A comparison of four cathepsins (B, L, N and S) with collagenolytic activity from rabbit spleen

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We have separated four cathepsins (B, L, N and S) from rabbit spleen. They are all collagen-degrading cysteine proteinases, with M_r values of 25250, 23500, 34000 and 30000 for cathepsin B, L, N and S respectively. Cathepsins B, N and S have isoelectric points of 5.4, 6.2 and 6.8 respectively, whereas cathepsin L exhibited multiple charge forms in the range 5.0–5.7. A comparison of their specific activity against a variety of protein and synthetic substrates shows many differences. These differences can be visually illustrated through isoelectric focusing and detection of enzymic activity with protein and synthetic-substrate overlays. By using an enzyme-linked immunosorbent assay based on the binding to chicken cystatin and detection with polyclonal and monoclonal antibodies to native cathepsins B and L, no cross-reactivity of the four native enzymes was observed. Studies on the co-operative or synergistic effect in degrading collagen indicated that, of the different combinations tested, only the combination of cathepsin B and N exhibited enhanced collagenolysis.

INTRODUCTION

The two most extensively studied lysosomal cysteine proteinases, cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15), have been well characterized and seem to be ubiquitous (for a review see Katunuma & Kominami, 1983). They degrade a variety of proteins, and as such have a role in intracellular protein turnover, as well as being implicated in extracellular-matrix degradation in disease states (for instance in tumour metastasis, muscular dystrophy, emphysema and arthritis) (Rochefort *et al.*, 1987; Kominami *et al.*, 1987; Burnett & Stockley, 1985; Etherington *et al.*, 1988).

We have been interested in those enzymes that are capable of degrading insoluble collagen in acidic micro-environments, intracellularly in the lysosome and extracellularly in local zones [for example, between the invading osteoclast and the underlying surface of the bone (Baron *et al.*, 1985; Silver *et al.*, 1988)]. Although collagen is resistant to non-specific proteolysis, cathepsin B (Etherington, 1974; Burleigh *et al.*, 1974) and cathepsin L (Kirschke *et al.*, 1982; Mason *et al.*, 1984) have been shown to cleave native collagen in such a way as to cause the depolymerization of the cross-linked fibre.

Two other collagenolytic enzymes have been tentatively identified, and have been named cathepsin N (Ducastaing & Etherington, 1978; Etherington, 1980; Etherington & Birkedal-Hansen, 1987) and cathepsin S (Locnikar *et al.*, 1981; Turk *et al.*, 1980). Their limited distribution (bovine spleen) and similar substrate specificities and physicochemical properties to cathepsin L have led to the conclusion that they may be tissue or species variants of cathepsin L (Kirschke & Barrett, 1985). Bovine cathepsin S has been shown to have different kinetic properties from cathepsin L (Kirschke *et al.*, 1984, 1986). The position of cathepsin N though has remained less clear, mainly owing to the difficulties in

assaying specifically for this enzyme. This has led to some uncertainty concerning its separate existence and the suggestion that it may be identical with cathepsin S (Kirschke & Barrett, 1985). More recently (Maciewicz *et al.*, 1987) we have compared the ability of these four cathepsins to cleave insoluble collagen and their degree of inhibition by chicken egg-white cystatin. The results indicated that cathepsins L and N have a high binding affinity for collagen with a high turnover rate, whereas cathepsins B and S bind less strongly to and cleave insoluble collagen more slowly. Except for cathepsin B, all are strongly inhibited by cystatin. In many respects cathepsins N and S are very similar to cathepsin L, and definitive proof that these three enzymes together with cathepsin B are all different enzymes needs to be obtained, since the minor cathepsins N and S may have a more specific role to play in the cell than either cathepsin B or cathepsin L.

In the present paper we show that the four collagen-degrading activities can be isolated simultaneously from one tissue source, rabbit spleen. To establish their identity we have compared these four enzymes by: (1) their ability to degrade a variety of synthetic and natural protein substrates; (2) their physicochemical properties such as molecular size and isoelectric point; (3) their immunological properties. From these results we conclude that cathepsins B, L, N and S are distinct and separate collagenolytic enzymes. A preliminary account of some of this work has been published (Maciewicz & Etherington, 1985; Etherington *et al.*, 1986).

