Purification of the neutral proteoglycan-degrading metalloproteinase from human articular cartilage tissue and its identification as stromelysin matrix metalloproteinase-3

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The 'neutral' proteoglycan-degrading metalloproteinase of human articular cartilage was purified 3500-fold by use of an anti-(matrix metalloproteinase-3) immunoglobulin G affinity column. Molecular masses of the latent and multiple active forms and specificity of action on casein, transferrin, gelatin and fibronectin were identical with those of authentic stromelysin (matrix metalloproteinase-3) from cultured human rheumatoid synovial fibroblasts. The optimum pH of this proteinase on proteoglycan monomer was pH 5.5, and on Azocoll, 6.2; digestion of fibronectin and gelatin was more extensive at pH 5.5 than at 7.5.

INTRODUCTION

Matrix metalloproteinases are a group of enzymes secreted by connective-tissue cells into the extracellular matrix in zymogen form. They can be activated by organomercurials and trypsin with a loss of 10–12 kDa in molecular mass. They are zinc enzymes which require Ca^{2+} for full activity. Okada *et al.* (1986) have recommended assigning numbers to members of the group. Thus collagenase would also be known as matrix metalloproteinase-1 (MMP-1), 'gelatinase' as MMP-2 and a proteinase that digests proteoglycan and other matrix components as MMP-3. Recently, Murphy *et al.* (1988) have concluded that MMP-3, stromelysin and collagenase-activating proteinase (Vater *et al.*, 1983) are all the same proteinase.

We reported in 1976 (Sapolsky *et al.*) that human articular-cartilage extracts contain metalloproteinase activities acting on proteoglycan at neutral and acid pH. In further work (Woessner & Selzer, 1984) these activities were resolved; the acid metalloproteinase had a pH optimum of 5.3 in Tris/maleate buffer, and the neutral metalloproteinase had a pH optimum of 7.5 in Tris/HCl buffer. The acid metalloproteinase has been purified to homogeneity and characterized (Azzo & Woessner, 1986).

In the present study we have purified the neutral metalloproteinase to homogeneity and have shown that it is identical with human MMP-3/stromelysin. A surprising finding is that stromelysin has an acid pH optimum with many substrates, a feature overlooked previously owing to use of a chelating buffer. The cartilage form of MMP-3/stromelysin is the first to be purified directly from tissue extracts, proving that it is a normal component of the living tissue, presumably derived from chondrocytes. This enzyme is implicated in the loss of proteoglycan from osteoarthritic cartilage (Martel-Pelletier *et al.*, 1984) and in the activation of latent collagenase (Murphy *et al.*, 1987).

MATERIALS AND METHODS

Materials

Transferrin, casein, 4-aminophenylmercuric acetate (APMA), Brij-35, 1,10-phenanthroline, phenylmethane-sulphonyl fluoride, bovine serum albumin, donkey anti-(sheep immunoglobulin) IgG-linked horseradish peroxidase, fibronectin and types III and IV collagens were purchased from Sigma Chemical Co. Post-mortem human patellas were obtained at autopsy and stored at -20°C . Crude medium containing MMP-3 was prepared from human rheumatoid synovial fibroblasts (Okada *et al.*, 1986); MMP-3 was purified from this medium by the method of Ito & Nagase (1988).

Enzyme assays

Proteoglycan-degrading activity was routinely assayed on [^3H]acetylated proteoglycan monomer from bovine nasal cartilage entrapped in polyacrylamide beads (Nagase & Woessner, 1980). Enzyme was added in a standard buffer (50 mM-Tris buffer, pH 7.5, containing 10 mM- CaCl_2 , 0.02% NaN_3 and 0.03% Brij-35) with or without addition of 1.5 mM-APMA to activate latent enzyme as described by Azzo & Woessner (1986). One unit is defined as the amount of enzyme that releases 1 μg of proteoglycan in 1 min at 37°C . Blanks were obtained by adding 1.5 mM-1,10-phenanthroline. Elastin-degrading activity was assayed in a mixture containing 1 mg of insoluble [^3H]elastin prepared as described by Boucek *et al.* (1979) and 0.1 unit of metalloproteinase in standard buffer, pH 7.5, at 37°C for 18 h. Type I collagen-degrading activity was assayed by the method of Dean & Woessner (1985) with 0.03 unit of proteinase.

