

## The purification and properties of cathepsin L from rabbit liver

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Cathepsin L was purified from rabbit liver by a method involving whole-tissue homogenization, pH precipitation, ammonium sulphate fractionation and chromatography on CM-Sephadex C-50, phenyl-Sepharose and Sephadex G-75. Pure enzyme was obtained without the necessity of laborious subcellular fractionation techniques. The  $M_r$  of the enzyme was determined to be 29000 by gel filtration, and affinity for concanavalin A-Sepharose indicated that it was a glycoprotein. A novel technique for detection of enzyme activity in agarose isoelectrofocusing gels showed that the enzyme existed in multiple isoenzymic forms with pI values ranging from 5.0 to 5.9. The enzyme catalysed the hydrolysis of azocasein, collagen and Z-Phe-Arg-NMec (where Z and NMec indicate benzyloxycarbonyl and *N*-methylcoumarin derivative respectively) optimally at pH 5.2, 3.3 and 6.0 respectively. In addition, cathepsin L was found to degrade benzoyl-Phe-Val-Arg-NMec and 3-carboxypropionyl-Ala-Phe-Lys-NMec. However, cathepsin B also cleaved all of these substrates. One major difference between these two enzymes was in their Michaelis constants for Z-Phe-Arg-NMec; cathepsin B had  $K_m$  75  $\mu\text{M}$  whereas that of cathepsin L was 0.7  $\mu\text{M}$ . Cathepsin L was inhibited by all of the usual chemical inhibitors of thiol proteinases as well as the more specific inhibitors Z-Phe-Phe-CHN<sub>2</sub>, Z-Phe-Ala-CHN<sub>2</sub>, compound E-64 and compound Ep-475. Active-site titration with compound E-64 showed that the purified sample contained 80% active protein, which had  $k_{\text{cat}}$  20 s<sup>-1</sup> for the substrate Z-Phe-Arg-NMec. Antibodies were raised to active cathepsin L, and these did not cross-react with cathepsin B, thus demonstrating that these two enzymes are immunologically distinct.

The lysosomal cysteine endopeptidases are probably the most active tissue proteinases responsible for intracellular degradation (Barrett & Kirschke, 1981). The major enzymes in this group are cathepsins B, H and L. In investigations of the degradation of extracellular matrix, cathepsins B and L have been shown to be capable of cleaving acid-insoluble collagen to yield soluble products (Etherington, 1974; Mason *et al.*, 1982; Kirschke *et al.*, 1982). Both of these enzymes can also cleave the synthetic substrate Z-Phe-Arg-NMec (Barrett, 1980; Kirschke *et al.*, 1982). However, cathepsin B can be distinguished from cathepsin L by its ability to cleave Bz-Arg-NNa. A specific substrate for cathepsin L has not yet been found. Cathepsin H is an endoaminopeptidase that can also degrade Bz-Arg-NNa. However, it can be distinguished from

cathepsin B by its ability to catalyse the hydrolysis of unblocked amino acid derivatives such as Arg-NNa and Arg-NMec. This enzyme is unable to degrade collagen or Z-Phe-Arg-NMec. Cathepsin B has been purified from both whole-tissue extracts and lysosomal preparations from many species (for references see Barrett & McDonald, 1980). Cathepsin L, however, has been isolated mainly from lysosomal extracts, as extracts from whole tissue often yield considerably less active enzyme, probably owing to the effect of inhibitors from the cytosol fraction (Towatari *et al.*, 1976; Kirschke *et al.*, 1977).

The present paper describes a method for preparing highly purified cathepsin L from extracts of rabbit liver. The specificity of this enzyme was examined for a range of synthetic and protein substrates and the results were compared with those reported for the enzyme obtained from rat liver lysosomes. A short communication on part of this work has been published previously (Mason *et al.*, 1982).

Abbreviations used: Z, benzyloxycarbonyl; NMec, *N*-methylcoumarin; NNa,  $\beta$ -naphthylamide; Bz, benzoyl; Tos, tosyl; Suc, 3-carboxypropionyl; IgG, immunoglobulin G.

## Materials

NMec derivatives were obtained from Bachem Feinchemikalien A.G. (Bubendorf, Germany). Insoluble collagen was obtained from the Millipore Corp. (Freehold, NJ, U.S.A.). Bz-Arg-NNa, Tos-Lys-CH<sub>2</sub>Cl ('TLCK') and azocasein were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The cysteine-proteinase inhibitor compound E-64 (L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucine 4-guanidinobutylamide) was purchased from Taisho Pharmaceuticals (Saitama, Japan) and compound Ep-475 (L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucyl-isopentylamine) was a gift from Professor K. Hanada (of that Company). Z-Phe-Phe-CHN<sub>2</sub> and Z-Phe-Ala-CHN<sub>2</sub> were gifts from Dr. E. Shaw (Brookhaven National Laboratory, Brookhaven, NY, U.S.A.). GelBond NF was obtained from Miles Laboratories (Slough, Berks., U.K.). Ampholines were bought from LKB (Bromma, Sweden). Chromatographic materials, standard proteins and agarose IEF were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Livers were obtained from freshly slaughtered rabbits and stored at -20°C until required.