MATERIALS AND METHODS

Purification of cathepsins

Spleens (50–100 g, collected from New Zealand rabbits and stored at -25°C) were partially thawed, minced

Abbreviations used: Ep-475, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(3-methyl)butane; Z-, benzyloxycarbonyl-; -NHMeC, 7-(4-methyl)coumarylamide; Bz-, benzyl-; e.l.i.s.a., enzyme-linked immunosorbent assay.

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and homogenized in 2 vol. of cold 50 mM-sodium acetate buffer, pH 5.0, containing 1 mM- Na_2EDTA and 0.25% (v/v) Triton X-100. The homogenate was stirred at 4 °C for 2 h and then centrifuged at 15000 *g* for 20 min. The supernatant was adjusted to pH 4.2 with 2 M-HCl and the precipitate was removed by centrifugation as above. The collagenolytic activity in the supernatant was concentrated by fractionation with $(\text{NH}_4)_2\text{SO}_4$ (20–65% satn.), and the pellet was taken up in and dialysed against 60 mM-sodium citrate buffer, pH 5.3, containing 1 mM- Na_2EDTA . Insoluble protein was removed by centrifugation and the supernatant was applied to a 20 cm $\times$ 2.6 cm column of Amberlite CG-50 (BDH Chemicals, Poole, Dorset, U.K.) equilibrated in the same buffer (Etherington, 1976). Washing of this column under isocratic conditions resulted in the elution of cathepsin B, followed by a mixture of cathepsins L and N. Cathepsin S was eluted with 0.15 M-sodium phosphate buffer, pH 6.2, containing 1 mM- Na_2EDTA . The three pools of enzyme activity were concentrated by precipitation with 75%-satd. $(\text{NH}_4)_2\text{SO}_4$. The resulting pellets were each dissolved in a small volume of 50 mM-sodium acetate buffer, pH 4.5, containing 0.1 M-NaCl and 1 mM- Na_2EDTA and chromatographed on a Sephadex G-75 column (4.8 cm $\times$ 80 cm) equilibrated with this buffer. Enzyme activity was determined, and enzyme pools were adjusted to 25%-satd. $(\text{NH}_4)_2\text{SO}_4$ and concentrated by hydrophobic chromatography on columns of phenyl-Sepharose. Elution from these columns was with 50% (v/v) ethylene glycol in 20 mM-sodium phosphate buffer, pH 5.8, containing 1 mM- Na_2EDTA . Samples were desalted on a small Sephadex G-25 column into 20 mM-Bistris buffer, pH 5.7, and fractionated in this buffer by f.p.l.c. in a Mono Q column. Cathepsins B and L were bound to this column and were eluted with 0.5 M-NaCl. Cathepsins N and S were not absorbed at this pH. The four separate enzyme pools obtained at this stage were further purified by using a Mono S column equilibrated in 50 mM-sodium acetate buffer, pH 5.0, and a gradient of 0–0.5 M-NaCl. The final purification step was preparative isoelectric focusing in an LKB 110 ml column: 0.32% (w/v) pH 3.5–10 Ampholine and 0.96% (w/v) pH 4–6 Ampholine were used with a stabilizing gradient of 0–75% (v/v) ethylene glycol. The proteins were focused according to the manufacturer's instructions. The enzymes were desalted in Sephadex G-25 and concentrated as before by hydrophobic chromatography on phenyl-Sepharose. When stored at 4 °C in this buffer they were stable for several months.

The concentration of each cathepsin was determined by active-site titration with Ep-475 (gift from Dr. K. Hanada, Taisho Pharmaceuticals, Saitama, Japan) by using the collagenolytic assay (Barrett & Kirschke, 1981; Maciewicz *et al.*, 1987).

Enzyme assays

Assays (rate and fixed time) for cathepsin activity against Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, Arg-NHMec, Bz-Phe-Val-Arg-NHMec and Bz-Gly-Gly-Arg-NHMec (Bachem, Bubendorf, Switzerland) were as previously described (Mason *et al.*, 1984).

Collagenolytic activity against insoluble bovine tendon type I collagen (Lorne Laboratories, Twyford, Berks., U.K.) was determined from the release of

hydroxyproline-containing peptides at pH 3.4 at 37 °C (Maciewicz *et al.*, 1987).

