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Collagenolytic cysteine proteinases of bone tissue

Cathepsin B, (pro)cathepsin L and a cathepsin L-like 70 kDa proteinase

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The aim of the work was to identify and characterize the cysteine proteinases of bone tissue, as these enzymes appear necessary for bone resorption. Three cysteine-dependent proteolytic activities were separated from a homogenate of mouse calvaria by a fractionation procedure involving $(NH_4)_2SO_4$ precipitation, gel filtration and ion-exchange chromatography. The first two are typical cathepsins B and L with respect to (1) their reactivity with anti-(cathepsin B) and anti-(cathepsin L) antibodies respectively, (2) their relative rate constants for inhibition by benzyloxycarbonyl-Phe-Phe-CHN_2 and L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucylamido-(4-guanidino)butane and (3) their enzymic properties, such as the higher activities of cathepsin L against collagen and gelatin as compared with cathepsin B, and the fact that benzyloxycarbonyl-Arg-Arg 4-methoxy-2-naphthylamide is hydrolysed only by cathepsin B. Cathepsin L was mainly recovered in its precursor form, as indicated by its apparent 40 kDa molecular mass and its relative stability at pH 7.2. The third enzyme is a cathepsin L-like proteinase with an apparent molecular mass of 70 kDa. It is immunoprecipitated by anti-(cathepsin L) antibodies, and appears as the 25 kDa band of mature cathepsin L in Western blots. It further resembles (pro)cathepsin L with regard to its activities against synthetic substrates and proteins such as collagen, and with regard to its response to various inhibitors. However, unlike (pro)cathepsin L, it is eluted as a 70 kDa protein on gel filtration (even in the presence of 1 % Brij or 1 M-NaCl), it is stable at pH values as high as 9, and it exhibits stronger affinity for phenyl-Sepharose. It might thus result from a strong complex between mature cathepsin L and another entity that confers stability at alkaline pH and favours hydrophobic interactions. This 70 kDa activity was also detected in mouse muscle and long bones of Ca^{2+}-deficient chicks but not in mouse liver, spleen or kidney.

INTRODUCTION

Bone resorption occurs at the contact of the ruffled border of the osteoclasts, in a sealed extracellular microenvironment that has many characteristics of a phagolysosome [1]. As reviewed elsewhere [2], the solubilization of the mineral at that level is due to the secretion of acid, which depends on the activity of carbonic anhydrase and proton pumps, whereas the degradation of the organic matrix (mainly collagen) occurs probably through the concerted action of lysosomal cysteine proteinases, lysosomal hydrolases and collagenase.

A role for lysosomal cysteine proteinases was initially considered because of the ability of cysteine proteinases (cathepsins B, L, N, S and T) of several tissues (spleen, liver, kidney, lung and placenta) to degrade native collagen at acid pH [3–10]. Direct evidence supporting their participation in bone resorption was provided by the demonstration that specific inhibitors of cysteine proteinases inhibit bone resorption in cultures of explanted bones [11–13] or of isolated osteoclasts [14], as well as *in vivo* [12]. Furthermore an ultrastructural study indicated that these agents inhibit the degradation of the organic matrix in the subosteoclastic resorption zone while allowing its demineralization to continue to some extent [15]. This suggested that the primary action of the inhibitors is on cysteine proteinases involved in the degradation of the collagenous bone matrix by the osteoclast.

Despite these observations, the information available about the nature of the cysteine proteinases present in bone remains limited [12,16]. The present work reports the characterization of three collagenolytic cysteine proteinases in this tissue: cathepsin B, cathepsin L, which is mainly recovered in its 40 kDa precursor form, and a 70 kDa proteinase that resembles cathepsin L but differs from it by its higher apparent molecular mass, its higher hydrophobicity and its stability at pH 9.

MATERIALS AND METHODS

Materials

Z-Phe-Phe-CHN_2 and rabbit anti-(rat cathepsin B) and anti-(rat cathepsin L) antibodies [17] were gifts from Dr. H. Kirschke and Dr. B. Wiederanders, Martin-Luther Universität, Halle, Germany. Rabbit antiserum against mouse cathepsin L [18] was a gift from Dr. M. Gottesman, National Cancer Institute, Bethesda, MD, U.S.A. Latex–Protein A (Protein A covalently bound to 0.8 μm latex particles) was given by Dr. J. M. Saint Remy and J. G. Gilles from our Institute. Rabbit chondrocytes [19] were kindly donated by Dr. V. Lefebvre from our laboratory. Z-Phe-Ala-CHN_2, Z-Arg-Arg-NH-NapOMe and Z-Phe-Arg-NH-Mec were from Bachem, Bubendorf, Switzerland. Brij 35, CHAPS, mannose 6-phosphate, pepstatin and cystatin were from Sigma Chemical Co., St. Louis, MO, U.S.A. CM-Sephadex, phenyl-Sepharose, Sephadex CL-4B, Polybuffer 74, Mono P and Superose 12 columns, as well as molecular-mass standards for gel filtration and electrophoresis, were from Pharmacia, Uppsala, Sweden. Centricon membranes were from Amicon, Danvers, MA, U.S.A. Ultrogel AcA 44 and Ultrogel AcA 54 were from LKB, Stockholm, Sweden. I-125 h.p.l.c. column was from Waters Associates, Milford, MA, U.S.A.

Abbreviations used: Z-, benzyloxycarbonyl-; -NH-Mec, 7-(4-methyl)coumarylamide; -NH-NapOMe, 4-methoxy-2-naphthylamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucylamido-(4-guanidino)butane.

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Phenylmethanesulphonyl fluoride was from Merck, Darmstadt, Germany. Casein (Hammersten quality) was from Nutritional Biochemicals, Cleveland, OH, U.S.A. The biotin–streptavidin–peroxidase reagents were from Amersham International, Amersham, Bucks., U.K. Other materials were from suppliers previously mentioned [12].

