Extracellular-matrix degradation at acid pH

Avian osteoclast acid collagenase isolation and characterization

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Osteoclasts degrade bone matrix, which is mainly type I collagen and hydroxyapatite, in an acidic extracellular compartment. Thus we reasoned that osteoclasts must produce an acid collagenase. We purified this enzyme, a 31 kDa protein, from avian osteoclast lysates (in 100 mM acetate/1 mM CHAPS/1 mM dithiothreitol, pH 4.4), fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, gelatin-affinity, cation exchange, and gel filtration. Fraction activity was measured using diazotized collagen or ^3H -labelled cross-linked collagen (decalcified and trypsin-treated metabolically L-[4,5- ^3H]proline-labelled bone) as substrates. Iodoacetate, leupeptin, antipain, pepstatin and mercurials inhibited collagenolysis by the isolated proteinase; mercurial derivatives could not be re-activated by dithiothreitol. Collagen degradation was maximal at pH 4.4; purified proteinase reproduced the collagenolytic activity of cell lysates. The N-terminal amino acid sequence from the isolated protein and its CNBr degradation fragments showed sequence similarity to mammalian cathepsin Bs, and near-identity with avian liver cathepsin B. Peptide substrate specificity of the osteoclastic

enzyme resembled those of mammalian cathepsin B and its avian liver counterpart, but degradation of low-molecular-mass substrates by the osteoclastic enzyme was slower, reflecting generally lower k_{cat} values. Further, k_{cat}/K_m varied less between arginine-containing substrates than for previously reported cathepsin Bs, indicating different substrate specificity of the osteoclast enzyme. Polyclonal antibody raised to a 25 kDa fragment of the enzyme recognized a single 31 kDa band in SDS/PAGE of osteoclast lysates blotted to poly(vinylidene difluoride), adsorbed collagenolytic activity of osteoclast lysates, and stained avian osteoclasts in tissue sections. Degenerate sense- and antisense-oligonucleotide primers, predicted from segments of primary amino acid sequence, amplified a 486 bp DNA fragment; this was cloned and sequenced. Of 162 amino acids encoded, 77% are identical with those of human cathepsin B; hybridization identified a 2.4 kb RNA in osteoclast lysates. We conclude that the major avian osteoclast collagenolytic enzyme is a cathepsin B, whose activity varies from other enzymes of its class.

INTRODUCTION

In air-breathing vertebrates, bone degradation is vital to growth, calcium homeostasis, and maintenance of skeletal strength. Major bone components are type I collagen and a basic calcium salt, hydroxyapatite [1]. A specific biochemical mechanism for bone collagen degradation has not been identified. On the other hand, bone mineral solubilization is known to be mediated by HCl secretion at the osteoclast's bone attachment site, the ruffled membrane [2]. The presence of acid secretion at the site of bone degradation suggested that bone collagenolysis proceeds at low pH. However, the enzyme degrading other extracellular collagen matrices, collagenase, is a zinc-containing enzyme active only at pH ~ 7. Moreover, bone collagen is highly cross-linked and insoluble, so that its physical dissolution by acid at biologically achievable pH is insignificant [3].

Nevertheless, bone collagen degradation is a major physiological process; so much so that bone degradation rate is clinically measured by urinary hydroxyproline, despite collagen breakdown in all other tissues. One hypothetical biochemical mechanism for bone collagen removal held that osteoblast-produced collagenase degrades the bone's organic matrix, while the osteoclast removes mineral [4]. However, we found that isolated osteoclasts degrade both the organic and inorganic components of bone, and that osteoclast lysates degrade bone collagen at

pH < 6, suggesting that the osteoclast produces an acid collagenase, active under conditions which are compatible with its acid mineral removal [5]. Other workers have shown that thiol-proteinase inhibitors block bone resorption [6,7], in keeping with the acid collagenolysis hypothesis, and indeed several thiol proteinases have been shown to reside in bone, although these proteinases have not been localized to osteoclasts.

The availability of highly enriched populations of avian osteoclasts [5] enabled us to directly address this issue. We purified a collagenolytic proteinase, a 31 kDa proteinase resembling cathepsin B, from the cell lysates. A 162-amino-acid segment of this enzyme is 77% identical with mammalian cathepsin Bs by cDNA cloning. Antibody to isolated enzyme adsorbed osteoclast lysate collagenolytic activity and demonstrated, by immunohistochemical staining, enzyme in osteoclasts *in situ*. These findings argue that this enzyme presides over osteoclastic bone collagen degradation.

METHODS

Cell isolation and lysates

Osteoclasts were isolated from medullary bone of calcium-starved laying hens (*Gallus domesticus*) as described previously [5]. Osteoclast-enriched fractions after serum sedimentation $[(1-2) \times 10^7/\text{bird}; \sim 2 \text{ g wet weight of cells with } \sim 90\% \text{ of nuclei}]$

Abbreviations used: Z-Arg-Arg-MeNap, benzyloxycarbonyl-L-arginyl-L-arginine 4-methoxy- β -naphthylamide; Bz-Arg-Nap, benzoyl-D,L-arginine β -naphthylamide; Bz-Arg-Nan, benzoyl-D,L-arginine *p*-nitroanilide; DTT, dithiothreitol; Z-Phe-Arg-NHMec, benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amido-4-methylcoumarin; Z-Pro-Ala-Gly-Pro-MeNap, benzyloxycarbonyl-L-prolyl-L-alanylglycyl-L-proline 4-methoxy- β -naphthylamide.

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in osteoclasts] were suspended in 100 mM sodium acetate/1 mM dithiothreitol/1 mM CHAPS, pH 4.4, and frozen (-80°C) until used. Under these conditions, acid collagenolytic activity was stable for several months, and erythrocytes, devoid of measurable proteolytic activity, were the only significant contaminating cells. Lysates were prepared by thawing $(1-3) \times 10^8$ osteoclasts and disrupting the mixture with a glass homogenizer.

Collagen-degradation assays

Collagenolysis was assessed as indicated in the Figures, generally with either diazotized skin collagen (Azocoll; Calbiochem), or decalcified and trypsin-treated (0.1%, 25°C , 60 min) rat bone that had been metabolically labelled with L-[4,5- ^3H]proline (cross-linked bone collagen) as described in [8]. The diazotized substrate was generally used in screening assays, and all such results were confirmed using the highly-cross-linked and non-denatured bone collagen. Non-collagenous proteins were removed from this bone by trypsin digestion, and it was also produced using the collagen-specific proline label [5]. However, results of assays using either substrate were essentially identical (see the Results section).

Unless specified, either assay was conducted by mixing enzyme sample (1–50 μl , depending on activity) with 100 μg of substrate in a total volume of 500 μl adjusted to 100 mM acetate/1 mM dithiothreitol/0.1 mM CHAPS, pH 4.4. Assay mixtures, in 1 ml polyethylene centrifuge tubes, were incubated (37°C , 18 h), and activity assessed as A_{524} (Azocoll) relative to a pepsin-solubilized total hydrolysis control, or as supernatant ^3H (cross-linked bone collagen) after centrifugal separation (10000 g, 2 min) relative to an HCl-hydrolysed (6 M, 110°C , 18 h) control.

