Biochem. J. (1985) **226**, 233–241
Printed in Great Britain

Human liver cathepsin L

Robert W. MASON, George D. J. GREEN* and Alan J. BARRETT
Department of Biochemistry, Strangeways Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

(*Received 28 August 1984/Accepted 19 October 1984*)

Cathepsin L was purified to apparent homogeneity from human liver obtained *post mortem*. It was necessary to treat the homogenate at pH 4.2 and 37°C to release active enzyme. The purification procedure involved ion-exchange chromatography on carboxymethyl-Sephadex and the Mono S column of a Pharmacia fast-protein-liquid-chromatography system. The enzyme was found to consist of two polypeptide chains of M_r 25000 and 5000. The larger chain was shown to contain the active-site cysteine residue. Human cathepsin L proved to be similar to the rat and rabbit enzymes in regard to kinetic constants for the substrate benzyloxycarbonylphenylalanylarginine 7-(4-methyl)coumarylamide and rates of inactivation by the active-site-directed reagents benzyloxycarbonylphenylalanylphenylalanyldiazomethane and benzyloxycarbonylphenylalanylalanyldiazomethane. Thus clear characteristics of cathepsin L are now emerging, and these should simplify the identification of the enzyme in other tissues and species.

Cathepsin L is of particular interest because it has a much higher specific activity in the degradation of a variety of physiological protein substrates, including collagen, than the other lysosomal proteinases (Barrett & Kirschke, 1981; Kirschke *et al.*, 1982). There are difficulties in the isolation of human cathepsin L, however, and there has been no full report of this. Thomas *et al.* (1980) briefly reported the detection of a cathepsin L-like enzyme in human kidney. The enzyme was separated from cathepsin B and shown to degrade azocasein and collagen, but no further characterization was reported. An enzyme called 'collagenolytic cathepsin' (later named 'cathepsin N'), has been found in human placenta (Evans & Etherington, 1978) and in human liver biopsies (Murawaki & Hirayama, 1980); it differed from cathepsin B and resembled cathepsin L in its low activity towards a range of synthetic substrates. Cathepsin N was shown to be a cysteine proteinase by its sensitivity to inhibition by iodoacetic acid, $HgCl_2$ and leupeptin, but, since collagen was the only substrate used, a full comparison of properties with cathepsin L is not possible.

Some characteristics of an enzyme described as human liver cathepsin L have been reported (Pagano & Engler, 1982). The purification procedure, not described fully, seems to have been similar to that used for the purification of cathepsin N (Evans & Etherington, 1978). Like human kidney cathepsin L, the enzyme was shown to hydrolyse azocasein, but had little activity against low-M_r synthetic substrates.

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with IUPAC–IUB Recommendations [*Biochem. J.* (1972) **126**, 773–780]. Additional abbreviations are: Cit, L-citrulline; Dns, 5-dimethylaminonaphthalene-1-sulphonyl; f.p.l.c., fast protein liquid chromatography (Pharmacia system); NHMec, 4-methyl-7-coumarylamide; SDS, sodium dodecyl sulphate. The inhibitors are: E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane (also D-isomer where indicated); Ep-420, DL-3-benzoxy-*trans*-2,3-epoxypropyl-isoleucyltyrosine methyl ester; Ep-459, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-amino)butane; Ep-460, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-benzyl-oxycarbonylamino)butane; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(3-methyl)butane. The abbreviations used for enzyme kinetic parameters are: $[I]_t$, total inhibitor concentration; $k_{cat.}$, catalytic constant; k_{+2}, rate constant for irreversible inhibition of enzyme–inhibitor complex; k'_{+2}, apparent second-order rate constant for inactivation, independent of substrate concentration; k''_{+2}, apparent second-order rate constant for inactivation in the presence of substrate; $K_{i(app.)}$, apparent inhibition constant in the presence of substrate; v_0, rate of reaction in absence of inhibitor; v_i, rate of reaction in presence of inhibitor.

* Present address: Friedrich Miescher Institut, P. O. Box 273, CH-4002 Basel, Switzerland.

The purpose of the present investigation was to purify cathepsin L from human liver and to compare its properties with those of the rabbit and rat liver enzymes. Preliminary reports of the results have been presented (Mason & Barrett, 1984*a*,*b*).

Experimental

Materials

Aminomethylcoumarin substrates were from Cambridge Research Biochemicals, Cambridge, U.K., Z-Phe-Cit-NHMec, Z-Phe-Ala-NHMec and Z-Phe-Met-NHMec being custom-synthesized.

Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were kindly given by Dr. E. N. Shaw, Friedrich Miescher Institute, Basel, Switzerland. Iodoacetic acid (specially purified for biochemical work) and Brij 35 (polyoxyethylene dodecyl ether) were supplied by BDH Chemicals, Poole, Dorset, U.K. 2-Hydroxyethyl disulphide was from Aldrich Chemical Co., Gillingham, Dorset, U.K.

