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Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective-tissue components

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A metalloproteinase, 'proteoglycanase', that degrades proteoglycan and insoluble type IV collagen as well as casein was purified to homogeneity from rabbit bone culture medium. The major form of this proteinase had a final specific activity of 2400μg of casein degraded/min per mg of enzyme protein, and M_r 24500 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis or 12500 by gel-filtration chromatography. It was active over the pH range 5.0–9.0 against a number of substrates, and the rates of degradation were almost constant over the whole of this range. The products generated from proteoglycan-aggregate degradation by this enzyme indicated cleavage at multiple chondroitin sulphate-binding sites along the protein core. In a new assay to detect degradation of insoluble type IV collagen, the proteoglycanase generated large fragments, probably by cleavage in the non-helical regions. The enzyme degraded laminin, fibronectin and procollagen, removing the extension peptides of the last-mentioned. It also cleaved the 'weak region' of the type III collagen helix in a manner analogous to trypsin. The synthetic substrate 2,4-dinitrophenyl-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2 was cleaved exclusively at the Gly–Ile bond. The proteoglycanase was inhibited by tissue inhibitors of metalloproteinases from rabbit bone culture medium, human amniotic fluid and bovine nasal-cartilage extracts, forming essentially irreversible inactive complexes. The importance of this tissue-derived enzyme, with such a wide-ranging degradative capacity, in normal and pathological connective-tissue matrix degradation is discussed.

The presence of a group of at least three separable latent metalloproteinases in media from various connective tissues in culture has been described by investigators in our laboratory and others (Sellers *et al.*, 1978; Vaes *et al.*, 1978). The activity against proteoglycan and casein (or azocasein) was more recently shown to be associated with the degradation of the basement-membrane components, type IV collagen, laminin and fibronection (Murphy *et al.*, 1981*b*). In the present paper we describe the purification of the major form of an enzyme from rabbit bone culture medium that degrades all these substrates. An analysis of its action on proteoglycan aggregate and insoluble type IV collagen in comparison with proteinases such as trypsin and elastase was made. It is known that the tissue metalloproteinases are inhibited by a specific tissue-derived inhibitor, 'TIMP' (Sellers *et al.*, 1979; Cawston *et al.*, 1981; Murphy *et al.*, 1981*a*). The nature of the interaction between this inhibitor and the purified proteoglycanase is described.

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Materials and methods

Materials

Most materials were as described previously (Cawston & Tyler, 1979; Murphy *et al.*, 1981*b*). Procion Red was obtained from Amicon, Stonehouse, Glos., U.K. DNP-peptide (2,4-dinitrophenyl-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2) was obtained from the Protein Research Foundation, Osaka, Japan. Insoluble type IV collagen was prepared from bovine anterior lens capsules by the method of Mainardi *et al.* (1980). Procollagens, type I from chick-embryo tendon fibroblasts and from

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human fibroblasts, type II from chick-embryo sternum chondrocytes and type III from human fibroblasts infected with rhabdomyosarcoma virus, were generously given by Dr. D. Prockop and Dr. F. Njieha, Rutgers Medical School, Piscataway, NJ, U.S.A. Type III collagen from human placenta was kindly given by Dr. C. Sear, Searle Research and Development, High Wycombe, Bucks., U.K. Human polymorphonuclear-leucocyte elastase was kindly given by Dr. J. Saklatvala and Dr. A. J. Barrett, Strangeways Laboratory, Cambridge, U.K.

Culture techniques

Rabbit calvariae were cultured and the medium was harvested as described by Sellers *et al.* (1978). Medium containing metalloproteinase activity (collagen-, gelatin- and casein-degrading activities) was pooled, freeze-dried and reconstituted in a small volume (Cawston *et al.*, 1981).

Enzyme assays

Collagen and gelatin degradation were assayed as described by Sellers *et al.* (1978), casein degradation by the method of Cawston *et al.* (1981), and proteoglycan degradation by using proteoglycan monomer entrapped in polyacrylamide beads, as described by Nagase & Woessner (1980). Degradation of insoluble type IV collagen was assayed by using [^{14}C]acetylated insoluble lens-capsule collagen. The collagen was labelled as a colloidal suspension in 25mM-sodium borate buffer, pH9.5, containing 400mM-$CaCl_2$ by the method of Cawston & Barrett (1979), and had a final specific radioactivity of about 3×10^5c.p.m./mg. The ^{14}C-labelled collagen was stored at −20°C as a dry solid and was stable over a period of at least 1 year. A suspension of 1mg/ml in 1mM-acetic acid/10mM-$CaCl_2$ was made before use, and 100μl portions were pipetted from the rapidly stirred suspension into Microfuge tubes. The assay was conducted in a final volume of 300μl of 50mM-Tris/HCl buffer, pH7.5, containing 10mM-$CaCl_2$, at 37°C. Undigested collagen was removed by centrifugation at 10000*g* in a microcentrifuge for 10min, and 200μl portions of the supernatant were taken for measurement of radioactivity. For measurement of total radioactivity 50μg of bacterial collagenase was included in the assay mixture. For comparison of the release of radioactivity with the release of hydroxyproline-containing peptides, the assay was scaled up to include 2mg dry wt. of collagen in a volume of 1ml. The hydroxyproline and radioactivity contents of the supernatant and the pellet (after papain digestion; Murphy *et al.*, 1982) were then determined. Assuming a hydroxyproline content of 12% for type IV collagen (Dixit, 1978), the dry weight of the preparation was equivalent to the content of collagen by weight. Conditions for the study of the degradation of solubilized basement-membrane components and procollagens were as described previously (Murphy *et al.*, 1981*b*), or are given in the Results section. Degradation of the synthetic heptapeptide DNP-peptide was assayed by the method of Woessner (1979).

