Human Cathepsin B1

PURIFICATION AND SOME PROPERTIES OF THE ENZYME

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1. Cathepsin B1 was purified from human liver by a method involving autolysis, fractional precipitation with acetone, adsorption on, and stepwise elution from, CM-cellulose and an organomercurial adsorbent, gel chromatography and finally equilibrium chromatography on CM-cellulose. 2. The early stages of the procedure, including the use of the organomercurial adsorbent, were suitable for the simultaneous isolation of cathepsin D. The two cathepsins were sharply separated on the organomercurial column, and particular attention was given to the method for the preparation and use of this adsorbent. 3. A method is described for the staining of analytical isoelectric-focusing gels for cathepsin B1 activity, as well as protein. By this method it was shown that cathepsin B1 was represented by at least six isoenzymes during the greater part of the purification procedure. After the gel-chromatography step this group of isoenzymes was obtained essentially free of other proteins, in good yield. The isoenzymes were resolved from this mixture by chromatography on CM-cellulose. The purified enzyme was stable for several weeks at slightly acid pH values in the absence of thiol compounds; it was unstable above pH 7. 4. The pI values of the isoenzymes of cathepsin B1 extended from pH 4.5 to 5.5, that of the major isoenzyme tending to increase from 5.0 to 5.2 during the purification procedure. Gel chromatography indicated a molecular weight of 27 500 for all of the isoenzymes, whereas polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate gave a value of 24 000. 5. An antiserum raised in sheep against the purified enzyme reacted specifically with the alkali-denatured molecule. Purified cathepsin B1 contained no material precipitable by an anti-(human cathepsin D) serum. 6. The enzyme hydrolysed several *N*-substituted derivatives of L-arginine 2-naphthylamide, as well as haemoglobin, azo-haemoglobin, azo-globin and azo-casein. Greatest activity was obtained near pH 6.0. 7. The sensitivity of human cathepsin B1 to chemical inhibitors was generally similar to that of other thiol proteinases. The enzyme was inactivated by the chloromethyl ketones derived from tosylphenylalanine, tosyllysine, acetyltetra-alanine and acetyldialanylprolylalanine. 8. The hydrolysis of α -*N*-benzoyl-DL-arginine 2-naphthylamide by extracts of human liver at pH 6 was attributable entirely to cathepsin B1.

Otto and his colleagues (Otto & Schepers, 1967; Otto, 1967; Otto & Bhakdi, 1969; reviewed by Otto, 1971) showed that the enzymic activity of 'cathepsin B' (Greenbaum & Fruton, 1957) was attributable to the action of two enzymes, now called cathepsins B1 and B2 (Barrett & Dingle, 1971). Cathepsin B1 is now generally accepted to be a thiol-dependent lysosomal proteinase, which is also active on several synthetic substrates and has a molecular weight of about 25 000.

Cathepsin B1 is known to occur in rat and several other species, but work on its purification has been confined largely to the bovine enzyme (Otto, 1967; Keilová & Keil, 1969; Snellman, 1969; McDonald *et al.*, 1970; Franklin & Metrione, 1972). The purpose of the present paper is to report a procedure for the preparation of human cathepsin B1 in a highly

purified state. The procedure, which makes use of an organomercurial-Sephadex adsorbent, has the advantages of allowing the simultaneous isolation of cathepsin D from the same tissue sample and seems to be applicable to other species.

The availability of pure cathepsin B1 makes possible a detailed study of its specificity of action on low-molecular-weight substrates and proteins, and the use of specific antisera to the enzyme may help to establish its physiological functions.

Materials

Human liver free from disease was removed in the course of post-mortem examinations and stored at -20°C until required.

Arquad 2C-50, a 50% (w/v) solution of crude didodecylammonium chloride also containing analogues of longer and shorter chain length, in aq. 50% (v/v) propan-2-ol, was obtained from Armour Hess Chemicals Ltd., Leeds LS1 4NR, U.K. When required for use, Arquad 2C-50 was diluted with 4 vol. of water, and the mixture was warmed until it became clear; it was used, while still clear, at about 30°C.

Bz - DL - Arg - NHC₁₀H₇ (α - *N* - benzoyl - DL - arginine 2-naphthylamide hydrochloride), α - *N* - benzoyloxycarbonyl-L-arginine 4-methoxynaphthylamide hydrochloride and α - *N* - benzoyl-DL-arginine 4-methoxyanilide hydrochloride were purchased from Bachem, Liestal, Switzerland. Albumin (bovine serum, crystallized), α -chymotrypsinogen A (bovine, 6×crystallized), ovalbumin (grade V), soya-bean trypsin inhibitor (type 11-S), Fast Garnet GBC, Tos - LysCH₂Cl (TLCK, 7 - amino - 1 - chloro - 3 - tosylamido - L - heptan - 2 - one), Tos - PheCH₂Cl (TPCK, 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one), 4-hydroxymercuribenzenesulphonic acid and *N*-ethylmaleimide were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. 5,5'-Dithiobis(2-nitrobenzoic acid), di-isopropyl phosphorofluoridate, casein and cytochrome *c* and myoglobin (both bovine) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Coomassie Brilliant Blue R 250, prepared by Imperial Chemical Industries, was supplied by G. T. Gurr, High Wycombe, Bucks., U.K. Cyanogen bromide and Hyflo Super-Cel were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Sephadex G-75 (superfine grade), Sepharose 4B and Blue Dextran were products of Pharmacia (Great Britain) Ltd., London W5 5SS, U.K., whereas CM-cellulose and DEAE-cellulose were CM-52 and DE-52 respectively from Reeve Angel Scientific Ltd., London EC4V 6AY, U.K. 4-Aminophenylmercuric acetate and pentachlorophenol were purchased from Ralph N. Emanuel Ltd., Wembley HA0 1PY, Middx., U.K. 2,2'-Dipyridyl disulphide, phenylmethanesulphonyl fluoride and puromycin dihydrochloride were from Calbiochem Ltd., London W1H 1AS, U.K.

Agarose - Gly - Gly - L - Tyr (*O* - Bzl) - L - Arg (lot no. 400/4) was purchased from Miles-Seravac (Pty) Ltd., Maidenhead, Berks., U.K. The chloromethyl ketones derived from *N*-acetyl-tetra-L-alanine (Ac-[Ala-],AlaCH₂Cl) and *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine (Ac-[Ala-]₂Pro-AlaCH₂Cl) were prepared and kindly given by Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, Ga., U.S.A. Iodoacetic acid and iodoacetamide were recrystallized from *n*-heptane before use. 2-Bromo-2-phenylacetophenone was purchased from Kodak Ltd., Kirkby, Liverpool L33 7UF, U.K. Leupeptin-Pr (propionyl-Leu-Leu-DL-ArgH) was kindly given by

the Banyu Pharmaceutical Co. Ltd., 7, 2-chome, Nihonbashi Honcho, Chuo-ku, Tokyo, Japan.

Bovine haemoglobin was prepared as described by Barrett (1970). Azo-haemoglobin and azo-casein were prepared by treating the proteins with diazotized sulphanilic acid, essentially as described by Charney & Tomarelli (1947), but the quantity of diazonium salt was doubled for haemoglobin. Azo-globin was prepared by pouring a 4% (w/v) solution of azo-haemoglobin into 10 vol. of 1.5% (v/v) conc. HCl in acetone at 0°C. The precipitate was dialysed against water and diluted to 4% (w/v).

