Bovine Spleen Cathepsin B1 and Collagenolytic Cathepsin

A COMPARATIVE STUDY OF THE PROPERTIES OF THE TWO ENZYMES IN THE DEGRADATION OF NATIVE COLLAGEN

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1. Bovine spleen cathepsin B1 and collagenolytic cathepsin were separated by chromatography on Amberlite IRC-50 and collagenolytic cathepsin was partially purified by chromatography on DEAE-Sephadex (A-50). 2. Collagenolytic cathepsin degraded insoluble tendon collagen maximally at pH 3.5 and at 28°C; mainly α -chain components were released into solution. At 28°C the telopeptides in soluble skin collagen were also cleaved to yield α -chain components. Collagenolytic cathepsin was thus similar to cathepsin B1 in its action against native collagen, but mixtures of these two enzymes exhibited a synergistic effect. 3. The addition of thiol-blocking compounds produced similar inhibition of collagenolytic cathepsin and cathepsin B1. The enzymes responded similarly to all other compounds tested except to 6-aminohexanoic acid, when collagenolytic cathepsin was slightly activated and cathepsin B1 was almost unaffected. 4. Leupeptin, which is a structural analogue of arginine-containing synthetic substrates, inhibited collagenolytic cathepsin as effectively as cathepsin B1. Collagenolytic cathepsin was shown to retain a low residual activity against α -N-benzoyl-DL-arginine *p*-nitroanilide during purification which was equivalent to 0.2% of the activity of cathepsin B1. 5. Cathepsin B1 and collagenolytic cathepsin could not be separated by affinity chromatography on organomercurial-Sephadex 4B. The two enzymes could be resolved on DEAE-Sephadex (A-50) and by isoelectric focusing in an Ampholine pH gradient. The pI of the major cathepsin B1 isoenzyme was 4.9 and the pI of collagenolytic cathepsin was 6.4. 6. From chromatography on Sephadex G-75 (superfine grade) the molecular weights were calculated to be 26000 for cathepsin B1 and 20000 for collagenolytic cathepsin. The difference in molecular weight was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

The thiol-dependent enzyme of tissue lysosomes, cathepsin B1, was first identified by Otto and his co-workers (Otto, 1967; Otto & Schepers, 1967), from the ability of this proteinase to inactivate certain enzymes of the glycolytic pathway. Cathepsin B1 was subsequently found to degrade various protein substrates, but the properties have been characterized principally through its action on synthetic substrates (Otto, 1971). Cathepsin B1 has been purified from bovine (Franklin & Mettrione, 1972; Keilová & Tomášek, 1973; Etherington, 1974) and human (Barrett, 1973; Swanson *et al.*, 1974) tissues. The properties of the human liver enzyme have been studied in great detail (Barrett, 1973).

Cathepsin B1 from both human liver (Burleigh *et al.*, 1974) and bovine spleen (Etherington, 1974) has been found to be especially effective in the dissolution of native insoluble collagen, when the action was directed to cleavage of the telopeptide region of the collagen subunits, which carry the inter-chain cross-links. The collagenolytic activity of the enzyme occurred at a lower pH than for general proteolysis and in a range where the substrate readily swelled.

This effect was believed to be necessary in order to facilitate access by the enzyme (Etherington, 1974; Burleigh *et al.*, 1974).

During the purification of cathepsin B1 from bovine spleens, collagenolytic activity was partially lost to the extent that the purified isoenzymes exhibited only about 5% of the corresponding activity in the original extract. On further examination of the chromatographic systems a second collagen-degrading enzyme was shown to be retained by the Amberlite IRC-50 column used in the initial purification of cathepsin B1. This enzyme was subsequently eluted after the pH of the developing buffer was raised. It was then detected only through its collagenolytic activity (Etherington, 1974). The enzyme has been designated 'collagenolytic cathepsin', the name that was originally used by Bazin & Delaunay (1966) before the identification of cathepsin B1 and the recognition of its specific collagenolytic activity.

The present paper examines the properties of collagenolytic cathepsin from bovine spleen and compares these with those of cathepsin B1 from the

same source. Collagenolytic cathepsin was also substantially purified and the physical properties of the two enzymes were compared.

Experimental

Materials

Bovine spleens were collected immediately after slaughter and stored at -25°C until required.

Calf skin soluble collagen was prepared as described by Jackson & Cleary (1967) and stored frozen as a 0.5% (w/v) solution in 0.01 M-acetic acid. Insoluble tendon collagen was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

