A catalytically active high- M_r form of human cathepsin B from sputum

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A cysteine proteinase from purulent sputum was partially purified by a method involving affinity chromatography on Sepharose–aminohexanoylphenylalanyl-glycinaldehyde semicarbazone. It was immunologically related to lysosomal cathepsin B from human liver and was similar in many, but not all, other aspects. It was catalytically active, as demonstrated by active-site-directed radioiodination, and hydrolysed three cathepsin B substrates, two with K_m values similar to those of lysosomal cathepsin B. In addition, the rates of inactivation of the sputum and lysosomal forms of the enzyme by L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane (Compound E-64) were very similar. However, the sputum enzyme differed from lysosomal cathepsin B in the following respects. Inhibition by chicken cystatin was much weaker for sputum cathepsin B than for the lysosomal enzyme. Sputum cathepsin B had greater stability at pH 7.5 and a higher apparent M_r , even after deglycosylation, than lysosomal cathepsin B. We conclude that the form of cathepsin B found in sputum is probably a truncated form of human procathesin B, with some differences in properties that could be of physiological importance.

INTRODUCTION

The cysteine proteinase cathepsin B (EC 3.4.22.1), in common with other lysosomal enzymes, is synthesized as a prepro form with an M_r larger than that of the mature enzyme. The M_r of the polypeptide portion of human procathesin B (i.e. after cleavage of the signal peptide) is calculated to be 35900 from its cDNA (Chan *et al.*, 1986), whereas that of the enzyme isolated from liver is 27600 on the basis of its amino acid sequence (Ritonja *et al.*, 1985).

In human fibroblasts, cathepsin B is processed intracellularly, with 5% of newly synthesized precursor being secreted (Hanewinkel *et al.*, 1987). Two extracellular high- M_r forms of cathepsin B have been described. One is inactive, but can be activated by incubation with pepsin at low pH (Mort *et al.*, 1983). The other was reported to be active against low- M_r synthetic substrates (Recklies *et al.*, 1982; Mort & Recklies, 1986), but the evidence for this was indirect, immunoreactivity being demonstrated in a partially purified preparation that also showed cathepsin B-like activity. This putative active form has only been demonstrated in tissue culture. Both the inactive and active forms had apparent- M_r values of 40000, consistent with procathesin B in its glycosylated state (Chan *et al.*, 1986). They also differed from the lysosomal form of the enzyme in their greater stability at neutral pH.

Cathepsin B-like activity has been detected in human bronchoalveolar-lavage fluid and sputum (Burnett *et al.*, 1983; Burnett & Stockley, 1985; Chang *et al.*, 1986). We now describe the partial purification and properties of the enzyme from bronchiectatic sputum.

EXPERIMENTAL

Materials

Z-Arg-Arg-NHnap dipicrate was a gift from Dr. C. G. Knight of the Tissue Physiology Department of this Laboratory, and was converted into the dihydrochloride by a method described by him (Knight, 1980). Z-Tyr-Ala-CHN₂, provided by Dr. E. N. Shaw, Friedrich Miescher Institute, Basel, Switzerland, was radioiodinated by Dr. R. W. Mason of this Department, who also provided human cathepsin L labelled with this reagent. N-Glycanase [peptide- N^4 -(N-acetyl- β -glucosaminyl)-asparagine amidase; EC 3.5.1.52] was a gift from Genzyme Corp., Boston, MA, U.S.A. Biotinylated donkey anti-(sheep/goat immunoglobulin) serum (affinity-purified) and horseradish peroxidase–streptavidin were from Amersham International, Amersham, Bucks., U.K. Compound E-64 (catalogue no. E3132), 2-naphthylamine hydrochloride, pepstatin A, 1,10-phenanthroline monohydrate, iPr₂P-F, agarose (type II) and Brilliant Blue G were from the Sigma Chemical Co., Poole, Dorset, U.K.