Methods

Assay of cathepsin B1

The procedure used as a routine was that of Barrett (1972b) in which the substrate is Bz-DL-Arg-NHC₁₀H₇ and free 2-naphthylamine is determined colorimetrically after coupling with Fast Garnet GBC (diazotized *o*-aminoazotoluene). One unit of activity hydrolysed 1 μ mol of substrate/min under the reaction conditions (so that the unit is 1000-fold larger than that of Barrett, 1972b). Enzyme preparations were diluted to 30 munits or less/ml before assay, with a solution containing 10 mM-sodium acetate buffer, pH 4.7, 1 mM-disodium EDTA and 1 mg of crystalline bovine serum albumin/ml. On some occasions, controls were run with 1 mM-4-hydroxymercuribenzenesulphonic acid in the incubation mixtures in place of cysteine and EDTA, to detect trypsin-like activity selectively.

Assay of proteolytic activity

Cathepsin D was assayed as described by Barrett (1970). The proteolytic activity of cathepsin B1 was detected by a modification of this method, in which reaction mixtures contained 1 mM-EDTA and 2 mM-cysteine, and were buffered at pH 6.0 with 0.10 M-sodium-potassium phosphate. Haemoglobin was 2% (w/v), and azo-haemoglobin, azo-globin and azo-casein were 1% (w/v), finally. Incubation was at 40°C for 30 min. The colour due to trichloroacetic acid-soluble reaction products was read at 280 nm, or at 366 nm for the azo-proteins.

Determination of protein

The concentration of protein solutions was determined either by measurement of *E*₂₈₀ or by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard, as indicated in the Results section. The dry-weight concentration of material insoluble in aq. 80% (v/v) acetone was also measured for samples encountered early in the purification procedure, which contained large amounts of particulate material.

Analytical isoelectric focusing

The method was that of Barrett (1970), and LKB carrier ampholines of pH range 5–7 were used at a final concentration of 1% (w/v). Sets of eight gels were stained for protein, after fixation for 1 h in 400 ml of 5% (w/v) trichloroacetic acid, with Coomassie Brilliant Blue R 250 in 400 ml of a buffered solvent prepared by dissolving 20 g of sodium formate in a mixture of formic acid (35 ml), ethanol (330 ml) and water (630 ml). After being left for 4 h with 100 µg of dye/ml, or overnight with 50 µg/ml, the gels were stored in water to which 1 g of 1,1,1-trichloro-2-methylpropan-2-ol (chlorbutol) and 1 mg of pentachlorophenol in 9 ml of butan-1-ol had been added per litre as preservatives; no destaining was necessary.

Whole gels, or half gels cut longitudinally, were stained for cathepsin B1 activity as follows. Each gel was incubated for 3–5 min at 40°C in 5 ml of 0.10 M-KH₂PO₄–Na₂HPO₄ buffer containing 1.33 mM-EDTA, 2.67 mM-cysteine and 4.0 mg of benzyloxycarbonyl-L-arginine 4-methoxynaphthylamide hydrochloride introduced in 100 µl of dimethyl sulphoxide. 4-Methoxynaphthylamine liberated within the gels by the action of cathepsin B1 was located by transferring the gels to a solution of Fast Garnet GBC (1 mg/ml) in 5 mM-4-chloromercuribenzoate–25 mM-EDTA, for 15 min at room temperature. Crimson bands on a pale-yellow background indicated the position of cathepsin B1 activity. The gels were stored in the preservative solution. The function of the 4-chloromercuribenzoate and EDTA in this procedure was exactly as described for the quantitative assay (Barrett, 1972b), but the reaction product was insoluble in the absence of BRIJ-35. In some experiments the substrate was replaced by Bz-DL-Arg-NHC₁₀H₇ or leucine 2-naphthylamide, and sometimes cysteine was replaced in the incubation mixture by 1 mM-4-hydroxymercuribenzenesulphonate.

Organomercurial–Sephacrose

Preparation. Sephacrose 4B was 'activated' by treatment with cyanogen bromide, by a method based on that of Axén & Ernback (1971), and then coupled with 4-aminophenylmercuric acetate in a procedure similar to that of Sluyterman & Wijdenes (1970). Sephacrose 4B gel (200 g) was well washed with water on a sintered-glass filter, and suspended in 200 ml of water. A solution of 8 g of cyanogen bromide in 320 ml of water was poured in, and the pH was rapidly raised to pH 11 by addition of 2 M-NaOH, with constant stirring. The pH was maintained for 6 min by addition of more alkali, and the gel was returned to the sintered-glass funnel and quickly washed with a large volume of 0.10 M-NaHCO₃, at 4°C. The activ-

ated Sephacrose was suspended in 400 ml of 10% (v/v) dimethyl sulphoxide, and 0.52 g of 4-aminophenylmercuric acetate in 40 ml of dimethyl sulphoxide was introduced slowly, with stirring. The mixture was stirred at 4°C overnight, transferred to a large chromatographic column and washed with 4 litres of 20% (v/v) dimethyl sulphoxide, so that the E_{260} of the effluent fell to 0.10.

Before use, the adsorbent was converted into its cysteine mercaptide form by treatment with 10 mM-cysteine in 50 mM-sodium acetate buffer, pH 5.5, containing 200 mM-NaCl and 1 mM-EDTA; it was then reconverted into the free mercurial form, and its capacity was determined, as described below.

Regeneration. After use (see the Results section for details) the adsorbent was reactivated from its cysteine mercaptide by treatment, in the chromatographic column, with 2 bed vol. of 10 mM-HgCl₂ in 20 mM-EDTA and 50 mM-sodium acetate buffer, pH 4.8. The flow rate was 20 ml/h per cm², and the excess of HgCl₂ was finally removed by a wash with several bed volumes of 1 mM-EDTA in 0.20 M-NaCl and 50 mM-sodium acetate buffer, pH 5.5.

Determination of capacity. The adsorbent was separated from excess of buffer on a sintered-glass filter, and about 1 g of the damp gel was weighed, resuspended in water and poured into a short chromatographic column of 1 cm diameter. The gel was treated with a few ml of 0.10 M-KH₂PO₄–Na₂HPO₄ buffer, pH 8.0, followed by 5 ml of the same buffer containing 1 mg of 5,5'-dithiobis-(2-nitrobenzoic acid)/ml and 2 mg of Na₂SO₃/ml, so that the adsorbent was saturated with 2-nitro-5-thiobenzoic acid. The gel was washed with a few ml of 50 mM-sodium acetate buffer, pH 4.8, and the 2-nitro-5-thiobenzoic acid was eluted with 2 mM-HgCl₂ in the same buffer. The effluent (5–10 ml) was collected after the application of the HgCl₂; it was diluted to about 25 ml, adjusted to 0.10 M-cysteine, adjusted to pH 8.5 with 1 M-NaOH and made up to exactly 50 ml with water. The E_{412} was measured, and the capacity of the adsorbent was calculated from the molar extinction of 13600 litre·mol⁻¹·cm⁻¹ given by Ellman (1959) for the anion of 2-nitro-5-thiobenzoic acid:

$$\text{Capacity } (\mu\text{mol/g}) = \frac{E_{412} \times 50}{13.6 \times \text{wt. of adsorbent (g)}}$$

As a routine, the same portion of gel was treated once more with 0.10 M-KH₂PO₄–Na₂HPO₄ buffer, pH 8.0, and the determination of capacity was repeated exactly as before. A result appreciably higher than the first value was an indication that the adsorbent had initially been incompletely regenerated. Newly prepared organomercurial–Sephacrose had a capacity of 2.5–3.5 µmol/g; this fell somewhat on repeated use. No deterioration in the quality of the separations obtained with adsorbent was noted,

but it was used only so long as the capacity remained above $1\text{ }\mu\text{mol/g}$.