## Methods

### Enzyme assays

Bz-Arg-NNa-hydrolysing activity was measured as described by Barrett (1976). Arg-NMec hydrolysis was measured essentially as described by Barrett (1980), but with 100mM-sodium phosphate buffer, pH 6.8, containing 1mM-EDTA, and 10mM-cysteine as activator. Measurement of Z-Phe-Arg-NMec hydrolysis was based on the assay method of Barrett (1980), with an incubation medium of 100mM-sodium phosphate buffer, pH 6.0, containing 1mM-EDTA, 10mM-cysteine, 10 μM-Z-Phe-Arg-NMec and enzyme in a total volume of 2ml. Incubation was at 37°C for 10min, after which time the reaction was stopped by the addition of 2ml of 100mM-sodium acetate buffer, pH 4.3, containing 100mM-sodium chloroacetate. For kinetic analyses initial rates at 37°C were determined from continuous recordings made on a Servoscribe 1S chart recorder attached to a Perkin-Elmer 3000 fluorescence spectrometer fitted with a thermostatically controlled cell holder. Excitation and emission wavelengths were 340nm and 460nm respectively. Activity against all other NMec derivatives was assayed similarly. The Michaelis constants were calculated several times with the use of a range of enzyme concentrations (3.3–33ng/ml). The reaction was started by the addition of substrate (0.3–50 μM).

Activity against insoluble collagen at pH 3.5 was assayed as described by Etherington (1972), but

with 10mM-cysteine as activator. Measurement of azocasein-hydrolysing activity was based on the method of Kirschke *et al.* (1977). For routine assays an incubation mixture of 100mM-sodium phosphate buffer, pH 6.0, containing 1mM-EDTA, 10mM-cysteine, 0.5% azocasein and enzyme in a total volume of 1.4ml was used. Incubation was for 3h at 37°C, and the reaction was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid. Acid-soluble material was estimated by measuring the absorbance at 366nm.

### Inhibition studies

For general inhibition studies, enzyme was preincubated with inhibitor for 5min and residual activity was measured by the methods described above. Active-site titration with compounds E-64 and Ep-475 was performed as described by Barrett & Kirschke (1982). A 25 μl volume of inhibitor at a range of concentrations was added to a series of tubes containing 25 μl of enzyme solution (8 μg/ml) and 50 μl of 100mM-sodium phosphate buffer, pH 6.0, containing 1mM-EDTA and 10mM-cysteine. After the mixtures had stood for 30min at room temperature, 25 μl of each sample was assayed for residual Z-Phe-Arg-NMec-hydrolysing activity by using the continuous monitoring method described above.

### Determination of protein

Protein concentrations were measured by the Coomassie Brilliant Blue G method of Sedmak & Grossberg (1977), with bovine serum albumin as standard.

### Purification of cathepsin L

A preliminary report on the purification of cathepsin L from rabbit liver has already been published (Mason *et al.*, 1982). This procedure has now been improved and extended as described below.

Rabbit liver (200g) was homogenized in 2 vol. of 100mM-sodium acetate buffer, pH 5.0, containing 1mM-EDTA and 0.2% Triton X-100 in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) at maximum speed for 2min at 4°C, and the resulting homogenate was stirred overnight at 4°C. Debris was removed by centrifugation at 15000g for 20min, and the extraction efficiency was improved by washing the pellet with a further volume of isolation buffer. The pooled supernatants were acidified to pH 4.2 with 2M-HCl, and precipitated protein was removed by centrifugation at 15000g for 20min. A 20–65%-saturation-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction of the supernatant was taken, and the precipitated protein was redissolved in 80ml of 20mM-sodium phosphate buffer, pH 5.8, containing 1mM-EDTA (Buffer A) and the pH re-adjusted to 5.8 with 1M-NaOH. Insoluble material

was removed by centrifugation at 27000g for 20 min.