CM-cellulose (CM52) was supplied by Reeve Angel Scientific Ltd., London EC4V 6AY, U.K. DEAE-Sephadex (A-50), Sephadex G-75 (superfine grade), Sepharose 4B and Blue Dextran 2000 were purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Amberlite IRC-50 was obtained from BDH Chemicals Ltd., Poole BH12 4NN, Dorset, U.K., who also supplied Bz-Arg-NHC₆H₄NO₂ (α -N-benzoyl-DL-arginine *p*-nitroanilide). Ampholine solutions and prepared thin-layer polyacrylamide-gel plates for electrofocusing were obtained from LKB Instruments Ltd., South Croydon CR2 8YD, U.K. The following were purchased from Sigma (London) Chemical Co., Kingston-on-Thames KT2 7BH, U.K.: Fast Garnet GBC; *p*-chloromercuribenzenesulphonic acid; 2,2'-dithiodipyridine; Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride); cytochrome *c*; whale myoglobin; ovalbumin (grade V). Bovine serum albumin (puriss) and Bz-Arg-NHC₁₀H₇ (α -N-benzoyl-DL-arginine 2-naphthylamide) were purchased from Koch-Light, Colnbrook SL3 0BZ, Bucks., U.K. Chymotrypsinogen A was supplied by Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K. 4-Aminophenylmercuric acetate was purchased from Ralph N. Emanuel Ltd., Wembley HA0 1PY, Middx., U.K., and 2-bromo-2-phenylacetophenone was purchased from Eastman Kodak Ltd., Kirkby, Liverpool L33 7UF, U.K.

Pepstatin and chymostatin were kindly given by Professor H. Umezawa, Institute of Microbial Chemistry, Shinagawa-Ku, Tokyo, Japan. Ac-(Ala)₃-Ala-CH₂Cl (*N*-acetyl-tetra-alanyl chloromethyl ketone) was a generous gift from Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, Ga., U.S.A. Freeze-dried cultures of different *Streptomyces* strains were obtained from the Department of Applied Biology culture collection, University of Bradford, through the generosity of Dr. T. Cross.

Methods

Extraction of cathepsins. The extraction procedure used previously for the purification of cathepsin B1

included an autolytic treatment at 37°C (Etherington, 1974). This step was found to cause the loss of a significant portion of collagenolytic cathepsin, and an alternative method was therefore developed for the extraction of this second enzyme. Spleens were partially thawed, stripped of fascia and major blood vessels and passed once through a meat grinder. About 1 kg of mince was added to 2 vol. of ice-cold 0.1 M-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol and disrupted with a Polytron (Kinematica G.m.b.H., Lucerne, Switzerland) homogenizer. Triton X-100 was then added to the homogenate (0.1%, v/v), which was stirred at 4°C for 16 h.

Debris was removed by centrifuging at 2800g for 45 min in an MSE Mistral 6L centrifuge. The supernatant was then adjusted to pH 3.8 with 2 M-HCl and the suspension stirred for 30 min. The precipitated protein was removed at 15000g for 20 min in an MSE High-Speed 18 centrifuge. Cathepsin B1 and collagenolytic cathepsin were precipitated with (NH₄)₂SO₄ and were collected in the 40–70% saturation fraction as previously described (Etherington, 1974). The crude enzyme mixture was suspended in a small volume of 1 mM-EDTA and 1 mM-2-mercaptoethanol and dialysed against the same solution. Insoluble material was removed by centrifugation. The enzymes were maintained at 4°C throughout the extraction procedure.

Column chromatography. The enzyme sample was run on to a column (5 cm $\times$ 20 cm) of Amberlite IRC-50 equilibrated to pH 5.3 with 0.09 M-sodium citrate buffer, containing 1 mM-EDTA and 0.5 mM-dithiothreitol, and the eluate fractions which contained the unadsorbed cathepsin B1 were pooled and precipitated with (NH₄)₂SO₄ at 90% saturation (Otto, 1967; Etherington, 1974). Collagenolytic cathepsin was then eluted from the column with 0.15 M-sodium phosphate buffer, pH 7.5, containing 1 mM-EDTA and 0.5 mM-dithiothreitol. The active fractions were pooled, dialysed against 1 mM-EDTA containing 1 mM-2-mercaptoethanol and freeze-dried. The crude dried enzyme was stored desiccated at -25°C .

Cathepsin B1 was further purified on a column (2.6 cm $\times$ 20 cm) of CM-cellulose and the predominant isoenzyme, Fraction I (Etherington, 1974), was similarly dialysed and freeze-dried, or stored frozen at -25°C in the presence of 33% (v/v) glycerol. Crude collagenolytic cathepsin was fractionated on a DEAE-Sephadex (A-50) column (2.6 cm $\times$ 20 cm) under the same experimental conditions as used for cathepsin B1 (Etherington, 1974).

A Sephadex G-75 (superfine grade) column (1.6 cm $\times$ 50 cm) equilibrated as described by Distelmaier *et al.* (1972) was calibrated with proteins (3 mg) of known molecular weight in a small volume (0.5 ml) of the same buffer. Blue Dextran 2000 and ³H₂O were used

for the location of the void volume V_0 and the total fluid volume V_t respectively.

The organomercurial-Sepharose 4B adsorbent of Sluyterman & Wijdenes (1970) was prepared as described by Barrett (1973). The affinity column (1.1 cm $\times$ 15 cm) was eluted and regenerated after each run with the solvent systems described by Barrett (1973).

The chromatographic separations were maintained at 4°C and column effluents were monitored as a routine at 280 nm with a Uvicord II Spectrophotometer (LKB). The protein content of the enzyme fractions was also determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The NaCl concentration in the effluent fractions was determined with a chloride titrator CMT10 (Radiometer, Copenhagen, Denmark).

Isoelectric focusing. Analytical isoelectric focusing was performed in polyacrylamide gel with an LKB Multiphor electrophoresis unit. Samples (5–10 μ g) were applied to a half-width gel containing Ampholines from pH 3.5 to 9.5. This was focused and then stained exactly as prescribed by the manufacturers.