Immunochemical methods

Two sheep were immunized with purified human cathepsin B1, one animal being injected with activatable enzyme, whereas the other received enzyme inactivated by carboxymethylation (prepared as described by Starkey & Barrett, 1973). On day 0, 1.5 mg of immunogen in 2.5 ml of 0.9% NaCl solution was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit 1, Mich., U.S.A.) and injected into two intramuscular sites. The procedure was repeated on day 15 and, for one animal, on day 30 as well: the animals were exsanguinated about 10 days after the final injection.

Sheep anti-(human cathepsin D) serum was prepared as described by Dingle *et al.* (1971).

Double immunodiffusion by the method of Ouchterlony (1967) was done with 1% (w/v) Meath agarose (from Medical and Biological Instrumentation Ltd., Ashford, Kent, U.K.) in 20 mM-KH₂PO₄-Na₂HPO₄ buffer, pH 6.0 or pH 7.0, containing 0.15 M-NaCl, or a buffer containing 21.6 mM-barbitone sodium and 21.6 mM-sodium acetate, adjusted to pH 8.2 with 1 M-HCl.

The method of Starkey & Barrett (1973), with benzyloxycarbonyl-L-arginine 4-methoxy-2-naphthylamide as substrate and Fast Garnet GBC as coupling reagent, was used in an attempt to detect activity of cathepsin B1 in precipitin lines.

Testing of potential inhibitors

A stock solution of purified cathepsin B1 (70 munits/ml) was adjusted to 2 mM-cysteine and 1 mM-EDTA, and left for 5 min. The activated enzyme was then diluted 1:3 (v/v) with 0.01% bovine serum albumin solution through which O₂-free N₂ had been bubbled for 30 min.

Portions of the dilute enzyme solution (0.5 ml, containing 120 pmol) were transferred to assay tubes and 1.5 ml of 0.10 M-KH₂PO₄-Na₂HPO₄ buffer, pH 6.0, alone or containing a potential inhibitor, was mixed with each. After preincubation for 5 min at 40°C, or 18 h at 4°C, 50 μ l of Bz-DL-Arg-NHC₁₀H₇ (40 mg/ml) in dimethyl sulphoxide was mixed in, to start the reaction. After a further 10 min the assays were concluded by the standard method (Barrett, 1972b). In positive controls, the pH 6 buffer contained no inhibitor. Reagent blanks were prepared for each inhibitor by introducing the diluted enzyme to a tube in which buffer, inhibitor and substrate had been incubated, immediately before the coupling reagent was added. It was also established that none of the inhibitors modified the colour yield (*E*₅₂₀) in the reaction of 2-naphthyl-

amine with Fast Garnet GBC under the conditions used. Some slight modifications in the composition of the incubation mixtures were necessitated by the properties of the inhibitors, as follows: 75 mM-N-(2-acetamido)-iminodiacetic acid-NaOH buffer, pH 6.0, replaced phosphate buffer for CaCl₂; 5% (v/v) propan-2-ol was used with Tos-PheCH₂Cl, 5% (v/v) ethanol with phenylmethanesulphonyl fluoride, 1.0–0.01% (v/v) methanol with the peptide chloromethyl ketones, and 1.25% (v/v) dimethyl sulphoxide with 2-bromo-2-phenylacetophenone and 2,2'-dipyridyl disulphide.

Results

Purification of cathepsin B1

Somewhat surprisingly, it was found that with some modifications the early stages of a method for the isolation of human cathepsin D (Barrett, 1970) gave excellent results also with cathepsin B1, but because of the instability of cathepsin B1 above pH 7, the entire purification was done below this pH value. The purification scheme, together with the additional steps by which cathepsin D may be isolated at the same time, is summarized in the form of a flow diagram in Scheme 1.

Extraction and autolysis. The capsule and other connective-tissue elements of human livers were removed. The tissue was passed through an electric mincer and stored at -20°C until required.

About 3 kg of tissue mince, having been thawed at 4°C overnight, was dispersed in 2 parts (v/w) of a solution of NaCl (10 g/litre), butan-1-ol (20 ml/litre) and disodium EDTA (1 mM) by use of a Silverson homogenizer. Arquad 2C-50, in the form of a 20% (v/v) emulsion (see the Materials section), was poured slowly into the homogenate with brisk stirring, until 50 ml had been introduced for every litre of homogenate. The mixture was adjusted to pH 6.5 by addition of 2 M-Tris-HCl buffer, pH 9.0, or 5 M-sodium formate buffer, pH 2.8, as appropriate. After 30 min the homogenate was centrifuged in the MSE Mistral 6L centrifuge at 1500g for 30 min at 15°C. The almost clear red supernatant solution was decanted from the firm pellets, adjusted to pH 4.5 with 5 M-sodium formate buffer, pH 2.8, and incubated overnight at 40°C.

Arquad 2C-50 proved to be a more efficient flocculating agent for the removal of particulate material than the calcium phosphate used previously (Barrett, 1970). Although the value of autolysis was fully demonstrated only when it was followed by fractionation with acetone, there was a consistent increase in the total activity of cathepsin B1 in this stage (Table 1); this was probably due to partial inhibition of the enzyme by one or more proteins that were destroyed in autolysis. The alternative

Scheme 1. Flow diagram summarizing the procedure for purification of cathepsins B1 and D from human liver. Experimental details are given in the text and quantitative results are presented in Table 1.

possibility, that autolysis activates an inactive precursor of cathepsin B1, cannot be excluded.

Acetone fractionation. The preparation was cooled to 0°C by stirring in a stainless-steel vessel, surrounded by an ice–NaCl freezing mixture. Acetone (0.90 vol.) cooled to –5°C in the same way was run in, with stirring, over a period of 10 min. The first precipitate was removed by centrifugation at 1500g for 15 min at 0°C, in the Mistral centrifuge and discarded. A further portion of acetone, equal in volume to the first, was cooled and added to the supernatant as before. The second precipitate, containing cathepsins B1 and D, was collected by filtration through Whatman no. 90 paper in the presence of filter aid (10g of Hyflo Super-Cel/kg of tissue used). The filtrate was discarded, and the filter cake was extracted four times by stirring with 5 mM-disodium EDTA (100 ml/kg of tissue, for each treat-

ment). The pooled extracts were dialysed overnight against water (20 vol.) at 4°C.

The fractionation with acetone after autolysis afforded a valuable purification of both cathepsins B1 and D, with reasonable recoveries (Table 1).