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with IUB–IUPAC Recommendations (1984); additional abbreviations are: Ahx, 6-aminohexanoyl; E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane; GlySc, glycinaldehyde semicarbazone; NHMec, 7-(4-methyl)coumarylamide; NHnap, 2-naphthylamide; iPr₂P-F, di-isopropyl fluorophosphate; HEDS, 2-hydroxyethyl disulphide; f.p.l.c., fast protein liquid chromatography (Pharmacia system); the abbreviations used for kinetic parameters are: K_m , the Michaelis constant; K_i (app), apparent dissociation constant of enzyme and inhibitor in the presence of substrate; K_i , the inhibition constant; [S], total substrate concentration; [I], total inhibitor concentration; k_{obs} , the apparent pseudo-first-order rate constant for inactivation; k_2 (app), apparent second-order rate constant for inactivation in the presence of substrate; k_2 , second-order rate constant for inactivation.

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Fast Garnet base (4-amino-2,3-dimethylazobenzene) was from Cambrian Chemicals, Croydon, Surrey, U.K. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Bachem Feinchemikalien A.G., Bubendorf, Switzerland. HEDS (98 %) was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Fisons (Loughborough, Leics., U.K.) provided $(\text{NH}_4)_2\text{SO}_4$ specially low in heavy metals and acrylamide specially purified for electrophoresis. *NN'*-Methylenebisacrylamide (specially purified for electrophoresis) and Nonidet P40 were from BDH, Dagenham, Essex, U.K. The nitrocellulose membrane sheets (Trans-Blot) were from Bio-Rad Laboratories, Watford, Herts., U.K., and the medical X-ray film was from Fuji Photo Film (U.K.) Co., London, U.K.

Protein determination

Protein concentrations were determined by the Bio-Rad dye-binding assay (Bio-Rad Laboratories), with bovine serum albumin as standard.

Cathepsin B assay

The enzyme sample was activated for 20 min at 40 °C in the presence of 50 mM-Mes/1 mM-EDTA/2 mM-cysteine, pH 5.5 (final volume 0.975 ml). Blank values were obtained by the inclusion of E-64 (10 μM) in duplicate incubations. Z-Arg-Arg-NHnap (25 μl of a 20 mM solution) was added and the incubation was continued for 15 or 30 min, after which time the reaction was stopped by the addition of 1 ml of the colour reagent (Barrett, 1976). One unit of enzyme activity is defined as that releasing 1 nmol of 2-naphthylamine/min under the assay conditions described.

Partial purification of cathepsin B from bronchiectatic sputum

Purulent-sputum samples were collected from patients with bronchiectasis (diagnosed radiologically). The samples were centrifuged at 50000 *g*, 3 °C for 90 min to obtain the sol phase. The supernatants were stored at -70 °C until sufficient quantities (20–30 ml) had been collected for preparation. They were then thawed, and the following proteinase inhibitors were added from their respective stock solutions: pepstatin (1 $\mu\text{g}/\text{ml}$ from a 100 $\mu\text{g}/\text{ml}$ solution in ethanol), 1,10-phenanthroline (0.1 mM from a 10 mM solution in ethanol), *iPr*₂P-F (1 mM from a 500 mM solution in propan-2-ol), HEDS (2 mM from a 100 mM solution in water). The material was clarified by centrifugation at 2000 *g*, and the supernatant was adjusted from a pH in the region of 8.0 (range 7.70–8.04) to 7.6 with 50 mM-Tris/HCl, pH 7.5. The following manipulations were done at 4 °C.

Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly, with stirring, to 40 % saturation. After 30 min, the mixture was centrifuged at 25000 *g* for 20 min. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant with stirring to 90 % saturation. After 30 min the mixture was again centrifuged at 25000 *g* for 20 min. The pellet was resuspended in 3 ml of 0.1 M-sodium phosphate buffer containing 0.02 % Brij 35, pH 6.3 (Buffer A) and dialysed against three changes of 100 ml of Buffer A.

The $(\text{NH}_4)_2\text{SO}_4$ fraction was clarified by centrifugation at 2000 *g*, and dithiothreitol was added to 2 mM. After 20 min at 0 °C it was applied to a column (6 ml bed vol.) of Sepharose-Ahx-Phe-GlySc (Rich *et al.*, 1986) in Buffer A in 5 ml. The flow (25 ml/h per cm^2) was interrupted for 3 h, and the column was then washed

with 2 bed vol. of Buffer A. Then 1 bed vol. of 0.05 M-Tris/HCl/0.5 M-NaCl/0.1 % Brij 35 in 20 % (v/v) glycerol, pH 7.5 (Buffer B), containing 2 mM-HEDS was applied to the column and the flow was again stopped. The column was left for 16 h, and elution was resumed with another 2 bed vol. of Buffer B containing 2 mM-HEDS. Fractions of volume 1 ml were collected throughout. Fractions containing activity against Z-Arg-Arg-NHnap eluted in Buffer B were combined and concentrated to 0.3 ml.