Isoelectric focusing was also performed in the 110 ml LKB column stabilized by a glycerol gradient. Sucrose was unsatisfactory for the density gradient, as it charred in the hydrolysis step used in the collagenolytic cathepsin assay. Ampholines of pH range 3–8 were incorporated to a concentration of 1%. The focusing time was 40 h, with a maximal voltage of 800 V for the latter 24 h. The column was maintained at 4°C with water circulated from a cooling unit. Fractions (1 ml) were collected and the pH was determined without delay.

Assay of enzymes. Collagenolytic activity and hydrolysis of Bz-Arg-NHC₆H₄NO₂ by cathepsin B1 were determined by using previously described methods (Etherington, 1974). Collagenolytic cathepsin was also tested for hydrolytic activity against Bz-Arg-NHC₆H₄NO₂ and Bz-Arg-NHC₁₀H₇ (Barrett, 1972). In the assay of collagenolytic activity one unit is defined as the amount of enzyme to release 1 μ mol of hydroxyproline in 1 min at 37°C. For the synthetic substrates one unit of activity is defined as the amount of enzyme to release 1 μ mol of chromogen in 1 min at 37°C. The effect of pH on the collagenolytic activity of the separated enzymes was investigated by the method used originally for crude preparations (Etherington, 1972, 1973).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Enzymic digests of calf skin soluble collagen and insoluble tendon collagen were performed at 28°C and at pH values between 3.0 and 6.0 as previously described (Etherington, 1974). Portions (50–100 μ g of protein in 30 μ l of solution) of digestion products were analysed by sodium dodecyl sulphate/

polyacrylamide-gel electrophoresis by the method of Sykes & Bailey (1971).

Preparation of egg-white papain inhibitor. The crude papain inhibitor was prepared by the method of Fossum & Whitaker (1968) and fractionated on Sephadex G-75 (superfine grade) as described by Keilová & Tomášek (1974). The effluent fractions were assayed for inhibition of cathepsin B1 and collagenolytic cathepsin. For this 0.1 ml of effluent was added to 0.1 ml of the respective enzyme and pre-incubated for 5 min at room temperature (22°C). Residual activity was then determined by the standard methods.

Testing of potential inhibitors. The cathepsins were dissolved initially in 1 mM-EDTA. Traces of mercaptoethanol remained in the freeze-dried enzymes and re-activation was found unnecessary. Samples were preincubated with each compound at pH 6.0 and 37°C for 5 min as described by Barrett (1973). Samples were then removed for the determination of the residual collagenolytic activity of each enzyme at pH 3.5. Residual cathepsin B1 activity at pH 6.5 was also determined with the Bz-Arg-NHC₆H₄NO₂ substrate. Blanks were set up for each compound, and where an organic solvent was required to dissolve the test compound before incorporation into the pre-incubation buffer, then a separate control was prepared from each modified buffer (see Barrett, 1973).

Leupeptin was obtained from a wild strain of *Streptomyces*. Initially 11 different strains were grown at 20°C in 1-litre flasks containing 100 ml of the medium described by Aoyagi *et al.* (1969). The shaken culture broths were sampled at intervals of 24 h and the supernatants tested for inhibition of trypsin activity against Bz-Arg-NHC₆H₄NO₂ (Erlanger *et al.*, 1961). One strain, *S. lavendulae* 106 (isolated from an Australian soil sample), showed particularly strong inhibition at 48 h. For the production of leupeptin from this strain 2.5 litres of broth were held in 10-litre rotating flasks at 20°C for 48 h. Leupeptin in the culture supernatant from two flasks (3300 ml) was purified as described by Umezawa (1972). Leupeptin was finally obtained as an aqueous solution containing 340 mg of inhibitor. An amino acid analysis (Moore & Stein, 1954) with a Jeol (Tokyo, Japan) JLC-6AH analyser gave the composition as Leu (1.0), Ile (0.078), Val (0.088), which was in close agreement with the values quoted by Umezawa (1972). The molarity of this inhibitor solution, which was stored at –25°C, was calculated for the acetyl derivative.

Results

Fractionation of cathepsin B1 and collagenolytic cathepsin

The extraction procedure originally used included an autolysis step, which decreased the bulk of the

non-enzymic protein. The recovery of cathepsin B1 was consistently high after autolysis, but collagenolytic cathepsin was occasionally obtained in very low yield. The yield of enzyme was improved considerably by omitting this autolysis step. Much of the contaminating protein was instead removed by acid precipitation at pH 3.8.

The two enzymes were totally separated on the column of Amberlite IRC-50. Collagenolytic cathepsin was adsorbed to the column, but cathepsin B1 emerged in the breakthrough peak. The major isoenzyme of cathepsin B1, Fraction I, was obtained from this crude enzyme preparation by chromatography on CM-cellulose (Etherington, 1974). The crude collagenolytic cathepsin was eluted from the Amberlite IRC-50 column at pH 7.5. Previously a pH 8.0 buffer had been used (Etherington, 1974), but the enzyme was desorbed just as effectively at this lower pH. The specific activity after this step was about 0.07 unit/mg. Since Otto (1971) has shown that cathepsin B1 is unstable near neutrality, it was important that this second enzyme should not be exposed to a pH that was higher than necessary.