Enzyme assays

The assays with Z-Arg-Arg-NH-NapOMe and Z-Phe-Arg-NH-Mec as substrate (respectively 'Z-Arg-Arg-NH-NapOMe hydrolase' and 'Z-Phe-Arg-NH-Mec hydrolase') were performed almost as described in refs. [20] and [21], but by measuring fluorimetrically the release of methoxy-β-naphthylamine [22]. K_m values were estimated from Eadie–Hofstee plots, with the use of seven substrate concentrations and linear-regression analysis.

Hydrolysis of gelatin was performed in a 0.1 ml reaction mixture, containing the enzyme preparation, 0.1 M-acetate buffer, pH 3.5, 5 mM-EDTA, 5 mM-cysteine and 0.05% [^{3}H]gelatin (1×10^5–2×10^5 c.p.m./mg, obtained by heat denaturation of acid-soluble guinea-pig skin collagen, radiolabelled as described [23]). Controls were run either with heat-inactivated enzyme or by substituting 5 mM-iodoacetamide for 5 mM-cysteine. After 2–4 h incubation at 37 °C, the reaction was stopped by addition of 0.1 ml of ice-cold 28% (w/v) trichloroacetic acid. The radioactivity of the trichloroacetic acid-soluble degradation products was measured in a liquid-scintillation counter. One unit of activity refers to the amount of enzyme that degrades 1 μg of gelatin into trichloroacetic acid-soluble fragments in 1 min.

Hydrolysis of casein was determined in a similar way to that of gelatin. The 0.1 ml reaction mixture contained the enzyme preparation, 0.1 M-acetate buffer, pH 5.5, 1 mM-EDTA, 4 mM-cysteine and 0.5% [^{3}H]casein (800000 c.p.m./mg, radiolabelled as described in ref. [23]). After 4 h incubation at 37 °C, the reaction was stopped by addition of 0.1 ml of ice-cold 10% (w/v) trichloroacetic acid and the radioactivity of the trichloroacetic acid-soluble degradation products was measured. One unit of activity refers to the amount of enzyme that degrades 1 μg of casein into trichloroacetic acid-soluble fragments in 1 min.

Depolymerization of collagen was determined by incubating 50 μg of acid-soluble guinea-pig skin collagen in 0.1 ml of 65 mM-acetate buffer, pH 4, containing 2 mM-dithiothreitol, 5 mM-EDTA and the enzyme preparation. Controls were obtained either with heat-inactivated enzyme or by substituting 2 mM-iodoacetamide for 2 mM-cysteine. After 18–24 h incubation at 30 °C, the reaction mixture was denatured by addition of 0.05 ml of 0.187 M-Tris/HCl buffer, pH 6.8, containing 6% (w/v) SDS, 15% (v/v) glycerol, 6 mM-EDTA, 1.5 M-urea, 0.15 M-dithiothreitol and 0.03% Bromophenol Blue, and by increasing the temperature to 100 °C for 2 min. The α-, β- and γ-components were separated by SDS/PAGE (with separating gels of 6% acrylamide concentration) [24] and stained with Coomassie Blue. Their relative concentrations were estimated by scanning the gels at 600 nm in a densitometer (Beckman model CDS-200). The activity was determined either from the ratio of α- and β-components (which is directly proportional to the amount of enzyme and to the incubation time) [6] or from the amount of α-components generated in 1 min (which is not linear with enzyme concentration or with time).

Homogenization and $(NH_4)_2SO_4$ fractionation

A batch of 200 newborn-mouse calvaria (either non-cultured or recovered from cultures routinely run for collagenase production; see ref. [25]) were homogenized as described previously [26] in 20 ml of 0.1 M-acetate buffer, pH 5.3, containing 0.1 M-Na_2SO_4. The large debris were removed by centrifugation at 7000 *g*-min and a 20–70%-satn.-$(NH_4)_2SO_4$ fraction was taken. The pellet was resuspended in 5 ml of 40 mM-acetate buffer, pH 5.3, containing 0.1 M-Na_2SO_4 and extensively dialysed against the same buffer. This 20–70%-satn.-$(NH_4)_2SO_4$ fraction contained nearly all the gelatinase activity, about 50% of the Z-Arg-Arg-NH-NapOMe hydrolase activity and 80–400% of the Z-Phe-Arg-NH-Mec hydrolase activity detected in the 7000 *g*-min supernatant.

Chromatography

'Preparative' gel filtration was routinely performed at 4 °C, in a column (90 cm × 1.6 cm) of Ultrogel AcA 54 equilibrated with 40 mM-acetate buffer, pH 5.3, containing 0.1 M-Na_2SO_4, at a flow rate of 15–20 ml/h. Then 1–5 ml of the 20–70%-satn.-$(NH_4)_2SO_4$ fraction was applied to the column and 5 ml fractions were collected. 'Analytical' gel filtration was performed on an I-125 or a Superose 12 column, connected to an h.p.l.c. pump (model 6000 A; Waters Associates), at flow rates of 1 or 0.5 ml/min respectively with or without recycling of the effluent. Samples (0.1–1 ml) were applied to the column and 0.5 ml fractions were collected. The columns were calibrated with Dextran Blue, BSA, ovalbumin, pepsin, soya-bean trypsin inhibitor and cytochrome *c* as molecular-mass standards.

CM-Sephadex chromatography was performed at 4 °C in a 20 cm × 1.6 cm column equilibrated with 10 mM-citrate buffer, pH 4.8. A 1–30 ml sample, dialysed against the same buffer, was applied to the column at a flow rate of 20 ml/h. The column was washed first with 30 ml of the starting buffer, then with about 60 ml of the same buffer containing 0.3 M-KCl and thereafter with 180 ml of a linear gradient of 0.3–0.6 M-KCl in the buffer.

Phenyl-Sepharose chromatography was performed at 4 °C in a 1 cm × 1.6 cm column equilibrated with a 20 mM-phosphate buffer, pH 5.8, containing 25%-saturation $(NH_4)_2SO_4$. A sample (usually 1 ml) equilibrated in the same buffer was applied to the column at a flow rate of 1.5 ml/h. The column was washed successively with about 25 ml of a decreasing $(NH_4)_2SO_4$ gradient in the phosphate buffer, with 10 ml of phosphate buffer without additives, and with 20 ml of phosphate buffer containing 50% (v/v) ethylene glycol.