The affinity-purified material was run on a TSK-G 2000 SW (LKB) gel-chromatography column (7.5 mm $\times$ 600 mm) in Buffer B, at a flow rate of 0.2 ml/min, and 0.5 ml fractions were collected in tubes containing sufficient 100 mM-HEDS to give a final concentration of 2 mM.

Determination of kinetic constants

Continuous rate assays with peptidyl coumarylamide substrates were used to determine K_m values, and inhibition constants were determined with chicken cystatin and E-64. For the measurement of K_m values with Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, sputum cathepsin B or human liver cathepsin B, was activated in 50 mM-Mes/1 mM-EDTA/0.005 % Brij 35/2 mM-dithiothreitol, pH 5.5, at 30 °C for 10 min. Pre-warmed water was added, followed by substrate (5–150 μM final concn.) to 3.0 ml. The initial rate of reaction was then determined in a temperature-controlled cell with a magnetic stirrer. Data collection was via a microprocessor linked to the fluorimeter (Perkin-Elmer LS-3B) (excitation 360 nm, emission 460 nm), and the K_m was calculated by non-linear regression analysis.

Chicken cystatin was form 2 purified as described by Anastasi *et al.* (1983), titrated with papain which had previously been standardized by E-64 titration (Zucker *et al.*, 1985). For the determination of the $K_{i(\text{app.})}$ for the reaction between the forms of cathepsin B and chicken cystatin, continuous rate assays similar to those described above were performed. The enzyme was activated as described above, and Z-Arg-Arg-NHMec (25 or 50 μM final concn.) was then added. The rate of substrate hydrolysis was recorded, after which inhibitor (ranging from 5×10^{-10} to 2×10^{-7} M final concn.) was added in a negligible volume. The initial rate was allowed to relax to the new steady state, which was then recorded. Non-linear regression analysis of the replot of fractional activity versus inhibitor concentration (Henderson, 1972) gave $K_{i(\text{app.})}$. The K_i was found from the $K_{i(\text{app.})}$ by the relationship:

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

The rate constant for inactivation by E-64 was also determined by continuous rate assays. The reaction was started by the addition of pre-activated enzyme to a reaction mixture containing the Mes/EDTA/Brij 35/dithiothreitol buffer system (above) with 25 or 50 μM -Z-Arg-Arg-NHMec and the inhibitor. The E-64 concentration was varied from 6.25 to 75 nM. The inhibitor was assumed to be a direct inactivator (Knight, 1986), and the value of $k_{\text{obs.}}$ was derived by non-linear regression (Stone & Hofsteenge, 1985). $k_{2(\text{app.})}$ (calculated as $k_{\text{obs.}}/[\text{I}]$) was constant over the range of inhibitor concentrations tested, and k_2 was found by the equation:

$$k_2 = k_{2(\text{app.})}(1 + [\text{S}]/K_m)$$

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed, with reduction, essentially as described by Bury (1981) in slab gels containing 12.5% (w/v) total acrylamide. The samples were prepared by precipitation with 12.5% (w/v) trichloroacetic acid, which concentrated and inactivated the enzyme samples.

Active-site-directed radioiodination of cathepsin B

The partially purified sputum enzyme (26 μ g of protein) was mixed with 2.5 nmol of Z-Tyr([125 I])Ala-CHN₂ in 1 ml of 50 mM-Mes buffer, pH 5.5, containing 2 mM-dithiothreitol. Human liver cathepsin B was labelled in the same way.