The crude collagenolytic cathepsin and the pure isoenzyme of cathepsin B1 were safely stored as freeze-dried powders at -25°C over silica gel. Freeze-drying caused an initial loss of activity to each enzyme, up to 25% for cathepsin B1, and about 10–15% of the activity of collagenolytic cathepsin was lost.

Crude preparations of cathepsin B1 (1.4 units) and collagenolytic cathepsin (0.4 unit) were each chro-

matographed on DEAE-Sephadex (A-50) (Fig. 1). Cathepsin B1 was separated into the two main isoenzyme forms as found previously. Fraction II was eluted with 0.12–0.15M-NaCl, and Fraction I, the major component, was eluted with 0.15–0.22M-NaCl, in agreement with the earlier data (Etherington, 1974). Collagenolytic cathepsin was just adsorbed under the conditions used and the enzyme was eluted from the column with 0.02–0.07M-NaCl. The recovery of collagenolytic activity from DEAE-Sephadex (A-50) was about 40% and the specific activity was now increased to 1.5 units/mg.

Gel chromatography

Crude collagenolytic cathepsin (0.12 unit) and cathepsin B1, Fraction I (0.065 unit), were chromatographed on Sephadex G-75 (superfine grade) to determine any difference in molecular size (Fig. 2). Inactive material was also separated from each enzyme at this step. Cathepsin B1 was eluted just before the position for chymotrypsinogen A (mol.wt. 21 600). The elution volumes of the other protein markers, cytochrome *c* (mol.wt. 13 400), myoglobin (mol.wt. 17 816), ovalbumin (mol.wt. 43 500) and bovine serum albumin (mol.wt. 65 400), were plotted against log molecular weight to provide the calibration graph (Andrews, 1965). The molecular-weight values for these proteins were taken from Edmundson (1965) and Smith (1970). The molecular weight of

Fig. 1. Chromatography of crude cathepsins on DEAE-Sephadex (A-50)

The column (2.6 cm $\times$ 20 cm) was equilibrated with 10 mM-sodium phosphate buffer, pH 6.5, containing 1 mM-EDTA and 0.5 mM-dithiothreitol. The enzymes were eluted by a linear gradient (300 ml) of 0–0.3 M-NaCl ($\square$) with a flow rate of 0.32 ml/min. (a) $\circ$, Cathepsin B1 (1.4 units); the bars show the volumes in which the fractions I and II were collected. (b) $\triangle$, Collagenolytic cathepsin (0.4 unit); the bar shows the volume in which the fraction was collected. —, % transmittance at 280 nm (protein).

Fig. 2. Determination of molecular weight by exclusion chromatography on Sephadex G-75 (superfine grade)

A column (1.6cm $\times$ 50cm) was equilibrated with 0.15M-sodium acetate, pH4.6, containing 0.1M-NaCl, 1mM-EDTA and 0.5mM-dithiothreitol. Samples (0.5ml) were pumped through the column at a rate of 0.1ml/min. (a) $\circ$, Cathepsin B1 (0.065 unit). The locations of V_0 , V_i and the marker proteins (bovine serum albumin, 1; ovalbumin, 2; chymotrypsinogen A, 3; myoglobin, 4; cytochrome c, 5) are indicated by arrows. (b) Δ , Collagenolytic cathepsin (0.12 unit). —, % transmittance at 280nm (protein).

Fig. 3. Isoelectric focusing in the 110ml column with an Ampholine pH gradient of 3.5-8.0

Proteins were focused at up to 800V over 40h; the column was then emptied at a rate of 1.0ml/min. (a) $\circ$, Cathepsin B1 (0.22 unit); (b) Δ , collagenolytic cathepsin (0.24 unit). —, % transmittance at 280nm (protein); $\square$, pH.

cathepsin B1 was calculated to be 26000, which was in close agreement with the previously reported values of 25000 (Otto, 1971) and 27000 (Franklin & Metri-

one, 1972). Collagenolytic cathepsin was eluted just before the position for myoglobin and the molecular weight was calculated to be about 20000.

Isoelectric point determination

Cathepsin B1 (0.22 unit) and collagenolytic cathepsin (0.24 unit) were focused separately in an Ampholine pH gradient of 3.5–8.0 (Fig. 3). The pH at the cathepsin B1, Fraction I, peak was 4.9, which was similar to the value of 5.0–5.2 found for the main human isoenzyme (Barrett, 1973). The recovery of activity was about 75%. A single peak of activity was found for collagenolytic cathepsin which was located at pH 6.4. The recovery of activity for this enzyme was near 50%. The enzyme was found to be unstable in the Ampholine solution at its isoelectric point and rapidly lost activity on storage.

Gel electrophoresis

Collagenolytic cathepsin was purified by chromatography on DEAE-Sephadex (A-50) and the enzyme analysed by electrophoresis in 7.5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate. Two protein bands were observed which moved ahead of cathepsin B1 (Plate 1). The faster moving component was always evident and was possibly a partially degraded form of the enzyme. Protein standards were separated in the same gel. Cathepsin B1 moved close to chymotrypsinogen A, and the collagenolytic cathepsin bands were located between chymotrypsinogen A and myoglobin, which confirmed the difference in molecular weight determined by exclusion chromatography.

