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RABBIT BONE COLLAGENASE INHIBITOR BLOCKS THE ACTIVITY OF OTHER NEUTRAL METALLOPROTEINASES

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SUMMARY

Rabbit bone explants produce a collagenase inhibitor during the first few days in culture and later produce neutral metalloproteinases, one of which is collagenase. We present evidence that the collagenase inhibitor also blocks the activity of the other neutral metalloproteinases (after activation by 4-aminophenylmercuric acetate), indicating that one inhibitor may function as a single regulator for all the metalloproteinases involved in connective tisue catabolism.

INTRODUCTION

Rabbit connective tissues in culture produce a specific collagenase in a latent form which has been shown to be a complex between active enzyme and a tissue inhibitor (1-3). Rabbit bones synthesize two other neutral metalloproteinases, distinct from collagenase, which degrade gelatin and cartilage proteoglycans respectively (4). These two enzymes have been called gelatinase and neutral metalloproteinase III (4), and are also found largely

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in latent forms; they can be activated by the thiol blocking reagent 4-aminophenylmercuric acetate, APMA**(4).

In this communication we report that the inhibitor of rabbit collagenase that is synthesized by bone explants during the first few days in culture also blocks the activity of the two other neutral metalloproteinases.

METHODS AND MATERIALS

Bone cultures. Bone explants from new born rabbits were cultured to produce latent metalloproteinases, and these enzymes were partially purified and activated with APMA as before (4).
Assays. Collagenase was assayed by the release of [^{14}C]-labelled peptides from thermally reconstituted, trypsin resistant fibrils of [^{14}C]-acetylated rat skin collagen (4). Gelatinase was measured as previously described (4), except that 100 µg gelatin was used for each assay. For collagenase and gelatinase one unit of enzyme is defined as 1 µg substrate degraded per minute at 37° (4). The neutral metalloproteinase III (4) was assayed using [^{35}S]-labelled cartilage proteoglycan aggregates (CPG) entrapped in polyacrylamide beads (5). The published method was modified so that the final incubation volume was 250 µl, containing CPG (approx. 30 µg) and enzyme. Degradation of the [^{35}S]-labelled proteoglycan proceeded via the formation of high molecular weight intermediates. Free radioactive sulphate was not released and the apparent molecular weights of the products of enzyme digestion were higher than those of the free chondroitin sulphate chains generated by the action of papain (Sellers, Murphy Meikle and Reynolds, unpublished data). The liberation of radioactivity from the beads following proteoglycan degradation was linear with respect to $\log_{10}$ of the enzyme concentration for a standard time. Inhibitor activity was assayed by adding inhibitor preparations to a known amount of either activated collagenase, or activated gelatinase, or activated neutral metalloproteinase III in the enzyme assay systems outlined above.
Column chromatography. Unconcentrated, pooled inhibitory culture medium was chromatographed on Ultrogel AcA54 (column 86 x 1.5cm) equilibrated with 50 mM-Tris/HCl, pH 7.4, 1 M-NaCl, 10 mM-$CaCl_2$, 0.05% (w/v) Brij-35 and 1%(v/v) butan-1-ol; 2.5 ml fractions were collected. The column was calibrated using bovine serum albumin (68,000), ovalbumin (44,500), carbonic anhydrase (29,000), soya-bean trypsin inhibitor (22,500) and cytochrome c (12,500) as the standards.
Materials. All materials have been fully described (2,4).

RESULTS

There was a lag of two days before enzyme activities could be detected in the culture medium of rabbit bone explants, and

**Abbreviations: 4-aminophenylmercuric acetate, APMA; cartilage proteoglycan aggregates, CPG; soyabean trypsin inhibitor, STI.