Chromatofocusing was performed with a Mono P column connected to an h.p.l.c. pump (Waters model 6000 A), at a flow rate of 1 ml/min. The gel was equilibrated with 25 mM-Bistris/iminodiacetic acid buffer, pH 7.1. The sample, diluted 4–10-fold in Polybuffer 74, pH 3.95, was applied to the column after the upper part of the gel had been rendered slightly acidic by eluting the column with 3 ml of Polybuffer. After application of the sample, the column was further eluted with Polybuffer.

The enzyme preparations were stored at −80 °C after dialysis against 50 mM-acetate buffer, pH 5, containing 1 mM-EDTA.

Active-site titrations

Active-site titrations were performed essentially as described in refs. [20] and [27]. The enzyme preparations were concentrated about 5-fold on Centricon membranes, preincubated with increasing amounts of E-64 and assayed with Z-Phe-Arg-NH-Mec as substrate. It was verified that increasing the amounts of the 40 kDa and 70 kDa proteinases led to a linear increase in the titration values.

Evaluation of rate constants for the inactivation of the proteinases by Z-Phe-Phe-CHN$_2$ and E-64

The apparent second-order rate constants were determined as described in ref. [27]. The cathepsin B and the 40 kDa and 70 kDa proteinases were preincubated at 30 °C in the assay buffer

for Z-Phe-Arg-NH-Mec hydrolysis, in the presence of respectively 12 μM- and 0.022 μM-, 0.027 μM-Z-Phe-Phe-CHN_2 or 0.19 μM-, 0.019 μM- and 0.019 μM-E-64. Samples (20 μl) were removed at seven different times and the residual activity was determined with Z-Phe-Arg-NH-Mec as substrate as described above. The data were analysed by using a computerized non-linear-regression program based on the steepest-descent technique [28].

Western blotting

SDS/PAGE was performed as reported in ref. [24]. Separating gels consisted of a gradient of 7–15% acrylamide (2.6% of this was as methylenebisacrylamide). The electrophoresed proteins were transferred to nitrocellulose in a Bio-Rad Transblot cell [29]. The nitrocellulose was washed [30] and the transferred proteins were allowed to react with rabbit antibodies against rat cathepsin L, rat cathepsin B or mouse cathepsin L. Thereafter the rabbit antibodies were allowed to react with biotin-coupled donkey antibodies raised against rabbit IgG and revealed by streptavidin–biotin–peroxidase.

Release of cysteine proteinases in the culture fluids

Embryonic mouse calvaria were cultured in medium 199 for 1, 2 or 3 days as previously described [12] and the conditioned media were assayed for the enzyme activities (see above).

RESULTS

Separation of proteinases by chromatography

Gel filtration (Fig. 1). The gelatinase and Z-Phe-Arg-NH-Mec hydrolase activities of the 20–70%-satn.-$(NH_4)_2SO_4$ fraction each resolved into three similar peaks on filtration on Ultrogel AcA 54: about 50% of these activities were recovered as 70 kDa proteins and the remainder as 40 kDa and 25 kDa proteins. The 25 kDa peak also contained all the Z-Arg-Arg-NH-NapOMe hydrolase activity of the 20–70%-satn.-$(NH_4)_2SO_4$ fraction. Filtration on other gels such as Superose 12 or I-125 resulted in similar separations. Moreover, the activities eluted as 70 kDa proteins still did so in a number of conditions susceptible to cleavage of non-covalent bonds, for instance when gel filtration was performed at high ionic strength (0.3 M-Na_2SO_4 or 1 M-NaCl), in the presence of detergent (1% Brij 35 or CHAPS), at pH 4, 5.3 or 8.5, in the presence of EDTA (10 mM) or mannose 6-phosphate (10 mM) and after sonication of the $(NH_4)_2SO_4$ fraction, or when running on Ultrogel AcA 44 a preparation of the 70 kDa proteinase that has been obtained after three successive chromatographies on Ultrogel AcA 54, CM-Sephadex and phenyl-Sepharose (see below).

Ion-exchange chromatography. The pool of the fractions containing the 25–40 kDa proteinases (Fig. 2*a*) and the pool of these containing the 70 kDa proteinase (Fig. 2*b*) were analysed separately on a CM-Sephadex column eluted with a KCl gradient. The Z-Phe-Arg-NH-Mec hydrolase and gelatinase activities of the 70 kDa were both eluted at about 0.4 M-KCl. So also were 70% of the gelatinase and of the Z-Phe-Arg-NH-Mec hydrolase activities of the 25–40 kDa pool, which became completely separated from the Z-Arg-Arg-NH-NapOMe hydrolase as well as from the remaining gelatinase and Z-Phe-Arg-NH-Mec hydrolase, all of which had been eluted already at 0.25 M-KCl. On further analysis of the CM-Sephadex fractions of the latter 25–40 kDa pool on Ultrogel AcA 54 (not shown), it appeared that the activities eluted at 0.4 M-KCl were mainly 40 kDa proteins and that those eluted at 0.25 M-KCl were mainly 25 kDa proteins. Gel filtration combined with CM-Sephadex chromatography thus allowed separation of (1) 70 kDa proteinase