Isoelectric focusing of the two enzymes in polyacrylamide gels revealed the individual protein zones more clearly than the column technique. Freshly prepared cathepsin B1 was located as a sharp band

near the anode. Several very weak minor bands were also found if a freeze-dried preparation was tested. Since cathepsin B1 loses some of its activity during freeze-drying the additional bands probably represent the inactive protein. Partially purified collagenolytic cathepsin was located as a single band on the cathode side of cathepsin B1. A number of additional minor bands were also observed from a concentrated sample.

Studies on the degradation of collagen

A pH–activity curve was constructed for both crude collagenolytic cathepsin and cathepsin B1, Fraction I (Fig. 4). Maximal activity was located at pH 3.5 for both enzymes, exactly as found originally for the crude mixed enzyme preparations (Etherington, 1972, 1973).

Calf skin soluble collagen was digested by crude collagenolytic cathepsin (0.011 unit/ml) for 5 h at 28°C in order to prevent thermal denaturation of the products. The salt-precipitated collagen was then analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 2). All the digests revealed a decrease in the content of β and γ components, and this was most noticeable in the pH range 3.5–5.0. Insoluble collagen was digested also at 28°C and only at pH 3.5. The salt-precipitable material was negligible after 5 h, but at 22 h the

Fig. 4. Effect of pH on collagenolytic activity

See the Experimental section for details. ●, Cathepsin B1; Δ, collagenolytic cathepsin.

Fig. 5. Digestion of insoluble bovine tendon collagen by mixtures of cathepsin B1 and collagenolytic cathepsin

Stock solutions of cathepsin B1 (collagenolytic activity, 5.5 munits/ml) and collagenolytic cathepsin (11 munits/ml) were mixed in various proportions. Δ, Activity of enzyme mixture; ----, sum of the individual contributions.

EXPLANATION OF PLATE I

Separation of cathepsin B1 and collagenolytic cathepsin by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by isoelectric focusing in polyacrylamide gels

(a) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of: (i) cathepsin B1; (ii) collagenolytic cathepsin; (iii) protein standards [(1) bovine serum albumin, (2) ovalbumin, (3) chymotrypsinogen, (4) myoglobin]. O denotes the origin.
 (b) Isoelectric focusing in polyacrylamide gel containing Ampholines of pH 3.5–9.5 of: (i) cathepsin B1; (ii) collagenolytic cathepsin; (iii) collagenolytic cathepsin concentrated approx. tenfold.

EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of collagen chains after digestion by crude collagenolytic cathepsin

(a) Digestion of acid-soluble collagen at 28°C for 5 h and at pH values between 3.0 and 6.0 by collagenolytic cathepsin (0.011 unit/ml). (b) Digestion of insoluble bovine tendon collagen at 28°C and pH 3.5 by collagenolytic cathepsin (0.011 unit/ml) for 5 h and 22 h. Untreated collagen (S) was used as the standard. O denotes the origins. The positions of the collagen chains (α , β , γ) are indicated.

Fig. 6. Chromatography on organomercurial-Sephadex 4B

The column (1.1 cm $\times$ 15 cm) was washed free of unabsorbed protein with 50 mM-sodium acetate buffer, pH 5.5, containing 0.2 M-NaCl and 1 mM-EDTA, at a flow rate of 0.32 ml/min; 10 mM-cysteine in the buffer was applied to the column as indicated by the arrow. (a) $\circ$, Cathepsin B1 (0.07 unit); (b) $\triangle$, collagenolytic cathepsin (0.03 unit). —, % transmittance at 280 nm (protein).

enzyme-solubilized collagen was readily recovered and shown to consist predominantly of α chains.

Cathepsin B1 and collagenolytic cathepsin were mixed in various proportions and then tested for their combined collagenolytic activity. Activity was enhanced when the enzymes were mixed (Fig. 5) and maximal activity was 40% greater than the sum of the individual contributions. This clearly indicated a synergism in the action of these two enzymes.

Chromatography on organomercurial-Sephadex-4B

Thiol proteinases are retained on a column of organomercurial-Sephadex-4B through the formation of a reversible mercaptide linkage, and can be displaced by the addition of cysteine. Human cathepsin B1 has been separated from non-thiol proteinases, such as cathepsin D, on this support and then recovered in almost 100% yield (Barrett, 1973). The affinity of collagenolytic cathepsin for this support was similarly tested. Crude enzyme (0.03 unit) obtained after chromatography on Amberlite IRC-50 was applied to the column and the activity was firmly bound under the starting conditions. Collagenolytic cathepsin was subsequently recovered with no measurable loss of activity when 10 mM-cysteine was passed through the column (Fig. 6). A portion of freeze-dried cathepsin B1 (0.07 unit) purified by chromatography on CM-cellulose was chromatographed under identical conditions. A small amount of inactive protein was unadsorbed and the enzyme was then readily displaced by cysteine.