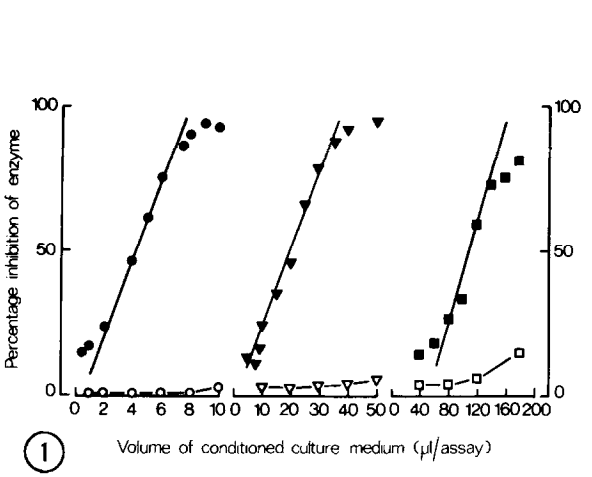
during this period the medium contained an inhibitor of specific collagenase (2). Such medium from the first two days of culture inhibited the activity not only of collagenase on collagen, but also of gelatinase and neutral metalloproteinase III on gelatin and cartilage proteoglycans respectively (Fig.1). There was no inhibition of these enzyme activities if equivalent amounts of culture medium from bone explants maintained in the presence of cycloheximide (0.2 mM) was used in the assays. Inhibition of all the enzyme activities (between 20% and 70%) was linear with respect to increasing volume of inhibitory medium.

When pooled inhibitory culture medium was chromatographed by gel filtration, fractions from a peak of molecular weight approx. 30,000 (Fig.2) inhibited the three enzyme activities. The serum components of the heat inactivated rabbit serum in the medium (5% v/v) produced essentially no inhibition of the enzymes. The calculated recoveries from the column chromatography were 80%, 76% and 95% for activities against collagenase, gelatinase and neutral metalloproteinase III respectively.

We showed previously (1,2) that the action of partially-purified preparations of rabbit bone collagenase inhibitor was destroyed by trypsin and by heat treatment, and antagonised by APMA. Conditions of trypsin pre-treatment and heat sufficient to destroy the inhibitory activity against collagenase also reduced the inhibition of gelatinase and neutral metalloproteinase III (Table 1). Furthermore, the inclusion of APMA (0.5mM) in enzyme assays greatly reduced the inhibition of gelatinase and neutral metalloproteinase III.

DISCUSSION

The present paper provides preliminary data that other rabbit bone neutral metalloproteinases may be regulated by a mechanism



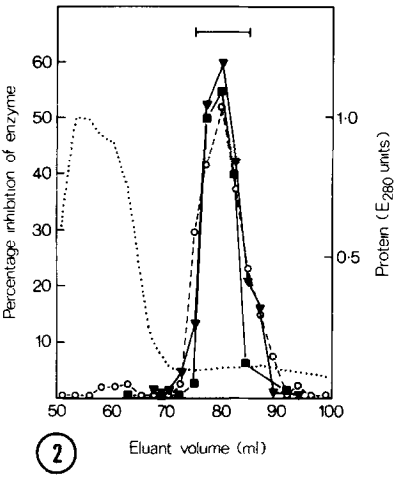


Fig. 1. The inhibition of collagenase, gelatinase and neutral metalloproteinase III by conditioned culture medium. Closed symbols show the percentage inhibition of either APMA-activated collagenase (0.05 unit) or APMA-activated gelatinase (0.047 unit) or APMA-activated metalloproteinase III (10 µg CPG released/18h), by medium from rabbit bone explants. Open symbols show the values obtained from medium of explants cultured with cycloheximide (0.2mM). Medium alone gave similar values. (●,○) Inhibition of of collagenase; (▼,▽) inhibition of gelatinase; (■,□) inhibition of neutral metalloproteinase III.