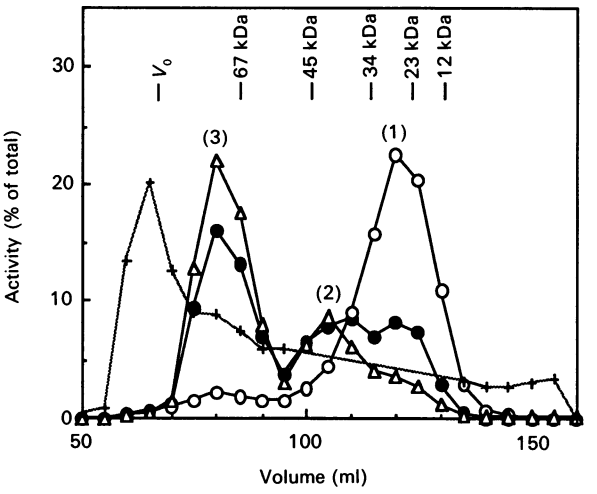

Fig. 1. Separation of bone cysteine proteinases by gel filtration

A 20–70%-satn.-$(NH_4)_2SO_4$ fraction of calvaria extract was applied to an Ultrogel AcA 54 column, previously calibrated as explained in the Materials and methods section. The cysteine proteinases eluted were evaluated by their activity against Z-Phe-Arg-NH-Mec (●), Z-Arg-Arg-NH-NapOMe (○) or gelatin (△), and the protein (+) by the absorbance at 280 nm. The activities in each fraction are expressed as percentages of the corresponding total recovered activities (i.e. respectively 122%, 100% and 175% for the activities against Z-Phe-Arg-NH-Mec, Z-Arg-Arg-NH-NapOMe and gelatin). The three peaks of activity are indicated by the numbers (1), (2) and (3).

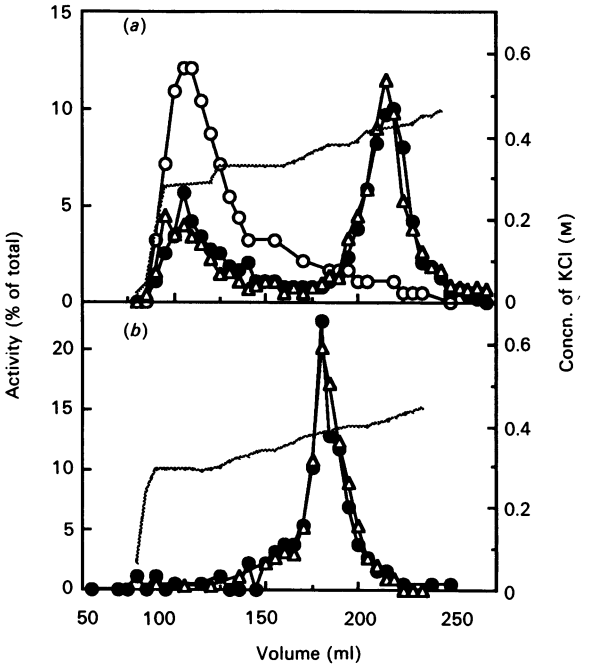

Fig. 2. Chromatography of bone cysteine proteinases on CM-Sephadex

(*a*) The pooled fractions of peaks (1) and (2) of the gel-filtration column (see Fig. 1) and (*b*) the pooled fractions of peak (3) were applied separately to a CM-Sephadex column. The elution of the cysteine proteinases was evaluated by their activity against Z-Phe-Arg-NH-Mec (●), gelatin (△) and, for the pooled peaks (1) and (2), Z-Arg-Arg-NH-NapOMe (○). The KCl gradient was monitored by the refractive index of the fractions (·····). The activities in each fraction are expressed as percentages of the corresponding total recovered activities [i.e. in (*a*) respectively 110%, 154% and 85% for the activities against Z-Phe-Arg-NH-Mec, Z-Arg-Arg-NH-NapOMe and gelatin; in (*b*) respectively 85% and 78% for the activities against Z-Phe-Arg-NH-Mec and gelatin].

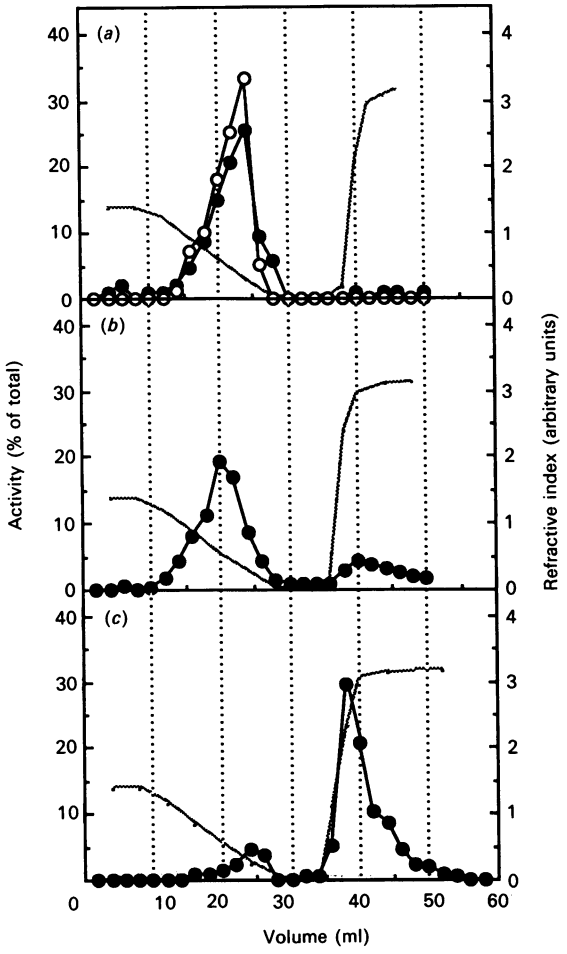

Fig. 3. Chromatography of bone cysteine proteinases on phenyl-Sepharose

Preparations of (*a*) 25 kDa, (*b*) 40 kDa and (*c*) 70 kDa proteinases were purified from calvaria extracts by $(NH_4)_2SO_4$ precipitation, Ultrogel AcA 54 gel filtration and CM-Sephadex chromatography and applied to a column of phenyl-Sepharose. Their elution was monitored by their activity against Z-Phe-Arg-NH-Mec (●) and, for the 25 kDa preparation, also against Z-Arg-Arg-NH-NapOMe (○). The concentrations of the buffers used for this elution were monitored by the refractive index of the fractions (·····). The activities in each fraction are expressed as percentages of the corresponding total recovered activities (i.e. respectively 76%, 85% and 79% for the activities against Z-Phe-Arg-NH-Mec of the 25 kDa, 40 kDa and 70 kDa proteinase preparations and 75% for the activity against Z-Arg-Arg-NH-NapOMe).

activities eluted at 0.4 M-KCl, (2) 40 kDa proteinase activities eluted at 0.4 M-KCl and (3) 25 kDa proteinase activities eluted at 0.25 M-KCl. They were called respectively 70 kDa, 40 kDa and 25 kDa proteinase preparations and were used for most experiments reported in this paper.