$(\text{NH}_4)_2\text{SO}_4$  was removed by filtration on a column of Sephadex G-25 equilibrated in buffer A. The pooled active fractions were applied to a column (22 cm  $\times$  4.8 cm) of CM-Sephadex C-50 pre-equilibrated in the same buffer. After washing with 2 column volumes of buffer A, the column was eluted with a linear gradient (0–0.6 M) of NaCl in buffer A (2  $\times$  900 ml). A peak corresponding to cathepsin L was eluted, with maximal activity at 0.32 M-NaCl. These fractions were pooled and adjusted to 25% saturation with  $(\text{NH}_4)_2\text{SO}_4$  before being applied to a column (10 cm  $\times$  1.6 cm) of phenyl-Sepharose that had been pre-equilibrated in buffer A containing  $(\text{NH}_4)_2\text{SO}_4$  at 25% saturation. After the column had been washed with 5–10 column volumes of this buffer, activity was eluted with 50% (v/v) ethylene glycol in buffer A. Active fractions were further purified by gel filtration on a column (80 cm  $\times$  4.4 cm) of Sephadex G-75 equilibrated in buffer A containing 100 mM-NaCl. Purified enzyme was concentrated by adding  $(\text{NH}_4)_2\text{SO}_4$  to 25% saturation, applying the solution to a small column (2 cm  $\times$  1.6 cm) of phenyl-Sepharose and, after the column had been washed, eluting the enzyme with 50% ethylene glycol in buffer A. Enzyme fractions were then taken back into buffer A by gel filtration on Sephadex G-25. Further purification was achieved by rechromatography on a column (20 cm  $\times$  2.6 cm) of CM-Sephadex C-50. Activity was eluted with a linear gradient (0–0.6 M) of NaCl (2  $\times$  250 ml) as described above, and the enzyme activity was concentrated on phenyl-Sepharose.

#### *Concanavalin A-Sepharose chromatography*

Highly purified enzyme was equilibrated into buffer A containing 0.2 M-NaCl, 1 mM- $\text{CaCl}_2$  and 1 mM- $\text{MnCl}_2$  on Sephadex G-25. This was then applied to a column (10 cm  $\times$  1.6 cm) of concanavalin A-Sepharose, and activity was eluted with a linear gradient (0–100 mM) of methyl D-mannoside (2  $\times$  50 ml).

#### *Isoelectrofocusing of samples*

Purified samples of enzyme were subjected to isoelectrofocusing in agarose gels. The gel solution was made up of 0.16 g of agarose IEF and 1.9 g of sorbitol in 15 ml of water. After the mixture had been boiled to dissolve the agarose, the solution was allowed to cool to 70°C. To this 0.5 ml of Ampholine pH 3.5–10 and 0.5 ml of Ampholine pH 4–6 were added. After mixing, the gel solution was quickly poured on to a sheet (11 cm  $\times$  12.5 cm) of Gelbond NF and allowed to set at 4°C for at least 1 h. Samples (5–10  $\mu\text{l}$ ) were then applied to the surface of the gel in strips (3 mm  $\times$  6 mm) of

Whatman no. 54 filter paper. Focusing was performed on a LKB flat-bed electrofocusing apparatus. Focusing time was usually 2 h, with a final potential of 1000 V for the last 45 min.

Activity within the gels was detected by immersing the complete gel into a solution of 100 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA, 10 mM-cysteine and 10  $\mu\text{M}$ -Z-Phe-Arg-NMec. After 5–10 min the gels were removed and photographed immediately under a lamp emitting u.v. light at a wavelength near to 340 nm.

#### *Antibody preparation*

A 180  $\mu\text{g}$  portion of rabbit liver cathepsin L (3 ml) purified as described previously (Mason *et al.*, 1982) was emulsified with an equal volume of Freund's complete adjuvant and injected into a young goat at multiple subcutaneous sites. Four repeat injections with 100  $\mu\text{g}$  of enzyme preparation (2 ml) emulsified with an equal volume of Freund's incomplete adjuvant were given at 3-weekly intervals. At 3 weeks after the final booster injection the animal was killed and the serum collected. IgG fractions were prepared by chromatography on DEAE-Sephacel equilibrated in 17.5 mM-sodium phosphate buffer, pH 6.3 (Levy & Sober, 1960). The unbound IgG fractions were concentrated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation and redissolved in 100 mM-sodium phosphate buffer, pH 6.0.

#### *Immunodiffusion techniques*

Double-diffusion analyses were carried out essentially as described by Ouchterlony (1962). Analyses were performed in 1% agarose gels in 100 mM-sodium phosphate buffer, pH 6.0, containing 3% (w/v) poly(ethylene glycol). After diffusion had been allowed to proceed for a specified time at 4°C, non-complexed protein was removed by blotting and rehydrating the gels several times. Precipitin lines, containing active enzyme, were detected under u.v. light as described above. Immunodiffusion after isoelectrofocusing was performed similarly by applying immune IgG fractions to wells cut in parallel to the focused samples.