Fig. 2. Gel chromatography of medium containing the inhibitory activty against metalloproteinases. Pooled unconcentrated culture medium (4 ml), containing no detectable metalloproteinases, was chromatographed on a calibrated column of Ultrogel AcA54 as described in the Methods section. Fractions were assayed for their ability to inhibit APMA-activated collagenase (0.05 units) APMA-activated gelatinase (0.047 units) and APMA-activated neutral metalloproteinase III (10 µg CPG released/18h). The results are expressed as percentage inhibition of the standard amount of enzyme. (○) Inhibition of collagenase; (▼) inhibition of gelatinase; (■) inhibition of neutral metalloproteinase III; (...) protein, E_{280}. The bar indicates the fractions that were pooled to determine inhibitor recoveries and to give the partially-purified inhibitor preparations used in Table 1.

similar to that for collagenase (4). Rabbit bone explants produce an inhibitor which blocks the actions of gelatinase and neutral metalloproteinase III, and has similar characteristics to those reported earlier for the tissue collagenase inhibitor (1,2). It is not possible to state categorically that there is just one species of inhibitor until it has been completely purified. There is the possibility that there could be a family of very closely

Table 1.

EFFECTS OF TRYPSIN, HEAT TREATMENT AND APMA ON THE INHIBITION OF COLLAGENASE, GELATINASE AND NEUTRAL METALLOPROTEINASE III BY PARTIALLY-PURIFIED INHIBITOR PREPARATIONS.

Treatment of Inhibitor	Inhibition of APMA activated Collagenase (.05 units)	Gelatinase (.047units)	Metalloproteinase III (10 µg CPG released/18h)
Buffer + buffer	50	55	50
Trypsin + STI	0	0	0
Buffer + STI	50	45	50
Buffer + heat	10	15	5
APMA solvent	45	50	50
APMA (0.5mM)	8	10	20

* Buffer was 50 mM-Tris/HCl, pH 7.4, 1 M-NaCl, 10 mM-$CaCl_2$, 0.05% (w/v) Brij-35, 1% (v/v) butan-1-ol.

** Addition of APMA (0.5 mM) to activated enzyme alone did not increase further the activity of the enzyme under the conditions of the assays.

Partially-purified inhibitor (AcA54 chromatography) was pre-treated with trypsin (10 µg/ml; 30 min, 37°C) followed by the addition of soyabean trypsin inhibitor, STI (50 µg/ml); controls received STI following pre-incubation with the buffer* (30 min, 37°C). Alternatively, a similar amount of inhibitor was heated to 90°C for 30 min before the enzyme assays. Also assays were conducted with an equal amount of inhibitor and enzyme in either the presence or absence of APMA (0.5mM)**. Results are expressed in terms of the percentage inhibition of the standard amounts of the enzymes.

related inhibitors, each specific for one metalloproteinase, but our initial attempts to purify the inhibitor, using several different chromatographic procedures, have yielded fractions that inhibit all of the enzymes.

Many cells and tissues in culture synthesise neutral metalloproteinases which can degrade gelatin and proteoglycans (4,6-11). These other enzymes, like collagenase, exist largely in latent

forms that can be activated either by limited proteolysis (4,9), or better, by APMA (4). Previously (12) we concluded that latent collagenases are enzyme-inhibitor complexes and showed that many connective tissues synthesize and secrete a potent inhibitor of collagenase during the early days of culture (2,3). We conclude from the present data that other latent metalloproteinases are complexes between enzyme and the "collagenase" inhibitor.

We have evidence that other connective tissues synthesize a similar group of metalloproteinases to those of rabbit bone (4), as well as synthesizing similar inhibitors. An inhibitor that was synthesized locally would provide an efficient regulation of the extra-cellular activities of the group of enzymes that are the most important in matrix catabolism. Together, collagenase and the other metalloproteinases have the ability to degrade all of the organic components of bone matrix. Our evidence so far suggests that the group of metalloproteinases are synthesized and secreted in a coordinate fashion; consequently studies designed to analyze the factors controlling the synthesis of inhibitor by bone and other connective tissues should give new insights into the control of resorption.

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