Phenyl-Sepharose chromatography (Fig. 3). Most (> 80–90%) of the cysteine proteinases of the 70 kDa and 40 kDa preparations and all the activity of the 25 kDa preparation were each eluted in a single main peak from a phenyl-Sepharose column. Interestingly, most of the 70 kDa proteinase bound more strongly to phenyl-Sepharose than did the other two preparations, but this was not useful for preparative purposes since the application of large samples to the column resulted in appreciable losses of enzymic activity.

Chromatofocusing on Mono P. The pH values at which the Z-Phe-Arg-NH-Mec hydrolase activities of each of these preparations were eluted from a Mono P column were all in the same range (results not shown). The 25 kDa proteinase was eluted at pH 5.3 in a single broad peak. Half of the 70 kDa proteinase was eluted in a peak at pH 5.4 and the other half came out more diffusely between pH 5 and pH 4. The 40 kDa proteinase was eluted in two main peaks, at pH 5.8 and 5.2, thus suggesting the existence of two main enzymic forms in the 40 kDa proteinase preparation.

Enzymic properties (Table 1, E–H)

Substrates. Preparations of 25 kDa, 40 kDa and 70 kDa proteinases all depolymerized collagen and degraded gelatin, casein and the synthetic substrate Z-Phe-Arg-NH-Mec, as summarized in Table 1 (part E). Higher activities were recovered in the preparations of the 70 kDa proteinase than in those of the 40 kDa and 25 kDa proteinases, although titrations with E-64 (an irreversible fast-reacting inhibitor of cysteine proteinases), performed on two different preparations of each of these proteinases, indicated that the 40 kDa and 70 kDa preparations contained about equal amounts of cysteine proteinase molecules (0.08 pmol/calvarium), but the 25 kDa preparation slightly more (0.14 pmol/calvarium). Z-Arg-Arg-NH-NapOMe, a synthetic substrate believed to be specific for cathepsin B [20], was significantly degraded only by the 25 kDa preparation.

pH optimum and K_m. The pH optima for the degradation of the synthetic substrates was approx. 6 for the 25 kDa proteinase and approx. 5.5 for the 40 kDa and 70 kDa proteinases (tested pH interval: between 4 and 7). The optimum pH for the degradation of proteins was more acidic: a value of 4 was found for the degradation of gelatin by a cell extract (tested pH interval: between 2.5 and 7) and a value of approx. 4.5, for the depolymerization of collagen by the three preparations of proteinases (tested pH interval: between 3.9 and 7.4). Almost no collagenolytic activity was detected at neutral pH. The K_m value of the 40 kDa and 70 kDa proteinases for Z-Phe-Arg-NH-Mec was much lower than that of the 25 kDa enzyme (Table 1, part F).

Stability at pH values above 7 (Fig. 4). Preparations of 25 kDa, 40 kDa and 70 kDa proteinases each behaved in a different way on preincubation at a pH above 7. The 25 kDa proteinase was rapidly inactivated at pH 7.3. In contrast, the 70 kDa proteinase was stable at this pH for preincubation periods up to 15 h, as well as for a preincubation at pH 9 for 1 h. Enzymic preparations of the 40 kDa proteinase had an intermediate behaviour.

Inhibition studies. Pepstatin (1.6 μM), phenylmethanesulphonyl fluoride (1 mM) and EDTA (5 mM), which inhibit respectively aspartic proteinases, serine proteinases and metalloproteinases, did not inhibit by more than 14% the hydrolysis of gelatin and by no more than 4% the hydrolysis of the synthetic substrates by each of the three proteinases preparations. In contrast, 0.4 μM-cystatin, a natural inhibitor of cysteine proteinases, inhibited almost completely the degradation of Z-Phe-Arg-NH-Mec by these proteinases. So also did several synthetic low-molecular-mass inhibitors, such as iodoacetamide (1.0 mM), leupeptin (0.01 μM), Z-Phe-Ala-CHN_2 (0.01 μM), Z-Phe-Phe-CHN_2 (10 μM) and E-64 (10 μM). As shown in Table 1 (part H), Z-Phe-Phe-CHN_2 inactivated the 25 kDa proteinases at a 10^3-fold lower rate than the 40 kDa and 70 kDa proteinases, whereas the inactivation rates obtained with E-64 did not appear that different.

Immunological properties

An incubation of the 40 kDa and the 70 kDa proteinase preparations with a suspension of latex–Protein A-bound anti-(mouse cathepsin L) antibodies resulted in the loss of at least 90% of their activity (Table 2). When a similar treatment was performed with the 25 kDa proteinase, or if antibodies of non-

Table 1. Overview of the properties of three bone cysteine proteinases

In part E, the activities were determined in several preparations (numbers given in parentheses) and shown as means ± S.D. In parts F and H, the values presented are means ± S.D. Abbreviation: N.D., not determined.