## **Results**

#### *Purification of cathepsin L*

The early stages of acid precipitation and  $(\text{NH}_4)_2\text{SO}_4$  fractionation were effective in the removal of endogenous inhibitors to give an apparent 5-fold increase in total activity (Table 1). The presence of such inhibitors therefore gave a distorted impression of the final purification and yield of the enzyme. A large peak of inactive protein was not absorbed by chromatography on CM-Sephadex C-50, and two peaks of collageno-

Table 1. *Purification of rabbit liver cathepsin L*  
For experimental details see the text. Activity was measured by hydrolysis of Z-Phe-Arg-NMec.

Step	Volume (ml)	Protein (mg)	Activity (nmol/min per ml)	Total activity ( $\mu$ mol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Initial extract	480	9000	54.6	26.2	2.9	1	100
pH 4.2 supernatant	480	4080	137	65.8	16.1	5.5	250
20%-satn.-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> supernatant	480	1920	128	61.6	32.1	11.0	235
65%-satn.-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate	145	870	870	126	145	50	480
1st CM-Sephadex	500	125	132	66.0	526	181	251
1st phenyl-Sepharose	31	35.5	997	30.9	870	300	118
Sephadex G-75	210	8.6	107	22.5	2610	900	86
2nd CM-Sephadex	60	0.19	100	6.0	31900	11000	23
2nd phenyl-Sepharose	20	0.16	275	5.5	34400	11800	20

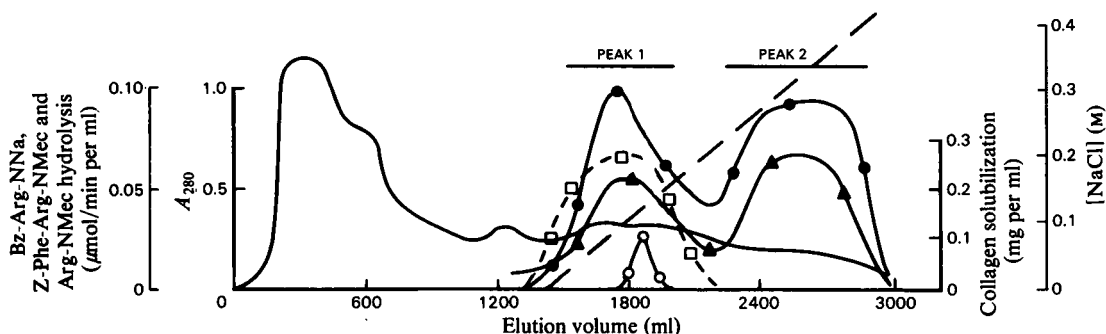


Fig. 1. *Chromatography of partially purified rabbit liver cathepsin L on CM-Sephadex*  
Activities against Bz-Arg-NNa ( $\square$ ), Arg-NMec ( $\circ$ ), Z-Phe-Arg-NMec ( $\bullet$ ) and collagen ( $\blacktriangle$ ) were measured as described in the Methods section. Protein was measured as  $A_{280}$  (—) and NaCl concentration of the effluent was also measured (---). Fractions indicated by the horizontal bars were pooled for further purification.

lytic activity were eluted by the salt gradient (Fig. 1). These two peaks also contained activity against Z-Phe-Arg-NMec, which is a substrate for both cathepsins B and L. Peak 1 contained peaks of activity towards Bz-Arg-NNa and Arg-NMec. However, these peaks did not coincide, indicating the presence of cathepsins B and H. These two enzymes can be separated by chromatography on DEAE-cellulose (Mason *et al.*, 1982). The second collagenolytic peak did not contain such activities and is therefore characterized as cathepsin L. This separation step is most important, as otherwise cathepsin B would interfere with the cathepsin L assays, since a specific substrate for cathepsin L has not yet been found. Hydrophobic chromatography on phenyl-Sepharose was found to be highly effective in concentrating cathepsin L with minimal loss of activity (Table 1). Also, less-hydrophobic contaminating proteins were unabsorbed, and this resulted in an increase in specific activity. We previously had found that much

cathepsin L activity was lost on concentration by ultrafiltration.

