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Neutral Metallo-Proteinases of Rabbit Bone

SEPARATION IN LATENT FORMS OF DISTINCT ENZYMES THAT WHEN ACTIVATED DEGRADE COLLAGEN, GELATIN AND PROTEOGLYCANS

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Rabbit bones in culture produce specific collagenase and neutral metallo-proteinase activity in latent forms that can be activated by either 4-aminophenylmercuric acetate or trypsin. Latent neutral metallo-proteinase activity was resolved by gel filtration into two enzymes, distinct from collagenase, that degrade gelatin and cartilage proteoglycans.

Collagen is the major structural component of connective tissues, and much research has been directed towards the characterization of enzymes, particularly specific collagenases, involved in its resorption (Harris & Cartwright, 1977). To understand the mechanisms of connective-tissue matrix turnover it is necessary to characterize the enzymes capable of degrading all the macromolecules of the matrix. Werb & Reynolds (1974) showed that neutral metallo-proteinase activity was synthesized and secreted along with collagenase by rabbit synovial cells in culture. This neutral proteinase activity was separated chromatographically from collagenase and it degraded gelatin and cartilage proteoglycans, but not collagen (Werb *et al.*, 1978).

Most connective tissues in culture produce collagenase in a latent form and we presented evidence that latent collagenases are complexes between active enzyme and a tissue inhibitor (Sellers *et al.*, 1977; Murphy *et al.*, 1977; Sellers & Reynolds, 1977; Reynolds *et al.*, 1977). In the present paper we report the production by rabbit bone in culture of latent neutral metallo-proteinase activity, distinct from specific collagenase, and its separation into two enzymes that degrade, when activated, gelatin and cartilage proteoglycans.

Materials and Methods

Materials

Azocoll was from Calbiochem, San Diego, CA 92112, U.S.A. Brij 35 (polyoxyethylene dodecyl ether) was from BDH, Atherstone, Warwick, U.K. Azocasein, prepared by the method of Charney & Tomarelli (1947), was a gift from Dr. A. Barrett (Strangeways Research Laboratory, Cambridge, U.K.). Sources of all other chemicals were described previously (Sellers *et al.*, 1977; Sellers & Reynolds, 1977).

Methods

Culture techniques. Bone explants from the cranial vaults of 1–3-day-old rabbits were cultured as before (Sellers & Reynolds, 1977); each dish had either one bone per 1.5ml of medium containing 5% (v/v) heat-treated rabbit serum (Burroughs Wellcome, Beckenham, Kent, U.K.) or two bones per 5ml of medium without serum.

Enzyme assays. (*a*) With collagen. These were done by measuring the release of ^{14}C-labelled peptides from thermally reconstituted trypsin-resistant fibrils of ^{14}C-labelled rat skin collagen (Sellers & Reynolds, 1977). The [^{14}C]acetylated rat skin collagen used in these experiments was a gift from Dr. T. Cawston (Strangeways Research Laboratory). One unit of collagenase hydrolyses 1μg of reconstituted fibrils/min at 35°C (Burleigh *et al.*, 1977).

(*b*) With gelatin. These were carried out essentially by the method of Harris & Krane (1972). ^{14}C-labelled gelatin was freshly prepared by the thermal denaturation of native ^{14}C-labelled collagen at 60°C for 30min. Incubations were made at 35°C in a final volume of 250μl containing enzyme preparation, Tris/HCl buffer, pH7.4 (15μmol), $CaCl_2$ (3μmol), NaCl (20μmol) and gelatin (50μg; 1400d.p.m.). One unit of gelatinase hydrolyses 1μg of gelatin/min at 35°C.

(*c*) With cartilage proteoglycans. ^{35}S-labelled cartilage proteoglycans were prepared and entrapped in polyacrylamide beads (Dingle *et al.*, 1977). Incubations with enzyme preparations were at 35°C in a final volume of 1ml containing Tris/HCl buffer, pH7.4 (50μmol), $CaCl_2$ (10μmol) and substrate (approx. 12.5μg of cartilage proteoglycan/assay;

9000d.p.m.). The reaction was stopped by adding 250μl of 5% (w/v) sodium dodecyl sulphate in 5% (v/v) HCl, and samples were centrifuged at 1250g to sediment the beads. Supernatant (200μl) was counted (Dingle *et al.*, 1977) to determine the radioactivity solubilized from the beads. One unit of enzyme hydrolyses 1 ng of cartilage proteoglycan/min at 35°C.

(*d*) With other substrates. Assays for neutral proteinase were also made at 35°C with either azocasein (Werb *et al.*, 1974) or Azocoll (Werb & Reynolds, 1974) as substrate. One unit of enzyme hydrolyses 1μg of substrate/min.