Assay of collagenolytic cathepsin with the synthetic substrates of cathepsin B1

Collagenolytic cathepsin appeared to exhibit an extremely weak activity against Bz-Arg-NHC₆H₄NO₂ when the Amberlite IRC-50 chromatograms were assayed. Initially this was attributed to traces of coloured material in the effluent fractions, but a careful check revealed a small progressive release of 4-nitroaniline from the cathepsin B1 substrate by crude collagenolytic cathepsin. When collagenolytic cathepsin was purified by DEAE-Sephadex (A-50) this weak hydrolytic activity was still detectable, but it was too low for accurate determination. Barrett (1972) has improved the sensitivity in the assay of cathepsin B1 by using Bz-Arg-NHC₁₀H₇ as substrate and coupling the released 2-naphthylamine to Fast Garnet GBC. In our laboratory 1 unit of cathepsin B1 by the standard assay for the release of 4-nitroaniline is equivalent to 37.4 units by Barrett's (1972) method for the release of 2-naphthylamine. Collagenolytic cathepsin (4.6×10^{-3} hydroxyproline unit/ml) released a measurable amount of 2-naphthylamine after 1 h of incubation, and this activity against Bz-Arg-NHC₁₀H₇ was calculated to be 1.25×10^{-3} unit/ml. This is equivalent to 3.34×10^{-5} 4-nitroaniline unit/ml if the ratio of these two activities is assumed to be the same as for cathepsin B1. Therefore 1 hydroxyproline unit of collagenolytic cathepsin exhibits 7.26×10^{-3} unit of cathepsin B1-like activity. Since for pure cathepsin B1, Fraction I, 1 hydroxyproline unit is equivalent to 3.3 4-nitroaniline units (Etherington, 1974) then collagenolytic

Fig. 7. Chromatography of chicken's-egg-white inhibitor on Sephadex G-75 (superfine grade)

Crude inhibitor (0.5 g) was fractionated on a column (2.2 cm $\times$ 52 cm) with 0.1 M-sodium acetate buffer, pH 5.0, at a flow rate of 0.16 ml/min. —, % transmittance at 280 nm (protein); $\circ$, inhibition of cathepsin B1 against Bz-Arg-NHC₆H₄NO₂ at pH 6.5; $\bullet$, inhibition of cathepsin B1 against insoluble collagen at pH 3.5; Δ , inhibition of collagenolytic cathepsin at pH 3.5.

cathepsin retains a calculated activity against Bz-Arg-NHC₆H₄NO₂ that is about 0.2% as effective as pure cathepsin B1.

Inhibition by chicken's-egg-white protein

The papain inhibitor in chicken's-egg white (Fossum & Whitaker, 1968) has been found to be inhibitory to two other thiol proteinases, cathepsin B1 (Keilová & Tomášek, 1974) and cathepsin C (Keilová & Tomášek, 1975). A chromatogram of the crude inhibitor (0.5 g) on a column of Sephadex G-75 (superfine grade) was tested for inhibition of cathepsin B1 activity against both Bz-Arg-NHC₆H₄NO₂ (0.14 4-nitroaniline unit/ml) and collagen (0.071 hydroxyproline unit/ml). Maximal inhibition was located by both assays at the side of the last major protein peak, as found previously (Keilová & Tomášek, 1974) (Fig. 7). Collagenolytic cathepsin (0.065 unit/ml) was also inhibited by this same protein fraction and the degree of maximal inhibition (66%) agreed well with the inhibition of collagenolysis by cathepsin B1 (62%). Both stock enzyme solutions had been adjusted to be of approximately equal collagenolytic activity.

Effect of potential inhibitors

Stock solutions of cathepsin B1 (0.056 unit/ml) or collagenolytic cathepsin (0.047 unit/ml) were used in all the inhibitor studies. Portions of each enzyme were preincubated with 3 vol. of 0.1 M-sodium phosphate buffer, pH 6.0, containing the appropriate inhibitor. The cathepsin B1 assays were then allowed to

proceed for 2 h, whereas the incubation time for the assay of collagenolytic activity was for 6 or 18 h.

Bovine and human cathepsin B1 have been shown to possess a thiol group that is essential for enzymic activity (Otto, 1971; Barrett, 1973). Inhibitors of thiol enzymes were tested against collagenolytic cathepsin, together with cathepsin B1, which was assayed against both insoluble collagen and Bz-Arg-NHC₆H₄NO₂. The full data are recorded in Table 1 and show that the thiol inhibitors iodoacetamide, 4-hydroxymercuriphenylsulphonic acid and 2,2'-dithiodipyridine inhibit both enzymes, thus establishing the existence of an essential thiol group in collagenolytic cathepsin. 2,2'-Dithiodipyridine was a more effective inhibitor of collagenolysis, which was determined at the lower pH of 3.5. Brocklehurst & Little (1973) have found that 2,2'-dithiodipyridine reacted with papain more effectively under mildly acidic conditions. Preincubation of cathepsin B1 with ZnSO₄ inhibited all activity against Bz-Arg-NHC₆H₄NO₂ at pH 6.5, owing to blocking of the enzyme's thiol group. Collagenolytic activity of both enzymes at pH 3.5 was unimpaired by the addition of Zn²⁺. EDTA was omitted from the buffer for the assay of this ion. Previously Zn²⁺ was found to exhibit no inhibition of collagenolysis with rat liver extracts (Etherington, 1972).