Deglycosylation

Radioiodinated sputum cathepsin B (0.87 μ g of protein) was mixed in a 50 μ l volume with 10 μ g of bovine serum albumin, divided equally between two tubes and precipitated with 12.5% (w/v) trichloroacetic acid. The pellets after centrifugation were washed with acetone, air-dried and boiled for 5 min in 0.5% SDS containing 100 mM-2-mercaptoethanol (10 μ l) and cooled. Then 320 mM-sodium phosphate/16 mM-1,10-phenanthroline hydrate/2% (v/v) Nonidet P40, pH 8.6 (20 μ l) was added, followed by 0.625 unit of *N*-glycanase to one of the tubes. The two tubes were incubated for 16 h at 37 °C, after which the protein was recovered by precipitation with trichloroacetic acid and subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography.

Immunological methods

Preparation of affinity-purified anti-(cathepsin B) IgG and anti-chymopapain IgG. Human liver cathepsin B for use as an antigen, and for coupling to CNBr-activated Sepharose 4B to provide an affinity column, was purified by two different methods, as described below, in order to diminish the chances of a common contaminant being present in the material for injection and in the material coupled to the affinity column.

Cathepsin B was obtained from human liver by the method of Schwartz & Barrett (1980). It was purified further by chromatography on the f.p.l.c. Mono Q HR10/10 column using a NaCl gradient (0–0.2 M) in 20 mM-sodium phosphate buffer/1 mM-EDTA, pH 6.0. Active fractions were pooled and then dialysed against 20 mM-sodium phosphate buffer/1 mM-EDTA, pH 6.0. The enzyme was inactivated by carboxymethylation of the active-site cysteine (10 mM-iodoacetic acid after activation with 2 mM-dithiothreitol) and 200 μ g was emulsified with complete Freund's adjuvant and injected intramuscularly into a sheep. Three booster injections of 100 μ g were given on weeks 2, 4 and 30. Serum was collected on week 33.

IgG was obtained by (NH₄)₂SO₄ precipitation and chromatography on DEAE-cellulose essentially as described by Davies *et al.* (1978). Fractions exhibiting a precipitating reaction with human liver cathepsin B in double immunodiffusion at pH 8.3 (see below) were combined.

Human liver cathepsin B was also purified by the method of Rich *et al.* (1986), followed by chromatography on the Mono Q column (see above); 35 mg of the pure enzyme was coupled to CNBr-activated

Sepharose 4B (35 ml) as described for papain (Anastasi *et al.*, 1983). Under these conditions (pH 9.0), cathepsin B is irreversibly denatured. The sheep IgG pool (see above) was applied to the immunoaffinity column, and the column was washed with 20 mM-sodium phosphate/0.5 M-NaCl, pH 7.3. Cathepsin B-directed IgG was then eluted from the column with 50 mM-diethylamine, pH 11.5, and 5 ml fractions were collected into tubes containing 0.65 ml of 2 M-potassium phosphate, pH 7.2. Those containing protein (monitored by A_{280}) were combined and dialysed against 10 mM-sodium/potassium phosphate/140 mM-NaCl buffer, pH 7.4, containing 0.01% NaN₃, then stored frozen. A 95 mg portion of cathepsin B-directed IgG was recovered from 1.35 g of protein applied to the affinity column (assuming $A_{280}^{1\%} = 14.0$).

Carboxymethylated chymopapain, purified by the method of Buttle & Barrett (1984), was used to raise an antiserum in a sheep. IgG was partially purified by (NH₄)₂SO₄ fractionation (Davies *et al.*, 1978).

Double immunodiffusion. The precipitin reaction of anti-(cathepsin B) IgG with native and denatured liver cathepsin B was tested by using 1% agarose in 0.1 M-sodium acetate buffer, pH 5.0, and in 90 mM-Tris/80 mM-boric acid, pH 8.3, respectively. Plates were allowed to develop for 24 h before being washed, dried and stained with Brilliant Blue G (Barrett, 1974).

Immunoblotting. Proteins separated by SDS/polyacrylamide-gel electrophoresis were transferred electrophoretically to nitrocellulose sheets (Towbin *et al.*, 1979). The sheets were washed twice in 10 mM-Tris/HCl/0.15 M-NaCl/0.05% Triton X-100, pH 7.4 (washing buffer), then blocked in two successive incubations containing 10% (v/v) horse serum in the same buffer (blocking buffer), before being washed again as described above. The sheets were incubated for 1 h at 25 °C, with agitation, in 15 ml of the blocking buffer containing 20 μ g of affinity-purified anti-(cathepsin B) IgG, then washed before incubation with agitation in a 1:500 dilution of biotinylated donkey antibody to sheep immunoglobulin in blocking buffer (15 ml) for 1 h. The washing step was repeated, and the sheets were incubated for 30 min in a 1:300 dilution of peroxidase-streptavidin. A final wash was followed by detection in a solution containing 10 mg of 4-chloro-1-naphthol in 0.5 ml of methanol and 24.5 ml of Tris/HCl buffer containing 0.15 M-NaCl, pH 7.4, and 10 μ l of H₂O₂ (30%, v/v).

