of cathepsin L purified by the present method (against insoluble collagen, for example) was about twice that obtained by the previous method (see below). Even so, our best preparations were found to be only 50–60% active by titration with E-64, so the specific activity calculated for fully active enzyme would be about 4-fold greater. The yield of cathepsin L remained unaltered at about 1 mg/100 rat livers used.

Action of cathepsin L on synthetic substrates

Cathepsin L differs from the related cathepsins B and H in its lack of activity towards such generally used substrates as Bz-Arg-$NPhNO_2$, Bz-Arg-NNap and Bz-Arg-OEt (Kirschke *et al.*, 1977*a*), but is active against Bz-Arg-NH_2 and Z-Lys-$OPhNO_2$ (Kirschke *et al.*, 1977*a*; Strewler & Manganiello, 1979). We have now found that it is also active against Z-Phe-Arg-NMec. The kinetic parameters of rat liver cathepsin L purified by the present method and human liver cathepsin B were determined under the conditions described under 'Methods' and are shown in Table 1. The concentrations of the enzymes were determined by titration with E-64 as described above.

The kinetic parameters for cathepsin B with Z-Arg-Arg-NNap agree closely with those determined previously by Knight (1980), when allowance is made for our method of determining effective enzyme concentration. Hydrolysis of this substrate by cathepsin L was barely detectable, and it is well suited to the assay of cathepsin B in the presence of cathepsins H and L (Kirschke *et al.*, 1980). It is now

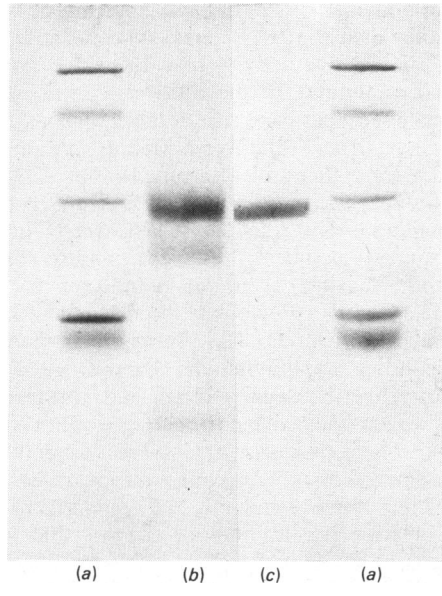

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of cathepsin L*

The samples, run on a 12.5% polyacrylamide gel slab, were: (*a*), standards (bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome *c*); (*b*), cathepsin L prepared and stored in the presence of glutathione; and (*c*), cathepsin L prepared as the mercury derivative, as described here.

Table 1. *Action of cathepsin L and cathepsin B on synthetic substrates*

Cathepsin L was isolated from rat liver as described under 'Methods', and assays were at pH 5.5 and 30°C. Cathepsin B was from human liver, and was assayed at pH 6.0, 30°C, for Z-Lys-$OPhNO_2$, and at 40°C with the methylcoumarylamides and the naphthylamide.

	Cathepsin L			Cathepsin B		
Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($10^{-3} \cdot M^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/k_m$ ($10^{-3} \cdot M^{-1} \cdot s^{-1}$)
Z-Arg-Arg-NNap	(Trace activity)			157	0.17	887
Z-Lys-$OPhNO_2$	19.8	0.010	1980	59	0.019	3084
Z-Phe-Arg-NMec	26.0	0.007	3700	1500	0.15	9868
Z-Arg-Arg-NMec	(Trace activity)			1344	0.39	3419

clear that Z-Arg-Arg-NMec is also suitable for this purpose, and is more convenient to use.

Z-Lys-OPhNO$_2$ is a sensitive (though inconvenient) substrate for cathepsins B and L; K_m is very low for both enzymes, as is to be expected in view of the efficient acylation of cysteine proteinases by such esters (Liu *et al.*, 1969).

Z-Phe-Arg-NMec proved to be an excellent new substrate for cathepsin L as well as for cathepsin B; for both enzymes $k_{cat.}/K_m$ was greater than for Z-Lys-OPhNO$_2$. It is striking, however, that K_m is 20-fold lower for cathepsin L than cathepsin B. This has the effect that, at low s values (~5 μM), the specific activities of the two enzymes are of similar order (like their $k_{cat.}/K_m$ values), whereas at higher s values the relative activity of cathepsin L is much less (corresponding to the ratio of $k_{cat.}$ values). It was this effect that gave rise to the erroneous earlier impression that Z-Phe-Arg-NMec is not a good substrate for cathepsin L (Barrett, 1980).