Chromatography on Sephadex G-75 gave a broad peak of activity with a low absorption at 280 nm. Peak activity was eluted at a point corresponding to an  $M_r$  value of 29000. After concentration and gel filtration of this sample, a 900-fold purification had been achieved. However, trace amounts of Bz-Arg-NNa-hydrolysing activity still remained, indicating the presence of some cathepsin B and/or cathepsin H. Rechromatography on CM-Sephadex C-50 removed this activity, along with a substantial quantity of contaminating protein. Strong protein-protein interactions in the crude extract were probably responsible for the initial poor separation on this resin. The final sample after concentration and gel filtration contained 160  $\mu$ g of protein, indicating a purification of 11800-fold and a yield of 20% by comparison with the original extract. The specific activity of the purified enzyme for the substrate

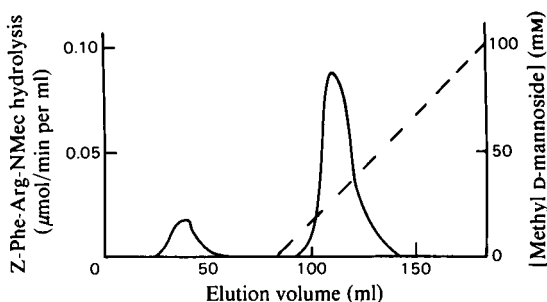


Fig. 2. Chromatography of purified rabbit liver cathepsin L on concanavalin A-Sepharose. For experimental details see the text.

Z-Phe-Arg-NMec was  $34.4 \mu\text{mol}/\text{min}$  per mg (Table 1).

#### Chromatography on concanavalin A-Sepharose

Approx. 90% of the cathepsin L activity was adsorbed on concanavalin A-Sepharose and subsequently eluted with 25 mM-methyl D-mannoside (Fig. 2), which indicated that cathepsin L existed mainly as a glycosylated protein. The unadsorbed fraction would not bind when re-applied to a freshly prepared column of concanavalin A-Sepharose, confirming that some of the enzyme was not glycosylated. However, the specific activities of both fractions were similar to that of the applied sample. Samples of each pool were retained for examination of their isoenzymic forms (see below).

#### Isoelectrofocusing of cathepsin L

It has previously been reported from this laboratory that there are three main isoenzymic forms of rabbit liver cathepsin L (Mason *et al.*, 1982). Flat-bed isoelectrofocusing in agarose gels of the purified enzyme was performed and activity against Z-Phe-Arg-NMec was located under u.v. light (Fig. 3). A complex pattern of isoenzymes with a range of pI values was seen. Furthermore, similar patterns were also seen with crude preparations of enzyme freshly extracted from rabbit liver and with both the bound and unbound fractions from concanavalin A-Sepharose. By contrast, crude preparations of rabbit liver cathepsin B, prepared as described elsewhere (Mason *et al.*, 1982), showed only one band of Z-Phe-Arg-NMec-hydrolysing activity. In addition, only the cathepsin B sample could be detected when Z-Arg-Arg-NMec, a specific substrate for this enzyme (Kirschke *et al.*, 1982), was substituted for Z-Phe-Arg-NMec.

#### Inhibition of cathepsin L

Purified rabbit liver cathepsin L was inhibited by iodoacetic acid, Tos-Lys-CH<sub>2</sub>Cl and leupeptin,

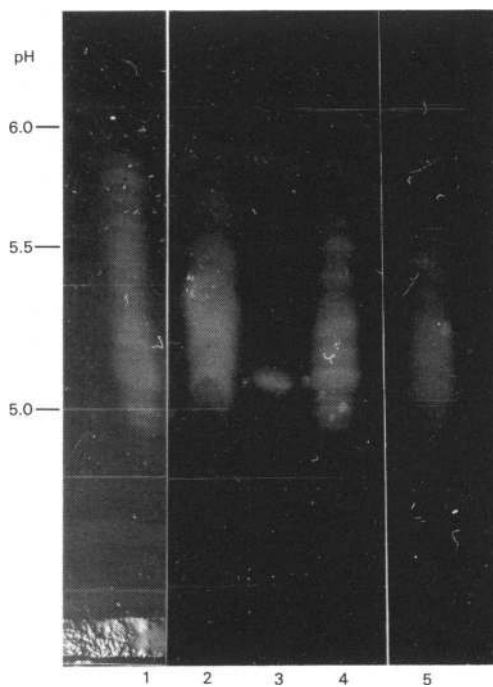


Fig. 3. Detection of Z-Phe-Arg-NMec-hydrolysing isoenzymes after isoelectrofocusing in agarose gels. pI values were determined by using a Pharmacia low-pI calibration kit. Track 1, purified cathepsin L, before chromatography on concanavalin A-Sepharose; track 2, freshly prepared crude extract of enzymes (prepared by whole-tissue homogenization, pH precipitation and  $(\text{NH}_4)_2\text{SO}_4$  fractionation); track 3, rabbit liver cathepsin B; track 4, cathepsin L fraction bound to concanavalin A-Sepharose; track 5, cathepsin L fraction not bound to concanavalin A-Sepharose.