CM-cellulose chromatography with stepwise elution. A quantity of CM-cellulose in its free acid form was suspended in water, adjusted to pH 4.0 with 1 M-NaOH and brought to a damp-dry state on a sintered-glass filter. The dialysed product of acetone fractionation (pH 4.0–4.3) was stirred for 30 min with 10g (damp weight) of the CM-cellulose/litre per E_{280} unit. The exchanger was separated on a sintered-glass filter, rinsed with 10 mM-sodium acetate buffer, pH 4.3, and packed as a short bed in a chromatographic column, in the same buffer. Cathepsins B1 and D were almost completely adsorbed on the ion exchanger, whereas some protein and much brown

Table 1. *Purification of human cathepsins B1 and D*

The yields of protein and enzymic activity are expressed per kg of tissue, and are mean values based on five separate preparations, each with about 3 kg of tissue. The value for protein in the first three stages is, more precisely, material insoluble in aq. 80% (v/v) acetone, whereas that for the later stages is the product of vol. (l) and E_{280} . As explained in the Results section, the specific activity of purified cathepsin B1 based on a Lowry *et al.* (1951) determination of protein was exactly twice the value derived from E_{280} .

	Cathepsin B1				Cathepsin D		
	Protein (g)	Activity (units)	Specific activity (units/ml/ E_{280} unit)	Yield (%)	$10^{-3} \times$ Activity (units)	Specific activity (units/ml/ E_{280} unit)	Yield (%)
Homogenate	203	419	0.0021	(100)	113	5.6	(100)
Arquad supernatant	68	309	0.0045	74	78	11	69
Autolysed extract	50	434	0.0087	104	60	12	53
Acetone (47–64%) fraction	1.26	381	0.302	91	55	44	49
CM-cellulose (stepwise elution)	0.54	224	0.415	54	26	48	23
Organomercurial–Sephadex							
Unadsorbed	0.22	5	—	1	23	105	20
Adsorbed	0.076	132	1.74	32	0.3	—	0.3
Sephadex G-75 superfine	0.037	101	2.73	24	—	—	—
CM-cellulose (equilibrium elution)	0.010	27	2.70	6	—	—	—
DEAE-cellulose	0.032	—	—	—	12	375	11

pigment were not adsorbed and were discarded in the filtrate.

The column was treated in a stepwise fashion, first with 200mM-sodium acetate buffer, pH4.3 (2 bed vol.), and then with 200mM-sodium acetate buffer, pH5.5, containing 200mM-NaCl. Two peaks of protein were eluted from the column with the 'fronts' of the two buffers; the first of these represented contaminating protein, and was discarded, whereas the second was rich in both cathepsins. The stepwise development of the column was convenient and rapid, and gave a highly concentrated product. Active fractions in the effluent could be recognized and pooled simply on the basis of the characteristic appearance of the record of E_{280} obtained with an LKB Uvicord II absorptiometer; no assays were required for individual fractions. The superior resolution that might have been achieved by a continuous gradient elution was not required at this stage in the purification. The quantitative results of the step are presented in Table 1.

Organomercurial–Sephadex chromatography. The pool of material eluted from CM-cellulose at pH5.5 was made 1mM with respect to disodium EDTA and run on to a column of organomercurial–Sephadex (50ml bed vol./litre per E_{280} unit). Unadsorbed protein, including cathepsin D, was eluted with 50mM-sodium acetate buffer, pH5.5, containing 0.20M-NaCl and 1mM-EDTA, at a flow rate of 20ml/h

per cm². The adsorbed proteins were eluted stepwise from the column as a sharp peak with 10mM-cysteine in the same buffer (Fig. 1). Assays of individual fractions typically showed that cathepsin B1 was distributed throughout the peak, and all fractions containing appreciable amounts of protein were combined. The pool was concentrated to 20ml under pressure of N₂ by use of an Amicon Diaflo apparatus fitted with a Millipore PSAC membrane. (Appreciable amounts of cathepsin B1 penetrated Amicon UM-10, UM-20E and PM-10 membranes). Because of the instability of cathepsin B1 in the presence of cysteine (see below), it was important to apply the concentrated pool to the next column promptly, or to change the buffer by further ultrafiltration.

The organomercurial column proved extremely efficient in the separation of cathepsins D and B1 (Table 1). As cathepsin D was almost completely unadsorbed, it can probably be concluded that the eight half-cysteine residues in the molecule (Barrett, 1971) form four disulphide bonds. The separation on this adsorbent was not so clear-cut when the buffer contained less NaCl, perhaps because of ion-exchange effects. Several batches of organomercurial–Sephadex prepared with 4-chloromercuribenzoate by the carbodi-imide method (Cuatrecasas & Anfinsen, 1971) proved to have a much lower capacity for both 2-nitro-5-thiobenzoic acid and protein than that prepared from 4-aminophenylmercuric acetate. It

Fig. 1. Organomercurial-Sephadex chromatography of cathepsin B1

Distribution of cathepsin B1 activity (—) and protein measured as E_{280} (----) in effluent from a column of organomercurial-Sephadex. After the bulk of the protein, including cathepsin D, had been eluted under starting conditions, cathepsin B1 was displaced by 10mM-cysteine (indicated by arrow). The fractions combined for further purification are indicated by the horizontal bar.

was surprising to find that 96% of the activatable cathepsin B1 in the sample applied to the column was bound to it, although most of this was certainly not in an active state; reduction with sulphite (Sluyterman & Wijdenes, 1970) was not required. Elution of cathepsin B1 with HgCl_2 , 4-chloromercuribenzoate or dithiothreitol was less satisfactory than with cysteine. H. Keilová (personal communication) has independently used organomercurial-Sephadex to purify bovine cathepsin B1 with success.

Chromatography on Sephadex G-75 (superfine grade). The concentrated pool of cathepsin B1 from organomercurial-Sephadex (20ml) was applied to a column (2.5cm $\times$ 85cm, 416cm³) of Sephadex G-75 (superfine grade), in 50mM-sodium acetate buffer, pH 5.5, containing 0.20M-NaCl and 1mM-EDTA. The column was run at 4ml/h per cm², and a typical elution profile is shown in Fig. 2. The major peak of protein, in which enzymic activity closely paralleled protein concentration, was preceded by several smaller peaks of inactive protein. The pool of highly purified cathepsin B1 from the column was quite colourless, some yellow material, probably not protein, having been greatly retarded.

Fig. 2. Sephadex G-75 gel chromatography of cathepsin B1

Distribution of cathepsin B1 activity (—) and protein measured as E_{280} (----) in effluent from a column of Sephadex G-75 (superfine). The fractions combined for further purification are indicated by the horizontal bar.

CM-cellulose equilibrium chromatography. The mixture of isoenzymes of cathepsin B1 contaminated by small amounts of inactive protein that was obtained by gel chromatography was resolved by rechromatography on CM-cellulose.

The sample (about 100mg of protein concentrated to 2ml by ultrafiltration) was equilibrated with 27mM-acetic acid-63mM-sodium acetate (pH 5.0) and was applied to a column (10cm $\times$ 1.2cm, 11cm³) of CM-cellulose equilibrated with the same buffer. Development of the column with the same buffer resulted in the progressive elution of isoenzymes of cathepsin B1 in order of decreasing isoelectric point (Fig. 3 and Plate 1). The fractions containing the major isoenzyme minimally contaminated with minor isoenzymes or inactive protein were combined and are referred to as purified cathepsin B1. The specific activity of purified cathepsin B1 was consistently found to be 2.6-3.2 units/ml per E_{280} unit. On the basis of a Lowry *et al.* (1951) determination of protein, it was 5.2-6.4 units/mg.