Enzyme purification

The concentrated culture medium (16 litres, concentrated 100-fold) was chromatographed on Ultrogel AcA-44 (Sellers *et al.*, 1978; Murphy *et al.*, 1981*b*) to partially separate gelatin-, proteoglycan- and type-I-collagen-degrading metalloproteinase activities. The last two activities did not separate well by this method and were generally pooled for subsequent purification. The pool was concentrated about 10-fold on an Amicon Diaflo apparatus fitted with a PM10 membrane, and the two activities, which were partially latent at this stage, were activated by incubation with 0.7mM-4-aminophenylmercuric acetate at 37°C for 4h. Type-I-collagen- and proteoglycan-degrading activities were then more rigorously separated by the following protocol, modified from the method of Cawston & Tyler (1979). In each case the sample was loaded in the column equilibration buffers described, which contained 0.03% toluene preservative. Column eluates were monitored for degradation of casein, proteoglycan, gelatin, type I collagen and type IV collagen.

(*i*) *DEAE-Sepharose chromatography.* The Ultrogel AcA-44 pool was chromatographed on a column (1.0cm × 10cm) of DEAE-Sepharose, equilibrated with 25mM-sodium cacodylate buffer, pH7.2, containing 5mM-$CaCl_2$ and 0.05% Brij 35. Both the type-I-collagen-degrading and the proteoglycan- and casein-degrading activities did not bind to this matrix, but a significant proportion of the remaining gelatinase activity bound.

(*ii*) *Heparin–Sepharose chromatography.* The pooled specific collagenase and proteoglycanase fractions from DEAE-Sepharose were chromatographed on a column (1.0cm × 7cm) of heparin–Sepharose equilibrated with 25mM-sodium cacodylate buffer, pH6.9, containing 5mM-$CaCl_2$ and 0.05% Brij. The casein-degrading activity passed through this column, but collagenase activity bound and was eluted with 0.5M-NaCl.

(*iii*) *Zinc-chelate–Sepharose chromatography.* The fractions containing proteoglycanase activity were pooled and run on a column (0.5cm × 7cm) of zinc-chelate–Sepharose equilibrated with 25mM-sodium borate buffer, pH8.0, containing 0.15M-NaCl, 5mM-$CaCl_2$ and 0.05% Brij 35. The column was eluted as described by Cawston & Tyler (1979). Proteoglycanase activity did not bind to the matrix, but contaminant proteinase and traces of specific collagenase activity were removed. Fractions con-

taining proteoglycanase activity were concentrated 20-fold on the Amicon Diaflo apparatus fitted with a UM2 membrane. During concentration up to 20% of the enzyme activity passed through the membrane, but this could be largely recovered by re-concentration of the filtrate. No difference in properties between the retained and the filtered enzyme, in terms of charge properties, M_r or enzyme activity, could be detected.

(*iv*) *Procion Red–agarose chromatography.* The concentrated pool was run on a column (0.5 cm × 45 cm) of Procion Red–agarose equilibrated with 25 mM-sodium cacodylate buffer, pH 6.8, containing 5 mM-$CaCl_2$ and 0.05% Brij 35. The elution profile of casein-degrading activity is given in Fig. 1 and is further described in the Results section.

Protein determinations

Pools of material from the enzyme purification were assayed by the fluorescence method of Weigele *et al.* (1972).

Inhibitor studies

Analysis of the effect of chemical inhibitors was made by their inclusion in the various assay systems described. Naturally occurring metalloproteinase inhibitors were purified by the method of Cawston *et al.* (1981) for rabbit bone inhibitor (TIMP), by the method of Murphy *et al.* (1981*a*) for human amniotic-fluid inhibitor and by modification of these methods for inhibitor from bovine nasal-cartilage extract (R. A. D. Bunning, unpublished work). One unit of inhibitor is defined as the amount required to give 50% inhibition of 2 units of collagenase in a collagen-fibril assay (Murphy *et al.*, 1977). Inhibitors were briefly preincubated with proteoglycanase (37°C, 10 min) before assay. For gel-filtration studies ^{125}I-labelled proteoglycanase (5000–10000 c.p.m.; 5–10 units) was preincubated with a slight excess of inhibitor for 30 min at 37°C. No enzyme activity was detectable in these samples. The incubation mixtures were chromatographed on a calibrated Ultrogel AcA-44 column (1.5 cm × 100 cm) in 25 mM-sodium cacodylate buffer, pH 7.2 containing 1 M-NaCl, 5 mM-$CaCl_2$, 0.05% Brij 35 and preservatives. Fractions were assayed for radioactivity as well as proteinase activity.

Iodination of proteins

N-Succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)-propionate was used to label samples of enzymes for the analysis of purity (Bolton & Hunter, 1973). The reagent was dried down under a stream of N_2, taken up in dry propan-2-ol and pipetted (5–10 μCi in 5–10 μl) into tubes containing up to 80 μl of the samples to be labelled in 20 mM-sodium borate buffer, pH 8.5, at 0°C.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

^{125}I-labelled protein samples from the proteoglycanase purification were electrophoresed on 12%-polyacrylamide gels, fixed, stained and radioautographed as described by Cawston *et al.* (1981). Products from the study of the degradation of various substrates were run on gels of various concentrations, as indicated (Murphy *et al.*, 1981*b*).