Antisera

Monospecific antiserum was prepared by injecting sheep at 2-week intervals (three times) with an emulsion of Freund's complete adjuvant and a solution containing

Abbreviations used: APMA, 4-aminophenylmercuric acetate; MMP-3, matrix metalloproteinase-3 (= stromelysin); PAGE, polyacrylamide-gel electrophoresis.

50 μ g of the 28 kDa form of purified human MMP-3. The IgG fraction of the antiserum was precipitated with 0–35 % -satd. $(\text{NH}_4)_2\text{SO}_4$, followed by DE-52 column chromatography in 0.1 M-Tris/HCl, pH 8.0. Anti-(human MMP-3) IgG was then attached to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions and used for affinity chromatography. Monospecificity of the antiserum was shown by double immunodiffusion against crude medium and by immunoprecipitation of radiolabelled medium using the methods of Vater *et al.* (1981) and by Western blotting (see below). In each case reactivity was limited solely to proenzyme and active enzyme.

Enzyme purification

Step 1. A 20 g portion of cartilage dissected from human patellas was homogenized in 1 M-guanidinium chloride in buffer, chromatographed on DEAE-Sephacel, concentrated on Amicon YM-10 membranes, and chromatographed on Ultrogel AcA 54 as described by Azzo & Woessner (1986).

Step 2. Partially purified enzyme solution from step 1 was passed through a anti-(human MMP-3) immuno-adsorbant column (4 ml) equilibrated with standard buffer containing 150 mM-NaCl at 9 ml/h and 24 °C. The eluate was recycled through the column, which was then washed with 8 ml of buffer. The bound materials were eluted stepwise with 3, 6 and 8 M-urea in buffer at a rate of 18 ml/h. Fractions were dialysed against standard buffer, assayed, and analysed by SDS/PAGE.

Activation of latent enzyme

Enzyme solutions were treated with 1.5 mM-APMA in standard buffer containing 100 mM-NaCl at 37 °C for 15 h. After activation, the enzyme was freed of APMA by passing through spin columns of Sephadex G-10 as described by Okada *et al.* (1986). For the pH-profile studies the columns were equilibrated with buffer-free solution containing 100 mM-NaCl, 10 mM- CaCl_2 , 0.02 % NaN_3 and 0.03 % Brij-35.

Western immunoblots

Samples (unboiled and unreduced) were resolved by SDS/PAGE and transferred on to Zeta probe filters (Bio-Rad) in a Transblot apparatus (Hoeffer) containing Tris/glycine buffer, pH 8.3 (Chin *et al.*, 1985). The filters were then blocked overnight (60 °C) with 10 % bovine serum albumin in Tris-buffered saline and treated with 22 μ g of anti-(human MMP-3) IgG/ml in Tris-buffered saline at 37 °C for 90 min. The filter was washed, treated for 90 min with the second antibody [donkey anti-(sheep immunoglobulin) IgG linked to horseradish peroxidase] (1:250, 37 °C), washed again and developed with 3,3'-diaminobenzidine (0.2 mg/ml in 0.1 M-Tris, pH 7.2, containing 0.042 % H_2O_2).

pH curve measurements

Activated enzyme (100 μ l aliquots) was passed through spin columns as described above and mixed with an equal volume of appropriate buffer (0.2 M) in the pH range 4–9. The final mixture contained 0.1 M-buffer, 10 mM- CaCl_2 , 0.03 % Brij-35, and 0.02 % NaN_3 . Proteoglycan beads were added and the incubation was for 18 h at 37 °C. The initial and final pH of each digestion mixture were determined by use of a microelectrode and averaged. Assays were done in duplicate and a blank of

1.5 mM-1,10-phenanthroline was included at each pH. Azocoll (1 mg in a 1.5 ml final assay volume) was digested using buffer-free enzyme, buffer, blanks and incubation conditions as for the beads. The substrates gelatin and fibronectin were digested at various pH values, and the products were examined by SDS/PAGE.

RESULTS AND DISCUSSION

Purification procedure

The neutral metalloproteinase was purified from human cartilage extracts by combining an earlier method of purification used for the acid metalloproteinase with an antibody affinity-chromatography step. In step 1 the enzyme was purified 20-fold by DEAE-Sephacel and molecular-sieve chromatography. This removed the proteoglycan and collagenase, but not acid metalloproteinase nor a great deal of unwanted protein (Fig. 1a, lane labelled 'ACA'). In step 2, about 80 % of the neutral-proteinase activity was bound to an anti-(MMP-3) affinity column and could be eluted with 3 M-urea. The acid proteinase did not bind to this column. This step increased the specific activity 175-fold. The overall purification was 3500-fold, with a recovery of 15 % of the initial activity. The specific activity of the pure enzyme at pH 7.5 was 438 units/mg of enzyme.