Gelatinase activity was assayed by a modification of the method of Harris & Krane (1972). Gelatin was prepared from acid-soluble rat tail tendon collagen, labelled with [^3H]acetic anhydride (Amersham International, Amersham, Bucks., U.K.), by heat denaturation at 45 °C for 30 min (Jackson & Cleary, 1967). The gelatin had a specific radioactivity of 300 000 c.p.m./mg. The assay mixture (0.225 ml) contained 0.1 M-sodium acetate buffer, pH 4.5, 1 mM- Na_2EDTA , 10 mM-cysteine and 100 μg of gelatin. The reaction was stopped with 75 μl of 45% (w/v) trichloroacetic acid, and the undegraded gelatin was pelleted in a Microfuge. A 200 μl sample of the supernatant was added to 4.5 ml of BDH Scintran cocktail and its radioactivity counted in a scintillation counter. Rates were determined within the linear range of 1–10% solubilization.

Azocasein-degrading activity was determined with 0.1% (w/v) azocasein solution in 0.1 M-sodium phosphate buffer, pH 6.0, containing 1 mM- Na_2EDTA and 10 mM-cysteine. In assays where 3 M-urea was included, the final pH was 5.0. The reaction was stopped by the addition of trichloroacetic acid to 5% (w/v), the mixture was centrifuged in a Microfuge and the absorbance of the supernatant was determined at 366 nm.

Generally cathepsins B and L were assayed with Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec respectively, cathepsin N was assayed with insoluble collagen and cathepsin S was assayed with azocasein/urea.

Inhibitors of cysteine proteinase activity were tested by using previously described methods (Mason *et al.*, 1984).

Isoelectric focusing and detection of enzyme activity

Analytical isoelectric focusing was performed in the LKB Multiphor electrophoresis unit. The gel contained 1.0% (w/v) agarose-IEF, 0.12% (w/v) sorbitol, 1.33% (w/v) pH 4–6 Ampholine and 1.33% (w/v) pH 6–8 Ampholine, and was cast on to GelBond NF film (FMC Corp., Rockland, ME, U.S.A.). Samples were applied with filter-paper applicators, and electrophoresis was carried out for 2 h at a constant current of 15 mA. Enzyme activity for both fluorogenic and protein substrates was detected by applying an overlay of buffered substrate solution in 1% (w/v) agarose/3% (w/v) poly(ethylene glycol) 6000. Buffered substrate solutions were as described above. These overlaid isoelectric-focusing gels were incubated in a moist atmosphere at 32 °C for several hours, as indicated in the Figure legends.

NHMec-hydrolysing activity was visually detected by using a u.v. lamp. Protein-degrading activity was observed as a clear zone after the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R 250 in 45% (v/v) methanol/5% (v/v) acetic acid. The location of collagenolytic activity was determined by sectioning the gel into narrow transverse segments (5 mm) and assaying these segments directly for activity against insoluble collagen as described above. The pH gradient was determined immediately after focusing from aqueous extracts of narrow strips cut from a gel run in parallel.

Immunological methods

Immunological cross-reactivity of cathepsins N and S against cathepsins B and L was tested by using the following antibodies: goat polyclonal antibody against rabbit cathepsin L (Etherington *et al.*, 1984), mouse monoclonal antibodies, CE2 and AF8, to native rabbit cathepsin B (Wardale *et al.*, 1986), and a rat monoclonal antibody (EG9/10) to native rabbit cathepsin L (R. A. Maciewicz, R. J. Wardale & D. J. Etherington, unpublished work). IgG was purified from the goat serum by chromatography on a Mono Q column in 20 mM-triethanolamine buffer, pH 8.0, with a 0–0.5 M-NaCl gradient. The IgG peak was eluted at 0.17–0.23 M-NaCl (Clezzardin *et al.*, 1985). IgG fractions from monoclonal supernatants were initially chromatographed on a Mono S column in 10 mM-sodium citrate buffer, pH 5.5, with a 0–0.5 M-NaCl gradient. The IgG peak, which was eluted at 0.12–0.2 M-NaCl, was then rechromatographed on the Mono Q column.