Enzyme purification

General conditions

Except as specified, all chromatography was performed at 4°C in 10 mM acetate/0.1 mM dithiothreitol/0.1 mM CHAPS, pH 4.4. Between procedures, samples were stored at -80°C . Concentration or desalting, where indicated, was performed at 4°C by dialysis against appropriate solutions or by vacuum dialysis, using 10–12 kDa cut-off Spectrapor cellulose membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

Chromatography

Gelatin-agarose (Sigma, ~ 4 mg/ml) affinity chromatography was performed using a 1.5 cm $\times$ 35 cm column. CM-cellulose ion-exchange chromatography (type 6B, ~ 120 $\mu\text{equiv.}/\text{ml}$ capacity; Sigma, St. Louis, MO, U.S.A.) was done on a 2 cm $\times$ 20 cm column after overnight washing in 10 mM acetate, pH 4.4, to pH < 4.8 . CM-silica chromatography was done with a 6 mm $\times$ 250 mm column packed with a methanolic slurry of substrate (ACCEL-CM; Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). Gel filtration was performed using a 7.5 mm $\times$ 300 mm Bio-Sil TSK 125 column (Bio-Rad, Richmond, CA, U.S.A.).

Protein determination and sequencing

For column steps, protein was estimated from A_{280} as 1.0 mg/ml per absorbance unit or A_{230} as 5.0 mg/ml per absorbance unit (less matched buffer absorbance). Other protein concentrations were determined by the method of Groves et al. [9].

Proteins were sequenced by Edman degradation from Coomassie Blue-identified bands on poly(vinylidene difluoride)

membranes electroblotted from polyacrylamide gels, with *in situ* CNBr cleavage when indicated, as described [10].

Preparation of antiserum and immune staining

For antibody production, enzyme activity concentrated by means of CM-cellulose (stage 6, Figure 2 below) was boiled for 30 min in 10 mM dithiothreitol, and subjected to preparative SDS/PAGE, with the 25 kDa band excised after identification by comparison with standards. This protein is a major fragment of the enzyme, as shown by N-terminal sequencing and CM-silica purification (described below; immunization with 31 kDa active enzyme was also performed, but it did not produce antibody).

A rabbit was bled to produce pre-immune control serum and then injected with 500 μg of purified 25 kDa antigen in Freund's complete antigen, and boosted with 250 μg of antigen at 2-week intervals. After 4 weeks, animals were test bled and antibody titre was determined by immunoprecipitation of activity at pH 6.5 (Figure 8 below). Antibody specificity was assessed by staining poly(vinylidene difluoride) blots of antigen and cell lysates by the unlabelled antibody technique of Sternberger et al. [11], except that non-specific adherence of immune reagents to the blotting medium was blocked by pre-incubation in non-fat dry milk (1%) for 20 min prior to unlabelled-antibody reaction, and 1% non-fat dry milk was also added to the primary and secondary antibody incubations (Figure 10 below). When sufficient titres of antibody to stain Western blots at 1:200 dilution were observed, a larger quantity of antiserum was obtained for the study of enzyme distribution (see the Results section). Antigen-containing cells in formalin-fixed avian medullary bone sections were demonstrated by the same technique.

Other enzyme assays

Diazotized casein, produced as described [12], was used as a non-specific protein substrate, the degradation of which was compared with collagen under identical conditions (pH 4.5, 37°C). Assay was as with diazotized collagen, except that 5% trichloroacetic acid was used to precipitate undegraded substrate (which is soluble) prior to centrifugation and determination of enzyme activity as supernatant A_{366} relative to A_{366} of total hydrolysis with excess pepsin.

Chromogen-linked oligopeptide substrates (Sigma) were assayed essentially as described by Barrett and Kirsche [13]. Briefly, for β -naphthylamide substrates, assays were run in 500 μl total volume over 18 h at 38°C , pH 6.0, in 100 mM phosphate/1 mM EDTA/1 mM dithiothreitol (DTT) at $38 \pm 0.5^{\circ}\text{C}$. Coupling with Fast Garnet was by addition of 500 μl of 0.2 mM Fast Garnet in 0.2% Brij 35, with 10 min for colour development and reading in 1 ml cuvettes as A_{520} with a Perkin-Elmer $\lambda 3\text{-B}$ dual-beam spectrophotometer. β -Naphthylamide standards were run to obtain absorbance/ μg β -naphthylamide under the conditions used. For benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NHMe) substrates, reaction mixes were set up and incubated in the same way as β -naphthylamide substrates, except that, owing to the great sensitivity of fluorometric assays, the assay was terminated at 60 min, and a portion of the reaction mixture (100 or 200 μl) was added to 3 ml of water in a fluorimeter cuvette for reading in an Aminco-Bowman Series II fluorimeter. The assumption was made that substrate is so significantly depleted. Fluorescence emission spectrum from 350 to 600 nm was made with excitation at 331 nm. Emission at 600 nm was subtracted as background. Standard curves were produced with intact substrate and NHMe. Substrate emission at 395 nm (starting material peak)

and ratio of substrate A_{365}/A_{350} was used to calculate background at 365 nm (hydrolysed peak). A_{365} background was converted into nM NHMec using the standard curve. This was converted into mol/time for calculation of V_{max} and $1/v$.

Artificial-substrate degradation was determined using stage-6 enzyme, as material processed further was not available in the necessary quantity. K_m and k_{cat} were calculated from least-squares fits of three-point Lineweaver-Burk plots of substrate degradation; the substrate ranges used and correlation coefficients are shown in Table 2 (below) with the results of this procedure. K_m is independent of [enzyme]. However [enzyme] must be known for determination of k_{cat} . The quantity of material made titration of enzyme impractical, so this concentration was calculated from protein concentration determined spectrophotometrically [9], the apparent molecular mass on SDS/PAGE (31 kDa; Figure 2 below), and the relative activities of the stage-6 enzyme and maximally active stage-7 enzyme (Table 1 below).

As a control to eliminate the possibility that contaminating aspartate proteinases might affect results using stage-6 enzyme, benzyloxycarbonyl-L-prolyl-L-alanylglycyl-L-proline 4-methoxy- β -naphthylamide (Z-Pro-Ala-Gly-Pro-MeNap) and benzoyl-D,L-arginine β -naphthylamide (Bz-Arg-Nan) results were repeated with enzyme pretreated using the thiol-specific alkylating agent iodoacetate (1 mM, 1 h, 25 °C). Iodoacetate pretreatment eliminated all measurable activity on these substrates.

RNA isolation and cDNA hybridization

For reverse transcription or hybridization, RNA was isolated from osteoclasts by guanidine thiocyanate extraction, CsCl-density-gradient centrifugation, and phenol/chloroform extraction as described in [14]. Formaldehyde-denatured total osteoclast RNA (9 μ g) was separated electrophoretically on 1%-agarose gel and transferred to nylon according to the manufacturer's instructions (Zeta-probe; Bio-Rad, Richmond, CA, U.S.A.). cDNA (described below) was labelled with [α - 32 P]dCTP (3000 Ci/mmol; ICN, Irvine, CA, U.S.A.) by random priming as specified by the manufacturer (Random Primed DNA labelling kit; Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Probe specific radioactivity was $> 10^8$ c.p.m./ μ g of DNA. After overnight pre-hybridization of RNA membranes (50% formamide/250 mM Na_2HPO_4 /250 mM NaCl/1 mM EDTA/2% SDS/0.3% sheared herring sperm DNA, pH 7.2, 37 °C), 10^7 c.p.m./40 ng of denatured probe (95 °C, 10 min, in 2 ml of 2 mg/ml herring sperm DNA) was hybridized for 6 h at 65 °C in 2.5 ml of 180 mM NaCl/18 mM citrate (pH 7.0)/0.1% SDS containing 0.1 mg/ml herring sperm DNA and 0.02% each of Ficoll (type 400; Pharmacia), BSA, and polyvinylpyrrolidone. After hybridization, membranes were rinsed for 20 min at the indicated temperatures in several changes of 30 mM NaCl/3 mM citrate/0.1% SDS, pH 7, followed by overnight autoradiography.