A homogeneous preparation of latent enzyme was obtained (Fig. 1a, lane '0 h'); this latent zymogen appeared as a doublet of 57 and 59 kDa. The zymogen could be activated with 1.5 mM-APMA in a time-dependent fashion. About 9 h were required for full activation at 37 °C. Activation was accompanied by the formation of intermediate species about 48 and 45 kDa (Fig. 1a, '6 h') and resulted in final products of 45 and 28 kDa (Fig. 1a, '16 h'). (These molecular-mass values are based on averages from several gels.) The molecular masses of the latent and active forms correspond closely to those reported for MMP-3 (Okada *et al.*, 1986, 1988; Wilhelm *et al.*, 1987).

Western immunoblotting (Fig. 1b) with anti-(MMP-3) antiserum revealed a single reactive band in crude extracts of cartilage. This corresponded to the latent form of MMP-3 as found in culture medium from human rheumatoid synovial fibroblasts (Fig. 1b, 'MED'). This same band is seen after the Ultrogel AcA 54 step and in the final purified preparation, where it corresponds to the major silver-stained band in Fig. 1(a). Activation with APMA shows the formation of protein bands corresponding to those seen on silver staining (Fig. 1a). Again, the final products have molecular masses of approx. 45 and 28 kDa, corresponding to the two forms of the active enzyme. Blotting of the pure latent and active enzyme with an anti-(human collagenase) antiserum did not reveal any bands, indicating that collagenase had been removed. In addition, no immunoreactive bands were seen at 35 kDa, where active acid metalloproteinase would be expected (Azzo & Woessner, 1986). The reactivity of the various latent and active forms of the enzyme with an antiserum raised against the 28 kDa form of MMP-3 is confirming evidence for the identity of the cartilage and synovial enzymes. Finally, the bands at 45 and 28 kDa were cut from the gel and electroeluted. Triton X-100 was added to precipitate SDS, and the extracts were assayed with proteoglycan beads to establish that the protein bands were accompanied by enzymic

Fig. 1. SDS/PAGE of cartilage metalloproteinase

(a) Silver stain (Wray *et al.*, 1981) of latent and activated proteinase separated on 10% (w/v) acrylamide gel by the method of Laemmli (1970). The lanes are as follows: ACA, cartilage extract carried through step 1 of purification, ending with ultrafiltration on Ultrogel AcA 53; 0 h, purified latent enzyme without APMA activation; 3–16 h, latent enzyme treated with 1.5 mM-APMA for 3–16 h. (b) Western immunoblot, performed as described in the Materials and methods section. Lanes are as follows: CRUDE, cartilage extract; MED, medium from cultured synovial fibroblasts; ACA, cartilage extract through Ultrogel AcA 54 step; MMM, molecular-mass markers (prestained and reduced; masses are shown on right-hand scale); PURE, purified latent form of cartilage enzyme; 3, 6, 9 and 16, period (h) of activation with APMA; 16 CONC, 5-fold concentration of the enzyme in the preceding lane.

activity. Both active forms of enzyme were fully inhibited by 1.5 mM-phenanthroline. The latent and active forms of pure enzyme were stable at 4° and 22 °C for as long as 6 months and 2 weeks respectively.

Degradation of protein substrates

The specificity of action of the purified cartilage proteinase was compared with that of authentic MMP-3 from human rheumatoid synovial cells using various protein substrates. Fig. 2 shows that the digestion patterns of casein, carboxymethyltransferin, gelatin of type I collagen and fibronectin are essentially identical for the two proteinases. The cartilage enzyme also digested type IV collagen (Fig. 2) and had a weak effect on type III collagen (results not shown; about 20%

digestion when 200 µg was incubated with 0.36 unit of enzyme for 16 h at 37 °C). The enzyme had no detectable action on native type I collagen at 30 °C or on insoluble amorphous [³H]elastin at 37 °C. These substrate-specificity patterns again indicate that the cartilage and synovial enzymes are identical. Similar specificities have been reported for stromelysin of rabbit origin (Galloway *et al.*, 1983; Chin *et al.*, 1985).

pH optimum

The cartilage proteinase digested proteoglycan in the bead assay over a broad pH range, with the optimum at pH 5.5 (Fig. 3). MMP-3 gave a similar curve for Azocoll digestion, with the optimum displaced to a pH of approx. 6.2. The digestion of gelatin by the two enzymes was