which are commonly used inhibitors of cysteine proteinases (Table 2).  $\text{HgCl}_2$  inhibition was low because the 10 mM-cysteine used as activator reverses this inhibition.  $\text{HgCl}_2$  is used as a reversible inhibitor in the purification of rat liver cathepsin L to prevent autolysis (Kirschke *et al.*, 1982). The effect of  $\text{HgCl}_2$  in the absence of cysteine could not be demonstrated, as the rabbit enzyme requires a thiol activator to be present. The diazomethyl ketone and epoxy inhibitors of cysteine proteinases (Leary & Shaw, 1977; Hanada *et al.*, 1978) were strong inhibitors of cathepsin L. Z-Phe-Phe-CHN<sub>2</sub> appeared to be slightly more potent than Z-Phe-Ala-CHN<sub>2</sub>, which is the converse of the reported action of these inhibitors on cathepsin B from bovine spleen (Watanabe *et al.*, 1979).

#### Active-site titration of cathepsin L

The inhibitor compounds E-64 and Ep-475 bind

Table 2. *Inhibition of rabbit liver cathepsin L*

To measure inhibition of Z-Phe-Arg-NMec hydrolysis, enzyme (20 ng) was preincubated with inhibitor at 37°C for 5 min and residual activity assayed for 10 min as described in the Methods section. For inhibition of collagenolytic activity, enzyme and inhibitor were preincubated similarly and residual activity was assayed for 3 h. Results are expressed as percentages of activity remaining compared with sample assayed in the absence of inhibitor.

Inhibitor	Z-Phe-Arg-NMec hydrolysing activity (%)	Collagen-degrading activity (%)
None	100	100
HgCl <sub>2</sub> (1 mM)	90	95
Iodoacetic acid (1 mM)	3	6
Tos-Lys-CH <sub>2</sub> Cl (1 μM)	5	3
Leupeptin (0.1 μM)	1	2
Z-Phe-Phe-CHN <sub>2</sub> (1 μM)	1	4
Z-Phe-Ala-CHN <sub>2</sub> (1 μM)	3	7
Compound E-64 (1 μM)	1	0

to cysteine proteinases irreversibly in a 1:1 molar ratio (Hanada *et al.*, 1978; Tamai *et al.*, 1981). Titration of the highly purified cathepsin L from rabbit liver gave a linear response with compound E-64. From this plot the concentration of enzyme in the purified sample was calculated to be 0.228 μM. Therefore, from an *M<sub>r</sub>* value of 29 000, the concentration of active enzyme was 6.6 μg/ml. On the basis of the assay with Coomassie Brilliant Blue, this was 80% of the total protein in the sample, indicating that the preparation contained 20% inactive protein. A similar titration curve was obtained with compound Ep-475 as inhibitor. From these results, on the basis of the concentration of active enzyme the *k<sub>cat</sub>* value was calculated to be 20 s<sup>-1</sup>. Kirschke *et al.* (1982) obtained a value of 26 s<sup>-1</sup> for lysosomal preparations of cathepsin L from rat liver.

#### Substrate specificity of cathepsin L

The main substrates used for the detection of cathepsin L are azocasein, collagen and Z-Phe-Arg-NMec. The pH optima of the purified rabbit liver cathepsin L for these substrates were found to be 5.2, 3.3 and 6.0 respectively (Fig. 4). The pH-activity profile for azocasein hydrolysis was much broader than those of the other two substrates, with at least 75% of the maximal activity being

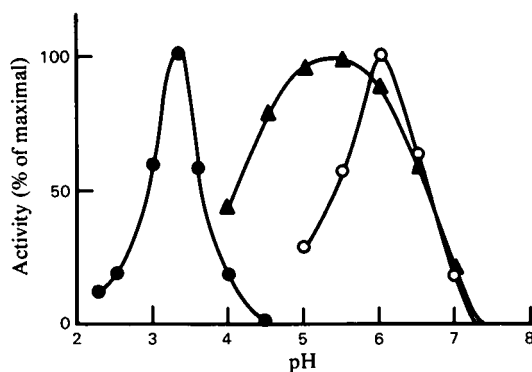


Fig 4. *pH-activity profiles for substrates of cathepsin L*. Activities are expressed as percentages of maximal activity. ▲, Azocasein hydrolysis; ○, Z-Phe-Arg-NMec hydrolysis; ●, collagen solubilization.

detectable in the pH range 4.5–6.0. At neutral pH, however, only a small percentage of azocaseinolytic activity remained. For the azocasein assay, the addition of 3M-urea to the incubation medium as described by Barrett & Kirschke (1981) did not alter the pH optimum but gave a higher yield of acid-soluble material. However, similar values of *A*<sub>366</sub> absorbance could be measured when either less trichloroacetic acid was used to precipitate protein or urea was incorporated into the trichloroacetic acid stop solution (results not shown). It would appear, therefore, that the urea has an effect on the solubility of the azocasein peptides rather than increasing the susceptibility of the azocasein to proteolysis by cathepsin L. The inclusion of urea in the incubation medium may be useful when contamination with cathepsin B is suspected, as urea has been reported to depress the azocaseinolytic activity of this enzyme (Kirschke *et al.*, 1982).