Reverse transcription, DNA amplification, cloning and sequencing

A 2.0 μ g portion of total osteoclast RNA was reverse-transcribed for 10 min at 25 °C and 30 min at 42 °C in the presence of 1 mM oligo(dT)₁₆, 1 mM (of each) dNTP, and 0.05 unit/ μ l Moloney murine reverse transcriptase (BRL), and 1 unit/ μ l RNAase inhibitor (Promega) in 220 μ l (total volume) of 50 mM Tris/5 mM MgCl_2 , pH 9. The reaction was stopped after 10 min at 95 °C.

DNA was amplified by thermocycling 20 μ l of the reverse-transcription mixture [the cDNA source, containing also oligo(dT)₁₆, the 3' primer] in 100 μ l total volume with 0.25 μ M degenerate 5' primer (see Figure 5 below), 2 units/ml *Taq*

polymerase (Promega) in 50 mM Tris/7 mM MgCl_2 , pH 9. This mixture was subjected to 30 cycles of denaturation (1 min at 94 °C), annealing (90 s at 37 °C), followed by a 90 s ramp to 72 °C and an additional 90 s for polymerization at 72 °C. After 30 cycles, the mixture was left for an additional 10 min at 72 °C. The resulting product contained DNAs of several hundred to thousands of bases, without any predominant or discrete product. This mixture was therefore re-amplified with both 3' and 5' degenerate primers (Figure 5 below) by the same protocol, except that each thermal cycle was 1 min at 95 °C, 1 min at 40 °C and 1 min at 72 °C, with an additional 7 min at 72 °C after the last cycle.

Discrete DNA bands from ethidium bromide-stained 1%-agarose gels were cut out, electroeluted into dialysis bags in 90 mM Tris/90 mM borate/2.5 mM EDTA, pH 8, adjusted to 300 mM with sodium acetate, precipitated in 70% (v/v) ethanol, and freeze-dried. DNA samples were reconstituted in sterile water, ligated into pre-cut PCR1000 plasmids (Invitrogen; containing kanamycin resistance and a cloning site interrupting a β -gal gene between a T7 promoter and pUC/M13 forward primer) with T4 ligase, and transformed into competent *Escherichia coli* cells (INV α F'), essentially as described in [15]. Positive clones were selected as white colonies on 5-bromo-4-chloro-indol-3-yl β -D-galactopyranoside (40 μ g/ml), kanamycin (50 μ g/ml) Luria-Bertani agar plates. Bacteria containing inserts were grown in Luria-Bertani medium with kanamycin, and plasmid prepared by alkaline lysis and poly(ethylene glycol) purification essentially as described in [15]. The presence of inserts was verified by cutting the plasmids with *Eco*RI and *Hind*III and revealing the inserts by agarose-gel electrophoresis.

DNA was sequenced by the dideoxynucleotide chain-termination method essentially as described in [15], using as primers T7 promoter and pUC/M13 forward primer oligo DNAs and the Klenow fragment of *E. coli* DNA polymerase I for chain extension.

Statistics

Where stated, error ranges are standard deviations (S.D.). Enzyme assays were performed in duplicate, except in column-fraction screening, where single assays were done.

RESULTS AND DISCUSSION

Enzyme purification

Collagenolytic activity purification is outlined in Table 1. Extraction of collagenolytic activity was optimized with respect to

Table 1 Purification of osteoclast acid collagenase

Purification stage	Protein	Activity*	Sp. activity*
1. Lysate	0.5 g	1800	3.6
2. 6 M urea/3 M NaCl	0.47 g	2300	5.0
3. 6 M urea	0.40 g	2300	5.8
4. $(\text{NH}_4)_2\text{SO}_4$	170 mg	960	5.6
5. Gelatin-agarose	11.3 mg	340	30
6. CM-cellulose	1.8 mg	570	320
7. H.p.l.c. gel filtration	140 μ g	130	920
8. H.p.l.c. cation exchange	80 μ g	14	170†

* Units are mg of collagen degraded (/mg of protein for specific activity); 18 h at 39 °C, pH 4.4.

† In step 8, 35–40 kDa contaminants are eliminated, but the enzyme is predominantly recovered as a cleavage fragment (for SDS/PAGE and sequence analysis, see Figures 2 and 4); for activity assays, stage-6 or -7 enzyme was used.

Figure 1 Column filtration purification steps

All proteinase assays shown were conducted at pH 4.5. (a) Gelatin-agarose fractionation of extracted collagenolytic activity (urea/NaCl extraction in 0.1% CHAPS/0.1 mM DTT, followed by desalting and NH_4Cl precipitation) from osteoclast lysates eluted in 3 column vol. of 10 mM acetate/1 mM DTT/0.01% CHAPS, pH 4.5. Absorbance of fractions at 280 nm indicates protein concentration (heavy solid line). Degradation (18 h, 37 °C by 30 μ l aliquots of fractions) of 100 μ g of Azocoll in buffer including DTT (see the Methods section) is given as A_{524} (broken line). Trichloroacetic acid-soluble absorbance from 100 μ g of diazotized casein similarly incubated with enzyme aliquots but in buffer with (Δ) or without ($\square$) 1 mM DTT is shown as A_{366} (thin continuous lines). Optical densities of totally degraded Azocoll and azocasein were 1.6 and 1.8 A units respectively. Azocoll degradation decreases by $\sim 70\%$ if performed in the absence of DTT (Table 3); note that azocasein degradation was not enhanced by DTT. Radiolabelled bone collagen degradation paralleled the Azocoll results in repetitions of this experiment, but was not performed in this instance. Fractions 15–18 were concentrated for further purification. (b) CM-cellulose fractionation of a gelatin-agarose activity peak. The column were pre-washed overnight with 10 mM acetate, pH 4.5, to give an effluent of pH < 4.8 , and the protein was loaded. The column was then eluted in 10 mM acetate/0.3 mM CHAPS/0.1 mM DTT, 4.5, for 2.0 column vol. and a linear 0–2 M NaCl gradient (made by adding 2.0 M NaCl with stirring to a fixed 100 ml volume of buffer, initially 10 mM acetate, as the buffer fed to the column) and from 2–3 column vol. (broken line). Absorbance at 230 nm indicates protein (solid heavy line). Azocasein, assayed as in (a), except that 100 μ l aliquots were used, is indicated by A_{366} (open triangles), whereas collagen degradation is indicated by solubilization of ~ 2000 c.p.m. of decalcified trypsin-treated ^3H -labelled bone collagen by 100 μ l aliquots of fractions (18 h, 37 °C, in acetate buffer with 1 mM DTT, pH 4.4 ($10^{-3} \times$ radioactivity, $\circ$)). In other runs, Azocoll degradation paralleled degradation of ^3H -labelled bone collagen, but was not performed in this instance. Fractions 20–24 were concentrated for further purification. (c) Gel-filtration fractionation of an activity peak from CM-cellulose in 10 mM acetate/150 mM NaCl. Protein is indicated by A_{230} (heavy line); collagenolytic activity is measured by Azocoll activity as in (a) above, expressed here as μ g of substrate solubilized by 100 μ l aliquots of fractions. Total substrate, determined by overnight digestion in 0.1% pepsin at pH 4.5, was 100 μ g, so the results also indicate percentage degradation ($\circ$). At this stage and beyond, a distinct azocasein-degrading peak was not seen. Low-activity azocasein degradation, paralleling collagen degradation and sensitive to the presence of DTT, is not shown (the purified enzyme displays limited general proteolytic ability; see the text). Positions of molecular-mass (M) standards are indicated below the arrowhead. Fractions 16 and 17 were used for sequencing, SDS/PAGE, assays and further purification. (d) CM-silica fractionation of activity from CM-cellulose. The column was washed with 2 M NaCl and approximately equilibrated with 10 mM acetate by a reverse acetate gradient (1 M to 10 mM) with final effluent pH of < 5.6 . Then h.p.i.c.-gel-filtration-purified enzyme was added, washed with 10 mM acetate, pH 4.5, and the column was eluted with a 0–2 M NaCl. The elution salt gradient was formed by adjusting ratios of 10 mM acetate, pH 4.5 and 2.0 M NaCl/10 mM acetate, pH 4.5, directly using dual pumps, delivering the low-salt buffer at a linearly decreasing rate and high-salt buffer at a complementary (increasing) rate; the portion from 0 to 1 M NaCl is illustrated (diagonal line). Protein is indicated by A_{230} (solid line). Aliquots of fractions (100 μ l) were assayed for degradation of Azocoll as in (a) above ($\circ$). Fraction 19 was used for sequencing, SDS/PAGE and activity assays.