Franklin & Mettrione (1972) succeeded in separating isoenzymes of bovine cathepsin B1 by equilibrium chromatography on DEAE-cellulose, and also with a shallow salt gradient on CM-cellulose at pH 5.0. Cathepsin B1 of sheep thyroid was resolved into three separate peaks by chromatography on CM-cellulose (Suominen & Hopsu-Havu, 1971).

Fig. 3. Chromatography of cathepsin B1 on CM-cellulose under equilibrium conditions

Distribution of cathepsin B1 activity (—) and protein measured as E_{280} (----) in effluent from a column of CM-cellulose eluted under equilibrium conditions as described in the text. The fractions combined as the final product are indicated by the horizontal bar, and the vertical strokes above indicate the approximate equivalent effluent volumes at which samples were taken from another separation run under identical conditions, for isoelectric focusing, with the result shown in Plate 1.

In some experiments, preparative isoelectric focusing in a sucrose gradient containing 1% (w/v) LKB Ampholines pH4–6 was tried as an alternative to CM-cellulose. The resolution of isoenzymes was good, but the total recovery of activity was less than 20%.

An attempt was made to purify cathepsin B1 by use of agarose-Gly-Gly-Tyr(O-Bzl)-Arg, as described for papain (Blumberg *et al.*, 1970). The enzyme was bound by the adsorbent, but was not eluted under the conditions described for papain, or by 1 mM-HgCl₂.

Purification of cathepsin D. Cathepsin D was simply isolated as a by-product of the purification of cathepsin B1, from the pool of protein that passed unadsorbed through organomercurial-Sephadex. The solution was dialysed against a buffer, pH 7.8, containing 15 mM-Tris and 10 mM-HCl, and was then stirred for 15 min with DEAE-cellulose (50 g of damp absorbent/litre per E_{280} unit) that had been equilibrated with the same buffer. The adsorbent was filtered off, and the filtrate was treated with a further portion

of adsorbent in exactly the same way. The second portion of adsorbent was then packed into a chromatographic column, and the first was packed on top of it. The elution of the column was started with 2 bed vol. of the buffer with which the adsorbent had been equilibrated, followed by a linear gradient increasing to 75 mM-Tris–50 mM-HCl–100 mM-NaCl over 10 bed vol. A bimodal peak of cathepsin D appeared about mid-way through the gradient (probably representing a partial separation of isoenzymes). The fractions with high specific activity were combined. The major isoenzymes of human cathepsin D were isolated by preparative isoelectric focusing as described previously (Barrett, 1970).

Use of the purification procedure with other species. A few experiments with liver of chicken and rabbit have indicated that cathepsin B1 from these species can be isolated by the procedure developed for the human enzyme. The properties of the enzymes from these species are similar in many ways to those of the bovine and human enzymes.

Some properties of purified cathepsin B1

Isoelectric focusing. Samples of cathepsin B1 (about 0.1 unit) were run in isoelectric focusing gels, and the distribution of activity was demonstrated as is described in the Methods section. The results were extremely consistent between individual gels and sets of gels, but at first their significance was questioned because it could be shown that there was a serious loss of activity during the preparation and running of the gels. Homogenization of gels for determination of their total cathepsin B1 activity directly after polymerization, and also after focusing, showed that an approximately 50% loss of activity occurred during each step, so that the overall recovery was only 25%. The situation was not improved by such expedients as including dithiothreitol or other thiol reagents, HgCl₂ or EDTA in the gels, or running the sample into the gels after polymerization. It was eventually concluded that the method was giving an accurate indication of the existence and relative quantities of a number of distinct isoenzymes of cathepsin B1 in these samples, because of the internal consistency of the results between many samples and preparations, the close agreement between the distribution of enzymic activity and protein with the most highly purified enzyme samples, and the results of CM-cellulose chromatography in conjunction with isoelectric focusing. The finding that neither pretreatment of enzyme samples with dithiothreitol nor inclusion of this reagent in isoelectric focusing gels altered the pattern of isoenzymes, indicates that mixed disulphides formed between enzyme molecules and low-molecular-weight thiols of different charges were not responsible for the spread of isoelectric points.

EXPLANATION OF PLATE I

Gel isoelectric focusing of samples from equilibrium chromatography of cathepsin B1 on CM-cellulose

Isoenzyme patterns revealed by isoelectric focusing of samples from the effluent of a column essentially identical with that described in Fig. 3. The samples were taken at the approximate equivalent effluent volumes indicated in Fig. 3, and the gels were stained for enzymic activity as described in the Methods section. The isoenzymes showed a progressive increase in pI with effluent volume.

EXPLANATION OF PLATE 2

Gel isoelectric focusing and immunodiffusion of cathepsin B1

(a) Two halves of an isoelectric-focusing gel in which partially purified cathepsin B1 (as eluted from organomercurial-Sephadex) had been run, and stained (i) for protein and (ii) for enzymic activity. A number of isoenzymes have been resolved, and most correspond to zones of protein, at this stage. (b) Two halves of an isoelectric-focusing gel in which purified cathepsin B1 had been run, and stained (i) for protein and (ii) for enzymic activity. Also shown (iii) is an identical gel that had been immersed in an antiserum specific for alkali-treated cathepsin B1, at pH 8.6. A double ring of immunoprecipitate is seen, by dark-ground illumination, corresponding to the double zone of protein seen in (i). (c) Immunodiffusion at pH 8.2 of (i) activatable cathepsin B1, (ii) carboxymethylated cathepsin B1, and (iii) cathepsin B1 inactivated by exposure to pH 8.2 for 3 h at 20°C, against (iv) a sheep antiserum raised by injection of carboxymethylated cathepsin B1. Each of the antigens was at pH 8.2 when applied to the plate. (d) Immunodiffusion at pH 6.0 of samples as in (c). Each of the antigens was at pH 6.0 when applied to the plate.

In preparative isoelectric-focusing experiments, the isoenzymes of cathepsin B1 were located in the range pH4.5–5.5, the major peak of activity being at pH5.0–5.2.

Because of the possibility that the multiple forms of the enzyme represented an artifact of autolysis, a sample of material at the stage preceding autolysis was run in analytical isoelectric-focusing gels. The result was technically not entirely satisfactory because of the large amount of inactive protein and coloured material present, but a pattern of activity with a good general similarity to that seen at later stages was clearly discernible. During the later stages of each preparation there were some marked changes in the relative intensity of certain bands of activity, and the major zone was displaced downwards (in correspondence with a change of pI from 5.0 to 5.2 observed in preparative isoelectric focusing); it is not yet clear whether these changes were the result of selective loss of some forms, or interconversion. Plate 2(a) shows the pattern of six or more distinguishable isoenzymes in analytical isoelectric focusing of cathepsin B1 purified to the stage of elution from organomercurial–Sephadex.

It is noteworthy that closely similar patterns of isoenzymes of cathepsin B1 were obtained in five preparations of the enzyme, although tissue from only three individuals was used in each preparation. This may mean that the isoenzyme patterns do not vary greatly between individuals. The existence of multiple forms of cathepsin B1 has been reported by Franklin & Mettrione (1972) for bovine spleen and is implied by the results of Suominen & Hopsu-Havu (1971) for sheep thyroid.