α-Methyl D-mannoside (grade III), cytochrome *c* (ox heart, type IIa), carbonic anhydrase (bovine erythrocyte), transferrin (human), albumin (bovine serum, crystallized), phosphorylase *a* (rabbit muscle, twice crystallized), insulin (bovine pancreas), soya-bean trypsin inhibitor (type II-S), E-64, mersalyl acid and Brilliant Blue G were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Aprotinin was given by Dr. E. Philip, Bayer A.G., Wuppertal, Federal Republic of Germany, and sheep IgG by Dr. M. E. Davies of this Department. Ep-475 and other epoxide analogues were given by Dr. K. Hanada, Taisho Pharmaceutical Co., Saitama, Japan. 6*N*-[^{3}H]-Acetyl-Ep-459 was prepared in this laboratory by Dr. C. Parkes and Dr. D. H. Rich.

Leupeptin was supplied by the Peptide Research Foundation, 476 Ina, Minoh-shi, Osaka 562, Japan. Ampholines were purchased from LKB Instruments Ltd., South Croydon, Surrey CR2 9PX, U.K. Sephadex G-25, CM-Sephadex C-50, phenyl-Sepharose and concanavalin A–Sepharose were from Pharmacia (G.B.) Ltd., Milton Keynes, Bucks. MK9 3HP, U.K., who also supplied the f.p.l.c. system.

Rat liver cathepsin L was a gift from Dr. H. Kirschke, Physiological Chemistry Institute, Halle (Saale), German Democratic Republic. Rabbit liver cathepsin L was prepared as described previously (Mason *et al.*, 1984). Azo-casein was prepared as described by Barrett & Kirschke (1981). Samples of human liver removed at autopsy, free from disease, were kindly contributed by Dr. P. Stovin, Papworth Hospital, Cambridge, U.K.; they were stored at −20°C until required for use.

Enzyme assays

Enzyme assays and active-site titrations were performed essentially as described by Barrett & Kirschke (1981). Thus, cathepsin L was routinely assayed with Z-Phe-Arg-NHMec (5 μM) at 30°C and pH 5.5, and cathepsins B and H with Z-Arg-Arg-NHMec (5 μM) and Arg-NHMec (5 μM) respectively. The molar concentration of active cysteine proteinase was measured by titration with E-64 or Ep-475. K_m values were determined by the method of Wilkinson (1961). Activity against azocasein was assayed in reaction mixtures (0.2 ml) containing 3 M-urea, stopped with 1 ml of 3% (w/v) trichloroacetic acid. All other conditions were exactly as described previously.

Measurement of rate constants for irreversible inhibition

Inhibition rate constants were determined by use of continuous assays in the presence of inhibitor and substrate as described by Tian & Tsou (1982). The reaction scheme is assumed to be as in eqn. (1):

$$\begin{array}{l} \qquad +S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \longrightarrow E+P \\ E \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad (1) \\ \qquad +I \overset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_{+2}} EI \end{array}$$

Enzymes were preincubated with 1 mM-dithiothreitol for 2 h at 4°C. Reaction mixtures, containing substrate (1 μM-Z-Phe-Arg-NHMec), inhibitor, 100 mM-sodium acetate buffer, pH 5.5, 0.01% Brij 35, 1 mM-EDTA and 1 mM-dithiothreitol, were preincubated for 5 min in a cuvette placed in a holder thermostatically controlled at 30°C. Reactions were started by the addition of activated enzyme (1.0 nM final concn.). Appearance of product was recorded continuously in a Perkin–Elmer LS-3 spectrofluorimeter standardized with 0.2 μM-aminomethylcoumarin, with excitation at 360 nm and emission at 460 nm, and the curves were analysed by the Guggenheim method as described by Jencks (1969) to determine the half-life of the enzyme. Linear-regression coefficients were usually greater than 0.998 and total times greater than four half-lives. Fast-reacting inhibitors were used at 100–1000 nM final concentration, and the total amount of substrate consumed during assays was below 15%. The observed (pseudo-first-order) rate of inactivation, $k_{obs.}$, was calculated as $0.695/t_{0.5}$. At a fixed substrate concentration, the apparent second-order rate constant for inactivation, k''_{+2}, (eqn. 2) was calculated as $k_{obs.}/[I]_t$ (Thompson & Blout, 1973). Provided that the substrate concentration did not vary greatly during the course of reaction, the effect of substrate on k'_{+2} would be

constant (eqn. 3 below; Tian & Tsou, 1982]:

$$E + I \xrightarrow{k''_{+2}} EI \quad (2)$$

$$k''_{+2} = k'_{+2}/(1 + [S]/K_m) \quad (3)$$

Measurement of K_i for leupeptin

K_i values for the inhibition of cathepsin L by leupeptin were determined as described by Green *et al.* (1984). Enzymes were used at a concentration of 0.01 nM and leupeptin in the range 1–15 nM. Apparent K_i values were determined from replots of the form $[I]_t/[1-(v_1/v_0)]$ versus v_0/v_1 (Henderson, 1972). The substrate concentration used was 10 μM, therefore corrections we made by use of the relationship:

$$K_i = K_{i(app.)}/(1 + [S]/K_m)$$

for simple competition. All measurements were made with less than 2% hydrolysis. The incubation temperature was lowered to 20°C because cathepsin L was too unstable at higher temperatures in the low concentrations needed for these measurements. At 20°C, 95% of enzymic activity remained after 40 min incubation of enzyme with activator and substrate.

Determination of protein

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard, and during the course of enzyme purification they were determined by measurement of A_{280}.