pH, reducing conditions, salt concentration and detergent solubilization to maximize yield from the limited cellular starting material. Buffers at pH > 7 resulted in rapid and irreversible loss of collagenolytic activity. Thus acetate buffers at optimum collagenolytic pH, ~4.5 [5], were used whenever possible. Collagenolytic activity in solution was increased 2–3 fold by the addition of detergents, with recoveries greater in the non-denaturing ionic detergent CHAPS (0.1%) than in Triton X-100, sodium deoxycholate or Brij-35 at a variety of concentrations. Barrett's alternative method of autolysis to solubilize acid proteinase enzyme activity [16] resulted in significantly reduced collagenolytic activity and was not used. Once solubilized, lower detergent concentrations allowed good retention of activity, and most steps were run at 0.01 or 0.001% CHAPS. Soluble collagenolytic activity was increased by extraction in salt and mild denaturants. Comparison of 0–3 M NaCl and 0–6 M urea extraction showed that 1 M NaCl and 2 M urea was optimal for initial extraction (3 h, 4 °C); solubilized activity was then desalted by dialysis against 2 M urea overnight without significant activity loss in the precipitate. Activity was protected by mild reducing conditions, and dithiothreitol (DTT) was therefore added to solutions. After solubilization and desalting, collagenolytic activity was concentrated by precipitation with 85% satd. $(\text{NH}_4)_2\text{SO}_4$.

Further purification used several column-chromatography steps. In each case, collagenolytic activity was found in one major peak, which was concentrated for the next step. Gelatin affinity chromatography at 4 °C and pH 4.5 separated two proteolytic peaks. One was weakly retarded and degraded collagen far less well at pH 4.5 than the general proteolytic substrate casein (Figure 1a); this non-specific proteinase peak was insensitive to DTT addition. In contrast, the more avidly retained second proteinase peak degraded collagen better than casein and required DTT for retention of activity. Only the collagenolytic peak was retained and further purified, although small amounts of caseinolytic activity typically were retained through the CM-cellulose step (Figure 1b). Protection of activity by dithiothreitol suggested that the collagenolytic enzyme is a thiol proteinase. Hence mercury-affinity purification [16] was attempted. Unfortunately this irreversibly inactivated the collagenolytic activity of osteoclast lysates; activity was not restored by dialysis against excess DTT.

Nevertheless, additional chromatographic separation steps allowed purification. Gelatin-agarose, CM-cellulose, and gel-filtration h.p.l.c. allowed recovery of ~0.1% of the starting protein with ~10% of the initial activity (Figure 1c). SDS/PAGE show that this material consisted of a ~31 kDa major protein, which was sequenced by Edman degradation, and several minor components. Further purification by CM-silica cation exchange was undertaken to confirm that the ~31 kDa protein was the active molecule (Figure 1d). This resulted in the loss of the bulk of the activity, but yielded a very pure preparation consisting of a 31 kDa minor band and a 25 kDa fragment not present in the preceding step (Figure 2).

Characterization of isolated enzyme

Reproduction of osteoclast lysate activity on bone collagen by the purified protein was tested. In 50 mM acetate/0.1 mM CHAPS/1 mM DTT at pH 4.4, 400 µg of osteoclast lysate protein degraded 60–90% (range) of 100 µg of Azocoll and a similar proportion of ^3H -labelled decalcified trypsin-treated bone collagen. A 10 µg portion of stage-8 protein degraded ~60% of Azocoll and $57 \pm 22\%$ of ^3H -labelled decalcified trypsin-treated bone collagen under the same conditions. Activity of stage-7

Figure 2 Appearance of enzyme preparations on polyacrylamide gels stained with silver

All samples were reduced in 10% SDS/5 mM DTT for 5 min at 100 °C prior to separation. Positions of molecular mass (*M*) markers in each gel are indicated. (a) Enzyme after $(\text{NH}_4)_2\text{SO}_4$ precipitation (stage 3) on SDS/10% PAGE. (b) Enzyme after CM-cellulose (stage 6) and after gel filtration (stage 7) on SDS/12% PAGE; a band at 37 kDa in the gel-filtration preparation was a medium artifact (compare with adjacent blank). A prominent band at 31 kDa is apparent by stage 6. (c) Enzyme after CM-silica separation (stage 8) on SDS/14% PAGE. A new prominent band at ~25 kDa is seen, with ~10% of the material retaining its original size. A high-molecular-mass doublet is a medium artifact (compare with adjacent blank). No other fractions eluted from CM-silica had measurable activity, and the smaller fragment appears to be a principle cleavage fragment of the 31 kDa enzyme (see the text).

Figure 3 pH dependence of bone collagen degradation by partially purified osteoclast acid collagenolytic activity

Portions (100 µg, 1400 c.p.m.) of 25–50 µm fragments of ^3H -labelled trypsin-treated decalcified bone were incubated in 100 mM acetate at the indicated pH with 10 µg aliquots of stage 5 enzyme (Table 1) for 23 h at 37 °C, and the soluble portion was counted for radioactivity after centrifugation (5 min at 10000 *g*).

enzyme was maximal, with total (>95%; no detectable difference from total hydrolysis using pepsin) degradation of 100 µg of Azocoll by <1 µg of enzyme; the loss of activity at stage 8 correlated with a relatively small amount of intact enzyme surviving the CM-silica h.p.l.c. column. However, this final step was designed to provide assurance that contaminants present in stage 6–7 enzyme at ~35–40 kDa did not mediate the observed collagen degradation, and the activity remaining was proportional to the amount of the intact (~31 kDa) enzyme (Figure 2). Collagenolysis was maximal at pH 4.5 (Figure 3).