Studies of proteoglycan-aggregate degradation

Proteoglycan aggregate was prepared from the articular cartilage of 2–3-year-old cattle. Cartilage (about 25 g wet wt.) from the metacarpal–phalangeal joints was sliced finely, washed in 0.15 M-NaCl, and extracted for 48 h in 220 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8 (containing 10 mM-sodium EDTA, 5 mM-benzamidine hydrochloride, 0.1 M-6-aminohexanoic acid and 1 μg of soya-bean trypsin inhibitor/ml). The extract was clarified by centrifugation and dialysed against 10 vol. of 50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors described above. The dialysed extract was adjusted to a density of 1.5 g/ml by addition of solid CsCl, and centrifuged at 34000 rev./min for 40 h at 12°C in the 8 × 34 ml vertical rotor of a Sorvall OTD-65 ultracentrifuge. The contents of the bottom two-fifths of each tube were combined and dialysed exhaustively at 4°C against water, 3 M-NaCl and finally water, for 48 h in each case. The dialysis residue was freeze-dried, and the product (A1 proteoglycan) was stored desiccated at −20°C. For enzyme studies A1 proteoglycan was dissolved by gentle mixing to a final concentration of 5 mg/ml in 0.1 M-Tris/HCl buffer, pH 7.4, containing 10 mM-$CaCl_2$ and 10 mM-$MgCl_2$. The solution was filtered through washed cotton-wool to remove any insoluble material, and hyaluronic acid (6 μl of 2.8 mg/ml) was added to ensure complete aggregate formation. The specific viscosity of the preparation was generally 4.0–4.5, as determined in a Cannon Manning Size 150 viscometer at 37°C. The proteoglycan aggregate was largely link-stabilized in that addition of hyaluronic acid oligosaccharides (average M_r 7500) accounting for 2% of the total uronic acid (Hardingham, 1979) resulted in less than a 15% fall in specific viscosity over 4 h at 37°C. Uronic acid was determined by an automated version (Heinegård, 1973) of the colorimetric method (Bitter & Muir, 1962), with glucuronolactone as standard. Protein was measured by an automated version (Heinegård, 1973) of the colorimetric method (Lowry *et al.*, 1951), with bovine albumin as standard. Galactosamine/glucosamine ratios were determined on samples hydrolysed in 8 M-HCl for 3 h at 95°C (Swann & Balazs, 1966) by using the short column of a Locarte automated amino acid analyser.

Results

Enzyme purification

The 4-aminophenylmercuric acetate-activated proteinase 'proteoglycanase' was purified from rabbit bone culture medium by using the column-chromatographic procedures described in the Materials and methods section. Table 1 summarizes the recovery of enzyme activity against casein, proteoglycan, type IV collagen, gelatin and type I collagen throughout the purification. Relative degradation of the first three substrates remained approximately constant during each column elution. Type-I-collagenase activity was substantially removed by heparin-Sepharose chromatography, and gelatinase activity by Ultrogel AcA-44 chromatography. The final step of the protocol involved concentration of the enzyme eluted from zinc-chelate–Sepharose and chromatography of this concentrated material on a dye–ligand column, Procion Red–agarose. The proteinase was eluted in three peaks from the Procion Red–agarose column (Fig. 1), and these were pooled separately. Most of the activity was slightly retarded on the matrix (pool I), and smaller, variable, amounts were retarded more efficiently (pool II, generally about 10%), or bound (pool III, generally about 2%). The last-mentioned form was eluted by inclusion of 0.5 M-NaCl in the column buffer. Gel-filtration analysis of the three activities showed that both the retarded forms I and II had M_r 12500 and the bound form consisted of a mixture of components of M_r 12500 and 45000. The distribution of activities against casein, proteoglycan, type IV collagen and gelatin were constant over the elution peaks in each case. Furthermore, the degradation products from each

Table 1. *Purification of rabbit bone proteoglycanase*

Concentrated medium from rabbit bone cultures was chromatographed on the various matrices as described in the Methods section. Eluate pools were assayed for degradation of casein, gelatin, proteoglycan, type IV collagen and type I collagen, as well as for protein. The activities against gelatin, proteoglycan and type IV collagen are expressed as a ratio of the activity against casein.

Stage	Casein-degrading activity (total units)	Specific activity (units/mg of protein)	Activity relative to activity with casein substrate: Gelatin substrate	Proteoglycan bead substrate	Type IV collagen substrate	Type I collagenase (total units)
Concentrated culture medium	17170	6.7	3.8	0.015	16	—
Ultrogel AcA-44 eluate	17000	33	6.5	0.014	43	5000
DEAE-Sepharose eluate	18865	181	5.8	0.012	48	6006
Heparin–Sepharose eluate	12848	182	5.7	0.014	48	62
Zinc-chelate–Sepharose eluate	11305	443	4.8	0.016	51	42
Procion Red–agarose I eluate	8204	2370	8.9	0.025	73	None detectable

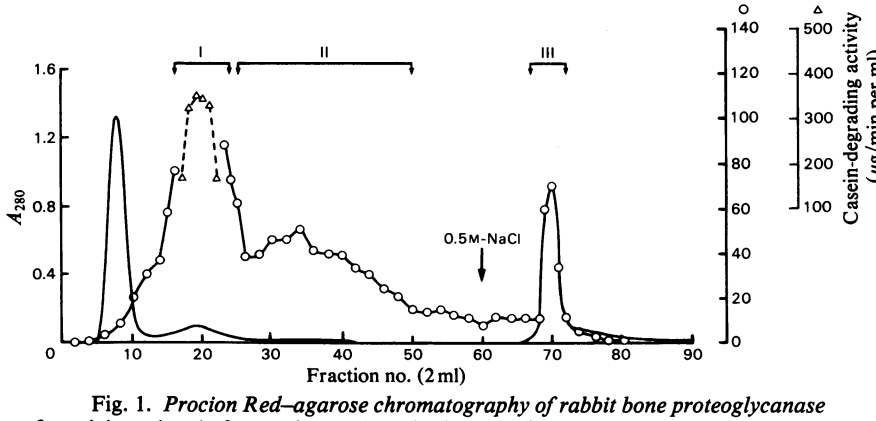

Fig. 1. *Procion Red–agarose chromatography of rabbit bone proteoglycanase*

The peak of activity eluted from zinc-chelate–Sepharose (see the Materials and methods section) was chromatographed on a column of Procion Red–agarose. Three peaks of proteinase activity towards [^{14}C]acetylated casein (O and Δ) were eluted. ——, Protein content (A_{280}).