Samples taken throughout the peak of activity eluted from Sephadex G-75 (superfine) and run in isoelectric-focusing gels showed an identical pattern of isoenzymes, so that there was no sign of any difference in molecular weight between isoenzymes. At this stage of purification the enzyme preparations showed a few minor bands of inactive protein, in addition to those with activity against Bz-DL-Arg-NHC₁₀H₇. The patterns of protein and enzymic activity obtained after chromatography on CM-cellulose under equilibrium conditions are shown in Plate 2(b). One of the two major bands of protein coincided with the major zone of enzymic activity whereas the second had no activity, and a diffuse minor zone of activity appeared where little protein was detectable. When halves of gels cut longitudinally were immersed in a 3:1 (v/v) mixture (pH8.6) of antiserum to cathepsin B1 (see below) and 2M-Tris-HCl buffer, pH9.0, both major bands of protein were seen to form immunoprecipitates (Plate 2b). Since the antiserum is specific for cathepsin B1 (in its alkali-denatured state), it was concluded that the inactive protein was not a contaminant, but inactivated cathepsin B1, probably that resulting from the large

loss of activity that occurred during the isoelectric focusing process.

Gel chromatography. The column of Sephadex G-75 (superfine) used in the purification of cathepsin B1 (see above) was calibrated with Blue Dextran, ovalbumin, chymotrypsinogen and cytochrome *c*. Cathepsin B1 was run on the column both alone and with the standards, in 50mM-sodium acetate, pH5.5, containing 0.20M-NaCl and 1mM-EDTA. The elution volume for cathepsin B1 was marginally less than that of chymotrypsinogen, and the molecular weight of the enzyme was determined to be 27500 by the method of Andrews (1965).

Gel electrophoresis. Purified human cathepsin B1 was run in electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). A single zone of protein was obtained with mobility corresponding to a molecular weight of 24000, in comparison with cytochrome *c* (mol.wt. 11700), myoglobin (mol.wt. 17200), chymotrypsinogen A (mol.wt. 25700) and ovalbumin (mol.wt. 43000) as standards (the molecular weights being taken from Weber & Osborn, 1969).

Since cathepsin B1 ran slightly ahead of chymotrypsinogen on Sephadex G-75 (superfine), but ran as if of lower molecular weight on sodium dodecyl sulphate–polyacrylamide gels, it would seem that one of the two proteins was behaving anomalously in one of the systems, although any such effect was too small to be positively identified by reference to the other standard proteins. Clearly, the results could also be explained by the existence of a second chain of very low molecular weight, in cathepsin B1. Franklin & Mettrione (1972) reported a molecular weight of 27000 for bovine cathepsin B1, and Keilová (1971), Snellman (1971) and Otto (1971) obtained values of 24000–25000.

The purified enzyme was run in disc electrophoresis (Davis, 1964), and one major protein zone was obtained with several minor zones of slightly lower mobility. No enzymic activity survived this electrophoresis at pH9, so it was not possible to decide whether the minor bands, as well as the major one, were due to forms of cathepsin B1. A single zone of protein, with enzymic activity, was obtained after electrophoresis in the system of Sakai & Gross (1967) at pH4.

Specific extinction coefficient. The protein content of purified enzyme preparations was determined by the method of Lowry *et al.* (1951), crystalline bovine serum albumin being used as standard. The average $E_{280}^{1\text{cm}}$ of solutions containing 1mg of protein/ml, on this basis, was 2.0.

Immunochemical properties. The antisera raised in sheep by injection of either purified cathepsin B1 or its carboxymethyl derivative produced a weak and diffuse precipitin reaction when run against the antigen in immunodiffusion plates buffered at pH7.0.

On barbitone-buffered plates at pH 8.2, however, a single, well-defined precipitin line was produced (Plate 2c). At this pH value, human cathepsin B1 was rapidly inactivated, and it therefore seemed possible that strong precipitin lines could be formed only with inactive enzyme. This possibility was supported by the finding that no precipitin lines at all were produced in immunodiffusion at pH 6.0. Arnon (1965) has reported the spontaneous dissolution of immunoprecipitates containing papain as a result of autolysis, but inactivation of cathepsin B1 with 4-chloromercuribenzoate or iodoacetate did not facilitate the formation of precipitin lines at pH 6 or 7. Exposure of the antigen to pH 8.2 for 3 h at 20°C, followed by readjustment of the pH to 6.0, however, allowed excellent precipitin lines to be produced at this pH value (Plate 2d). Immunoprecipitates formed with large amounts of active enzyme at pH 7.0 could not be shown to have any trace of cathepsin B1 activity at pH 6.0 when tested by the procedure of Starkey & Barrett (1973). It thus appears that the antiserum is specific for an alkali-inactivated form of cathepsin B1 formed in low yield at pH 7, but much more rapidly at pH 8–9. The carboxymethylated form of cathepsin B1 was used to immunize one animal because it is known that active cathepsin B1, but not this inactive derivative, is bound by human α_2 -macroglobulin *in vitro* (Starkey & Barrett, 1973), although the carboxymethyl-enzyme probably has a similar conformation to the active enzyme, since it, too, reacted with the antisera only at pH 8. If a similar binding to α_2 -macroglobulin occurred in the sheep, it might result in the rapid and selective elimination of the active enzyme from the circulation so that it could not act as immunogen. Since the antiserum raised in response to the carboxymethyl-enzyme also showed reactivity only with the alkali-denatured enzyme this explanation does not seem to be tenable. Rather, it seems likely that both activated enzyme and its carboxymethyl derivative were rapidly converted into their alkali-denatured state at physiological pH values in the animals immunized, and that these altered forms were the only effective immunogens. This situation would be somewhat analogous to that encountered in the attempt to raise antisera against active pepsin (Arnon, 1967).

All of the peaks of activity against Bz-DL-Arg-NHC₁₀H₇ separated in preparative isoelectric focusing gave precipitin lines showing complete immunological identity in immunodiffusion against one of the antisera at pH 8. Purified cathepsin B1 gave no reaction in immunodiffusion against sheep anti-(human cathepsin D) serum, even at high concentration; it was concluded that cathepsin D does not contaminate cathepsin B1 prepared in this way.

Stability. The purified enzyme (4–6 mg/ml) in sodium acetate buffer, pH 5.5, or sodium phosphate buffer, pH 6.0, each 0.05 M and containing 1 mM-

EDTA, was stored at 4°C. Some preparations initially of relatively low specific activity showed a roughly 2-fold increase in specific activity during storage; the reason for this is not understood, but Dr. J. K. McDonald (personal communication) had described a similar effect with bovine spleen cathepsin B1 freshly dissolved from the freeze-dried state. With the human enzyme the change did not involve any significant alteration in the pattern of isoelectrically focused isoenzymes. Other preparations maintained a specific activity in the order of 5 units/mg for several weeks, and the half-time for loss of activity was a matter of months. Samples stored with 1 mM-HgCl₂ also showed good stability, and were completely reactivatable. Under any of the satisfactory storage conditions the enzyme was not in a truly 'active' state, that is to say it did not show activity when assayed in the absence of a thiol reagent. The enzyme was markedly unstable on storage in the presence of cysteine or dithiothreitol; the reason for this was not established, but autolytic degradation may have occurred.