Recognition of the partially purified sputum enzyme by anti-(cathepsin B) IgG. Two sheets of nitrocellulose were soaked overnight at 4 °C in 15 ml of 10 mM-sodium/potassium phosphate/140 mM-NaCl, pH 7.4, containing 5 mg of either affinity-purified anti-(cathepsin B) IgG or anti-chymopapain IgG. Horse serum (to 10%, v/v) was then added, and the sheets were incubated for a further 6 h before being rinsed in water, followed by electroblotting buffer [25 mM-Tris/192 mM-glycine/0.1% SDS in 20% (v/v) methanol, pH 8.3].

Radioiodinated human liver cathepsins L and B, and labelled partially purified sputum cathepsin B, were individually mixed with 5 μ g of bovine serum albumin, then subjected to SDS/polyacrylamide-gel electrophoresis. After electrophoresis the proteins were electroblotted on to, or through, the prepared nitrocellulose

sheets; any protein passing through was retained on a second, untreated, sheet. The blots were rinsed and autoradiographed with a fluorescent screen.

RESULTS

Partial purification of cathepsin B from bronchiectatic sputum

Cathepsin B-like activity was assayed with Z-Arg-Arg-NH₂NPap and detection of 2-naphthylamine after coupling to an azo dye (Barrett, 1976). Despite its lower sensitivity, this assay was preferred to the more convenient assay with Z-Arg-Arg-NH₂Mec because of interference (both positive and negative) to fluorimetry by substances present in crude sputum samples.

Preliminary attempts at purification of the sputum enzyme on a column of Sepharose-Ahx-Phe-GlySc resulted in poor yields, as the bulk of the activity failed to bind to the column. It was subsequently found that the yield could be improved by a prolonged incubation on the column, and (NH₄)₂SO₄ precipitation was adopted as

a means of partially purifying and concentrating the sample so that it could be applied in less than one column volume. Nevertheless we were still unable to achieve binding of all the enzyme(s) responsible for activity against Z-Arg-Arg-NH₂NPap to the column (see Fig. 1*a*). When material that had passed straight through the column was reappplied under the same conditions, a similar proportion to that in the first run was bound. This indicates a poor affinity between enzyme and ligand, rather than the presence of multiple proteinases active against Z-Arg-Arg-NH₂NPap in the inhibitor-treated sputum sample. The recovery of greater than 100% of activity after affinity chromatography (despite the failure of all the enzyme to bind to the column) suggests the presence of a reversible inhibitor in the sputum samples (see Table 1).

The bound activity could be eluted from the affinity column in Buffer B. It was found, however, that the material was recovered in a smaller volume after an overnight incubation in elution buffer containing 2 mM-HEDS. Affinity chromatography resulted in a 265-fold increase in specific activity.

Fig. 1. Partial purification of cathepsin B from bronchiectatic sputum

(*a*) The (NH₄)₂SO₄ fraction was activated and run on the Sepharose-Ahx-Phe-GlySc column as described in the Experimental section. The arrow represents the point at which the flow was stopped after the column was filled with Buffer B containing 2 mM-HEDS. The material enclosed by the bar, with high specific activity against Z-Arg-Arg-NH₂NPap, was eluted the following day, pooled, concentrated and applied to the gel-filtration column in (*b*). (*b*) The pool from the affinity column was applied to the TSK-G2000 SW column and eluted as described in the Experimental section. The column had been calibrated with a mixture of Blue Dextran 2000 (*V*₀), bovine serum albumin (*M*_r 68 000), egg albumin (42 000), bovine chymotrypsinogen A (27 500), horse cytochrome *c* (12 700) and potassium ferricyanide (*V*_i). Sputum cathepsin B, which was eluted at a position corresponding to *M*_r 37 000 (marked by the bar), was collected and characterized.