To date the only synthetic substrate found for cathepsin L is Z-Phe-Arg-NMec (Kirschke *et al.*, 1982). This substrate is, however, also degraded by cathepsin B. Therefore, in an attempt to find an alternative substrate, several analogues of Z-Phe-Arg-NMec were tested. Two of these were cleaved by cathepsin L, namely Bz-Phe-Val-Arg-NMec and Suc-Ala-Phe-Lys-NMec (Table 3). These were degraded at a much lower rate than Z-Phe-Arg-NMec, and were also degraded by cathepsin B. An important difference between the activities of cathepsins B and L against Z-Phe-Arg-NMec is in their Michaelis constants. The value for cathepsin L was found to be 0.7 μM, whereas that for cathepsin B was much higher, at 75 μM. Thus the relative contribution of cathepsin L to recorded activity in a mixture of the two enzymes will be greatest at low substrate concentrations.

Table 3. Substrate specificity of cathepsins L and B

Substrate concentration was 10  $\mu\text{M}$  in all cases. Assays were for 10 min under standard conditions with a fixed amount of enzyme. Activities are expressed as percentages of Z-Phe-Arg-NMec hydrolysis.

Substrate	Activity (%)	
	Cathepsin L	Cathepsin B
Z-Phe-Arg-NMec	100 ( $K_m$ 0.7 $\mu\text{M}$ )	100 ( $K_m$ 73 $\mu\text{M}$ )
Bz-Phe-Val-Arg-NMec	9 ( $K_m$ 6 $\mu\text{M}$ )	80 ( $K_m$ 6 $\mu\text{M}$ )
Z-Gly-Pro-Arg-NMec	0	2
Suc-Ala-Phe-Lys-NMec	6 ( $K_m$ 5 $\mu\text{M}$ )	30 ( $K_m$ 8 $\mu\text{M}$ )
Z-Gly-Pro-NMec	0	0
Suc-Gly-Pro-Leu-Gly-Pro-NMec	0	0
Z-Arg-Arg-NMec	1	70 ( $K_m$ 25 $\mu\text{M}$ )

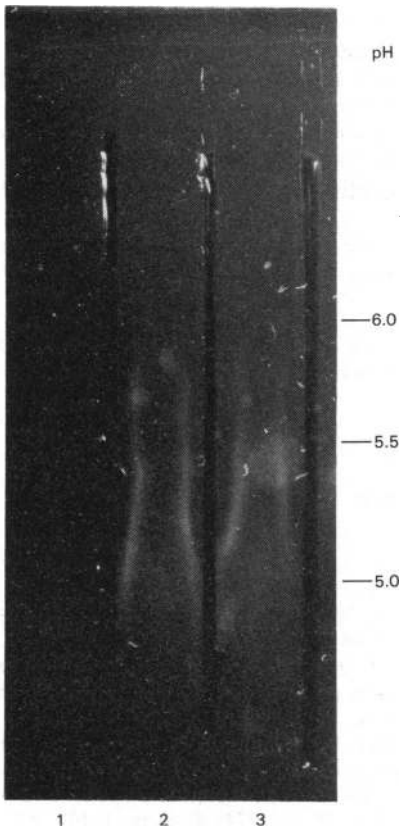


Fig. 5. Direct detection of enzyme-antibody complexes formed in agarose gels after isoelectrofocusing and immunodiffusion by Z-Phe-Arg-NMec-hydrolysing activity. Immune IgG was applied to the troughs for immunodiffusion after isoelectrofocusing of the samples as in Fig. 3. Track 1 contained a cathepsin B preparation; tracks 2 and 3 contained separate preparations of cathepsin L. Unbound proteins were removed before the staining for activity as described in the Methods section.

against purified IgG by the Ouchterlony immunodiffusion technique, and after the gels had stood for 4 days at 4°C enzymic activity was located in the precipitin lines by the methods described above. Active enzyme was detected in a single precipitin line (results not shown). No cross-reaction with rabbit liver cathepsin B could be detected.

Immunodiffusion of the isoenzymes after isoelectrofocusing gave a single precipitin line containing active enzyme that appeared to include most of the different isoenzymic forms (Fig. 5). This indicates that antibody has been raised to all of these forms, suggesting that they are derived from a common protein. However, the enzyme-antibody complex contained insufficient protein for detection by Coomassie Brilliant Blue R.