Activation of latent enzymes in culture media. Conditions for activation with either 4-aminophenylmercuric acetate or trypsin were as for rabbit bone collagenase (Sellers *et al.*, 1977). Medium incubated with 4-aminophenylmercuric acetate (1mM; 6–8h at 25°C) was cooled to 4°C and dialysed for 24h against two changes of 1 litre of 50mM-Tris/HCl (pH7.4)/1M-NaCl/10mM-$CaCl_2$/0.05% (w/v) Brij 35/1% (w/v) butan-1-ol.

Column chromatography. Culture media (4ml) were chromatographed on a calibrated column (86cm×1.5cm) of Ultrogel AcA 54. Samples were eluted in 50mM-Tris/HCl (pH7.4)/1M-NaCl/10mM-$CaCl_2$ containing either 0.02% (w/v) NaN_3 or 1% (v/v) butan-1-ol as preservative and in the presence or absence of 0.05% Brij 35. Columns were eluted at a flow rate of 13ml/h, and fractions of volume 2.5–3ml were collected. Molecular weights of the enzymes were calculated by using bovine serum albumin (mol.wt. 68000), ovalbumin (44500), carbonic anhydrase (29000), soya-bean trypsin inhibitor (22500) and cytochrome *c* (12500) as standards (Sellers & Reynolds, 1977).

Results

Latent neutral proteinase activity of rabbit bone explants

We showed previously that rabbit bone explants in culture produce latent collagenase after a lag of 1–4 days (Sellers *et al.*, 1977; Sellers & Reynolds, 1977). A similar pattern was also observed when neutral proteinase activity was assayed with gelatin, cartilage proteoglycans, azocasein or Azocoll as substrate. Cultures were maintained for up to 8 days, during which time no active collagenase or active neutral proteinase was detected. Activation of culture media with either 4-aminophenylmercuric acetate or trypsin resulted in degradation of all the substrates. For each culture the onset of the production of neutral proteinase activity coincided with that of collagenase. Explants cultured in the presence of cycloheximide (0.2mM) did not produce neutral proteinase activity.

Effect of inhibitors on rabbit bone neutral proteinase activity

Latent neutral proteinase activity in three independent pools of culture media was activated by incubation with either 4-aminophenylmercuric acetate or trypsin. The activated culture media were preincubated for 1h at 25°C in the appropriate assay buffer with potential inhibitors before the residual neutral proteinase activity was assayed. The metal-chelating agents 1,10-phenanthroline (1mM) and EDTA (10mM) inhibited the degradation of collagen (80–100%), gelatin (60–80%) and cartilage proteoglycans (70–90%) under the assay conditions. Incubation with di-isopropyl phosphorofluoridate (2mM), phenylmethanesulphonyl fluoride (2mM) or soya-bean trypsin inhibitor (0.1mg/ml) was without effect. The degradation of both Azocoll and azocasein was inhibited by the metal-chelating agents and not by di-isopropyl phosphorofluoridate.

Column chromatography of culture media containing latent neutral proteinase activity

Culture media were chromatographed on a calibrated column of Ultrogel AcA 54 (see under 'Methods'). Assays of column fractions either in the presence of 4-aminophenylmercuric acetate or after trypsin treatment showed that the latent neutral proteinase activity was resolved into two components, one that degraded gelatin and one that degraded cartilage proteoglycans. Both these activities were separated from specific collagenase, and a typical chromatogram is shown in Fig. 1.

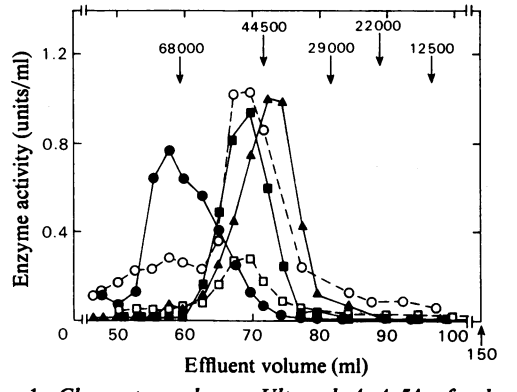

Fig. 1. *Chromatography on Ultrogel AcA 54 of culture medium containing latent collagenase and latent neutral proteinase activity*

Culture medium was chromatographed as described under 'Methods' and samples of fractions were assayed for enzyme activity against various substrates in the presence of 4-aminophenylmercuric acetate (1mM). Substrates degraded: ▲, collagen; ●, gelatin; ■, cartilage proteoglycan; □, azocasein; ○, Azocoll. Values above the arrows are the molecular weights of the standards.