Enzyme immunoprecipitation was as described by Wardale *et al.* (1986), with the following modifications. The enzyme preparations were labelled in their active centre by using iodo[2-¹⁴C]acetic acid (Amersham International), then isoelectrofocussed in an agarose gel as described above. Iodo[2-¹⁴C]acetate-labelled and unlabelled cathepsins migrated to the same isoelectric point. The isoelectric-focusing gel pieces containing labelled cathepsins were then used in the immunoprecipitation experiment.

Cystatin sandwich e.l.i.s.a. was carried out with poly(vinyl chloride) micro-titre plates (high-activated, Titertek; Flow Laboratories, Irvine, Ayrshire, U.K.) by the method of Voller & Bidwell (1975), as modified below. The plates were coated with 100 μ l (1 μ g) of chicken egg-white cystatin per well (Turk *et al.*, 1983) in 10 mM-sodium phosphate buffer, pH 6.0, containing 0.09 M-NaCl. In this and all subsequent binding steps the plate was shaken at setting 4 on a plate shaker (Flow Laboratories) for 1 h at room temperature, followed by three washes with 20 ml of 10 mM-sodium phosphate buffer, pH 6.0, containing 0.5 M-NaCl and 0.5% Tween-20 (PBS-6 medium). Then 100 μ l of doubling dilutions of each cathepsin starting with 0.1 μ M (i.e. 10 pmol) in PBS-6 medium were then added to each well, followed by 100 μ l of the specific anti-cathepsin antibodies described above and then 100 μ l of 1:1000 dilution of the respective peroxidase-conjugated anti-(animal IgG) antibody (Sigma Chemical Co., Poole, Dorset, U.K.). Peroxidase activity was detected by incubation at 20 °C for 5 min with 100 μ l of the following solution: 0.0025% (v/v) H₂O₂ and 3,3',5,5'-tetramethylbenzidine (1 mg dissolved in 100 μ l of dimethyl sulphoxide) diluted in 10 ml of 50 mM-citric acid/125 mM-Na₂HPO₄ buffer, pH 5.0. The colour intensity in each well was then measured at 450 nm with an e.l.i.s.a. plate reader (Titertek; Flow Laboratories).

Determination of M_r by exclusion chromatography

The M_r values of the cathepsins were estimated by using Sephadex G-75 (superfine grade) in a silicone-treated column (1.6 cm $\times$ 75 cm) in 50 mM-sodium acetate buffer, pH 4.5, containing 1 mM-Na₂EDTA and 0.1 M-NaCl and calibrated with proteins of known M_r . Eluted proteins were determined by absorbance at 280 nm, and enzymes were detected with the appropriate substrate.

RESULTS AND DISCUSSION

Purification

The four enzymes, cathepsins B, L, N and S, were isolated from rabbit spleen as summarized in Scheme 1. The purpose of this investigation was to identify all the types of collagen-degrading cathepsins in a single tissue source, with the use of one purification scheme. The spleen was chosen for the separation of these enzymes in this study, as it had been employed previously for the purification of both cathepsin N (Etherington, 1980) and cathepsin S (Locnikar *et al.*, 1981; Turk *et al.*, 1980). No attempt was made to determine the percentage purification of the individual enzymes because of the complexity of the scheme and the time scale involved. The salient features of this scheme are: (1) cathepsins B and S can be separated completely from each other and from cathepsins L and N on Amberlite; (2) cathepsins L and N can be isolated from one another by their differential binding to Mono Q; (3) isoelectric focusing removes any remaining cathepsins B and L from the cathepsin N and S preparations, and, except for cathepsin L, results in single enzyme peaks.

Clearly there are simpler methods for the purification of only cathepsin B or cathepsin L (Mason *et al.*, 1985; Rich *et al.*, 1986), but they are not adequate for the separation of all collagenolytic cathepsins. Indeed, several attempts have been made to demonstrate the existence of other collagenolytic cathepsins (Lynn & Labow, 1984; Kirschke *et al.*, 1986), but they have failed to find as many as the present method. Perhaps the inclusion of an autolysis step may be detrimental to certain cathepsins.