Edman degradation sequences of the 31 and 25 kDa proteins, and CNBr digests of the 31 and 25 kDa proteins are shown in Figure 4. Sequences were similar to those of mammalian cathepsin Bs [17–20], and the 25 and 31 kDa bands had identical CNBr sequences. This, and the SDS/PAGE results showing appearance of the 25 kDa protein with disappearance of the 31 kDa protein (Figure 2), indicated that the 25 kDa protein is part of the larger molecule. The difference between the start points of the sequences (50 amino acids; Figures 4a, 4b and 5) is consistent with the difference in size observed on SDS/PAGE (6 kDa), confirming this hypothesis. N-terminal Edman-degra-

Figure 4 Edman degradation sequences of the ~ 31 kDa and ~ 25 kDa isolated proteins (stages 7 and 8)

(a) N-terminal sequence from the ~ 31 kDa protein. (b) N-terminal sequence of the ~ 25 kDa protein. (c) and (d) Sequence from either ~ 25 or ~ 31 kDa proteins after CNBr digestion. Consensus results from several runs are shown; however, primary sequences are ambiguous in several places. These uncertain residues are listed with question marks. Note, however, that most of sequences (a) and (c) are contained in the cDNA sequence of the cloned portion of the enzyme (underlined regions of Figure 5) because its 5' and 3' ends correspond to deduced degenerate primers derived from these fragments, and the second CNBr cleavage fragment (c) is entirely contained within the cloned fragment (amino acids 68–79, also underlined in Figure 5). The initial methionine residues (parentheses) were not seen but rather assumed to be present from the cleavage technique used. However, their presence was confirmed in the cDNA (Figure 5). Residues from which degenerate probes for cDNA amplification were constructed are underlined in this Figure. Positions of fragments are given using the numbering scheme of Meloun et al. [17], with comparisons to reported bovine, rat, human, and chicken cathepsin B sequences [17–21]. Positions at which sequences differ are boxed.

dation data, and a second CNBr cleavage fragment (from Met⁶⁹) are contained within a cloned segment (see below; sequence underlined in Figure 5). Note that the N-terminal sequence essentially matches the reported sequences of chicken liver cathepsin B fragments (Figure 4a) [21]. Ambiguity of the primary N-terminal sequence was consistent with partial degradation, as most secondary sequence matched with the same sequence ± 1 –3 residues. However, CNBr digest sequences showed two different sequences corresponding to two methionine residues (Figures 4c and 4d).

cDNA analysis and hybridization

Regions of amino acid sequence with minimal degeneracy were calculated for the available primary sequence by multiplying the number of possible codons for adjacent amino acids. Two segments with low degeneracy and also containing few

ambiguities (underlined in Figure 4) were synthesized as sense (5') and antisense (3') degenerate oligonucleotide primers. By identity with previously cloned mammalian cathepsin Bs, these would bracket a hypothetical cDNA of ~ 470 bases. Two rounds of PCR, the first using oligo(dT)₁₆ and the synthetic degenerate 5' DNA as primers and the second round using both of the synthetic degenerate (5' and 3') DNAs as primers, amplified 486 and ~ 300 bp oligonucleotides, which were cloned into plasmid vectors and sequenced by dideoxynucleotide chain termination (see the Methods section and Figure 5). The ~ 300 bp oligonucleotide contained stop codons in all reading frames, and was discarded. Of 162 amino acids encoded in the longer isolate, 77% were identical with human cathepsin B [18,19]. The osteoclastic cathepsin B varies more from mammalian cathepsin Bs than mammalian cathepsin Bs from each other, where ~ 85% of amino acids are identical between species [17]. Only minimal data were obtained regarding the remainder of the

9 K O W P N C P Thr Ile Ser Glu Ile Arg Asp Gln
 1 AAA CAA TGG CCA AAT TGC CCC ACC ATC AGC GAG ATC AGA GAC CAG
 Human Glu Q W P Gln C P T I Lys E I R D Q
 24 Gly Ser Cys Gly Ser Cys[†] Trp Ala Phe Gly Ser Val Glu Val Ile
 46 GGC TCC TGC GGC TCT TGC TGG GCT TTT GGT TCT GTG GAA GTG ATT
 G S C G S C W A F G Ala V E Ala I
 39 Ser Asp Arg Ile Cys Val His Thr Asn Ala Lys Val Ser Val Glu
 91 TCG GAC AGA ATC TGT GTT CAC ACC AAC GCC AAG GTG AGC GTG GAG
 S D R I C Ile H T N --- --- V S V E
 54 Val Ser Ala Glu Asp Leu Leu Ser Cys Cys Gly Phe Glu Cys Gly
 136 GTG TCG GCG GAG GAT TTG CTG TCA TGC TGT GGC TTC GAG TGC GGC
 V S A E D L L Thr C C G Ser Met C G
 69 Met Gly Cys Asn Gly Gly Tyr Pro Ser Gly Ala Trp Arg Tyr Trp
 181 ATG GGG TGC AAT GGT GGT TAT CCT TCT GGT GCA TGG AGG TAC TGG
 Glu G C N G G Y P Ala Glu A W Asn Phe W
 84 Thr Glu Arg Gly Leu Val Ser Gly Gly Leu Tyr Asp Ser His Val
 226 ACA GAG AGG GGC CTC GTG TCT GGG GGT CTC TAT GAC TCC CAT GTG
 T Arg Lys G L V S G G L Y Glu S H V
 99 Gly Cys Arg Pro Tyr Thr Ile Pro Pro Cys Glu His His Val Asn
 271 GGC TGC GCC GGG TAC ACC ATC CCA CCC TGT GAG CAC CAC GTC AAT
 G C R P Y Ser I P P C E H H V N
 114 Gly Ser Arg Pro Pro Cys Thr Gly Glu Gly Gly Glu Thr Pro Arg
 316 GGC TCC CGG CCA CCT TGC ACT GGG GAG GGA GGA ACC CCC AGG
 G S R P P C T G E G --- Asp T P Lys
 128 Cys Ser Arg His Cys Glu Pro Gly Tyr Ser Pro Ser Tyr Lys Glu
 361 TGC AGC CGG CAC TGT GAA CCT GGC TAC TCG CCT TCC TAC AAG GAG
 C S Lys Ile C E P G Y S P Thr Y K Gln
 143 Asp Lys His Tyr Ala Ser His Ile Tyr Gly Val Pro Arg Ser Glu
 406 GAC AAG CAC TAC GGC AGT CAC ATC TAC GGT GTC CCT CGC AGC GAG
 D K H Y Gly Tyr Asp Ser Y Ser V Ser Asn S E
 158 Lys Glu Ile M A E I Y V N Q P
 451 AAG GAA ATC TAC CGG CTC TAG ATG CAG TTG GTT GGT
 K Asp I M A E I Y Lys N Gly P

Figure 5 cDNA and deduced amino acid sequences of the osteoclast's cathepsin B-like enzyme