**Discussion**

Previously cathepsin L has been purified from lysosomal extracts of rat liver, since homogenates of whole rat liver yield considerably less active enzyme, owing presumably to high concentrations of cytosol inhibitors (Kirschke *et al.*, 1977). In the present paper we report that, by using tissue homogenates of whole rabbit liver as starting material, cathepsin L can be purified to a high specific activity. In part, this may be due to the dissociation of much of the enzyme-inhibitor complexes during the early purification steps, as revealed by the initial increase in activity.

Cathepsin L from rabbit liver was found originally to occur as three isoenzymic forms (Mason *et al.*, 1982). The direct detection of Z-Phe-Arg-NMec-hydrolysing activity in isoelectrofocusing gels had revealed that there is a whole range of minor isoenzymic forms of cathepsin L, with pI values ranging from 5.0 to 5.9. Similar patterns of isoenzymes were found in freshly prepared extracts from either whole tissue or lysosomes. It would therefore appear that these multiple forms exist *in vivo*. There is no conclusive evidence available as yet for determining whether

*Antibodies to cathepsin L*

Polyclonal antibodies to rabbit liver cathepsin L were raised in a goat. The enzyme was tested

or not all of these represent genetically different proteins, but it is likely that at least some of them are the result of limited proteolysis either *in vitro* or *in vivo*.

Rabbit liver cathepsin L is very similar to rat liver cathepsin L in many respects. Both enzymes are probably glycoproteins, as revealed by their ability to bind to concanavalin A-Sepharose. The unbound fraction may be the result of limited hydrolysis of the enzyme by lysosomal hydrolases removing the carbohydrate groups. The specific activity of these enzymes for Z-Phe-Arg-NMec are very similar, and both are inhibited by compound E-64. The  $K_m$  for the rat liver enzyme is reported to be  $7\ \mu\text{M}$  (Kirschke *et al.*, 1982), as opposed to  $0.7\ \mu\text{M}$  found for rabbit liver. In our laboratory we have found rat liver enzyme prepared from tissue homogenates to have a similar  $K_m$  to the rabbit liver enzyme (D. J. Etherington & M. A. J. Taylor, unpublished work). A. J. Barrett (personal communication) has recalculated the  $K_m$  for rat cathepsin L and obtained a lower value of  $2.1\ \mu\text{M}$ .

pH-activity profiles vary according to the substrate used. The pH optimum for the solubilization of collagen was found to be 3.3, which is similar to the value found previously for other cysteine proteinases (Etherington, 1980). The major reason for this low value is probably that the collagen substrate is highly swollen at low pH values, and hence cleavage sites are more readily available to enzymic hydrolysis below pH4. The pH optimum for Z-Phe-Arg-NMec is higher than for the general proteinase substrate azocasein. This could be due to the easier accessibility of the small- $M_r$  substrate into the cleavage site of the enzyme. For protein substrates, accessibility will be restricted by ionic interactions with more sites on the enzyme. A similar shift in pH optima according to the size of substrate has been described for cathepsin B (Otto, 1971).

Of the range of small- $M_r$  NMec derivatives tested, Z-Phe-Arg-NMec was the most readily hydrolysed. Cathepsin L purified from rat liver has also been reported to have low activity against other synthetic substrates (Barrett & Kirschke, 1981), and a study by Katunuma *et al.* (1983) has failed to find a specific substrate for rat liver cathepsin L. Such specificity is rather surprising when one considers the rather generalized proteolytic activity of cathepsin L on peptides with known sequences (Kargel *et al.*, 1980). More-specific substrates may be found by altering the C-terminal leaving group, if the enzyme has specific binding sites on the amino side of the cleavage site. It has been suggested that the inhibitor compound E-64 may bind to such sites on the enzyme (Barrett *et al.*, 1982).

Previous work on cathepsin B has indicated that

this enzyme is not a suitable antigen, and antibodies can be raised only to the denatured enzyme (Barrett, 1973). However, it has been reported that antibodies could be raised to the active enzyme if it were conjugated to bovine serum albumin (Pierart-Gallois *et al.*, 1977). Results given in the present paper show that antibodies can be raised to active rabbit liver cathepsin L in goats by conventional techniques. The antibody preparation appeared to react with most of the isoenzymic forms of cathepsin L. The precipitin lines were continuous, indicating that only one enzyme-antibody complex had been formed. Therefore all of these isoenzymic forms are probably derived from a common protein.

From these studies it would therefore appear that rabbit liver cathepsin L is very similar to the enzyme found in rat liver. However, the rabbit liver enzyme is far less susceptible to irreversible inhibition in tissue extracts than is the rat enzyme. This smaller degree of inhibition simplifies the isolation of cathepsin L from rabbit liver by eliminating the necessity of cellular-fractionation techniques.

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