The much higher $k_{cat.}$ value of Z-Arg-Arg-NMec as compared with that of Z-Arg-Arg-NNap for cathepsin B confirms that the nature of the arylamide leaving group is important for this enzyme. It has previously been found that a nitroanilide is much less susceptible than the corresponding naphthylamide (Barrett, 1972), but the difference between -NNap and -NMec substrates was not detected in the earlier work of Barrett (1980); this is because $k_{cat.}$ values depended on measurements of protein concentration rather than on titration of active enzyme, and comparisons were made between preparations of enzyme that varied significantly in activity. Moreover, due allowance was not made for the strong inhibitory effect of dimethyl sulphoxide at concentrations of a few percent.

Activity towards proteins

The action of cathepsin L was studied with two protein substrates: azocasein, a widely used test substrate susceptible to most proteinases, and collagen, which is very resistant to most proteinases but is degraded in the acidic environment within lysosomes of some cells (Barrett & Saklatvala, 1981).

Azocasein. As can be seen in Table 2, azocasein proved to be far more rapidly degraded by cathepsin L than cathepsin B. The difference could be even further enhanced by the use of incubation mixtures, containing 3 M urea, at pH 5.0; this increased the activity of cathepsin L, but decreased that of cathepsin B, so that the difference in specific activities exceeded 250-fold (S. Riemann, H. Kirschke, B. Wiederanders, A. Brouwer, E. Shaw & P. Bohley, unpublished work).

Collagen. The degradation of insoluble collagen (bovine achilles tendon) at 37°C, at pH 3.5 or 6.0, was measured as described under 'Methods'. The release of hydroxyproline was linearly dependent on the incubation time and on the enzyme concentration up to 30% degradation.

As is shown in Table 2, cathepsin L was 5.6–9.0-fold more active in the solubilization of tendon collagen than was cathepsin B, and the enzymes were 5.3–8.4-fold more active at pH 3.5 than at pH 6.0.

Our cathepsin L preparation solubilized tendon collagen at 12.9 mg/min per mg of protein at pH 3.5

Table 2. *Activity of rat cathepsins L and B on azocasein and collagen*

Assays were performed as described under 'Methods', 1 unit corresponding to digestion of 1 mg of azocasein, or solubilization of 1 mg of collagen from the insoluble bovine tendon collagen (13.5% hydroxyproline).

		Specific activity (units/μmol)	
Substrate	pH	Cathepsin L	Cathepsin B
Azocasein	6.0	283	11.2
Azocasein in 3 M-urea	5.0	718	2.8
Collagen	6.0	68.7	12.3
(insoluble)	3.5	584	65.2

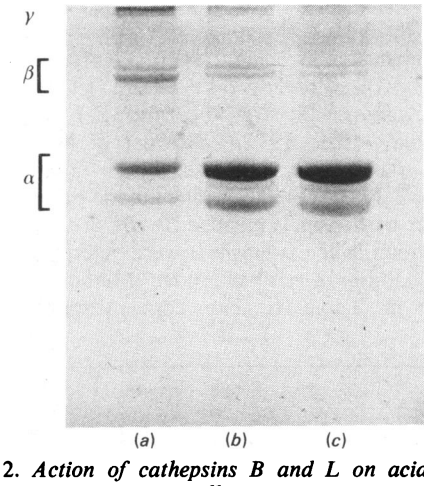

Fig. 2. *Action of cathepsins B and L on acid-soluble collagen*

The incubations (1 mg of collagen/300 μl of mixture) were for 30 min at 24°C in sodium formate buffer, pH 3.5, containing 1 mM-dithiotreitol and 1 mM-EDTA. Samples run on the 7% polyacrylamide gel in the SDS system were: (*a*), collagen control; (*b*), collagen with cathepsin B (4 μg/mg of collagen); and (*c*), collagen with cathepsin L (2.5 μg/mg of collagen) (the quantities of enzyme being determined by active-site titration).