Isoelectric focusing

Samples of human liver cathepsin L were subjected to isoelectric focusing in agarose gel and subsequently stained for Z-Phe-Arg-NHMec-hydrolysing activity as described previously (Mason *et al.*, 1984). Immediately after focusing, a strip of gel was cut into 5 mm segments, each of which was immersed overnight at 4°C in 1 ml of water, and the pH then determined by use of a glass micro-electrode. The activity of the eluates towards Z-Phe-Arg-NHMec was measured to confirm the results obtained by staining the gels directly.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Bury (1981), with the same reference proteins. Separating gels of 12.5% total acrylamide concentration (2.6% of this as methylenebisacrylamide) were used. Gels were stained for protein with Brilliant Blue G as described previously (Bury, 1981).

For active-site labelling of cathepsin L with 6,*N*-[^{3}H]acetyl-Ep-459, samples were preincubated with an equimolar concentration of inhibitor in the presence of 1 mM-dithiothreitol for 1 h at 37°C. Protein was then precipitated with 10% (w/v) trichloroacetic acid, and run on SDS/polyacrylamide gels as usual. Bands were cut from the stained gels for determination of radioactivity as described by Goodman & Martzura (1971).

Purification of human cathepsin L

Frozen liver (1.4 kg) was partially thawed, cut into small pieces and homogenized in 2 vol. of extraction buffer [1% NaCl/0.1% EDTA/2% (v/v) butan-1-ol]. Insoluble material was removed by centrifugation (1350 *g* for 30 min) and the supernatant was adjusted to pH 4.2, incubated at 37°C for 4 h, and left overnight at 4°C. Precipitated protein was removed by centrifugation as described above, and then a 20–80%-satn.-$(NH_4)_2SO_4$ fraction was taken. All operations (except centrifugation) up to and including this step were performed in a class I microbiological safety cabinet. The pellet was resuspended in 200 ml of 20 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA (buffer A), and insoluble material was removed by centrifugation (20000 *g* for 30 min). The supernatant was transferred into buffer A by gel chromatography through Sephadex G-25. The sample was applied to a column (25 cm × 4.4 cm) of CM-Sephadex C-50, which was washed with 2 bed volumes of buffer and eluted with a linear salt gradient to 1.0 M-NaCl (1900 ml total volume). Typically, cathepsin H was eluted at 80 mM-, cathepsin B at 150 mM- and cathepsin L at 400 mM-NaCl.

Fractions containing cathepsin L were combined and adjusted to 25% saturation with $(NH_4)_2SO_4$. The enzyme was then adsorbed to a column (10 cm × 1.6 cm) of phenyl-Sepharose, which was washed with 25%-satd. $(NH_4)_2SO_4$ in buffer A before cathepsin L was eluted with 25% (v/v) ethylene glycol in buffer A.

Active fractions were combined and equilibrated into 20 mM-sodium malonate buffer, pH 5.5, containing 1 mM-EDTA, and then chromatographed on the Mono S (HR5/5) column of a Pharmacia f.p.l.c. system, with a linear gradient (0–0.5 M; 25 ml) of NaCl in the same buffer. Active fractions were diluted 5-fold in 20 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA, and reapplied to the Mono S column equilibrated with this buffer. A similar NaCl gradient was applied. Peak activity from the two column runs was eluted at 360 and 380 mM-NaCl respectively.

Results and discussion

Purification

Human cathepsin L was purified 3500-fold, on the basis of total activity against Z-Phe-Arg-

NHMec after acid extraction (Table 1). Approximately 700 μg of purified enzyme was obtained from 1.4 kg of liver; this was found to be 40% active by titration with E-64. The apparent yield of enzyme was 5.1%, but this value is an underestimate, since cathepsin L is responsible for less than 25% of the total Z-Phe-Arg-NHMec-hydrolysing activity in the original extract (see below).

Acid treatment. The incubation of a crude homogenate of human liver at low pH before purification of cathepsin L proved to be the most critical step in the procedure. Cathepsin L exists in human liver homogenate in a 'latent' form, and omission of the acid treatment resulted in a negligible yield of the active enzyme. Conditions of acid treatment giving maximal generation of active cathepsin L were determined by two different methods. The first involved measuring the ratio of activities against Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec. Z-Arg-Arg-NHMec is cleaved by cathepsin B but not by cathepsin L (Barrett & Kirschke, 1981), whereas Z-Phe-Arg-NHMec is hydrolysed by both enzymes, so that an increase in the Z-Phe-Arg-NHMec/Z-Arg-Arg-NHMec ratio was taken as a measure of increased cathepsin L activity. Later, when it was established that human liver cathepsin L is irreversibly inhibited by Z-Phe-Phe-CHN_2 more than two orders of magnitude faster than cathepsin B (Table 3 below), the contribution of cathepsin L to the Z-Phe-Arg-NHMec-hydrolysing activity was determined by measuring the decrease in activity caused by this compound. Preincubation of fractions with 0.5 μM-Z-Phe-Phe-CHN_2 and 1 mM-dithiothreitol for 10 min at 30°C before assay inhibited cathepsin L by 95%, whereas cathepsin B was inhibited by less than 10% (determined by inhibition of Z-Arg-Arg-NHMec hydrolysis) by this treatment.