Table 1. Partial purification of cathepsin B from bronchiectatic sputum

Protein was determined by the Bio-Rad dye-binding assay. The unit of enzyme activity is defined in the Experimental section.

Stage	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Clarified sputum	350	371	1.06	100	1
(NH ₄) ₂ SO ₄ fraction	310	197	0.64	53	0.6
Sepharose-Ahx-Phe-GlySc chromatography	1.74	489	281	132	265
TSK-G 2000 SW chromatography	0.30	208	693	56	654

Fig. 2. SDS/polyacrylamide-gel electrophoresis of fractions from stages in the purification of sputum cathepsin B: autoradiography and deglycosylation of the radioiodinated enzyme

SDS/12.5 %-(w/v)-polyacrylamide-gel electrophoresis (with reduction) was used to analyse; a, the $(\text{NH}_4)_2\text{SO}_4$ fraction from bronchiectatic sputum; b, M_r standards; c, material purified by chromatography on a column of Sepharose-Ahx-Phe-GlySc (see Fig. 1a); d, the pool with high specific activity recovered from the gel-filtration column (Fig. 1b). Partially purified sputum cathepsin B (lane d) was radioiodinated with Z-Tyr(^{125}I)-Ala-CHN $_2$, electrophoresed as described above and autoradiographed with (lane e) or without (lane f) prior treatment with *N*-glycanase. See the Experimental section for details.

A further 2.5-fold increase was achieved by gel filtration (Table 1). As Fig. 1(b) shows, this step removed a substantial proportion of contaminating protein, but with a 58 % loss of activity (Table 1). The enzyme was eluted with an apparent M_r of 37 000; it was collected and stored in Buffer B containing 2 mM-HEDS. Under these conditions there was no loss of activity during 1 month.

The progress of the purification was monitored by SDS/polyacrylamide-gel electrophoresis. Fig. 2 (lanes a and c) shows that many of the proteins present in the $(\text{NH}_4)_2\text{SO}_4$ fraction were removed by affinity chromatography. Seven or eight protein bands were still visible, however (lane c). Some contaminating proteins were removed by gel filtration. Even so, the material used for characterization of the cysteine proteinase still contained a number of protein species, ranging in M_r from 60 000 to 25 000 after reduction (lane d).

Active-site-directed labelling with Z-Tyr(^{125}I)-Ala-CHN $_2$

The peptidyl diazomethanes react uniquely with the active-site cysteine residue of cysteine proteinases, causing irreversible inactivation (Shaw, 1984). Z-Tyr(I)-Ala-CHN $_2$ has been shown to be a particularly rapid inactivator of cathepsins B and L (Crawford *et al.*, 1988), so the radioactive analogue of this compound was used to label partially purified sputum cathepsin B. Labelling resulted in complete loss of activity against Z-Arg-Arg-NH Nap (not shown) and the appearance, after SDS/

polyacrylamide-gel electrophoresis and autoradiography, of a single band at M_r 36 000 (Fig. 2, lane f).

Removal of *N*-linked oligosaccharides

Treatment of the radiolabelled cysteine proteinase (see above) with *N*-glycanase followed by SDS/polyacrylamide-gel electrophoresis (with reduction) and autoradiography showed a shift in apparent M_r to 33 000 from 36 000 (Fig. 2, lanes e and f).

Identification of cathepsin B by immunoprecipitation

The affinity-purified anti-(cathepsin B) IgG gave a precipitin line against human liver cathepsin B in double immunodiffusion at pH 8.3. However, at pH 5.0 no reaction was detected (result not shown). It was concluded that the antibody preparation precipitated human liver cathepsin B denatured by incubation in alkaline media, but not the native enzyme. This is normal for antisera raised against cathepsin B (Barrett, 1973; Mort *et al.*, 1981a; Wardale *et al.*, 1986). At pH 8.3, only one line (of total identity with human liver cathepsin B) was formed when the antibody was tested against a clarified extract of human liver. A reaction of partial identity was formed between human and bovine liver cathepsins B (result not shown).