The instability of the enzyme to alkaline pH was demonstrated as follows. Purified cathepsin B1 (0.5 unit in 0.1 ml) was mixed with an equal volume of 0.5 M-Na₂HPO₄ (final pH 8.5) and left for 1 h at 20°C before being diluted to 2.0 ml and readjusted to pH 5.0 with 5 M-sodium formate buffer, pH 2.8. No activity was detectable after this treatment (i.e. less than 0.5% of the activity of a sample diluted to the same volume without exposure to alkaline pH). Sensitivity to neutral and alkaline pH has been reported for cathepsin B1 from bovine spleen by Otto (1971) and Franklin & Mettrione (1972).

Specificity and pH-dependence. It was reported previously that human cathepsin B1 hydrolyses benzyloxycarbonyl-L-arginine 2-naphthylamide and the corresponding 4-methoxy-2-naphthylamide, in addition to the benzoyl derivatives used in the present paper, but that benzoyl-DL-arginine 1-naphthylamide showed negligible hydrolysis (Barrett, 1972b). It has since been found that benzoyl-DL-arginine 4-methoxyanilide is also hydrolysed, although at a rather slow rate.

Purified cathepsin B1 degraded haemoglobin and azo-haemoglobin, azo-globin and azo-casein to fragments soluble in dilute trichloroacetic acid. In one preparative isoelectric-focusing experiment, proteolytic activity against azo-globin was shown to parallel activity against Bz-DL-Arg-NHC₁₀H₇ in the three largest peaks of activity. The pH optimum for digestion of azo-haemoglobin was close to 6.0, as was that for Bz-DL-Arg-NHC₁₀H₇ (Barrett, 1972b). A rather steep decline in activity above pH 7 was attributable to irreversible inactivation of the enzyme during the assay.

Inhibitors. Purified cathepsin B1 at high concentration was activated with cysteine and EDTA

and then diluted with an O₂-free 0.01% solution of crystalline bovine serum albumin (see the Methods section). The final concentrations of cysteine and EDTA in the incubation mixture were thus lowered to 125 and 62 μ M respectively, and that of the enzyme

was 60nM. A preliminary experiment showed that this method of minimizing the possible protection of the enzyme by the activators permitted the full activity of the enzyme to be expressed. The results are presented in Table 2.

Table 2. *Effect of potential inhibitors on human cathepsin B1*

The enzyme (120pmol in 2.0ml) was preincubated at pH6.0 with each compound for 5min at 40°C, before the 10min incubation with Bz-DL-Arg-NHC₁₀H₇. The values are means of at least two determinations. See the Methods section for details.

Compound	Final concn. (mM)	Inhibition (%)
4-Hydroxymercuribenzenesulphonic acid	0.1	100
	0.01	25
HgCl ₂	0.1	100
	0.01	55
HgCl ₂ +EDTA	0.1+1.0	96
	0.01+1.0	52
ZnSO ₄	0.1	96
	0.01	4
ZnSO ₄ +EDTA	0.1+1.0	15
	0.01+1.0	12
Sodium aurothiomalate	1.0	100
	0.1	92
CaCl ₂	10	39
	1.0	3
Iodoacetic acid	1.0	100
Iodoacetamide	1.0	97
N-Ethylmaleimide	1.0	99
2,2'-Dipyridyl disulphide	1.0	100
KCN	10	8
NaAsO ₂	1.0	40
NaAsO ₂ +cysteine+EDTA	1.0+2.0+1.0	0
Tos-PheCH ₂ Cl	0.1	100
	0.01	90
Tos-LysCH ₂ Cl	0.1	100
	0.01	100
Ac-[Ala-] ₃ AlaCH ₂ Cl	0.1	100
	0.01	100
	0.001	100
Ac-[Ala-] ₂ Pro-AlaCH ₂ Cl	0.1	100
Phenylmethanesulphonyl fluoride	1.0	70
2-Bromo-2-phenylacetophenone	0.1	100
Di-isopropyl phosphorofluoridate	1.0	24
6-Aminohexanoic acid	10	6
	100	9
Puromycin	1.0	7
Leupeptin-Pr	0.002	72
	0.0002	9
Soya-bean trypsin inhibitor	1.0mg/ml	50
	0.1 mg/ml	1

Powerful inhibition by 4-hydroxymercuribenzenesulphonate, Hg^{2+} and Zn^{2+} is clearly consistent with the classification of cathepsin B1 as a thiol proteinase, and the ability of EDTA to relieve inhibition by Zn^{2+} , but not Hg^{2+} , has also been described for papain (Sluyterman, 1967). As has been reported by Snellman (1971), the inhibition of cathepsin B1 by Ca^{2+} is greater than can be accounted for by binding to the essential thiol, and it seems that one or two carboxyl groups may be responsible for binding this cation. In separate experiments (A. J. Barrett, unpublished work) the inactivation of human cathepsin B1 by iodoacetamide at pH 6 was found to be distinctly slower and more variable in its completeness than that by iodoacetate, as has been reported for bovine cathepsin B1 (Snellman, 1971) and papain (Glazer & Smith, 1971). In our hands, cyanide is somewhat inhibitory to human cathepsin B1, and it does not activate the bovine enzyme (Greenbaum & Fruton, 1957), suggesting that the activatable form of cathepsin B1 differs in an important respect from that of papain (Klein & Kirsch, 1969) although its greatest effect on papain occurs above the pH limit for stability of cathepsin B1 (Kimmel & Smith, 1954). The degree of inhibition of human cathepsin B1 by arsenite was variable, and not so great as to lead us to conclude that the enzyme has a closely positioned pair of essential thiol groups, as did Misaka & Tappel (1971) for bovine cathepsin B. The abolition of the effect of arsenite by cysteine and EDTA also suggested that the effect was not due to the specific blocking of a dithiol.

Cathepsin B1 was inhibited by 2,2'-dipyridyl disulphide, as is papain (Brocklehurst & Little, 1970), presumably through the formation of a mixed disulphide involving the essential thiol.

Both $\text{Tos-PheCH}_2\text{Cl}$ and $\text{Tos-LysCH}_2\text{Cl}$ inhibit cathepsin B1 at first reversibly and then irreversibly, but $\text{Tos-LysCH}_2\text{Cl}$ reacted much more rapidly at a given concentration (P. M. Starkey & A. J. Barrett, unpublished work). Papain and ficin are also inhibited by both reagents (Glazer & Smith, 1971). Both of the *N*-acetylpeptide chloromethyl ketones inactivated cathepsin B1. The activity of these rather specific inhibitors of pancreatic elastase (Powers & Tuhy, 1972) further underlines the broad specificity of cathepsin B1.

Phenylmethanesulphonyl fluoride was not a very potent inactivator of cathepsin B1 after 5 min of preincubation, and the effect did not increase after incubation overnight at 4°C. This reagent inhibits papain completely (Whitaker & Perez-Villaseñor, 1968). Partially purified cathepsin B1 that was 70% inhibited by phenylmethanesulphonyl fluoride showed an unaltered pattern of zones of activity after analytical isoelectric focusing, so that no selective irreversible inactivation of particular isoenzymes had taken place. The partial inhibition of human cath-

epsin B1 by a rather high concentration of diisopropyl phosphorofluoridate (which did not increase during incubation at 4°C overnight) may well be due to the presence in the reagent of an impurity, as was found for the inhibition of ficin and bromelain (Gould & Liener, 1965; Murachi & Yasui, 1965). Similarly, the partial inhibition by soya-bean trypsin inhibitor at 1 mg/ml, but not at 0.1 mg/ml, was probably non-specific.