Action of the enzymes on synthetic and protein substrates

The specific-activity values for cathepsins B, L, N and S against a number of peptide substrates are presented in Table 1. There are several notable differences in their specificity. The high values obtained for cathepsin B against Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec and for cathepsin L against Z-Phe-Arg-NHMec and to a lesser extent against Bz-Phe-Val-Arg-NHMec are consistent with those reported in the literature for cathepsins from other species (Barrett & Kirschke, 1981; Kirschke *et al.*, 1984). Rabbit cathepsins S and N, however, had comparatively low activities towards all of the synthetic substrates tested. None of these preparations contained any cathepsin H or trypsin/plasminogen activator/urokinase, since they were inactive against their specific substrates, Arg-NHMec and Bz-Gly-Gly-NHMec respectively. The four cathepsin preparations were focused in agarose isoelectric-focusing gels and their locations were determined by incubation in different peptide substrates. The results are shown in Fig. 1. The fluorescence seen in the cathepsin N lane at the pI of cathepsin L is most probably due to a slight contamination of the cathepsin N preparation with cathepsin L. Unlike bovine cathepsin S (Kirschke *et al.*, 1984), the rabbit enzyme had only a low activity against Bz-Phe-Val-Arg-NHMec.

The activity of the four cathepsins towards a variety of protein substrates and their location on agarose isoelectric-focusing gels by using these substrates are shown in Table 2 and in Figs. 2 and 3 respectively. The four enzymes exhibit different specificities toward these

Scheme 1. Scheme of purification procedures employed for rabbit cathepsins B, L, N and S

Numbers in parentheses in the Mono S step refer to the concentration of NaCl required for elution, and in the isoelectric-focusing step to the pI obtained by focusing in ethylene glycol.

Table 1. Degradation of various synthetic substrates by cathepsins B, L, N and S

Enzymes were tested in the presence of 10 mM-cysteine as described in the Materials and methods section, and the activities are expressed as mol of substrate hydrolysed/min per mol of cathepsin. The concentration of each enzyme was determined by titration with Ep-475 by using the collagen assay. Abbreviation: N.D., none detected.

Synthetic substrate	Cathepsin ...	Activity (mol/min per mol)			
		B	L	N	S
Z-Phe-Arg-NHMec (10 μ M)		460	928	15.3	1.7
Z-Arg-Arg-NHMec (10 μ M)		207	17.8	3.4	N.D.
Bz-Phe-Val-Arg-NHMec (10 μ M)		487	54.4	22.0	1.2
Arg-NHMec (10 μ M)		N.D.	N.D.	N.D.	N.D.
Bz-Gly-Gly-Arg-NHMec (10 μ M)		N.D.	N.D.	N.D.	N.D.

protein substrates. Results for cathepsins B and L are in line with those reported previously (Kirsche *et al.*, 1982; Katunuma & Kominami, 1983), and show that cathepsin L is many-fold more active an enzyme towards protein substrate, mol per mol, than is cathepsin B. Cathepsins N and S could be distinguished mainly by their respective actions against insoluble tendon collagen, where the

former exhibited a much higher specific activity. Like bovine cathepsin N, the rabbit enzyme had a low activity against azocasein/urea and no detectable activity against azocasein in the absence of urea (Etherington, 1980). There were many differences between cathepsins L and N, with cathepsin N having a markedly lower activity towards azocasin and gelatin than towards collagen (but

Fig. 1. Location of cathepsins B, L, N and S on isoelectric-focusing gels with synthetic-substrate overlays in agarose gel

Cathepsins B, L, N, and S (0.36, 0.17, 1.2 and 1.5 pmol respectively) were applied to the isoelectric-focusing gel as indicated. The substrates were 15 μ M-Z-Arg-Arg-NHMec (panel a), 15 μ M-Z-Phe-Arg-NHMec (panel b) or 15 μ M-Bz-Phe-Val-Arg-NHMec (panel c). The photographs were taken after 7 min (panel a), 32 min (panel b) or 21 min (panel c) incubation at room temperature. The final pH of the focused gel is indicated on the left-hand side of panel (a).

Table 2. Degradation of various protein substrates by cathepsins B, L, N and S

Details are similar to those for Table 1, except that the activities are expressed as nmol of substrate equivalents degraded/min per mol of cathepsin.