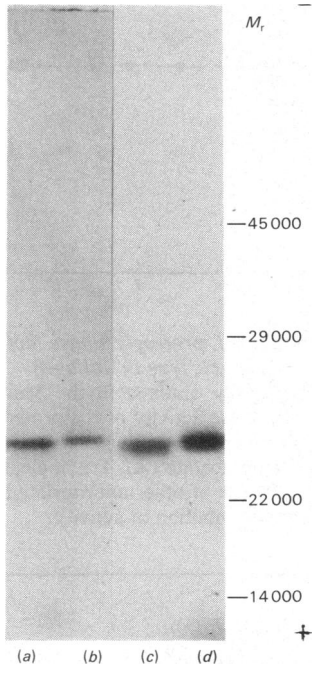

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteoglycanase*

The proteoglycanase eluted from the Procion Red–agarose column was ^{125}I-labelled and subjected to electrophoresis on a sodium dodecyl sulphate/12%-polyacrylamide gel. The gel was stained for protein with Coomassie Brilliant Blue [(*a*) peak II and (*b*) peak I] and was radioautographed [(*c*) peak II and (*d*) peak I].

substrate, as analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, appeared to be identical in each case, with similar pH optima (see below). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the two retarded forms I and II migrated as single bands of M_r 24500 and 24000 respectively (Fig. 2). The migration patterns were not modified by the absence of reducing agents. The bound form (peak III) was not homogeneous, but had a major band of protein at M_r 45000 (results not shown). This form was always less than 10% of the total enzyme activity and was not studied further. Portions of the major (M_r 24500) form of the enzyme were electrophoresed at pH8.6 with 0.05% Brij instead of sodium dodecyl sulphate in the electrophoresis buffers (Murphy *et al.*, 1981*a*), and the lanes were sliced, eluted and assayed for enzyme activity, or stained for protein with Coomassie Blue. No protein could be detected, but a single, somewhat broad, peak of enzyme activity was found about half-way down the gel. The eluates were labelled with ^{125}I, by using the Bolton & Hunter (1973) reagent, and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. Each active fraction from the original gel slices contained a single band of radioactivity with M_r 24500, which coincided with a single band staining with Coomassie Blue. The purified proteinase had a specific activity of 2370μg of casein degraded/min per mg of protein, 11250μg of glycosaminoglycan (about 87mg of proteoglycan) released from proteoglycan beads/18h per mg of protein, 3.9μg of type IV collagen solubilized/min per mg of protein and 32μg of gelatin degraded/min per mg of protein, which represented a 350-fold purification with 48% recovery of activity.

Inhibitors of proteoglycanase

The activity of the purified enzyme against all substrates was inhibited completely by 1,10-phenanthroline (2mM) as well as by EDTA (5mM). It was partially inhibited by thiol compounds, such as cysteine (10mM) and dithiothreitol (1mM), but not by the serine proteinase inhibitors di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride and soya-bean trypsin inhibitor, nor by the aspartate proteinase inhibitor pepstatin. Cysteine proteinase inhibitors, such as 4-aminophenylmercuric acetate or 4-chloromercuribenzoate, which activate the latent form of this enzyme (Sellers *et al.*, 1978), were slightly inhibitory (25% inhibition at 0.7mM). The proteoglycanase is a member of a group of tissue metalloproteinases that are inhibited by naturally occurring metalloproteinase inhibitors, purified from rabbit bone culture medium (TIMP; Sellers *et al.*, 1979; Cawston *et al.*, 1981) human amniotic fluid (Murphy *et al.*, 1981*a*) and bovine nasal-cartilage extracts (Roughley *et al.*, 1978; R. A. D. Bunning & G. Murphy, unpublished work). With casein as the assay substrate, inhibition was linear up to about 75% abolition of enzyme activity and was unaffected by the presence of 4-aminophenylmercuric acetate in the assay mixture (0.7mM). More-detailed studies with the human amniotic-fluid inhibitor showed that the inhibitions of enzyme activity against gelatin and insoluble type IV collagen substrates were also linear up to 75%. Total inhibition was difficult to attain in all cases, a plateau being obtained at about 90% inhibition. The amount of inhibitor required to give 50% inhibition of equivalent amounts of enzyme in each assay were not the same (Table 2). This may be due to a variable effect of substrates on the stability of the enzyme–inhibitor complex. By using ^{125}I-labelled purified enzyme, which retained enzyme activity, it was shown that both enzyme activity and radioactivity were eluted as a single peak on an Ultrogel AcA-44 gel-filtration column (M_r 12500). After incubation with a slight excess of the above inhibitors, the complex was eluted from the column

Table 2. *Inhibition of rabbit bone proteoglycanase by human amniotic-fluid inhibitor of metalloproteinases: effect of different assay substrates*

Various amounts of purified inhibitor (50 units/ml) were complexed with the fully purified enzyme and assayed against the various substrates as described in the Materials and methods section. From a plot of percentage inhibition against inhibitor concentration, the amount required for 50% inhibition was calculated. The results are expressed as a ratio of the volume of inhibitor required to inhibit by 50% 1 unit volume of enzyme.

Substrate	Volume ratio inhibitor/enzyme
Casein	37
Gelatin	1.3
Type IV collagen	1.2

as a major peak of radioactivity of higher M_r 43 500 with TIMP (M_r 34400), 41000 with human amniotic-fluid inhibitor (M_r 29000) and 33000 with bovine nasal-cartilage inhibitor (M_r 22000). A negligible amount of enzymic activity was associated with this peak, and treatment with 4-aminophenylmercuric acetate had no effect on this activity. A small amount of radioactivity, with negligible enzyme activity, was eluted in the original position (M_r 12500) even when a larger excess of inhibitor was used. Formation of the complex was prevented in each case by pretreatment of the proteoglycanase with 2mM-1,10-phenanthroline.