Properties	25 kDa proteinase	40 kDa proteinase	70 kDa proteinase
A. Apparent molecular mass (kDa) (gel filtration)	25	40	70
B. Concn. of KCl (M) for elution from CM-Sephadex	±0.25	±0.40	±0.40
C. pI (Mono P column)	5.3	5.2 and 5.8	5.4
D. Affinity for phenyl-Sepharose	+	+	+++
E. Activity against			
Collagen, pH 4 (ng/min per calvarium) (3)	1.5±0.5	6±4.3	14.4±2.8
Gelatin, pH 4 (ng/min per calvarium) (4)	14.6±3.8	22.5±16	48.9±16.6
Casein, pH 5.5 (2)	+	++	++
Z-Arg-Arg-NH-NapOMe, pH 6 (mmol/min per calvarium), (3)	2.3±1.1	Traces	Traces
Z-Phe-Arg-NH-Mec, pH 5.5 (pmol/min per calvarium) (7)	19.7±19.1	20.3±11.8	51.2±35.5
F. K_m for			
Z-Arg-Arg-NH-NapOMe (μM)	213±39	N.D.	N.D.
Z-Phe-Arg-NH-Mec (μM)	> 20	2.2±0.4	3.8±0.9
G. Stability at alkaline pH	No	+	++
H. Rate constant for inhibition by			
Z-Phe-Phe-CHN_2 ($M^{-1}\cdot s^{-1}$)	219±318	239000±33000	325000±39600
E-64 ($M^{-1}\cdot s^{-1}$)	57400±4800	223000±12300	390000±13100
I. Western blotting with anti-(cathepsin L) (kDa)	N.D.	25 and 40	25
K. Presumed identification (see the Discussion section)	Cathepsin B	Procathepsin L	'Cathepsin L-like 70 kDa proteinase'

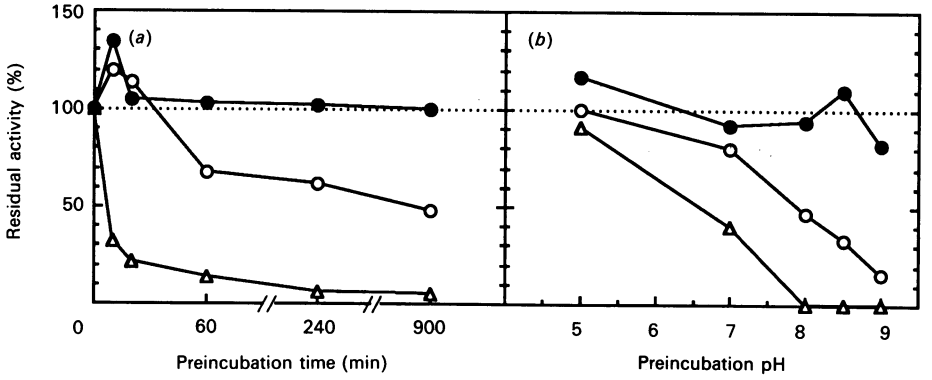

Fig. 4. pH-stability of the bone cysteine proteinases

(*a*) Preparations of 25 kDa (△), 40 kDa (○) and 70 kDa (●) proteinases were preincubated at 37 °C in a 25 mM-Tris buffer, pH 7.3, containing 0.01% Brij. At the indicated times, samples were removed and assayed for their activity with Z-Phe-Arg-NH-Mec as substrate. (*b*) Preparations of 25 kDa (△), 40 kDa (○) and 70 kDa (●) proteinases were preincubated at 37 °C for 1 h at the indicated pH. The pH was adjusted at pH 5 with 25 mM-acetate, at pH 7 with 25 mM-Bistris/HCl, and at pH 8 and above with 25 mM-Tris/HCl. All the buffers contained 0.01% (w/v) Brij 35. After this preincubation, the residual activities of the preparations were determined with Z-Phe-Arg-NH-Mec as substrate. The activities are expressed as percentages of the corresponding activities of non-preincubated samples

immunized rabbits were substituted for the anti-(cathepsin L) antibodies, no more than 13% of the activities was lost. This shows that the activities of the 40 kDa and the 70 kDa preparations (but not that of the 25 kDa preparation) are due to proteinases that are immunologically related to cathepsin L.

In order to analyse more accurately the molecular mass of the proteinases that were recognized by the anti(cathepsin L) antibodies, preparations of the 40 kDa and 70 kDa proteinases were submitted to Western blotting after SDS/PAGE and revealed with antibodies raised against rat cathepsin L (results not shown) or mouse cathepsin L (Fig. 5). Both gave similar immunoblots. Western blots of the 40 kDa proteinase preparation showed three close bands of about 40 kDa (two of which were only weakly stained) and one of about 25 kDa, thus suggesting that this preparation contains several proteins immunologically related to cathepsin L but differing in size. Interestingly, immunoblots of the 70 kDa proteinase preparation, which had been termed 70 kDa because of its behaviour on gel filtration (see above), usually showed only one band of 25 kDa, corresponding to the low-molecular-mass band of the 40 kDa preparation (Fig. 5). Occasionally, however, a faint 70 kDa band was also visible in addition to the 25 kDa band (not shown).

Anti-(cathepsin B) antibodies did not reveal any signal in dot-blots performed on the 40 kDa and 70 kDa proteinase preparations, but reacted well in those performed on the 25 kDa preparation (results not shown).

Release of cysteine proteinase activities in bone culture fluids

Culture fluids collected after the culture of parathyrin-treated embryonic mouse calvaria exhibited collagen-depolymerizing activity, gelatin-degrading activity and Z-Phe-Arg-NH-Mec hydrolase activity (K_m for Z-Phe-Arg-NH-Mec: 3±0.9 μM), all of which were abolished by 5 mM-iodoacetamide but not by 1 mM-phenylmethanesulphonyl fluoride or 5 μg of pepstatin/ml, thus

Table 2. Immunoprecipitation with anti-(cathepsin L) antibodies

Latex–Protein A was saturated either with antibodies against mouse cathepsin L or with control antibodies of non-immunized rabbits, and thoroughly washed in a buffer containing 20 mM-Bistris, pH 6.5, 0.15 M-NaCl and 0.5% (w/v) BSA. A 0.125% suspension of this latex–Protein A–IgG was made in 200 μl of the same buffer and incubated at 37 °C with the 25 kDa, 40 kDa and 70 kDa proteinases. After 1 h, the tubes were centrifuged and the activity of the proteinases was assayed in the supernatant with Z-Phe-Arg-N-Mec as substrate. Controls without latex–Protein A–IgG were run in parallel. The activities are expressed as percentages of the activities of these controls.