(which corresponds to the value of 584mg/μmol of active enzyme in Table 2, after adjustment for 52.7% activity by titration, and M_r 24000). Thus the activity of rat cathepsin L is about 10-fold greater than the value of 1.16mg/min per mg reported for untitrated bovine spleen cathepsin N (Ducastaing & Etherington, 1978).

The products of cleavage of the insoluble collagen by cathepsin L were shown by SDS/polyacrylamide-gel electrophoresis to be mainly α-chains, and the nature of the action of the enzyme on collagen was further examined by use of acid-soluble collagen from rat tail tendon (Fig. 2).

Cathepsin L, like cathepsin B, caused a decrease in the amount of the cross-linked β- and higher components, as compared with the control, and an increase in the uncross-linked α-chains. Almost no fragments of lower M_r than the α-chains were detected. We concluded from these results that cathepsin L resembles cathepsins B and N (Burleigh *et al.*, 1974; Etherington, 1974) in acting primarily (if not exclusively) on the terminal non-helical peptides of type I collagen that are involved in the cross-linking of the molecules.

Cathepsin H from rat or human liver (up to 60μg/mg of collagen) did not show any action on collagen (soluble or insoluble) at pH3.5 or 6.0, as judged by these methods.

Conclusions

The best methods we known for the isolation of cathepsins B, H and L, which appear to produce homogeneous preparations of constant specific activity, do not yield fully active material, as judged by active-site titration. For example, cathepsin L prepared by the present method was 50–60% active. Similar reservations doubtless apply to preparations of lysosomal cysteine proteinases in other laboratories (which have not been of higher specific activity), so we feel that activities against the various substrates are best expressed on the basis of molar concentration of titratable enzyme.

The inactivity of cathepsin L against all of the really convenient synthetic substrates tried previously has impeded research on the enzyme. Z-Phe-Arg-NMec has now proved to be an excellent and convenient substrate for cathepsin L, but there remains a need for a good substrate that is not also cleaved by cathepsin B.

The very low K_m of Z-Phe-Arg-NMec for cathepsin L may well be attributable to the existence of a non-productive binding mode, since $k_{cat.}/K_m$ is of the same order as that for cathepsin B. Z-Arg-Arg-NMec now appears to be the substrate of choice for the assay of cathepsin B (see also Barrett & Kirschke, 1981).

There is now little doubt that there is an intracellular route for collagen degradation involving the endocytosis of collagen by macrophages and fibroblasts, and its degradation in the lysosomal system (Barrett & Saklatvala, 1981). The collagenolytic activity of lysosomal enzymes at low pH has previously been attributed to cathepsin B (Burleigh *et al.*, 1974; Burleigh, 1977) and cathepsin N (Etherington, 1972, 1974, 1980). It is now apparent, however, that cathepsin L is at least as active against collagen as either of these, on a protein or molar basis.

In some respects cathepsin N resembles cathepsin L. Thus it is a lysosomal cysteine proteinase very active against collagen at acid pH, and with little action on synthetic substrates (Etherington, 1980). The enzymes are also similar in M_r and sensitivity to inhibitors, so it has seemed that they might be identical. This now seems less likely, however. Azocasein is a very good substrate for cathepsin L, but is reported to be almost unaffected by cathepsin N (Etherington, 1980); furthermore, cathepsin N does not hydrolyse Z-Lys-OPhNO$_2$ and does not inactivate aldolase (Etherington, 1980); in both respects the enzyme differs markedly from cathepsin L (Strewler & Manganiello, 1979; Towatari *et al.*, 1978). Also, it now appears that cathepsins L and N have different specificities of action on the insulin B-chain (Kärgel *et al.*, 1980). Another collagenolytic cysteine proteinase is the 'BANA-hydrolase' of Singh & Kalnitsky (1980) (BANA is α-*N*-benzoyl-DL-arginine β-naphthylamide hydrochloride). This enzyme resembles cathepsin H in many of its properties, but clearly differs from human and rat cathepsin H in that they lack collagenolytic activity.

Our present results do not in themselves give any direct indication of the likely physiological role of cathepsin L, but the very strong proteolytic activity of the enzyme would be consistent with the possibility that cathepsin L plays a major role in the lysosomal digestion of cellular proteins (Bohley *et al.*, 1980) and of collagen and other endocytosed proteins.

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