Protein substrate	Cathepsin ...	Activity (nmol/min per mol)			
		B	L	N	S
Collagen (11.5 μ M- $[\alpha$ -chain] ₃)		37.5	1660	1140	32.9
Azocasein, pH 6.0 (45.4 μ M)		0.060	4.17	N.D.	N.D.
Azocasein/urea, pH 5.0 (45.4 μ M)		0.048	13.0	1.92	0.658
Gelatin (4.2 μ M- α -chain)		0.49	2.22	0.095	0.035

still lower than that of cathepsin L). Cathepsin S was identified from its comparatively high activity against azocasein/urea relative to its collagenolytic activity. Overall, the specific-activity values for this enzyme were much lower than those found for bovine cathepsin S (Kirschke *et al.*, 1984, 1986). One possible explanation is that the very high enzyme concentration in the preparation indicated by active-site titration was, perhaps, not entirely due to this cysteine proteinase. Clearly these four enzymes seem to fall into two groups: those that cleave collagen with a high specific activity (cathepsins L and N) and those that have a low collagenolytic activity (cathepsins B and S).

Effect of inhibitors on collagenolytic activity

The use of several specific inhibitors of cysteine proteinase on the action of cathepsins B, L, N and S, when incubated with native collagen, were tested, and the results are shown in Table 3. It is apparent that all are cysteine proteinases, by their failure to cleave collagen in the absence of cysteine. As shown previously, chicken egg-white cystatin will inhibit all the enzymes, but a larger amount is necessary for complete inhibition of

cathepsin B (Maciewicz *et al.*, 1987). Peptidyl-diazomethane inhibitors are known to exhibit a high degree of specificity for individual cysteine proteinases (Green & Shaw, 1981). The inhibitory effect of 0.5 μ M-Z-Phe-Phe-CHN₂ was found to be a discriminating factor in the characterization of the four cathepsins, as cathepsins B and S were only poorly inhibited, compared with the almost complete inhibition of cathepsins L and N. This result is similar to that found for rat cathepsins B and L, and bovine cathepsin S, where 0.1 μ M-Z-Phe-Phe-CHN₂ only inhibited cathepsin L (Kirschke & Shaw, 1981; Kirschke *et al.*, 1984).

Co-operativity in degrading collagen

Previously it was shown that collagenolysis was accelerated by using a combination of bovine cathepsins B and N. This synergistic action, showing an increase in degradation of about 40%, suggested that the two enzymes could be cleaving at different sites in the collagen telopeptides (Etherington, 1976, 1980). When a similar experiment was performed, no deviation from the expected combined collagenolytic amount was seen, except with the combination of cathepsins B and N. Here

Fig. 2. Location of cathepsins B, L, N and S on isoelectric-focusing gels with protein substrate overlays in agarose gel

Panel (a): 0.3, 0.6, 2.4 and 12 pmol of cathepsins B, L, N and S respectively were applied to the isoelectric-focusing gel and then overlaid with 0.2% (w/v) gelatin. Panels (b) and (c): 108.8, 2.6, 7.2 and 9.0 pmol of cathepsins B, L, N and S respectively were applied to the isoelectric-focusing gel and overlaid with 0.2% (w/v) azocasein (panel b) or with 0.2% (w/v) azocasein in the presence of 3.5 M-urea (panel c). The gels were incubated at 32.5 °C for 17 h and the enzymes were located as described in the Materials and methods section. The final pH of the focused gel is indicated on the left-hand side of panel (a).

Fig. 3. Location of insoluble-collagen-degrading activity of samples of cathepsins B, L, N and S after isoelectric focusing

Cathepsins B, L, N and S (72.5, 3.4, 4.8 and 6.0 pmol respectively) were applied to the isoelectric-focusing gel. After focusing, the gel was cut into 0.5 cm transverse strips and then incubated in the presence of insoluble collagen for 16 h at 37 °C.

the activity was enhanced when the enzymes were mixed, and the activity obtained was 30% greater than the sum of the individual contributions. This is not as much as with the bovine enzymes, but clearly the same type of synergism can occur with these two rabbit cathepsins.

Determination of M_r

M_r values were determined by chromatography on Sephadex G-75. Cathepsin B exhibited an M_r of 25250, whereas the larger subunit of cathepsin L was eluted at M_r 23500, a typical value for these enzymes (Barrett & McDonald, 1980; Mason *et al.*, 1985). Cathepsin N had the largest M_r at 34000, very similar to that (34500)

observed for human cathepsin N (Etherington, 1980). The M_r obtained for rabbit cathepsin S (30000) was much larger than the value (24000) obtained for the bovine enzyme as determined by SDS/polyacrylamide-gel electrophoresis (Turk *et al.*, 1980; Kirschke *et al.*, 1986), although the latter may represent a reduced form of the enzyme.