Although Tos-Lys-CH₂Cl and Ac-(Ala)₃-Ala-CH₂Cl are active-site-specific compounds for trypsin (Shaw & Springhorn, 1967) and elastase (Powers & Tuhy, 1973) respectively, they will also alkylate essential thiol groups in enzymes (Whitaker & Perez-Villaseñor, 1968). Both cathepsin B1 and collagenolytic cathepsin were completely inhibited by

Table 1. *Effect of potential inhibitors on bovine cathepsin B1 and collagenolytic cathepsin*

Stock solutions of cathepsin B1 and collagenolytic cathepsin were preincubated at pH 6.0 with the respective compounds and residual activity was determined against Bz-Arg-NHC₆H₄NO₂ at pH 6.5 or collagen at pH 3.5. Full details are given in the Experimental section.

Compound	Final concn. (mM)	Inhibition (%)		
		Cathepsin B1		Collagenolytic cathepsin
		Bz-Arg-NHC ₆ H ₄ NO ₂ pH 6.5	Collagen pH 3.5	Collagen pH 3.5
4-Hydroxymercuribenzenesulphonic acid	2.0	81	84	97
Iodoacetamide	5.0	100	100	100
2,2'-Dipyridyl disulphide	2.0	85	100	100
	0.2	23	69	50
ZnSO ₄ *	1.0	100	0	0
	0.1	97	0	0
CaCl ₂ *	10.0	9	0	0
Tos-Lys-CH ₂ Cl	0.1	96	100	100
Ac-(Ala) ₃ -Ala-CH ₂ Cl	0.1	94	100	100
	0.01	92	100	90
	0.001	52	33	53
Phenylpyruvic acid	10.0	7	45	31
6-Aminohexanoic acid	50.0	9	16	-34
	10.0	5	0	-6
2-Bromo-2-phenylacetophenone	0.2	14	82	73
	0.02	4	17	36
Pepstatin	0.1	9	0	0
Chymostatin	0.1	84	100	100
	0.01	41	68	100
Leupeptin	0.2	100	100	100
	0.02	72	46	82
	0.002	1	19	6

* EDTA omitted from the assay buffer.

each compound, with Ac-(Ala)₃-Ala-CH₂Cl being the more effective at very low concentration.

2-Bromo-2-phenylacetophenone has been shown to inhibit cathepsin B1 in low concentration (Barrett, 1973), but inhibition was demonstrated at the higher enzyme concentration used here only in the assays for collagenolytic activity, when both enzymes responded similarly. The poor solubility of 2-bromo-2-phenylacetophenone precluded any further assessment of this compound.

Phenylpyruvic acid had no action on the hydrolysis of Bz-Arg-NHC₆H₄NO₂ by cathepsin B1, but was found to be slightly inhibitory to collagenolysis by both enzymes, as experienced with unfractionated extracts of rat liver (Etherington, 1972). Previously 6-aminohexanoic acid was also found to enhance activity, but for the separated bovine enzymes only collagenolytic cathepsin was found to be activated and there was no significant effect on cathepsin B1, as found previously by Barrett (1973) for the human enzymes.

Leupeptin is a potent inhibitor of cathepsin B1 (Barrett, 1973) and the leupeptin isolated from a wild strain of *Streptomyces* in this laboratory inhibited

collagenolytic cathepsin as effectively as it blocked the action of cathepsin B1. Chymostatin is a less specific inhibitor (Umezawa, 1972). It gave total inhibition of collagenolysis by both enzymes and was slightly less effective against cathepsin B1 when this was assayed colorimetrically. The fungal inhibitor, pepstatin, which appears to be specific for acid proteinases such as cathepsin D (Barrett & Dingle, 1972), had no action on either cathepsin B1 or collagenolytic cathepsin.

Discussion

The previously reported observation (Etherington, 1974) that extracts of bovine spleen contain two collagen-degrading enzymes has now been examined in greater detail. Cathepsin B1 has been purified to homogeneity in several laboratories and a number of its properties have been fairly well established (Barrett, 1973; Keilová & Tomášek, 1973; Etherington, 1974; Otto & Riesenköning, 1975). The second collagen-degrading enzyme, collagenolytic cathepsin, could be separated from cathepsin B1 by chromatography on DEAE-Sephadex (A-50) or Sephadex G-75

(superfine grade). The best preparations of collagenolytic cathepsin were still contaminated by small amounts of other proteins. By using isoelectric-focusing techniques the pI of collagenolytic cathepsin was found to be 6.4, whereas the major isoenzyme of bovine cathepsin B1 had a pI of 4.9. The pI values of the human cathepsin B1 isoenzymes were all below 5.6 (Barrett, 1973).

Cathepsin B1 was shown previously to degrade collagen by an initial cleavage in the telopeptide region, which thus released the interchain cross-links stabilizing the fibril (Etherington, 1974; Burleigh *et al.*, 1974). Collagenolytic cathepsin was also shown to degrade collagen in a similar manner, and with insoluble collagen as the substrate both enzymes exhibited maximal activity in a sharp peak at pH 3.5, as found originally with crude extracts of rat liver (Etherington, 1972) and involuting rat uterus (Etherington, 1973). Although the two enzymes exhibited very similar activities against collagen, the existence of identical specificities was cast into doubt when it was observed that on mixing the two enzymes the resultant combined activity was greater than the sum of each. This synergism was found to enhance collagenolysis by up to 40% above the individual contributions. However, whether the enzymes cleaved different bonds in the telopeptides or whether the same bonds were cleaved in the α_1 and α_2 chains, but at different rates, is not known at the present time.