Maximal activation of cathepsin L in the homogenate was found to occur at pH 4.2, with 90% of this amount at pH 4.0 and 4.5. Below pH 3.9 and above pH 5.0, yields of both cathepsin L and cathepsin B were greatly diminished. The release of active enzyme was also highly dependent on temperature and incubation time. The conditions of 4 h at 37°C followed by 16 h at 4°C were chosen for convenience, but a similar amount of activity could be released by incubation for 24 h at 25°C. The presence of pepstatin, an inhibitor of aspartic proteinases (1 μg/ml), or reversible inhibitors of cysteine proteinases (1 mM-mersalyl acid or 1 mM-2-hydroxyethyl disulphide) during the acid treatment did not affect the release of active enzyme.

Under the chosen conditions of acid treatment, the activity attributable to cathepsin L increased over 10-fold, to account for 25% of the total Z-Phe-Arg-NHMec hydrolysis in the standard assay. Cathepsin B activity also increased, but only by 1.5–2.0-fold.

The reason for the inactivity of cathepsin L in the homogenate is not known. Possibly the enzyme is complexed by cystatins A and B, the cytosolic inhibitors of cysteine proteinase that are present in human liver cells (Davies & Barrett, 1984). These inhibitors bind cathepsin L much more tightly than cathepsin B, but would not normally be destroyed by the conditions of acid treatment used here (Green *et al.*, 1984). In view of the results with the inhibitors of proteinases, it also seems unlikely that the liberation of cathepsin L activity is a consequence of the action of cysteine or aspartic proteinases. Working with extracts of rabbit muscle, Matsumoto *et al.* (1983) have shown a decrease in the activity of inhibitors of cysteine proteinases as a result of incubation at acid pH, but were also unable to demonstrate any enzymic involvement.

Further purification. Once the enzyme was extracted, its purification proved relatively simple. Human cathepsin L bound very tightly to cation-exchange media, although its pI is in the range 5.7–6.3 (see below). This property, which has previously been described for cathepsin L of rat and rabbit liver (Kirschke *et al.*, 1977; Mason *et al.*, 1984) led to the use of three cation-exchange columns under different conditions to purify the enzyme. Anion-exchange columns could not be used because of the

Table 1. *Purification of human liver cathepsin L*

Activity was measured against Z-Phe-Arg-NHMec as described in the Experimental section.

Step	Total protein (A_{280})	Total activity (μmol/min)	Specific activity (μmol/min per A_{280} unit)	Purification factor	Yield (%)
Acid extraction	60025	254	0.004	1	100
$(NH_4)_2SO_4$ fractionation	14590	240	0.016	4	94
CM-Sephadex	110	69	0.63	148	27
Phenyl-Sepharose	60.5	50.8	0.84	210	20
Mono S					
pH 5.5	2.73	19.1	7.01	1656	7.5
pH 5.0	0.85	12.9	15.1	3565	5.1

instability of cathepsin L above pH 5.5 (Fig. 2 below). The phenyl-Sepharose column served mainly to concentrate the sample; all protein applied was bound, but some coloured material remained on the column after elution of the enyzme.

Physicochemical properties

Relative molecular mass. SDS/polyacrylamide-gel electrophoresis of the enzyme under non-reducing conditions showed two bands of protein at M_r 25000 and 29000 (Fig. 1). After reduction, two bands were seen, at M_r 25000 and 5000. These results suggest that human liver cathepsin L comprises forms of M_r 29000 and 25000, of which the larger consists of two chains linked by disulphide bonds. There was no evidence for the existence of a single-chain M_r-29000 form in any of our preparations. It is not yet clear whether cathepsin L occurs as a two-chain or single-chain form *in vivo*, since the isolation procedure requires a step that might permit autolytic cleavage. Having used a different purification procedure for the isolation of an enzyme described as cathepsin L, Pagano & Engler (1982) saw only a single diffuse band of protein on SDS/polyacrylamide-gel electrophoresis with reduction, which corresponded to M_r 30000.

We found that only the M_r-25000 and -29000 chains were labelled with 6,*N*-[^{3}H]acetyl-Ep459 (Fig. 1), indicating that these contain the active site, in agreement with the labelling of the large chain of rat liver cathepsin L by Dns-Ala-Ala-Phe-CHN_2 and Z-Phe-Phe-$^{14}CHN_2$ (Kirschke *et al.*, 1984*a*). Cathepsin H from rat liver has also been shown to occur as a two-chain form, with the active site in the larger chain (Takio *et al.*, 1980). In contrast, cathepsin B of several species has been