Cross-reaction of anti-(cathepsin B) and anti-(cathepsin L) antibodies to native cathepsins B, L, N and S

To test for immunological cross-reactivity we developed a specific sandwich e.l.i.s.a. method based on the ability of these native cathepsins to bind very tightly

Table 3. Effect of various cysteine proteinase inhibitors on collagenolytic activity

Enzyme inhibitors were tested, at the final concentrations of inhibitors indicated, in the assay with insoluble collagen. The results are expressed as percentage of activity remaining compared with samples assayed in the absence of inhibitor.

Inhibitor	Cathepsin ...	Activity remaining (%)			
		B	L	N	S
None + cysteine (10 mM)		100	100	100	100
None - cysteine		0	0	0	0
Ep-475 (10 μ M) + cysteine		0	0	0	0
Z-Phe-Phe-CHN ₂ (0.5 μ M) + cysteine		72	17	19	61
Cystatin (100 nM) + cysteine		49	0	0	0
Cystatin (1 nM) + cysteine		100	30	33	39

Fig. 4. Cross-reaction of anti-(cathepsin B) and anti-(cathepsin L) antibodies to native cathepsins B, L, N and S by cystatin-antibody sandwich e.l.i.s.a.

Panel (a): detection of cathepsin L (■ and □), with anti-(cathepsin L) rat monoclonal antibody EG9/10 (□) or goat polyclonal antibody (■), and detection of cathepsin B (△), cathepsin N (+) and cathepsin S (x) with anti-(cathepsin L) goat polyclonal antibody. Similarly results were obtained for cathepsins B, N and S by using the anti-(cathepsin L) rat monoclonal antibody EG9/10. Panel (b): detection of cathepsin B (▲ and △) with anti-(cathepsin B) mouse monoclonal antibody CE2, (△) or AF8 (▲) and detection of cathepsin L (□), cathepsin N (+) and cathepsin S (x) with anti-(cathepsin B) mouse monoclonal antibody CE2. Similar results were obtained for cathepsins L, N and S by using the monoclonal antibody AF8.

to chicken egg-white cystatin (Maciewicz *et al.*, 1987) and then be detected through the binding of our specific monoclonal and polyclonal antibodies (Mason *et al.*, 1984; Wardale *et al.*, 1986). The results in Fig. 4(a) show that with both mono- and poly-clonal antibodies to native cathepsin L there was no binding to cathepsins B, N and S even at concentrations approx. 30 times that at which cathepsin L could be detected. Similarly, with two different monoclonal antibodies to cathepsin B (Fig. 4b) there was no binding to cathepsins L, N and S at concentrations equivalent to 4 times the amount at which cathepsin B could just be detected. Since cathepsins N and S are more similar to cathepsin L than B, we also tested whether anti-(cathepsin L) antibody could detect these cathepsins in an uncomplexed form. Samples of iodo[2-¹⁴C]acetate-labelled cathepsins were mixed with anti-(cathepsin L) IgG-tresyl-Sepharose and the percentage of radioactivity that was bound was found to be 5, 73, 10.7 and 13 respectively for cathepsins B, L, N and S. This again indicated that there was negligible cross-reaction between cathepsin L and the other three

cathepsins. These results confirm our previous observation that only cathepsin L produced any significant precipitation arc with antibody to cathepsin L (Maciewicz & Etherington, 1985).

Our results show that at least four cathepsins with collagenolytic activity can be separated simultaneously from one tissue source. They differ in their physicochemical properties, in their ability to degrade a variety of substrates and in their sensitivity to various inhibitors of cysteine proteinases, and they are immunologically unrelated. Cathepsin B can be distinguished by its ability to degrade Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec, and cathepsin L by its ability to cleave Z-Phe-Arg-NHMec but not substrates lacking a hydrophobic residue in P₂. Cathepsins N and S can be identified by their ability to cleave insoluble collagen and azocasein respectively, but not synthetic substrates.

We thank the Arthritis and Rheumatism Council of Great Britain for their financial support and R. J. Wardale for

help in preparation of the monoclonal antibody to rabbit cathepsin L.

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Received 26 February 1988/19 May 1988; accepted 31 May 1988