Characterization of proteoglycanase activity

The purified proteoglycanase was found to degrade casein and DNP-peptide over a broad range of pH, with optimal activity around pH 7–9. The enzyme showed unusual characteristics when assayed against proteoglycan encased in polyacrylamide beads, insoluble type IV collagen and gelatin, with almost optimal activity over the pH range 5–9 (e.g. insoluble type IV collagen; Fig. 3). Kinetic analysis of the degradation of insoluble substrates is not straightforward and was not attempted; with all large polypeptide substrates, changes in pH may effect their susceptibility to degradation. Viscometric analysis of proteoglycanase degradation of proteoglycan aggregate indicated that a very rapid initial loss in specific viscosity of the substrate occurred at all pH values. Relatively minor differences in the final specific viscosity attained were due to slight differences in the size of the products (results not shown). Proteoglycanase degradation of soluble type IV collagen was analysed by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis and found to occur equally well at pH 5 and pH 7.5, generating the same products (results not shown).

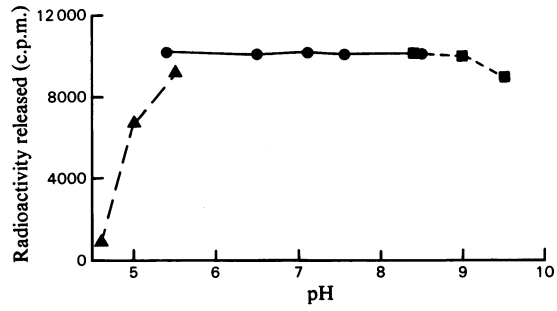

Fig. 3. *pH profile of proteoglycanase activity against insoluble type IV collagen*

By using the assay outlined in the Materials and methods section, the activity of the proteoglycanase against insoluble type IV collagen was assayed. The buffers used were acetate (▲), Tris/maleate (●) and borate (■). The use of other appropriate buffers did not modify the distribution of activity.

Proteoglycan degradation

Although the degradation of proteoglycan by this enzyme was monitored as a routine by the bovine nasal-cartilage proteoglycan polyacrylamide-bead assay, a more detailed study of the action on purified proteoglycan aggregate from bovine articular cartilage was also performed. By viscometric analysis, it was found that a rapid fall in viscosity of the proteoglycan could be observed in the presence of the metalloproteinase. The specific viscosity observed after a 6h incubation was about 25% of the initial value and similar to that obtained after 6h in incubations with 200μg of trypsin. Analysis of the products by chromatography on Sepharose CL-2B showed that as the incubation proceeds hexuronate-positive material is lost from the V_0 peak (proteoglycan aggregate) and appears in the included fractions 24–32 (Fig. 4). This is consistent with the progressive cleavage of chondroitin sulphate-rich peptides from the linear polysaccharide-attachment region of the core protein. The first detectable degradation products are of similar size to those produced by limited digestion of proteoglycan aggregate by trypsin, and are probably composed of peptides each substituted with at least five to ten chondroitin sulphate chains (Heinegård & Hascall, 1974). The shift in profile observed between 60min and 24h is consistent with further cleavage of such peptides. In the limit digestion more than 90% of the hexuronic acid was present as non-aggregating chondroitin sulphate–peptides. Analysis of the protein content of fractions from Sepharose CL-2B chromatography of the 24h-digestion products showed that about 50% of the proteoglycan protein was apparently undegraded and recovered in fractions 13–19 (Fig. 4). The remainder of the protein

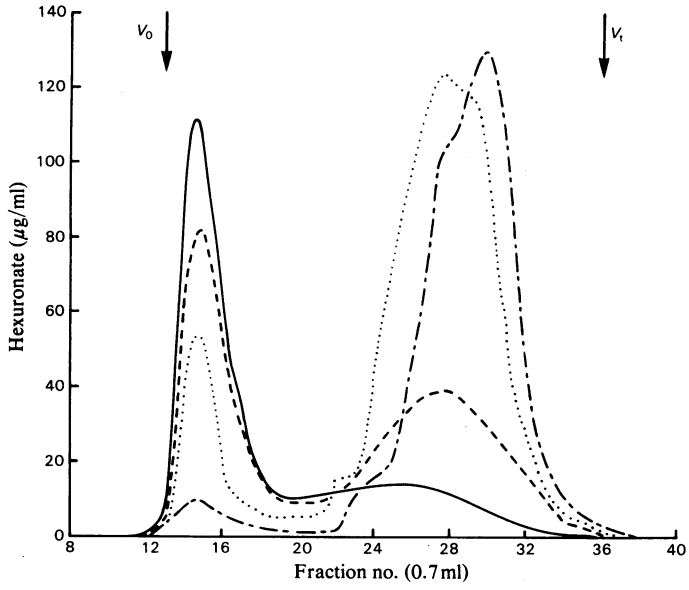

Fig. 4. *Analysis of the degradation products from proteoglycanase action on bovine articular-cartilage proteoglycan aggregate*

Proteoglycanase (9 units) was incubated with proteoglycan in a viscometer, as described in the Materials and methods section. Samples were removed at 0min (——), at 10min (30% decrease in specific viscosity (– – – –), at 60min (60% decrease in specific viscosity (·····) and at 24h (limit digest, 70% decrease in specific viscosity (-·——·-), and analysed on a column of Sepharose CL-2B under associative conditions (as hexuronic acid-containing material eluted).

was associated with the chondroitin sulphate–peptides in fractions 26–32. Fractions 13–19 were combined, dialysed against water and freeze-dried for analysis. The galactosamine/glucosamine molar ratio was determined as 0.54, compared with 2.7 for the substrate, consistent with the removal of more than 80% of the chondroitin sulphate chains during 24h proteolysis. The material in fractions 13–19 was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in comparison with the fractionated products from trypsin limited digestion. In both cases the two forms of link protein were observed (M_r 40000–45000), and also a broad band of diffuse protein (M_r 65000–100000 approx.) probably due to hyaluronic acid-binding region containing variable amounts of keratan sulphate. Column fractions were also assayed for hyaluronate-binding region by radioimmunoassay, which confirmed that all the binding-region protein was recovered in the V_0 peak, in association with hyaluronate. These results show that digestion of link-stabilized proteoglycan aggregate with proteoglycanase under conditions producing 90% removal of chondroitin sulphate–peptides does not result in marked degradation of the hyaluronic acid-binding region or either form of link protein. The results do not exclude the possibility of limited proteolysis of these proteins occurring during the incubation period. The effect of the rabbit bone proteoglycanase on proteoglycan aggregate is therefore similar to the effect of trypsin.