Table 1. *Apparent molecular weights of latent rabbit bone neutral metallo-proteinases*

Samples of three independent pools of culture media containing latent neutral proteinase activity were chromatographed on a calibrated column of Ultrogel AcA 54 (see under 'Methods'). Medium A and Medium B contained 3% and 5% (v/v) heat-treated rabbit serum respectively and were eluted in the absence of non-ionic detergent. Medium C contained no serum and was eluted in buffer containing 0.05% (w/v) Brij 35. Samples of the fractions were assayed for the various enzyme activities in the presence of 4-aminophenylmercuric acetate. For Azocoll, the values in parentheses are apparent molecular weights of the minor Azocoll-degrading activity.

	$10^{-3}\times$ Apparent mol.wt.		
Substrate	Medium A	Medium B	Medium C
Collagen	40.8	44.5	41.8
Gelatin	75.7	73.1	71.4
Cartilage proteoglycans	53.6	55.7	50.0
Azocasein	53.6	55.7	50.0
Azocoll	53.6 (75.7)	55.7 (73.1)	50.0 (71.4)

Azocasein was degraded only by the cartilage proteoglycan-degrading component. Azocoll was degraded by both the neutral proteinase components, although 70% of the total Azocoll degradation was associated with the cartilage proteoglycan-degrading activity.

The apparent molecular weights of the resolved latent neutral proteinase components were calculated (Table 1). There was no significant difference between chromatograms of culture media containing heat-treated rabbit serum eluted in the absence of non-ionic detergent (Medium A and Medium B), and chromatograms of culture media without serum eluted with 0.05% (w/v) Brij 35 in the buffer (Medium C). Estimates of apparent molecular weight (±S.E.M. for seven preparations) were 43700 (±1180) for latent collagenase, and 51070 (±1000) and 70900 (±1290) for latent cartilage proteoglycan-degrading activity and latent gelatin-degrading activity respectively. The differences in apparent molecular weight were highly significant ($P<0.01$).

When culture media were activated with either 4-aminophenylmercuric acetate or trypsin before chromatography, under similar conditions to those above, there was a significant fall in the apparent molecular weights of all the enzyme activities but these activated forms could not be clearly resolved into distinct components.

Discussion

We have shown that rabbit bone explants synthesize and secrete not only a specific collagenase but also neutral proteinase activity capable of degrading gelatin, cartilage proteoglycan, azocasein and Azocoll. Like collagenase, this activity was in a latent form that could be activated with either 4-aminophenylmercuric acetate or trypsin. Degradation of all the substrates was inhibited by metal-chelating agents and was insensitive to inhibitors of serine proteinases, suggesting strongly that the neutral proteinase activity was due to one or more metallo-proteinases. Since the neutral proteinase activity was uncovered by using 4-aminophenylmercuric acetate, the participation of a thiol proteinase was unlikely.

When culture media containing latent collagenase and latent neutral proteinase activity were chromatographed on Ultrogel AcA 54 the neutral proteinase activity was separated into two components, which degraded gelatin and cartilage proteoglycans respectively. It was not possible, however, to resolve these enzyme activities unambiguously if they had been activated before chromatography.

The gelatin-degrading enzyme did not degrade collagen, cartilage proteoglycans or azocasein, but degraded Azocoll to a small extent. Because of its relative specificity we have called this enzyme 'gelatinase'. Its properties suggest that it is similar to the enzyme in human synovium (Harris & Krane, 1972).

The other neutral metallo-proteinase degraded cartilage proteoglycans, azocasein and Azocoll, but neither collagen nor gelatin. In terms of μg of substrate degraded/unit time the order of susceptibility was Azocoll > azocasein > cartilage proteoglycans. We have tentatively called this less-specific enzyme neutral metallo-proteinase III to distinguish it from either collagenase or gelatinase. Neutral metallo-proteinase III activity was responsible for 70% of the Azocoll-degrading activity of the culture media and has properties that suggest it may be similar to the cartilage proteoglycan-degrading activities from rabbit macrophages and synovial cells (Vaes *et al.*, 1977) and from extracts of human cartilage (Sapolsky *et al.*, 1976).

The production of gelatinase and neutral metallo-proteinase III in latent forms suggests that these enzymes may be regulated similarly to collagenase (Sellers *et al.*, 1977; Murphy *et al.*, 1977; Reynolds *et al.*, 1977). This hypothesis is substantiated by the observations that activation of latent gelatinase and latent neutral metallo-proteinase III result in decreases in the apparent molecular weight of the enzymes, as was shown previously for collagenase (Sellers *et al.*, 1977). It seems likely that rabbit bones synthesize and secrete neutral metallo-proteinases, whose activities are under common control, and which together are capable of completely degrading the extracellular matrix. It remains for future work to determine whether neutral metallo-proteinase III can be resolved further, and to ascertain the detailed properties and molecular weights of activated gelatinase and activated neutral metallo-proteinase III.

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