6-Aminohexanoic acid has sometimes been thought to be an inhibitor of cathepsin B (Ali, 1964), but we did not find that it had a significant effect. Puromycin is an inhibitor of such naphthylamidases as leucine naphthylamidase (Barrett & Poole, 1969) and dipeptidyl aminopeptidase II (McDonald *et al.*, 1968), but had little effect on cathepsin B1. The potent inhibition of human cathepsin B1 by the peptide aldehyde, leupeptin-Pr (propionyl-Leu-Leu-DL-ArgH) is consistent with the report that the hydrolysis of benzoylarginine amide by a preparation of pig liver was inhibited by this compound (Ikezawa *et al.*, 1971).

Contribution of cathepsin B1 to the naphthylamidase activity of human liver homogenate

Experiments were made to decide how much of the activity of human liver at pH 6 in the hydrolysis of naphthylamide substrates is due to cathepsin B1.

Assays in which whole homogenate prepared as described for the purification of cathepsin B1 was incubated with $\text{Bz-DL-Arg-NHC}_{10}\text{H}_7$ or α -*N*-benzyloxycarbonyl-L-arginine 4-methoxy-2-naphthylamide showed that the hydrolysis of the two substrates was completely inhibited by 1 mM-4-hydroxymercuribenzenesulphonate in the absence of cysteine and EDTA.

The two arginine substrates, together with leucine 2-naphthylamide, were used in the staining of isoelectric-focusing gels, as described in the Methods section. The hydrolysis of all three substrates was completely abolished by 4-hydroxymercuribenzenesulphonate. Each of the bands of activity against one of the arginine substrates was also detectable with the other, and these zones were all considered to be due to isoenzymes of cathepsin B1, because of their similar antigenic and other properties, as was explained above. In contrast, a single band of activity against leucine naphthylamide was obtained; this had a higher isoelectric point than any of the forms of cathepsin B1, and did not have activity against the arginine substrates.

The failure to detect trypsin-like naphthylamidase activity resistant to the thiol-blocking reagent may indicate that no such enzyme exists in human liver, but could equally well be due to the low pH (6.0) at which the assays were made. The complete absence of leucine naphthylamidase activity in association with zones of cathepsin B1 is further evidence for the

conclusion that cathepsin B1 has no action on the leucine substrate, in agreement with Barrett & Poole (1969) and other workers (see Distelmaier *et al.*, 1972).

Discussion

The purification of cathepsin B1 from bovine spleen and liver has been undertaken by several workers (Otto, 1971; Keilová, 1971; McDonald *et al.*, 1970; Snellman, 1969), but it remained for Franklin & Mettrione (1972) to apply criteria of homogeneity to preparations of the enzyme. Otto (1971) has shown that the enzyme is also present in rat liver, and it is clear from our work that it occurs in liver of chicken, rabbit and man. There now seems little doubt that cathepsin B1 will be found very generally in the tissues of higher animals.

A group of proteins with the property of hydrolysing Bz-DL-Arg-NHC₁₀H₇ and α -N-benzyloxycarbonyl-L-arginine 4-methoxy-2-naphthylamide at pH6, but separable by isoelectric focusing, was obtained in the purification procedure. These have been considered to be isoenzymes of cathepsin B1, rather than separate enzymes, because they were all inactivated at alkaline pH values as well as by 4-hydroxymercuribenzenesulphonate, and had the same molecular weight, and closely similar pI values. Moreover, they gave a reaction of complete immunological identity in immunodiffusion at pH8.2. The nature of the structural differences underlying the differences in pI between the isoenzymes has not been elucidated, but the substitution of only very few charged amino acid residues would be required. The possibility that the isoenzymes contain different amounts of sialic acid, as is the case with some other lysosomal enzymes (Barrett, 1972a), seems to be precluded by the fact that no hexosamine has been detected in amino acid analysis of human cathepsin B1 (A. J. Barrett, unpublished work), and it is therefore apparently not a glycoprotein.

It appears that the two *N*-substituted arginine naphthylamides are specific substrates for cathepsin B1, at pH6.0, in human liver, in contrast to the situation with rat liver (de Lumen & Tappel, 1972; Distelmaier *et al.*, 1972).

'Cathepsin B' was at one time considered to resemble trypsin, but it is now clear that cathepsin B1 fits well into the 'thiol proteinase' group of enzymes. This conclusion has been supported for human cathepsin B1 by its pH optimum and activation characteristics (Barrett, 1972b), its insensitivity to pepstatin, the peptide inhibitor of carboxyl proteinases (Barrett & Dingle, 1972), and by its sensitivity to inhibitors as reported here. Thiol proteinases already known include papain, ficin and bromelain, from the plant kingdom, and the bacterial clostripain

and streptococcal proteinase. Cathepsin B1 is the first well-characterized thiol proteinase from the tissues of man and higher animals. It will be of interest to discover whether cathepsin B1 has sequence homologies with the plant or bacterial enzymes.

By far the best known of the thiol proteinases is papain, and some close resemblances between cathepsin B1 and papain have been discerned (Keilová & Turková, 1970; Barrett, 1972a). Cathepsin B1 differs from papain, however, in its instability at alkaline pH values, and this is perhaps related to the difference between the isoelectric point in the region of pH5, for cathepsin B1, and that of pH8.75 for papain (Glazer & Smith, 1971). The specific activity of purified cathepsin B1, with Bz-DL-Arg-NHC₁₀H₇ as substrate, is not more than half that of crystalline papain (Barrett, 1972b), which is likely to have contained some inactive enzyme (Sluyterman & Wijdenes, 1970). There are indications (P. M. Starkey & A. J. Barrett, unpublished work) that the general proteolytic activity of human cathepsin B1 is lower, as a function of its naphthylamidase activity, than is that of rabbit cathepsin B1 or papain. Nevertheless, the endopeptidase activity of the human enzyme is significant and it has a papain-like action on cartilage proteoglycans (Morrison *et al.*, 1973), collagen (Burleigh, 1973) and other proteins (A. J. Barrett, unpublished work).

Two of the methods that we have used in this work may be valuable in the study of other thiol proteinases. The 'post-coupling' method for location of zones of cathepsin B1 activity has also been used for the demonstration of cathepsin B1 and papain in precipitin lines on immunodiffusion plates (Starkey & Barrett, 1973), and may even be suitable for the histochemical location of thiol proteinases.

The organomercurial-Sephrose adsorbent effected an exceptionally sharp separation of two groups of proteins, presumably differing in that only one contained thiol groups free to react with the adsorbent. This type of separation is likely to be of much value in protein chemistry.

The availability of purified and characterized human cathepsin B1 makes possible experiments designed to establish what part it may play in the lysosomal catabolism of proteins.

The pure enzyme has also been used in the identification of α_2 -macroglobulin as the principal inhibitor of cathepsin B1 in human serum (Starkey & Barrett, 1973).

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