Degradation of other substrates

Analysis of the degradation of the synthetic substrate DNP-peptide showed that the enzyme degraded 0.06μmol/min per mg of protein. By using the method described by Woessner (1979), only glycine and leucine were detectable by amino acid analysis after hydrolysis of the DNP-peptide degradation products of the reaction. It was concluded that the principal cleavage site was the Gly–Ile bond; this was observed at both high and low percentage breakdown of the substrate. Degradation of the ^{14}C-labelled casein substrate was linear up to 30% lysis of the substrate to trichloroacetic acid-soluble fragments (Murphy *et al.*, 1981*a*). The casein used contained both α- and β-chains (Davies & Law, 1977). Analysis of the degradation products of this mixture and of the isolated α- and β-chains by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the β-chain (M_r 29000) was completely degraded at 30% lysis as detected by release of radioactive peptide. The α-chain (M_r 34000) was more slowly broken down, with the

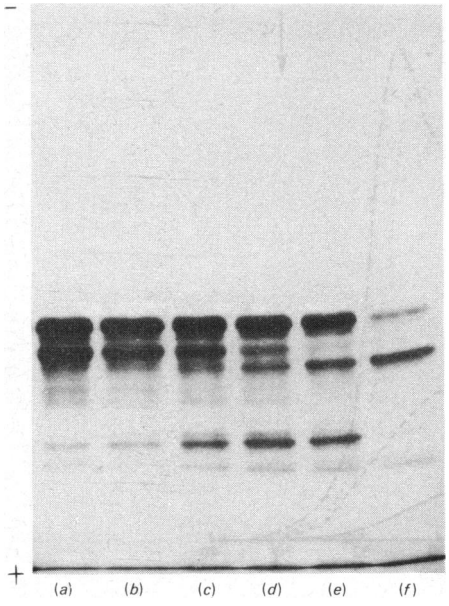

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the products of proteoglycanase digestion of casein with time*

Casein (10 μg) was incubated with proteoglycanase as described in the Materials and methods section for various lengths of time at 37°C: (*a*) 0h; (*b*) 1h; (*c*) 2h; (*d*) 4h; (*e*) 8h; (*f*) 34h. The incubation mixture was subjected to electrophoresis on a sodium dodecyl sulphate/12%-polyacrylamide gel under reducing conditions, and the protein bands were detected by staining with Coomassie Brilliant Blue.

formation of an intermediate, M_r 28000, form (Fig. 5).

Degradation of other connective-tissue macromolecules

The ability of a metalloproteinase activity from rabbit bone culture medium to degrade, not only casein, but also solubilized components of the basement membrane, such as laminin, fibronectin and type IV collagen, was described previously (Murphy *et al.*, 1981*b*). From the analysis of the action of the metalloproteinase purified in the present work against these substrates it is concluded that this was the same enzyme, since the products observed in each case were identical with those obtained with the crude material. The action of the enzyme on pepsin-solubilized type IV collagen at 27°C removes all high-molecular-weight bands, as observed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, producing large amounts of a band of M_r 150000 (Fig. 6). Very limited further breakdown of the collagen occurred. Purified

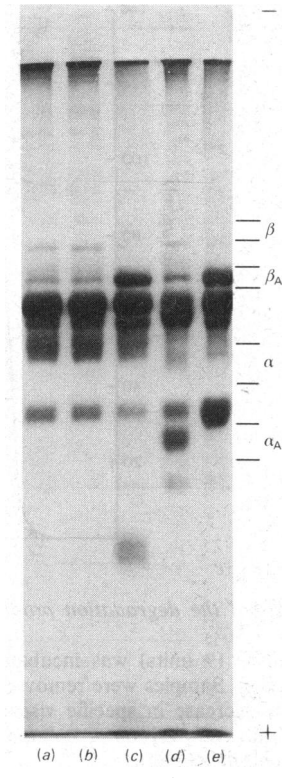

Fig. 6. *Degradation of soluble type IV collagen by proteoglycanase, elastase and trypsin*

Soluble type IV collagen (30 μg) from bovine anterior lens capsule was (*a*) unincubated, (*b*) incubated alone, (*c*) incubated with proteoglycanase (1.2 μg), (*d*) incubated with trypsin (50ng) or (*e*) incubated with elastase (10ng) for 20h at 25°C, and the products were analysed by electrophoresis on a sodium dodecyl sulphate/5.5%-polyacrylamide gel under reducing conditions. The mobilities of type I collagen β- and α-chains (M_r 200000 and 100000) and their specific collagenase cleavage products, β_A and α_A (M_r 150000 and 75000) are indicated as molecular-weight markers.

human polymorphonuclear-leucocyte elastase had a similar effect on the substrate, whereas trypsin had the ability to degrade a limited number of the high-M_r bands, but produced bands of low M_r (75000) as well (Fig. 6). No low-molecular-weight material was detectable with pore-limit gradient gels. Purified bacterial collagenase degraded all the bands completely (results not shown). Viscometric analysis of the action of the proteoglycanase on solubilized type IV collagen showed that 6 units (casein) of enzyme produced a 50% decrease in relative viscosity in 1.5mg of soluble type IV collagen (0.5ml) in 96h at 25°C.