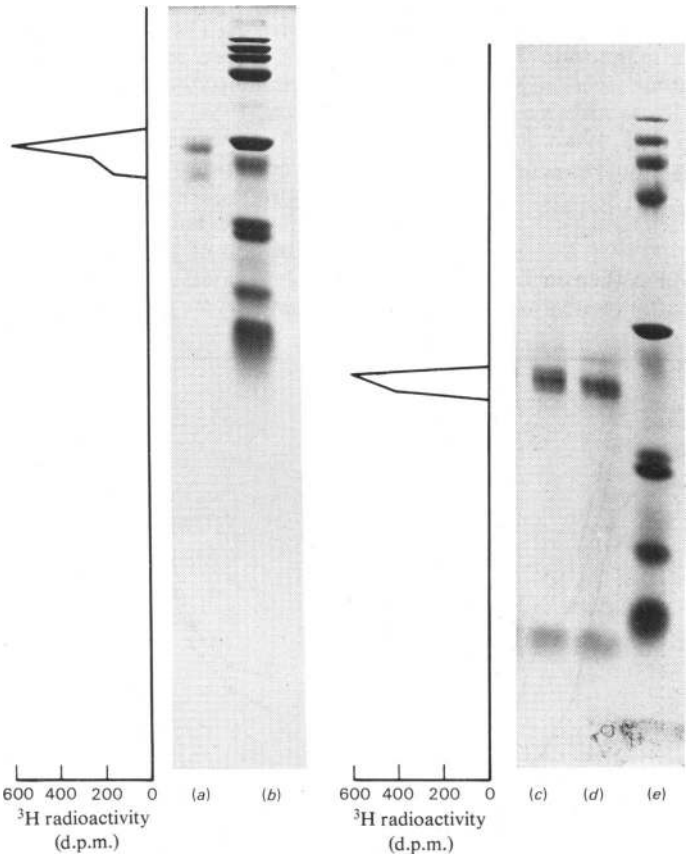

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of human cathepsin L*

SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions was in 12.5% (w/v)-acrylamide gels as described in the Experimental section. Active-site labelling with 6,*N*-[^{3}H]acetyl-Ep-459 was as described in the Experimental section. Incorporation of ^{3}H label is represented graphically alongside each gel. Approx. 0.2 μg of enzyme (determined by active-site titration) was applied to each lane. (*a*) Cathepsin L, unreduced; (*c*) and (*d*) cathepsin L, reduced; (*b*) and (*e*) reference proteins.

shown to have a light chain containing the catalytic cysteine residue that is formed by cleavage of the single-chain form of the protein near the *N*-terminus (e.g. Takio *et al.*, 1980). Sequence data have shown that the essential cysteine residues of papain and a number of homologous cysteine proteinases, including cathepsins B, H and S, are near the *N*-terminus (Barrett *et al.*, 1984).

Multiple isoelectric forms. Staining of isoelectric-focusing gels containing human cathepsin L for activity hydrolysing Z-Phe-Arg-NHMec revealed a major band at pH 5.9, with several minor bands in the range pH 5.7–6.3. We conclude that human liver cathepsin L consists of multiple forms with isoelectric points in the range 5.7–6.3. A similar range of isoenzymic forms has been found for both the rat and rabbit liver enzymes (Kirschke *et al.*, 1977; Mason *et al.*, 1984).

Behaviour on concanavalin A–Sepharose. When the purified cathepsin L was applied to a column of concanavalin A–Sepharose, 90% passed straight through, and the remaining 10% could be eluted with 200mM-α-methyl D-mannoside. The enzymes from rat liver and rabbit liver have also been shown to bind partially to this gel (Barrett & Kirschke, 1981; Mason *et al.*, 1984). It may be that heterogeneity of glycosylation is a general property of cathepsin L.

Catalytic activity

Stability. Human liver cathepsin L retained at least 70% of its activity after incubation at 37°C for 24h in the pH range 4.5–5.5 (Fig. 2*a*). At pH 7.0, however, 85% of activity was lost after only 15min incubation. Rat and rabbit liver cathepsin L are similarly unstable near to neutral pH (Kirschke *et al.*, 1980; Etherington *et al.*, 1984). This instability was shown to be partially responsible for the decline in activity above pH 6.0 (Fig. 2*b*). When hydrolysis of this substrate was measured during the first 30s of incubation, a significant amount of activity could be detected at pH 8.0. Similar results have been found for human cathepsin B and rabbit cathepsin L (A. J. Barrett, R. W. Mason & D. J. Etherington, unpublished work).

Action on peptidyl-NHMec substrates. The activities of cathepsin L from human, rabbit and rat liver against a range of synthetic substrates were determined (Table 2). The substrate concentrations were low (0.5 μM), so that the activities determined may be expected to be approximately proportional to values for $k_{cat.}/K_m$. Of those tested, Z-Phe-Arg-NHMec was clearly the best substrate, and values of K_m and $k_{cat.}$ were similar for all three enzymes. The values are in reasonable agreement with those reported previously for the rat and rabbit enzymes (Barrett & Kirschke, 1981; Mason *et al.*, 1984).

The specific activity of the enzyme from human liver purified by Pagano & Engler (1982) for the hydrolysis of Z-Phe-Arg-NHMec has not been reported, but it can be determined indirectly. Titration of the preparation with E-64 indicated that it contained 45% of active protein, and yet 1 μM-enzyme was used in assays with 25 μM-Z-Phe-