	Activity (% of controls)		
	25 kDa proteinase	40 kDa proteinase	70 kDa proteinase
Non-immune serum	97	87	100
Anti-(cathepsin L) serum	89	10	5

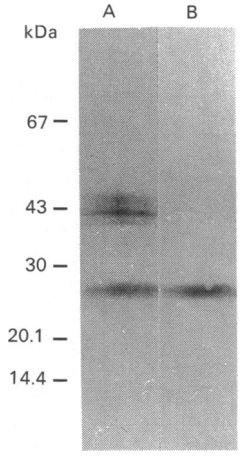

Fig. 5. Western blots of the 40 kDa and 70 kDa proteinase preparations

The 40 kDa (track A) and 70 kDa (track B) proteinases were purified from calvaria extracts by $(NH_4)_2SO_4$ precipitation, Ultrogel AcA 54 gel-filtration and CM-Sephadex chromatography. E-64 was added at a concentration of 10 ng/ml to the purified fractions (in order to prevent autodegradation), which were then concentrated 25-fold on Centricon filters, immunoblotted with the anti-(mouse cathepsin L) antibody and stained as described in the Materials and methods section.

indicating that they were due to cysteine proteinases. On gel-filtration or phenyl-Sepharose chromatography of the culture fluids, the Z-Phe-Arg-NH-Mec hydrolase activity behaved typically as the 40 kDa proteinase of the bone extract and did not exhibit the elution profiles characteristic for the 70 kDa proteinase (results not shown). It thus appears that the 40 kDa proteinase is released from the tissue, whereas no such release could be demonstrated for the 70 kDa proteinase. In confirmation of our previous results [12], release of 25 kDa Z-Arg-Arg-NH-NapOMe hydrolase (identified as cathepsin B; see below) from the bones could be detected only when the cultures were performed at a slightly acid pH.

Relative amounts of 70 kDa Z-Phe-Arg-NH-Mec hydrolase activity in various tissues

Extracts of various tissues and cells were screened for the presence of a 70 kDa Z-Phe-Arg-NHMec hydrolase by gel filtration, and for some of them by phenyl-Sepharose chromatography (Table 3). These two independent techniques gave concordant estimations. Elution profiles typical of the 70 kDa proteinase were obtained from mouse muscle, cultured rabbit chondrocytes, bone marrow and bone tissue of various origins. The proportion of Z-Phe-Arg-NH-Mec hydrolysis due to the 70 kDa proteinase was as high as 80% in chick long bones. Although extracts from liver, spleen, kidney and peritoneal macrophages exhibited Z-Phe-Arg-NH-Mec hydrolase activity, only traces of activity were detected in the fraction where the 70 kDa proteinase was expected.

Table 3. Relative amounts of the 70 kDa Z-Phe-Arg-NH-Mec hydrolase activity in various tissues

Mouse calvaria were taken from 5-day-old mice, other mouse tissues or cells from adult animals, and chick tissues from Ca^{2+}-deprived chicks. Tissues or cells were homogenized in 0.1 M-acetate buffer, pH 5, with a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 6×10^6 *g*-min and the supernatants were subjected to gel filtration on an I-125 column and, for some of them, on a phenyl-Sepharose column, as explained in the Materials and methods section. The activity of the fractions was assayed against Z-Phe-Arg-NH-Mec and Z-Arg-Arg-NH-NapOMe. The amount of the 70 kDa proteinase was evaluated by the percentage of activity Z-Phe-Arg-NH-Mec recovered either in the fractions eluted between 15 and 18 min after the injection of the sample on h.p.l.c. gel filtration (the peak fraction of the 70 kDa proteinase was obtained after 17 min, that of the 25 kDa proteinase after 20 min) or in the fractions eluted by 50% ethylene glycol on phenyl-Sepharose chromatography. The numbers of experiments that were performed are given in parentheses.

	70 kDa proteinase (% of the total Z-Phe-Arg-NH-Mec hydrolase activity)	
	Gel filtration	Phenyl-Sepharose chromatography
Mouse liver	5 (3)	5 (1)
Mouse spleen	3 (2)	–
Mouse kidney	10 (1)	4 (1)
Mouse muscle	57 (1)	58 (2)
Mouse bone marrow	32 (2)	–
Chick bone marrow	49 (1)	–
Mouse calvaria	68 (1)	70 (1)
Mouse tibia, metaphysis	31 (2)	–
Chick calvaria	79 (1)	–
Chick long bones	79 (3)	–
Mouse peritoneal macrophages (cultured)	10 (4)	–
Rabbit chondrocytes (cultured, five passages)	35 (2)	22 (1)

DISCUSSION

The present work allowed the isolation and characterization of three forms of cysteine proteinases from bone tissue. Their properties are summarized in Table 1.

Cathepsin B

The 25 kDa proteinase can be identified as cathepsin B as it exhibits typical features of this enzyme [31]: ability to hydrolyse Z-Arg-Arg-NH-NapOMe (with a typical K_m), lack of stability at pH values above 7, reactivity with anti-(cathepsin B) antibodies,

and, as compared with cathepsin L, a high K_m for Z-Phe-Arg-NH-Mec [20,32], a lower rate of inhibition by Z-Phe-Phe-CHN_2 [33] and a lower activity with collagen [9].

(Pro)cathepsin L

Immunoprecipitation demonstrated that most of the activity of the 40 kDa proteinase preparation is immunologically related to cathepsin L. Western blotting with anti-(cathepsin L) antibodies of two different origins further showed that the 40 kDa proteinase preparation contains two main immunoreactive constituents of about 40 and 25 kDa corresponding respectively to the precursor and to the mature forms of cathepsin L [34]. The K_m of the 40 kDa enzyme preparation for Z-Phe-Arg-NH-Mec and its rate constant for inactivation by Z-Phe-Phe-CHN_2 and E-64 were in the range of values determined for cathepsin L of various species [35] and for procathepsin L (purified procathepsin L appears active [36]). Furthermore, the pH-stability experiments showed that part of the 40 kDa proteinase preparation is inactivated at pH 7.2, whereas the other part remains stable for up to several hours at this pH, just as reported respectively for the mature and the precursor forms of cathepsin L [36]. It may thus be concluded that the 40 kDa proteinase is a cathepsin L, recovered mainly in its precursor form when freshly isolated from bone, but partly converted into its mature form under some conditions. Mature cathepsin L could not be detected either in homogenates of other tissues [33,37] unless they were preincubated at acidic pH [33] or subjected to special experimental procedures [37,38].