Cathepsin B1 can cleave amide, arylamide (Otto, 1971; Barrett, 1972) and ester (Otto, 1971) links with arginine and as a routine was assayed most conveniently by using a synthetic substrate. Collagenolytic cathepsin was different from cathepsin B1 in that it appeared to have negligible activity against Bz-Arg-NHC₆H₄NO₂ (Etherington, 1974). An attempt was therefore made to inhibit cathepsin B1 selectively with leupeptin. This fungal inhibitor blocks those enzymes which possess an amidase or esterase activity against arginine-containing substrates. Trypsin, papain, plasmin, kallikrein and thrombokinase were all effectively inhibited by leupeptin (Umezawa, 1972). This inhibitor was composed of several related compounds, the major component of which had the structure Ac-L-Leu-L-Leu-L-argininal. The L-argininal residue was an essential feature of the inhibitor and this competed with the substrate for the active site of the enzyme (Umezawa, 1972). When leupeptin was tested against collagenolytic cathepsin it was found, somewhat surprisingly, to inhibit this enzyme as effectively as it inhibited cathepsin B1. The finding prompted a closer examination of the action of collagenolytic cathepsin against the arginine-containing synthetic substrates. Collagenolytic cathepsin, after substantial purification on a column of DEAE-Sephadex (A-50), was found to retain a low residual activity

against Bz-Arg-NHC₆H₄NO₂ and Bz-Arg-NHC₁₀H₇, which was calculated to be 0.2% of the activity from an equivalent amount of cathepsin B1.

Collagenolytic cathepsin was established as a thiol proteinase from its response to the various thiol-blocking agents. The inhibitory effects of the other compounds tested were also similar with both enzymes, except for 6-aminohexanoic acid. Cathepsin B1 was unaffected, whereas collagenolytic cathepsin was slightly activated by this compound. Both enzymes were inhibited by the chicken's-egg-white papain inhibitor of Fossum & Whitaker (1968). This protein blocks the action of several thiol proteinases (Keilová & Tomášek, 1974, 1975) and in the present investigation both enzymes were found to be inhibited to an equivalent extent.

Although it was possible to assay cathepsin B1 in the presence of collagenolytic cathepsin by using Bz-Arg-NHC₆H₄NO₂ as substrate, it was not possible to assay collagenolytic cathepsin specifically. The existence of a synergistic effect also precluded the use of a subtractive analysis for this enzyme. Since collagenolytic cathepsin had a molecular weight that was smaller than that of cathepsin B1 by about 7000, the question arose whether collagenolytic cathepsin was a biological artifact caused by autolysis of cathepsin B1 or by the action of another tissue proteinase.

The collagenolytic cathepsin of cirrhotic liver and subcutaneous granuloma originally described by Bazin & Delaunay (1964, 1966) was probably a mixture of the enzyme with cathepsin B1. More recently Bazin & Delaunay (1971) have examined the properties of partially purified collagenolytic cathepsin from the rat subcutaneous granuloma. The enzyme was shown to be a thiol proteinase and these authors claimed that it had no activity against Bz-Arg-NHC₆H₄NO₂. In a parallel study of human placenta extracts (D. J. Etherington, unpublished work) the existence of both collagenolytic cathepsin and cathepsin B1 has been confirmed.

The catabolic removal of collagenous structures is believed to involve an interplay of several different enzyme systems. Histological examination of the uterus at post-partum involution has revealed the presence of macrophages, containing phagolysosomes, laden with collagenous fragments (Parakkal, 1969, 1972; Brandes & Anton, 1969). The fragments appear to arise from the limited action of an extracellular collagenase on the collagen fibril at physiological pH values. From recent studies of tooth eruption and resorption in the dog the presence of a second neutral proteinase has been confirmed, and this is believed to augment the action of the collagenase by disrupting the partially weakened fibril (Woessner & Cahill, 1974). Tissue macrophages engulf these manageable fragments and then digestion is completed intracellularly by the action of the

lysosomal enzymes. Cathepsin B1 and collagenolytic cathepsin degrade the remaining native collagen molecules to polypeptides, which then become susceptible to other proteinases within the lysosome. This two-stage mechanism for collagenolysis has been discussed in detail in several review articles (Woessner, 1973; Harris & Krane, 1974a,b,c).

An alternative mechanism has been proposed by Ten Cate & Syrbu (1974) and Ten Cate & Deporter (1975) from their studies of fibroblasts in the developing periodontal ligament. They have observed individual cells attempting to phagocytose intact collagen by engulfing the ends of the fibrils. When a fibroblast has ingested a large portion of the fibril it is then severed at the level of the cell membrane by an enzyme, presumably collagenase secreted from the rim of the cellular invagination. The membrane fuses and the phagolysosome passes into the interior of the cell, where digestion is completed by cathepsins, with presumably cathepsin B1 and collagenolytic cathepsin providing the initial action.

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