Amino acids (top line) are numbered to correspond with the longest (bovine) of the previously reported cathepsin B sequences [17–21]. Underlined portions indicate sequence corresponding to primary sequence by Edman degradation (Figure 4). The portion of the molecule corresponding to amino acids 1–8 was not cloned; there is one insertion relative to the bovine sequence (Gly, residue 124a, asterisk). Nucleotide numbering (middle line) is for this cDNA. Deduced amino acids different from human are *italic*; and human protein sequence [18] is shown for comparison (bottom line) as single-letter code, except for amino acids differing from the osteoclast, which are in three-letter code. The avian liver sequence is known for amino acids 1–36 and 50–66 [21]; the only difference with this deduced sequence, Ala → Ser at amino acid 34, is shown in **bold face**. Portions of the clone corresponding to primer sequences are given as single-letter code; primers used in DNA amplification were composed of all possible DNA sequences corresponding to these protein sequences in equal proportions. Additional deduced amino acids are shown as three-letter code. †, active site [17].

molecule. However, all previously sequenced mammalian cathepsin Bs have an additional conserved sequence around histidine¹⁹⁹ that includes a methionine at position 196 [17]. This would be predicted to produce a third CNBr cleavage fragment. Such a fragment was not observed, suggesting that additional significant differences exist in the remainder of the protein. Limited N-terminal sequence data from chicken liver [21] show one amino acid difference with the cloned segment (Figure 5). At high stringency, the isolated osteoclast cDNA hybridized to total avian osteoclast RNA at a single band of ~ 2.4 kb (Figure 6). A faint lower-molecular-mass band also seen in Figure 6 decreased with increasing stringency and matched with a high-abundance RNA by ethidium bromide staining; it was thus attributed to non-specific binding, probably to rRNA. The significance of these findings is discussed below.

Figure 6 Northern blot analysis, as detailed in the Methods section, showing that labelled cDNA recognizes a ~ 2.4 kb osteoclast RNA

After pre-hybridization, osteoclast RNA on nylon was hybridized with ³²P-labelled cDNA (Figure 5) for 6 h at 65 °C, washed for 20 min at 56 °C and autoradiographed overnight. Positions of RNA standards are indicated.

Substrate preference of the isolated enzyme, and effects of temperature and pH

Optimum pH for osteoclast lysate degradation of bone collagen was 4.5 and fell by ~ 50 % at pH values one unit lower or higher (Figure 3), in essential agreement with our previous report [5]. This pH-dependence did not vary measurably with purification.

Activity on artificial substrates was measurable at pH 6 (Table 2), the standard conditions for these determinations [13], although this enzyme isolate degraded artificial substrates very poorly and long incubations (18 h; see the Methods section) were necessary. This reflects the substrate degradation rates for these artificial substrates (k_{cat} values), which were generally lower by ~ 10-fold than in the previous report for avian hepatic cathepsin B [21] or mammalian cathepsin Bs [13]. Contrariwise, Bz-Arg-Nap and benzoyl-D-L-arginine *p*-nitroanilide (Bz-Arg-Nan) are relatively poor substrates for this enzyme isolate, and Arg-Arg, Gly-Pro or Bz-Arg substrates are better, as in previous cathepsin B determinations. Phe-Arg-NHMe has a relatively high K_m , but a very good k_{cat} , allowing sensitive determinations; Phe-Arg-NHMe degradation was similar at pH 4.5, with an ~ 25 % increase in activity. Relative to avian liver cathepsin B, K_m values were also reduced, and consequently k_{cat}/K_m ratios were all within the range of the previously reported values [21]. These enzyme isolates diverge, however, in arginine substrate specificity. k_{cat}/K_m for the various arginine substrates tested varied only ~ 25-fold for the osteoclast enzyme, contrasting dramatically with the ranges of ~ 1000-fold seen with avian liver cathepsin B [21] and of almost 100 000-fold with mammalian cathepsin B [13].

The ability of cathepsin B to cleave glycolamides is well documented [22]. However, in the helical collagen sequence, proline, hydroxyproline, and glycine predominate, suggesting that proline residue cleavage may also be important. In keeping with the hypothesized role for this enzyme in acidic collagen degradation, we found that Pro-Ala-Gly-Pro-MeNap was cleaved to release naphthylamide as actively by the avian osteoclast enzyme as Z-Arg-Arg-MeNap substrate (Table 2). Further, in contrast with other thiol proteinases, pepstatin inhibits activity of the avian osteoclast enzyme towards both low- and high-molecular-mass substrates (Tables 2 and 3).

Table 2 Artificial-substrate degradation by the isolated osteoclast acid collagenase in 100 mM phosphate/1.0 mM EDTA/1 mM DTT at 38 ± 0.5 °C

All determinations were at pH 6.0, except the last, which is a comparison of Z-Phe-Arg-NHMec activity at pH 4.5. All assays were performed with a single stage-6 isolate of enzyme. Least-squares fits of three-point Lineweaver-Burk plots are reported with the molar ranges tested and correlation coefficients (R^2) of the least-squares fits. Enzyme molarity was calculated from protein concentration (using a molecular mass of 31 kDa) and the ratio of activity of stage-6 preparations to maximal activity (see the Methods section).

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($10^3 s^{-1} \cdot M^{-1}$)	[S] range (μM)	R^2
Bz-Arg-Nap	0.56	55	10	40–160	0.94
Z-Arg-Arg-MeNap	1.04	16.7	62	20–100	0.98
Z-Pro-Ala-Gly-Pro-MeNap	1.1	15	73	20–100	0.99
Bz-Arg-Nan	0.70	26	27	25–100	0.82
Z-Phe-Arg-NHMec	27	233	116	40–200	1.0
+ 20 $\mu g/ml$ pepstatin	2.1	160	13	40–200	0.97
pH 4.5	35	151	231	40–200	0.98

Table 3 Avian osteoclast collagenase activity in the presence of proteinase inhibitors, Ca^{2+} and DTT

All assays used stage-6 enzyme and were performed using Azocoll in 50 mM acetate/0.1 mM CHAPS/1 mM DTT, pH 4.5, except as noted. Because of partial denaturation consequent to diazotization, results for each reagent were confirmed using 3H -labelled bone collagen, which gave similar results (not shown). Assays were quantified by A_{524} with respect to controls after 18 h incubation at 37 °C. This Table summarizes several separately run assays; where no errors are shown, these indicate results of single determinations, but were typical of multiple experiments. Where errors are shown, $n = 2$.

Reagents added	Concentration	Activity (% of control)
DTT	0	34
	0.1 mM	58
	0.3 mM	66
	1 mM	100 ± 10*
	3 mM	133
Ca^{2+}		
As $CaCl_2$	10 mM	128
As $Ca_3(PO_4)_2$	10 mM	104
HgCl ₂	100 μM	0†
Phenylchloromercuribenzoate	50 μM	0†
EDTA	2 mM	93 ± 8
Phenylmethanesulphonyl fluoride	100 μM	79 ± 5
Iodoacetic acid	100 μM	0
Pepstatin	1 $\mu g/ml$ (1.5 μM)	29
	5 $\mu g/ml$ (8 μM)	11
	10 $\mu g/ml$ (15 μM)	5
Leupeptin	1 $\mu g/ml$ (2 μM)	26
	5 $\mu g/ml$ (11 μM)	19
	10 $\mu g/ml$ (21 μM)	0
Antipain	1 $\mu g/ml$ (2 μM)	67
	5 $\mu g/ml$ (8 μM)	38
	10 $\mu g/ml$ (17 μM)	5

* Control definition; error ranges for controls of all experiments summarized fall within this range.