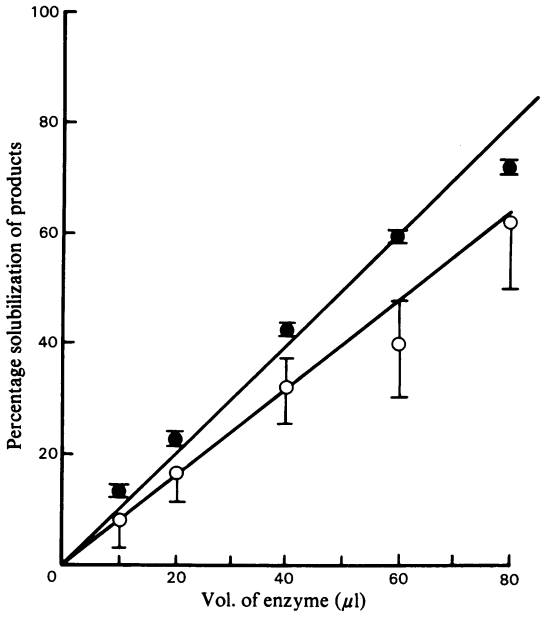

Fig. 7. *Release of hydroxyproline-containing materials and ^{14}C-labelled peptides by proteoglycanase action on [^{14}C]acetylated insoluble type IV collagen*

Insoluble type IV collagen, labelled by [^{14}C]-acetylation as described in the Materials and methods section, was incubated with different volumes of crude proteoglycanase at 37°C for 20h. The undigested collagen pellet and the supernatant after centrifugation were analysed for hydroxyproline (○) and ^{14}C-radioactivity content (●) as described in the text. The data points shown are the average of two estimations.

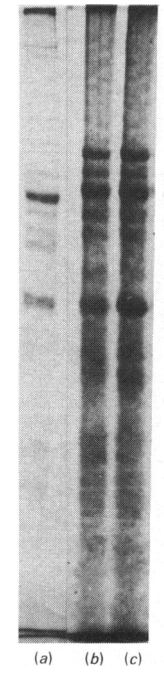

Fig. 8. *Analysis of the products of the degradation of insoluble type IV collagen by proteoglycanase*

Insoluble type IV collagen (^{14}C-labelled; 80μg) was incubated with (*b*) purified proteoglycanase (0.04 unit of type-IV-collagen-degrading activity) and (*c*) crude proteoglycanase from Ultrogel AcA-44 gel filtration of rabbit bone medium (0.06 unit) for 20h at 37°C. The supernatant from each incubation was analysed by electrophoresis on a sodium dodecyl sulphate/6%-polyacrylamide gel under reducing conditions. No detectable material was released from collagen incubated without enzyme. The band pattern of pepsin-solubilized type IV collagen is shown in (*a*). Identical band patterns were obtained if the gels were subsequently radioautographed.

The proteoglycanase was also able to solubilize insoluble type IV collagen at 37°C, as measured by the release of hydroxyproline into the incubation medium. ^{14}C-labelling of the collagen with [^{14}C]-acetic anhydride produced a convenient assay for this activity, since it was shown that release of radioactivity correlated with the release of hydroxyproline (Fig. 7). Release of ^{14}C-labelled products was always greater than release of hydroxyproline, which was probably due to the presence of ^{14}C-labelled collagenous proteins not removed in the preparation of the insoluble type IV collagen. Analyses of the nature of the products of degradation of insoluble type IV collagen by the proteoglycanase by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis on 5% (or 5–12%-gradient) gels were performed at 27°C and 37°C at different total percentage substrate lysis. In each case only small amounts of large-size material (M_r 75000–150000) could be detected both by Coomassie Blue staining and by radioautography, some of which was similar in size to the bands found in pepsin-solubilized type IV collagen from the same source (Fig. 8). Elastase and trypsin also produced small amounts of high-M_r bands in a similar fashion (results not shown). All the bands appeared to be collagenous in nature, since they were degraded by purified bacterial collagenase. The proteoglycanase was also shown to have the ability to act as a procollagen peptidase for procollagens types I, II and III (see, e.g., Fig. 9), removing both the *N*- and the *C*-terminal peptides at 25°C. Furthermore, the enzyme cleaved type III collagen at one locus at 25°C, producing a fragment of about the same size as that produced by trypsin cleavage of this collagen (Fig. 9).

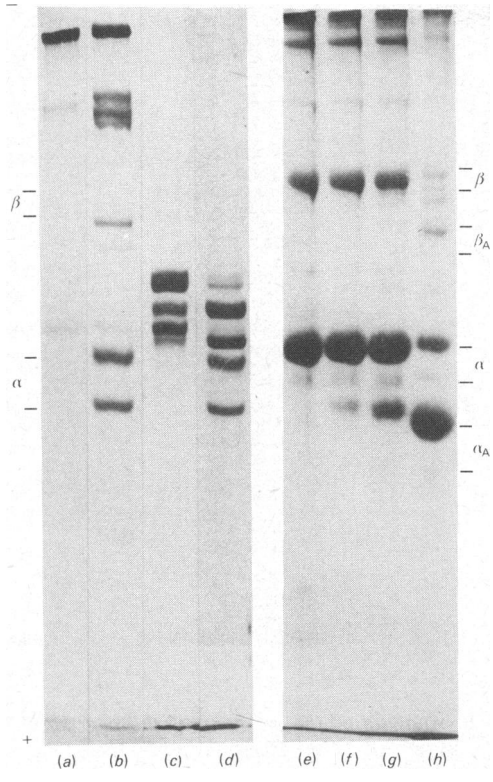

Fig. 9. *Degradation of type I procollagen and type III collagen by proteoglycanase*

Type I procollagen from chick-embryo fibroblasts (5 μg) was incubated (*a* and *c*) alone and (*b* and *d*) with proteoglycanase (10 μg) for 20h at 25°C. The reaction products were analysed by electrophoresis on a sodium dodecyl sulphate/5%-polyacrylamide gel under non-reducing (*a* and *b*) or reducing conditions (*c* and *d*). Similar products were generated at 37°C, but the final collagen chains were generated more quickly. Type III collagen (25 μg) was incubated (*e*) alone, (*f*) with proteoglycanase (120ng), (*g*) with proteoglycanase (1.2 μg) and (*h*) with trypsin (250ng) for 20h at 25°C. The products were analysed by electrophoresis on a sodium dodecyl sulphate/5.5%-polyacrylamide gel under reducing conditions. The mobilities of type I collagen β- and α-chains and their specific collagenase-cleavage products β_A and α_A are given as molecular-weight markers.