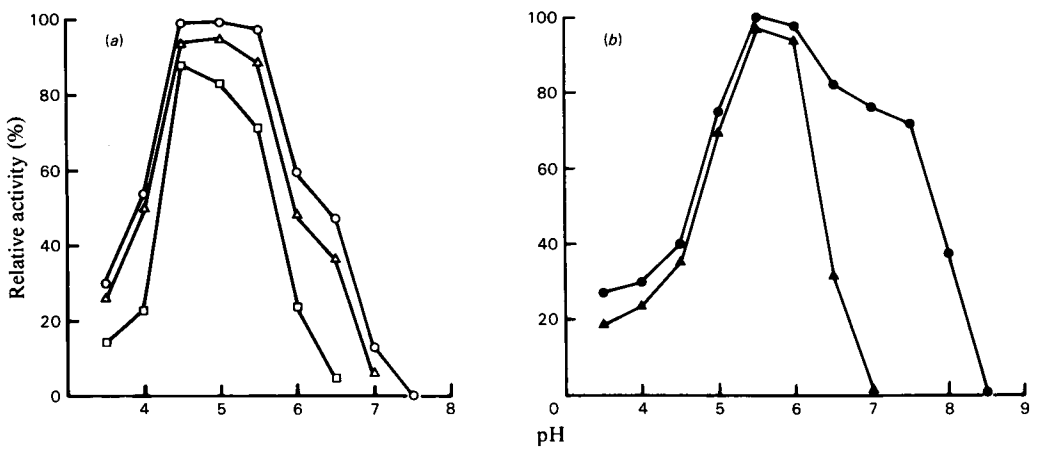

Fig. 2. *Stability of human liver cathepsin L and its effect on the pH optimum of Z-Phe-Arg-NHMec hydrolysis*
(*a*) Human liver cathepsin L was incubated in the absence of thiol activator at 37°C in 100mM-buffer at a range of pH values (see below) for 15min (○), 1h (△) or 24h (□). Activity was then measured under standard assay conditions. (*b*) Z-Phe-Arg-NHMec hydrolysis was measured at 30°C over a range of pH values either for a standard 10min assay (▲) or by recording continuously over the first 30s of hydrolysis (●). Buffers were sodium formate (pH 3.5–4.0), sodium acetate (pH 4.0–5.0), sodium malonate (pH 5.0–6.0), sodium phosphate (pH 6.0–7.5) and glycylglycine hydrochloride (pH 7.5–8.5).

Table 2. *Activity of cathepsin L against synthetic substrates*
All assays were at 30°C, pH 5.5, with 0.5 μM-substrate. Results are expressed as a percentage of activity for Z-Phe-Arg-NHMec, values in parentheses being K_m values (μM) and $k_{cat.}$ values (s^{-1}) respectively.

Substrate	Source of enzyme ...	Activity (%) Human liver	Rabbit liver	Rat liver
Z-Phe-Arg-NHMec		100 (2.4, 17)	100 (1.8, 20)	100 (4.5, 26)
Z-Arg-Arg-NHMec		<1	<1	<1
Arg-NHMec		<1	<1	<1
Z-Phe-Ala-NHMec		<1	<1	<1
Z-Phe-Cit-NHMec		44	38	44
Z-Phe-Met-NHMec		12	5	10

Arg-NHMec, at 40°C for 15 min (Pagano *et al.*, 1984). Under these conditions the maximum concentration of our enzyme preparation that could be used for linear assays was 0.4 nM, which hydrolysed 20% of the substrate. This implies that the specific activity of our preparation is at least 500 times higher than that of the enzyme used by Pagano *et al.* (1984). An explanation for this discrepancy could lie in the use of a different purification procedure; Pagano & Engler (1982) used a pH 7.5 step to elute the enzyme from a carboxymethylcellulose column, but we have shown that human liver cathepsin L is very unstable above pH 5.5 (Fig. 2*a*). The enzyme of Pagano and co-workers also differed from ours in having an affinity for α-cysteine proteinase inhibitor several orders of magnitude weaker (Pagano *et al.*, 1984; Gounaris *et al.*, 1984). We conclude that the enzyme purified by Pagano & Engler (1982) was either a severely modified form of cathepsin L or a different enzyme.

The data of Table 2 give some indications as to the requirements of the specificity subsites of cathepsin L. Z-Arg-Arg-NHMec, a highly specific substrate for cathepsin B, was not hydrolysed by cathepsin L. Analogues of Z-Phe-Arg-NHMec with citrulline or methionine at P_1 proved to be substrates, but Z-Phe-Ala-NHMec was not hydrolysed. Thus, on the basis of these limited comparisons, there appears to be a requirement for a non-polar hydrophobic residue at P_2, and a long aliphatic chain that may or may not carry a charge, at P_1. These conclusions are consistent with results from other laboratories. Mason *et al.* (1984) found with the rabbit enzyme that only the peptidyl-NHMec compounds with phenylalanine or valine in P_2 and arginine or lysine in P_1 were substrates. Furthermore, Katunuma *et al.* (1983) found that Z-Tyr-Met-2 naphthylamide was a substrate for rat liver cathepsin L.

Action on azocasein. The proteolytic nature of human liver cathepsin L was confirmed by its hydrolysis of azocasein. Under the conditions described by Barrett & Kirschke (1981) for the assay of rat liver cathepsin L in the presence of urea, the specific activity of azocasein hydrolysis for the human enzyme was found to be 520 μmol/min per mg. This is similar to the value found previously for the rat liver enzyme. However, assays involving azocasein require strict adherance to experimental procedures, since the quantities of trichloroacetic acid-soluble peptides measured under slightly different assay conditions have been shown to vary considerably (Mason *et al.*, 1982). For this reason, specific activity against azocasein should not be relied on in the characterization of cathepsin L.