† Mercurials were added to enzyme without DTT and incubated for 15 min at 4 °C, after which 1 mM DTT was added; no recovery of activity was observed at DTT up to 20 mM; mercurials had no effect in the presence of excess DTT.

Osteoclast enzyme collagen degradation is as effectively inhibited by pepstatin as it is by antipain and leupeptin, whereas the avian liver enzyme was unaffected by 10 μM pepstatin [21]. The effect on K_m of pepstatin addition was minimal (Table 2). The findings of Z-Pro-Ala-Gly-Pro-MeNap cleavage with similar rate to Z-Arg-Arg-MeNap, and the relatively narrow range in k_{cat}/K_m for arginyl substrates, suggest that pepstatin competes to various extents with protein or artificial peptide substrates because this

Figure 7 Degradation of type I collagen by the purified enzyme

Disappearance of salt-extracted guinea-pig collagen [41] (1 mg of type I collagen; left lane) and appearance of degradation products when incubated with ~ 100 ng of stage-7-purified enzyme at pH 4.4, demonstrated on SDS/8%/10%-PAGE stained with silver. The enzyme is seen as a faint band not present in the collagen control (first lane) in a no-collagen control (arrow, second lane). The enzyme cannot be distinguished reliably from collagen fragments in the mixed degradation assays, however. The middle lanes show degradation at 31 °C, and the right at 37 °C, over the indicated times. At 25 °C, degradation was insignificant. Although some discrete breakdown products in the 20–50 kDa range are seen, there is no predominant cleavage product, showing that many sequences in the collagen are hydrolysed at this pH by this enzyme. Complete collagen degradation is also mediated by stage-8 enzyme (Table 2).

enzyme's peptide specificity differs from those of other cathepsin Bs.

Additional Ca^{2+} , DTT, and proteinase-inhibitor effects on collagenolytic activity are shown in Table 3. Dithiothreitol protected collagenolytic activity; Ca^{2+} (10 mM) left activity largely unaffected. Chelating agents and serine-proteinase inhibitors were not effective.

The purified enzyme degrades a large excess of saline-extracted isolated guinea-pig type I collagen to heterogeneous low-molecular-mass fragments, at pH 4.5 and 31 or 37 °C, but no activity was seen at 25 °C (Figure 7). The dependence of this soluble collagen degradation on temperature at pH 4.5 confirms that osteoclast cathepsin B collagen degradation is predicated on collagen denaturation. All collagen degradation proceeded without accumulation of distinct (meta)stable intermediates, such as the roughly 1/4, 3/4-split TC^A and TC^B fragments characteristic of interstitial collagenase activity. Under these denaturing conditions, collagen would also be sensitive to aspartic

Table 4 Effect of albumin in solution on degradation of 100 μ g of insoluble collagen by stage 4 and stage 8 osteoclast collagenolytic enzyme at pH 4.4

Total degradation at stage 4 was $\sim 80\%$, and at stage 8 $\sim 10\%$ of total substrate. Substrate was titrated collagen at stage 4 and Azocoll at stage 8. Results are means $\pm$ S.D., $n = 2$ (stage 4) or 4 (stage 8).

Albumin (μ g/ml)	Collagen degradation (% of control)	
	140 μ g, stage 4	167 ng, stage 8
0	100 $\pm$ 10	100 $\pm$ 16
100	92 $\pm$ 11	81 $\pm$ 30
500	—	90 $\pm$ 40
600	59 $\pm$ 1	—

proteinases such as pepsin. Despite the unusual competitive inhibition of this cathepsin B by pepstatin (discussed above), the possibility of contaminating aspartic-proteinase activity causing the observed effect is eliminated by the uniform elimination of collagenolytic activity by thiol-proteinase inhibitors (Table 3). Further, degradation of artificial substrates, including Z-Pro-Ala-Gly-Pro-MeNap, was eliminated by iodoacetate treatment (see the Methods section).

The absence of a discrete intermediate degradation fragment, the observation of many fragments < 50 kDa on partial digestion and total degradation to low-molecular-weight fragments with extended time at 37 °C show that the collagen helix is cleaved at a variety of sites by this enzyme. At neutral pH this would be surprising, as collagen possesses a long helical region resistant to most proteinases; however, collagen is subject, as are all proteins, to denaturation by low pH or other factors. Thus, in the context of the low pH at which this enzyme operates, susceptibility of the helix to degradation at multiple sites would be expected. Indeed, mammalian cathepsin B has been described as a collagen-helix-cleaving enzyme [24] present in bone. pH optima for cathepsin B degradation of various proteins, including collagens, are reported by various investigators to be 3.5–5 (summarized by Barrett [22]).

Furthermore, diazotized collagen was a preferred substrate for this enzyme and was degraded more efficiently at pH 4.5 than was soluble diazotized casein (Figure 1), despite the fact that the collagen was present as an insoluble pellet. Similarly, albumin, even in substantial excess, did not proportionately reduce insoluble bone collagen degradation by the enzyme at pH 4.4, a finding not affected by purification (Table 4). Both albumin and casein were degraded by the enzyme, but at lower rates than was collagen (results not shown). Thus the osteoclast collagenolytic proteinase appeared to be a non-specific proteinase closely related to cathepsin B, for which insoluble collagen is a better substrate than some soluble proteins.

Immune confirmation of osteoclastic origin, role in collagenolysis and size of active enzyme

Antibody to the 25 kDa enzyme fragment adsorbed collagenolytic activity from osteoclast lysates, recognized osteoclasts in sections of avian bone by peroxidase-antiperoxidase staining and identified a 31 kDa band in osteoclast lysates by Western analysis (Figures 8, 9 and 10). These results confirm that the enzyme is required for effective collagenolysis by osteoclast lysates and that the enzyme is present in osteoclasts. Furthermore, the predominant form of the enzyme in osteoclast

Figure 8 Titre of antibody adsorbing collagenolytic activity from osteoclast supernatants (Azocoll digestion, given as $A_{524} \pm$ S.D.; vertical axis) produced by several injections with 25 kDa antigen, with respect to pre-immune rabbit serum (PICS, left)

A 100 μ l portion of undiluted antibody was allowed to react with 25 μ l of osteoclast lysate at pH 6.5, at 37 °C for 30 min, followed by precipitation of antibody with 5 mg of insoluble staphylococcal protein A (Sigma), after which the collagenolytic activity of supernatants was measured (with 1 mg of Azocoll in 50 mM acetate/1 mM β -mercaptoethanol/0.1 mM CHAPS, pH 4.4, for 18 h at 37 °C). Serum after 11 injections was used for Western blots and immunohistochemical staining (Figures 9 and 10).

lysates is similar in size to the enzyme as isolated at stage 7 (~ 31 kDa; Figure 2).