Immunoblot analysis of human liver cathepsin B run in SDS/polyacrylamide-gel electrophoresis with reduction demonstrated that the antibody recognized both the single-chain (M_r 29 000) form of the enzyme and the heavy chain (M_r 25 000) of the two-chain form. There

Fig. 3. Recognition of the partially purified sputum enzyme by anti-(cathepsin B) IgG

Human liver cathepsin L (lanes 1), human liver cathepsin B (lanes 2) and sputum cathepsin B (lanes 3) were labelled with Z-Tyr(^{125}I)-Ala-CHN $_2$ and subjected to SDS/polyacrylamide-gel electrophoresis with reduction. The proteins were transferred on to, or through, a nitrocellulose sheet previously soaked in anti-(cathepsin B) IgG (sheet a) or anti-chymopapain IgG (sheet c), and proteins passing through these sheets were collected on untreated sheets (b and d respectively). The sheets were autoradiographed and developed. See the Experimental section for details.

was no visible reaction with the light chain. The antibody recognized both the single chain and the heavy chain of bovine cathepsin B, and the single chain (the only form present) of sheep cathepsin B. No reaction was detected with human liver cathepsin L (results not shown). It was concluded that the antibody was monospecific for (denatured) cathepsin B and that forms of cathepsin B from various mammalian species possess common epitopes, as is also the case with cathepsin L (Mason, 1986).

Identification of the sputum cysteine proteinase as cathepsin B

Binding of the sputum enzyme to the monospecific antibody preparation raised against human liver cathepsin B was tested by electrophoretic transfer of the radiolabelled enzyme (Fig. 3, lanes 3) on to a sheet of nitrocellulose that had been soaked in the antibody, then saturated in horse serum (Fig. 3, sheet a). Radiolabelled human liver cathepsins B (lanes 2) and L (lanes 1) were treated the same way. Proteins passing through the nitrocellulose sheet were collected on a second, untreated, sheet (sheet b). In order to test for non-specific interactions between the enzymes and the antibody-treated sheet of nitrocellulose, the experiment was repeated with an antibody preparation directed against a plant cysteine proteinase, chymopapain, in place of the anti-(cathepsin B) IgG (sheet c). Both antibodies had been raised in sheep. It can be seen that the single-chain form of human liver cathepsin B (M_r 30000; lane a2) and the cysteine proteinase from sputum (M_r 36000; lane a3) both bound specifically to the anti-(cathepsin B) IgG; neither was retained by the anti-chymopapain-IgG-containing sheet. A small fraction of the sputum enzyme passed through, however, and was collected on the second sheet (lane b3). Some of the label associated with the light chain of human liver cathepsin B (M_r 5000) was retained non-specifically by the antibody-treated sheets (lane 2 on sheets a and c); this polypeptide is not recognized by the antibody to cathepsin B (see above). The heavy chain of the two-chain form of human liver cathepsin B does not contain the active-site cysteine residue (Ritonja *et al.*, 1985), and was therefore not detected by autoradiography. The heavy chain of human liver cathepsin L (M_r 25000), containing the active-site cysteine residue (Mason *et al.*, 1985) and therefore labelled, was not retained by either of the antibody-treated sheets, and in both cases was recovered on the second, untreated, sheets (lanes b1 and d1). From these experiments it was concluded that the sputum enzyme is a high- M_r form of cathepsin B.

Kinetic parameters and rate constants of inhibition

These were measured at pH 5.5 (the pH optimum of sputum cathepsin B; Burnett & Stockley, 1985) and 30 °C. K_m values with Z-Arg-Arg-NHMeC were 46 μ M for the sputum enzyme and 30 μ M for human liver cathepsin B. K_m values with Z-Phe-Arg-NHMeC were 71 μ M and 51 μ M respectively.

k_2 for the inactivation of sputum cathepsin B by E-64 was $1.6 \times 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$ and, for human liver cathepsin B, $1.5 \times 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$. The K_i for the inhibition of sputum cathepsin B by chicken cystatin was found to be 29 nM. This is more than an order of magnitude higher than the

K_i of 0.81 nM that was found for the human liver enzyme.