Discussion

The metalloproteinase proteoglycanase was purified to homogeneity in an active form from the pooled culture medium of young rabbit skull bones. Like collagenase and gelatinase, two other metalloproteinases produced by connective tissues in culture, the proteoglycanase occurs in a latent form. Because it always undergoes auto-activation on purification, we activated the fractions containing the latent metalloproteinase after the initial Ultrogel AcA-44 gel filtration, thereby obtaining a reproducible purification procedure. By sodium dodecyl sulphate / polyacrylamide - gel electrophoresis the pure proteoglycanase has M_r 24500. By gel-filtration analysis the purified enzyme has M_r 12500 (a small percentage, always less than 10%, has M_r 45000), and we conclude that it must have behaved anomalously on gel matrices. This conclusion is supported by the fact that it is not fully retained by an Amicon membrane with a cut-off of M_r 1000. The high M_r of 50000 for crude latent proteoglycanase by gel-filtration analysis is also puzzling (Sellers *et al.*, 1978), but may be due to an effect of contaminating proteins on its mobility.

The purification to homogeneity of the proteoglycanase has enabled us to analyse its precise ability to degrade individual connective-tissue components and to begin to assess its potential significance in physiological remodelling and pathological processes. The action of the proteoglycanase on purified proteoglycan was found to be similar to that of other proteinases, such as trypsin, elastase and cathepsin G (Roughley, 1977; J. D. Sandy, unpublished work). Reports of metalloproteinases from connective-tissue culture media with similar activities have also been made by Peeters-Joris *et al.* (1981). It appears that the chondroitin sulphate-attachment region of the core protein is susceptible to cleavage by a range of neutral proteinases, whether the proteoglycan is in monomeric or aggregate form. In contrast, the hyaluronic acid-binding region and link proteins are relatively insensitive to proteolysis. This difference has also been observed on treatment of rat chondrosarcoma proteoglycan aggregate with a neutral metalloproteinase isolated from chondrocyte cultures (Morales & Kuettner, 1982). Presumably the hyaluronate-bound proteins are relatively protected from proteolysis as a result of their globular nature (Perkins *et al.*, 1981) and close associations with each other and with hyaluronic acid (Franzén *et al.*, 1981).

The ability of the proteoglycanase to attack globular regions of collagenous molecules is demonstrated by the solubilization of type IV collagen and the removal of both the *N*-terminal and the *C*-terminal peptide extension regions of types I, II and III procollagens. It is not known whether the cleavage of these procollagens produces collagens identical with those produced by the *N*-terminal and *C*-terminal proteinases (Miyahara *et al.*, 1982) *in vivo* or *in vitro*. It is not possible to distinguish between collagen produced by proteoglycanase digestion of procollagen and collagen produced by normal processing (i.e. mature extracted collagen) when compared on sodium dodecyl sulphate/poly-

acrylamide-gel electrophoresis. Processing of procollagen with a mixture of partially purified *N*-terminal and *C*-terminal proteinases yields a band corresponding to disulphide-bonded *C*-terminal propeptide trimer (Miyahara *et al.*, 1982), and we found that a similar product is generated by the action of the proteoglycanase.

In contrast with collagenase, the proteoglycanase degrades a wide variety of substrates, but it seems to have a similar specificity for the amino acid residues at the cleavage site. Collagenase has been shown to cleave Gly–Leu and Gly–Ile bonds in collagens (Gross *et al.*, 1980), and gelatinase from human skin-fibroblast cultures (Seltzer *et al.*, 1981) and a metalloproteinase from extracts of involuting rat uterus (Woessner, 1979) have both been shown to specifically cleave the Gly–Ile bond of the synthetic heptapeptide DNP-peptide. DNP-peptide was cleaved by the proteoglycanase almost exclusively at the Gly–Ile bond, and this specificity probably explains its ability to degrade gelatin. It is not yet known whether the enzyme has the ability to cleave bonds other than Gly–Ile in macromolecular substrates. Sellers *et al.* (1979) have shown that all three metalloproteinases from rabbit bone cultures are inhibited by the tissue inhibitor TIMP, which is synthesized and secreted by connective tissues in culture. By using purified materials we have shown in the present work that the proteoglycanase forms a tight complex with specific metalloproteinase inhibitors purified from a number of sources. The complexes were not dissociable by treatment with either trypsin or organomercurials under the conditions required to activate latent enzyme. Similar observations have been made for collagenase (Cawston *et al.*, 1983). Since the enzyme–inhibitor complex formed is irreversible, it means that significant amounts of inactivated enzyme could be largely undetectable in tissues and culture media, distorting any attempted correlation between enzyme production and resorptive processes. It is known that neither latent metalloproteinases nor chemically inactivated forms (1,10-phenanthroline-inhibited) can bind to the inhibitor (T. E. Cawston & G. Murphy, unpublished work).

Previously the proteoglycanase described in the present paper was referred to as metalloproteinase III (Sellers *et al.*, 1978). We now think that it is more appropriate to call it proteoglycanase, since, by analogy with collagen and collagenase, proteoglycans are the major component of most connective tissues that will be degraded. Presently, however, we do not know if this is the most important role of the enzyme in connective-tissue degradation. Because the proteoglycanase has been shown to degrade a variety of connective-tissue macromolecules, including both soluble and insoluble type IV collagen, it could prove to have an important role in the degradation of basement membranes, and in events such as tumour invasion and angiogenesis. It seems likely that the proteoglycanase will be able to degrade the core protein of heparan sulphate proteoglycan of basement-membrane structures, as well as the collagenous component and laminin. Furthermore, this proteinase has a rather unusual ability to degrade substrates over a very wide range of pH values, and would be able to act not only in the extracellular milieu but also in the pericellular environment, where it has been thought that proteinases with a more acidic pH profile might be more effective (see, e.g., Vaes, 1981).

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