All osteoclasts were labelled by antibody to the enzyme, although the degree of antigen reactivity varied. Some mononuclear cells also were recognized, albeit weakly, by the antibody (Figure 9a); the nature of these cells was not studied. The strongest staining observed was of selected osteoclasts on bone (Figure 9b). In most cells, staining was distributed throughout the cytoplasm. It is important to note, however, that osteoclasts are strongly autolytic, and thus staining distribution may not reflect living intracellular antigen distribution. In some cases, staining at cell–bone interfaces was observed (arrows, Figure 9b). Other proteinases may also be involved in osteoclast degradative processes, and these would be undetected by this technique. Indeed, at least one additional abundant proteinase was detected (but not characterized) in early stages of osteoclast acid-collagenase purification (Figures 1a and 1b). Nevertheless, these results are compelling evidence that a cathepsin B is rate-limiting in collagenolysis by avian osteoclasts.

Comparison with other findings related to bone collagen degradation

In the present study, we purified and characterized collagenolytic activity from avian osteoclasts and found it to depend on a cathepsin B. The unusual properties of this enzyme reconcile results of several previous studies. Histochemical study [25] did not show cysteine- or aspartic-proteinase involvement in extracellular-matrix dissolution, raising the possibility that an osteoclastic collagenase might be unique. Our results are consistent with poor histochemical staining for cathepsin B using low-molecular-mass substrates; the isolated cathepsin B had markedly reduced k_{cat} values on arginyl substrates (Table 2). Similarly, Maciewicz and Etherington [26] noted high affinity and turnover rate on collagen for cathepsin Bs from rabbit spleen, whereas activity towards artificial substrate was poor. Thus a possible explanation is that these uncharacterized cysteine proteinases are cathepsin B variants. In support of this possibility, multiple processing forms [27], multiple alleles and chromosomal locations [28] and various glycosylation states [29] are reported for cathepsin B, and Mach et al. [30] recently demonstrated cell-line-specific variation in cathepsin B processing.

Figure 9 Identification of osteoclasts by antibody to the collagenolytic enzyme

Panels **A** and **B**, avian medullary bone stained by antibody to the 25 kDa fragment of enzyme, at 1:10 dilution, after immunization and 10 booster doses; panel **C**, medullary bone control treated identically but using pre-immune serum from the same animal at 1:10 dilution. Low-power photomicrographs (panels **A** and **C**) show that osteoclasts (OC) are identified by the antibody (brown staining with peroxidase), but not pre-immune serum. Some mononuclear cells stain as well (arrowheads). A high-power photomicrograph (panel **B**) shows osteoclasts on bone stained strongly by antibody; focal staining at the cell/bone interface is also seen (arrows). Scale: osteoclast nuclei are $\sim 4 \mu\text{m}$ across.

Figure 10 Demonstration of antibody specificity by Western analysis

An antibody was raised to the 25 kDa major fragment of the enzyme (see the Methods section) and used to stain poly(vinylidene difluoride) blots of osteoclast lysate and purified enzyme using the unlabelled antibody technique of Sternberger et al. [11]. Reaction with pre-immune serum ('PICS'; left lane), at 1:200 dilution, is compared with serum from a rabbit immunized and boosted ten times, also at 1:200 dilution ('Immune'; middle lane), on 20 μ g of osteoclast lysate (stage 3) to show specificity of the reaction. Reaction of immune serum with stage-7 enzyme ('Enzyme'; right lane) demonstrates that the immune serum is recognizing the enzyme in the lysate mixture. Positions of molecular-mass (M) markers (right) were determined in cut segments of membrane stained with Coomassie Blue and reassembled. Variable non-specific reaction of pre-immune serum and other protein (weak band in PICS at $\sim$ 50 kDa) was also seen in some cases, but did not cause unreasonably high backgrounds or precipitate activity (Figures 8 and 9).

Burleigh, Barrett and Lazarus described native collagen degradation by cathepsin B in 1973 [24]. Vaes [31] and his co-workers [6] showed that a variety of acid-proteinase inhibitors block osteoclastic activity. Recently, Delaissé, Ledent, and Vaes isolated from bone cathepsins B and L, and an additional cathepsin L-like proteinase [7], although cells producing these enzymes were not identified. Evidence for a role for cysteine proteinases in collagen turnover was also published by Etherington [32] and his co-workers. Silver, Murrills and Etherington also showed that the zone of contact of osteoclasts and substrate had low pH by micro-electrode measurements [33], confirming the preliminary results (1984) of Fallon [34], and supporting the physiological relevance of findings that cysteine proteinases degrade the matrix collagens of cartilage [35] and bone [36]. Curiously, the latter study found that Ca^{2+} stimulated collagen degradation $\sim$ 2-fold at 50–75 mM. We found that 10 mM Ca^{2+} did not effect osteoclast collagen degradation, although the high concentrations used by Etherington and Birkedahl-Hansen [36] were not tested. After isolating cysteine proteinases of $\sim$ 22–30 kDa with variable collagenolytic capacities. Maciewicz and Etherington [26] noted that definitive proof of the difference of this 'cathepsin N' from cleavage products of other cathepsins was needed; with this caveat, our present findings are similar.

Proteinases unrelated to cathepsin B have been hypothesized to play roles in bone degradation. The aspartic proteinase cathepsin D has been presented as an osteoclast activator [37]. Although bone collagen degradation is found here to depend on a cathepsin B, a DTT-insensitive proteinase consistent with cathepsin D is present in large quantities in osteoclast isolates. We noted that the large quantity of caseinolytic activity separate from the purified protein (Figures 1a and 1b) was not DTT-sensitive or inhibited by thiol-proteinase inhibitors (results not

shown), suggesting that this may be cathepsin D. Collagenase, a neutral-pH zinc proteinase pivotal to degradation of extracellular matrix in most tissues, was hypothesized to mediate bone collagen turnover. Osteoblasts are capable of producing collagenase [4,38], and a report of collagenase at the osteoclast attachment has been presented [39]. We found no detectable collagenase in adult rat bone [40], and the present finding that a cathepsin B is the primary collagenolytic enzyme of bone supports our earlier result. Collagenase or other proteinases may, however, play accessory roles in bone degradation that would be undetected by the methods of the present study.

Observed differences in the properties of hepatic and osteoclastic cathepsin B (Tables 2 and 3; [21]) could reflect either variation of enzyme processing or a structural difference in enzyme sequence. Until more of the structure of avian liver cathepsin B is known, the possibility that these enzymes are derived from different genes must be considered. Whereas most of the avian cathepsin B sequence is reported only from the present work, it appears likely that a common gene produces this and other avian cathepsin Bs. There is only one difference (Ala $\rightarrow$ Ser) between our deduced cDNA sequence and 58 overlapping amino acids of avian hepatic cathepsin B reported from Edman degradation ([21] and Figure 5). This conservative difference may be due either to an error in DNA amplification, which was accomplished using sequential PCR amplification and is therefore subject to error rates of $\sim$ 1 in 10^3 – 10^4 , to an error in Edman-degradation sequencing or to individual variation in the enzyme, for which there is precedent in other species [28]. The finding of only one hybridizing RNA at $\sim$ 2.4 kb in osteoclasts (Figure 6) indicates that, if related enzymes exist, they are either not abundant in the osteoclast, have significantly divergent DNA sequence or are not distinguishable by size from the osteoclastic enzyme. Furthermore, antibody to the isolated osteoclast enzyme identifies marrow mononuclear cells as well as osteoclasts (Figure 9a). Thus our findings are most consistent with the osteoclastic enzyme being cathepsin B, with variation in antigenicity and substrate preference that is most easily attributed to cell-specific enzyme processing, probably in post-translational processing or by proteolytic cleavage.

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