DISCUSSION

The cysteine proteinase from purulent sputum obviously shares many properties with lysosomal cathepsin B isolated from a solid tissue. Their immunological cross-reactivity, taken together with their hydrolysis of the same substrates and inhibition by the same inhibitors, strongly implies that the two enzymes are closely related. However, the difference in K_i for the inhibition of the two enzymes by chicken cystatin clearly demonstrates that sputum cathepsin B exhibits some modifications in properties, when compared with the lysosomal form of the enzyme.

Although the K_m values for the hydrolysis of the peptidyl-NHMeC substrates and k_2 for the inactivation by E-64 were very similar for the two forms of cathepsin B, the values reported here for human liver cathepsin B are significantly different from those previously reported. Thus K_m values for Z-Arg-Arg-NHMeC and Z-Phe-Arg-NHMeC reported here (30 μ M and 51 μ M) are markedly lower than reported values of 170 μ M and 250 μ M respectively (Kirschke & Barrett, 1987). k_2 for the inactivation by E-64 ($1.5 \times 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$) is much faster than has previously been found (e.g. $0.89 \times 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$; Rich, 1986). Most kinetic determinations with cathepsin B have been done in phosphate buffer at pH 6.0. We used Mes at pH 5.5, and therefore attribute the differences to an as-yet-unexplained buffer effect.

Cathepsin B from sputum differs fundamentally from the mature form of the enzyme in two respects. The sputum enzyme is stable over a period of weeks or months at pH 7.5, whereas human liver cathepsin B is unstable at this pH (Barrett, 1973). Sputum cathepsin B also has a significantly higher M_r than the enzyme from human liver, even after the removal of *N*-linked oligosaccharides. The M_r of the deglycosylated sputum enzyme (33000 by SDS/polyacrylamide-gel electrophoresis and autoradiography) is intermediate between that of the full-length proenzyme [35900 by cDNA sequence analysis (Chan *et al.*, 1986)] and the liver enzyme [27600 by amino acid sequencing (Ritonja *et al.*, 1985)]. The simplest interpretation of this finding, bearing in mind the immunological cross-reactivity of the sputum enzyme with human liver cathepsin B, is that the sputum enzyme is a partly processed form of procathepsin B. An extracellular form of human cathepsin B, M_r 35000, has previously been identified in the culture medium of tumour explants. This form was considered to be formed by limited proteolysis of the glycosylated (M_r 40000) precursor (Mort & Recklies, 1986).

It has been suggested that the processing of procathepsin B is a multi-step process, and that the intermediates may possess distinct biological properties (Chan *et al.*, 1986; Docherty *et al.*, 1984). The altered affinity for chicken cystatin as well as the stability at neutral pH could be provided by moieties present on the *N*-terminal extension of sputum cathepsin B.

The physiological significance, if any, of the presence of cathepsin B in purulent sputum remains to be established. The amount of the enzyme present is still not known. However, if one assumes the same turnover rate of Z-Arg-Arg-NHMeC by this enzyme and cathepsin B isolated from human liver, then the activity in purulent

sputum is equivalent to approx. 50 ng of active enzyme/ml or 1.35 nM. This value will be an underestimate of the total amount of enzyme present if, as seems likely, the enzyme in sputum is partially inhibited.

The pH optimum of the sputum enzyme is 5.5, with negligible activity remaining at and above pH 7.0 (Burnett & Stockley, 1985). The pooled sputum samples we examined had pH values above 7.0. Occasionally, the pH of purulent sputum may fall as low as 6.7 (Burnett *et al.*, 1983). Even so, it is likely that cathepsin B would require a more acidic environment for significant activity. Such local environments are thought to occur around metabolically active cells (Etherington, 1980).

The active high- M_r (40 000) form of human cathepsin B from mammary-gland-explant media was reportedly not bound by α_2 -macroglobulin, and it was therefore concluded that this form of the enzyme is unlikely to have activity against protein, as opposed to low- M_r synthetic substrates (Mort & Recklies, 1986). However, the active form of the enzyme of M_r 33 000 generated by pepsin treatment of ascites fluid was bound by α_2 -macroglobulin (Mort *et al.*, 1981b). Lysosomal cathepsin B is known to be active against important structural proteins, such as collagen (Burleigh *et al.*, 1974) and proteoglycan (Roughley, 1977). Such extracellular proteins are therefore potential substrates for the sputum enzyme.

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