Fig. 2. Substrate specificity of cartilage metalloproteinase compared with that of MMP-3

Substrates were incubated at 37 °C with purified active metalloproteinase and MMP-3 for various times. The reaction was stopped by adding 20 mM-EDTA, and an amount equivalent to 25 μ l of the original digest was applied to the gel after the mixture was boiled with SDS and with mercaptoethanol (except for fibronectin). Lane 1, substrate incubated without enzyme; lane 2, substrate incubated with cartilage metalloproteinase; lane 3, substrate digested with MMP-3. The substrate (weight/ml, proteinase units/ml, incubation time and % polyacrylamide) were: casein (212 μ g, 0.72 unit, 6 h, 12.5%); CM (reduced and carboxymethylated)-transferrin (240 μ g, 0.36 units, 90 min, 12.5%); gelatin (type I collagen, 240 μ g, 0.36 unit, 3 h, 7.5%); fibronectin (2 mg, 1.75 units, 16 h, 7.5%) and type IV collagen (200 μ g, 0.36 unit, 16 h, 5%).

Fig. 3. pH profile of proteoglycan monomer digestion by purified cartilage metalloproteinase and of Azocoll by MMP-3

The preparation of the curves is described in the Materials and methods section. Duplicate assays were done at each point. Closed symbols indicate proteoglycan digestion. The buffers were: ●, sodium acetate; ■, Mes/HCl; ▲, Tris/HCl; *, Tris/maleate. Open symbols indicate gelatin digestion; the buffers were as above, except for □ (Mops/HCl).

much more extensive at pH 5.5 than at pH 7 or 7.5 (Fig. 4). Similarly, fibronectin was digested by the cartilage enzyme more extensively at pH 5.5 than at pH 7.5 (results not shown).

These results were unexpected for two reasons: Okada *et al.* (1986) had shown a pH optimum of 7.8–8.0 for MMP-3 acting on reduced and carboxymethylated trans-

ferrin, and we had previously shown a pH optimum at 7.5 for proteoglycan digestion by the cartilage neutral proteinase (Azzo & Woessner, 1986). However, the latter result was obtained by use of Tris/maleate buffer in the acid region. This buffer apparently acts as a weak chelator, since replacement with acetate or Mes buffer gives a 6-fold increase in activity at pH 5.5 (Fig. 3). Galloway *et al.* (1983) reported a flat curve between pH 5 and 9 for the digestion of proteoglycan, gelatin and type IV collagen by rabbit proteoglycanase (stromelysin). Since they used Tris/maleate buffer over much of this range, the true pH optima may have been obscured.

A pH profile for proteoglycan digestion almost identical with that of Fig. 3 was reported by Campbell *et al.* (1986) for partially purified proteoglycanase obtained from cultured human cartilage stimulated by mononuclear-cell factor; this suggests that their enzyme is also MMP-3/stromelysin and confirms the chondrocytic origin of the enzyme in cartilage. The acid pH optimum of MMP-3/stromelysin on various substrates of the connective-tissue matrix raises the question of the pH at which it normally functions in the tissue. It is possible that the proteinase may function in the vicinity of the cell surface, where the pH may fall to acid values (Dingle & Knight, 1985).

In conclusion, these results on the purification and properties of the purified neutral metalloproteinase of cartilage show it to be identical with human MMP-3 from synovial fibroblasts (Okada *et al.*, 1986) and similar to rabbit stromelysin (Chin *et al.*, 1985) in the molecular mass of the various latent and active forms and in substrate specificity. Moreover, recent studies by Murphy *et al.* (1987) and Ito & Nagase (1988) demonstrate that MMP-3/stromelysin is essential for the full activation of procollagenase. It is, therefore, likely that the collagenase-activating proteinase isolated from bovine cartilage by Treadwell *et al.* (1986) is also MMP-

Fig. 4. Gelatin digestion at various pH values

Gelatin was incubated with proteinase for 4 h under the conditions given in Fig. 2. Portions were then subjected to SDS/PAGE and the bands were stained with Coomassie Blue. The pH was controlled with acetate (4.5), Mes (5.5) and Tris (7 and 7.5) buffers. Each pair of channels shows cartilage enzyme (CE) left and MMP-3 at equal concentrations. Controls with 1,10-phenanthroline were also run, but are not shown, since digestion was completely blocked.

3/stromelysin. The preparation of neutral proteinase reported by Sapolsky & Howell (1982) would correspond to the 28 kDa form of this enzyme.

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