Irreversible inhibition. In assays to determine second-order rate constants for irreversible active-site-directed inhibition of cathepsin L, it was found to be essential to pre-activate the enzyme fully. Otherwise, inhibition did not proceed to completion at the expected rate, so that reaction mixtures appeared to contain a second enzyme being inhibited much more slowly. This was the reason for the 2 h pre-activation at 4°C described in the Experimental section.

The apparent second-order rate constants of inhibition in the presence of substrate (k''_{+2}) for Z-Phe-Phe-CHN_2, Z-Phe-Ala-CHN_2, Ep-460 and Ep-475 were found to be independent of inhibitor concentration in the range 100–800 nM, as found previously for cathepsin B (Barrett *et al.*, 1982). However, values were dependent on substrate concentration, and a plot of $1/k''_{+2}$ against [S] for inhibition by Z-Phe-Phe-CHN_2 was linear (Fig. 3). This provided a separate estimate of the K_m for Z-Phe-Arg-NHMec, which confirmed the results obtained by the Wilkinson (1961) method (Table 2). Similar results were obtained for Z-Phe-Ala-CHN_2, Ep-460 and Ep-475, demonstrating that a second-order rate constant for inactivation, which is independent of substrate, k'_{+2}, can be calculated from eqn. (3). Values for k''_{+2} were obtained routinely at a substrate concentration of 1 μM, and k'_{+2} values were recalculated according to this equation.

E-64 and its analogues have been shown to be

potent active-site-directed irreversible inhibitors of human cathepsin B and rat liver cathepsin L (Barrett *et al.*, 1982). The fastest reacting of these epoxides found for human liver cathepsin L were Ep-460 and Ep-475 (Table 3). The values of k'_{+2} determined in the present study were similar to those obtained for rat liver cathepsin L (Barrett *et al.*, 1982). The rates of inactivation of cathepsins B and L by E-64 and its analogues are not sufficiently different to allow the two enzymes to be distinguished in crude mixtures. However, the diazomethane inhibitors Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 are shown to inhibit human liver cathepsin L very rapidly, at least two orders of magnitude faster than cathepsin B, confirming results for the rat liver enzyme (Kirschke & Shaw, 1981). Z-Phe-Phe-CHN_2 has been used selectively to inhibit cathepsin L in crude tissue extracts of rat liver (Riemann *et al.*, 1982). The apparent second-order rates of inactivation of rabbit liver cathepsin L by Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were determined to be 62300 and 74000 $M^{-1} \cdot s^{-1}$ respectively.

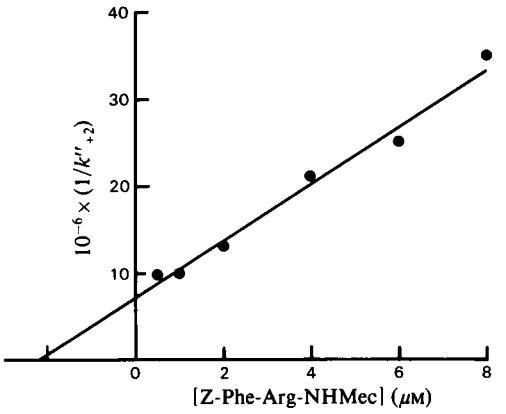

Fig. 3. *Influence of substrate on determination of apparent second-order rate of inactivation (k''_{+2}) of human cathepsin L*
Apparent second-order rates of inactivation (k''_{+2}) of human liver cathepsin L by Z-Phe-Phe-CHN_2 at several concentrations of substrate were determined as described in the text. Rates of inactivation independent of substrate concentration (k'_{+2}) were determined from the reciprocal of the intercept on the *y*-axis. An estimate of K_m is indicated by the intercept on the *x*-axis.

Reversible inhibition by leupeptin. Leupeptin was found to be a reversible inhibitor of cathepsin L from human, rat and rabbit liver. It was evident that maximal inhibition of the enzyme fractions had not been obtained after 40min incubation. However, spontaneous inactivation of the enzymes during more prolonged incubations would lead to an erroneously low estimate of K_i, so we decided to measure inhibited rates after 40min to obtain a maximal estimate of K_i. $K_{i(app.)}$ values for the human, rat and rabbit enzymes were determined to be 1.6, 1.4 and 1.8nM respectively. These gave K_i values of 0.3, 0.4 and 0.3nM when corrected. Since the values are maximal estimates, a K_i of less than 1nM for inhibition by leupeptin can be taken as a characteristic of cathepsin L. In contrast, the values for human liver cathepsins B and H were found to be 7nM and 6.9μM respectively (Knight, 1980; Schwartz & Barrett, 1980).

Conclusions

Our results show that cathepsins L from three different species have very similar properties. Criteria for the identification of the enzyme can now be considered to be as follows: (1) M_r in the range 25000–29000, often with two chains of M_r 25000 and 5000; (2) high affinity for the substrate Z-Phe-Arg-NHMec, with a K_m in the range 1–

Table 3. *Inhibition of cathepsins B and L by E-64 and its analogues and peptidyl diazomethanes*
The apparent second-order inhibition rate constants (k'_{+2}) for human liver cathepsin L were measured at 30°C and at pH5.5. Results are mean values of at least three separate determinations. Other values were obtained from Barrett *et al.* (1982).