Cathepsin L-like 70 kDa proteinase

The 70 kDa proteinase preparation has several properties in common with the (pro)cathepsin L described above; so are its almost complete immunoprecipitation with anti-(cathepsin L) antibodies, its enzymic properties towards various substrates, its rate of inactivation by cysteine-proteinase inhibitors and its affinity for CM-Sephadex. However, the 70 kDa proteinase preparation differs from (pro)cathepsin L by its higher molecular mass on gel filtration (even when this was performed under various dissociating conditions, such as high ionic strength or in the presence of detergents), its greater affinity for phenyl-Sepharose and its greater stability at high pH values. It also appears to have a distinct tissue distribution, and unlike procathepsin L, it could not be detected in the culture fluids of bone explants (whether treated or not with parathyrin).

A clue for understanding the relation between the 70 kDa proteinase and cathepsin L might be provided by the Western blots of the 70 kDa proteinase preparation. They show that the immunoreactive component of this preparation appears essentially as the 25 kDa mature form of cathepsin L. Thus the 70 kDa proteinase might be a strong complex (resistant to 1% Brij or 1 M-NaCl) between mature cathepsin L (not the precursor form) and another entity, which would dissociate under the conditions of SDS/PAGE used for Western blotting. The nature of this cathepsin L-binding entity is not known but some of its presumed properties may be noted: it would have an apparent molecular mass of about 45 kDa, confer a greater stability to cathepsin L at pH values as high as 9 and favour its binding on hydrophobic residues. Such properties may be relevant to those of a membrane-bound cathepsin L that has recently been observed [34,39,40]. However, the molecular properties of this binding have not yet been elucidated. In this respect, it should be noted that the cathepsin L-binding entity of the 70 kDa proteinase appears distinct from the 46 kDa cation-dependent mannose 6-phosphate receptor [41], since the 70 kDa proteinase did not dissociate in the presence of 10 mM-EDTA or 10 mM-mannose 6-phosphate.

We are not aware of other reports about cathepsin L complexes similar to that proposed here, but there are several descriptions of cysteine proteinases with characteristics similar to those of the bone 70 kDa proteinase. Z-Phe-Arg-NH-Mec-hydrolysing activities of about 68 kDa were detected in muscle [42,43] and spleen [10] but have not characterized in detail yet. Our present work confirmed the existence of the muscle enzyme, but not that of the spleen enzyme. The cysteine proteinase isolated from a human pancreatic cancer cell line [44] has a number of properties in common with the bone 70 kDa proteinase: it hydrolyses Z-Phe-Arg-NH-Mec, is stable above pH 7, has a molecular mass of 68 kDa, as judged by gel filtration, and dissociates on SDS/PAGE into components recognized by anti-(cathepsin L) antibodies. However, in this latter case, two components (and not one as for the bone enzyme) with molecular masses corresponding to the mature and the precursor form of cathepsin L were detected, and unlike the bone enzyme it was secreted into the culture fluids. A serine-rich 68 kDa cysteine proteinase has also been isolated from human amnion–chorion [45] and from rabbit V2 carcinoma cells [46]. It is stable above pH 7 and exhibits strong affinity for phenyl-Sepharose like the bone 70 kDa proteinase, but it did not dissociate into components of lower molecular mass on SDS/PAGE. It is also worth noting that the largest molecular mass expected from DNA sequences for cathepsin L is about 40 kDa [47]; it is therefore unlikely that these proteinases of about 70 kDa are pre-proenzymes.

Thus, at present, the best interpretation of the data seems to be that the bone 70 kDa proteinase is made of a catalytic subunit identical with mature cathepsin L and of another, still unidentified, subunit that stabilizes the enzymic activity at alkaline pH and favours hydrophobic interactions.

Relations with bone resorption

Previous work has shown that the role played by cysteine proteinases in bone resorption is related to the degradation of collagen occurring in the subosteoclastic lacuna [14,15]. Cathepsin B, (pro)cathepsin L and the 70 kDa cathepsin L-like proteinase described in this paper have properties compatible with such a role. All three enzymes degrade collagen molecules and gelatin, and the optimum pH for this activity is in the range of the pH values expected in the subosteoclastic resorption zone [48]. It has repeatedly been shown that cathepsin L degrades collagen and gelatin at a much higher rate than cathepsin B (see, for instance, ref. [9]). The data presented here illustrate this again. Besides, other molecular targets for cathepsins B and L may be considered: they may degrade other constituents of the bone matrix, such as proteoglycans [49], they may mediate the maturation of important lysosomal enzymes [50,51], and cathepsin B may activate procollagenase [52], another enzyme that is critical for bone resorption (reviewed in [2]) and that also acts at the level of the subosteoclastic resorption zone [53,54].

To exert these degradative activities against the extracellular matrix, the proteinases need to be extracellular. Our present data show that cathepsin B and procathepsin L are present in the extracellular fluids, and our previous work had already shown that cathepsin B accumulates in the culture fluids of bone explants in good correlation with their resorption [12], as do a series of other lysosomal hydrolases [55]. In contrast, the 70 kDa cathepsin L-like proteinase could not be detected in the culture fluids, even after stimulation with parathyrin. There is, however, a possibility that the enzyme is a plasma-membrane-bound cathepsin L (see above), as has been reported for cancer cell lines [39,40] the ability of which to 'invade' tissue is reminiscent of the ability of the osteoclasts to penetrate into the bone matrix. Although it is clearly not specific for bone, it is of interest that the highest proportion of 70 kDa Z-Phe-Arg-NH-Mec hydrolase

activity was found in long bones of Ca^{2+}-deprived chicks, a tissue particularly rich in osteoclasts.

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