Inhibitor	Enzyme ...	k'_{+2} ($M^{-1} \cdot s^{-1}$)		
		Human liver cathepsin L	Rat liver cathepsin L	Human liver cathepsin B
E-64 (L)		47800	96000	89000
E-64 (D)		2400	2700	1900
Ep-459		32500	27500	69500
N-Acetyl-Ep-459		8700	–	41000
Ep-460		152000	231000	175000
Ep-420		57400	12700	49400
Ep-475		114500	206000	298000
Z-Phe-Phe-CHN_2		136000	160000	185
Z-Phe-Ala-CHN_2		160000	70000	1220

4 μM; (3) hydrolysis of Z-Phe-Arg-NHMec, with a $k_{cat.}$ of approx. 20 s^{-1}; (4) negligible activity against substrates lacking a hydrophobic residue in P_2; (5) rapid and irreversible inhibition by Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2, several orders of magnitude faster than cathepsin B; (6) reversible inhibition by leupeptin with a K_i of less than 1 nM; and (7) instability at neutral pH. Some of these criteria have recently been used independently by Kirschke *et al.* (1984*b*) in distinguishing cathepsin S of ox spleen from cathepsin L.

We are most grateful to Dr. P. Stovin and his staff for providing post-mortem samples of human liver. We also thank all of our colleagues for stimulating discussions.

References

Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. & Hanada, K. (1982) *Biochem. J.* **201**, 189–198

Barrett, A. J., Nicklin, M. J. H. & Rawlings, N. D. (1984) *Symp. Biol. Hung.* **25**, 203–217

Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254

Bury, A. F. (1981) *J. Chromatogr.* **213**, 491–500

Davies, M. E. & Barrett, A. J. (1984) *Histochemistry* **80**, 373–377

Etherington, D. J., Mason, R. W., Taylor, M. A. J. & Wardale, R. J. (1984) *Biosci. Rep.* **4**, 121–127

Evans, P. & Etherington, D. J. (1978) *Eur. J. Biochem.* **83**, 87–97

Goodman, D. & Martzura, H. (1971) *Anal. Biochem.* **42**, 481–486

Gounaris, A. D., Brown, M. A. & Barrett, A. J. (1984) *Biochem. J.* **221**, 445–452

Green, G. D. J., Kembhavi, A. A., Davies, M. E. & Barrett, A. J. (1984) *Biochem. J.* **218**, 939–946

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 562–564, McGraw-Hill Book Co., New York

Henderson, P. J. F. (1972) *Biochem. J.* **127**, 321–333

Katunuma, N., Towatari, T., Tamai, M. & Hanada, K. (1983) *J. Biochem. (Tokyo)* **93**, 1129–1135

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., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S. & Bohley, P. (1980) *Ciba Found. Symp.* **75**, 15–35

Kirschke, H., Kembhavi, A. A., Bohley, P. & Barrett, A. J. (1982) *Biochem. J.* **201**, 367–372

Kirschke, H., Bohley, P., Fittkau, S. & Wiederanders, B. (1984*a*) *Symp. Biol. Hung.* **25**, 147–153

Kirschke, H., Locnikar, P. & Turk, V. (1984*b*) *FEBS Lett.* **174**, 123–127

Knight, C. G. (1980) *Biochem. J.* **189**, 449–453

Mason, R. W. & Barrett, A. J. (1984*a*) *Biochem. Soc. Trans.* **12**, 1111

Mason, R. W. & Barrett, A. J. (1984*b*) in *Intracellular Protein Catabolism V (Proceedings of the Meeting at Airlie, Virginia, June 1984)* (Khairallah, E. & Bond, J. S., eds.), A. R. Liss, New York, in the press

Mason, R. W., Taylor, M. A. J. & Etherington, D. J. (1982) *FEBS Lett.* **146**, 33–36

Mason, R. W., Taylor, M. A. J. & Etherington, D. J. (1984) *Biochem. J.* **217**, 209–217

Matsumoto, T., Okitani, A., Kitamura, Y. & Kato, H. (1983) *Agric. Biol. Chem.* **47**, 2365–2371

Murawaki, Y. & Hirayama, C. (1980) *Clin. Chim. Acta* **108**, 121–128

Pagano, M. & Engler, R. (1982) *FEBS Lett.* **138**, 307–310

Pagano, M., Esnard, F., Engler, R. & Gauthier, F. (1984) *Biochem. J.* **220**, 147–155

Riemann, S., Kirschke, H., Wiederanders, B., Brouwer, A., Shaw, E. & Bohley, P. (1982) *Acta Biol. Med. Ger.* **41**, 83–88

Schwartz, W. N. & Barrett, A. J. (1980) *Biochem. J.* **191**, 487–497

Takio, K., Towatari, T., Katunuma, N. & Titani, K. (1980) *Biochem. Biophys. Res. Commun.* **97**, 340–346

Thomas, G. T., Hughes, K. T. & Davies, M. (1980) *Biochem. Soc. Trans.* **8**, 596–597

Thompson, R. C. & Blout, E. R. (1973) *Biochemistry* **12**, 44–47

Tian, W.-X. & Tsou, C.-L. (1982) *Biochemistry* **21**, 1028–1032

Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332