Purification of rabbit bone inhibitor of collagenase

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1. Rabbit bones in tissue culture synthesize an inhibitor of collagenase during the first 4 days of culture. 2. The inhibitor was purified by a combination of gel filtration, concanavalin A–Sepharose chromatography, ion-exchange chromatography and zinc-chelate affinity chromatography. 3. The purified inhibitor migrated as a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and had a mol.wt. of 28 000. 4. The inhibitor blocked the activity of the metalloproteinases collagenase, gelatinase, neutral proteinase III (proteoglycanase), human leucocyte collagenase and gelatinase, but not thermolysin or bacterial collagenase. The serine proteinases plasmin and trypsin were not inhibited. 5. The inhibitor interacted with purified rabbit bone collagenase with 1:1 stoichiometry. 6. The inhibitory activity was lost after incubation for 1 h at 90°C, after treatment with trypsin (250 µg/ml) at 37°C for 30 min and after reduction and alkylation.

Connective tissues in culture synthesize metalloproteinases capable of digesting collagen and proteoglycan (Sellers *et al.*, 1978). Much interest has been shown in the way in which the tissues control the extracellular activity of these enzymes, to allow remodelling of the macromolecules when necessary, but to prevent irreversible breakdown of the matrix. The metalloproteinases are usually secreted in a latent form and require activation before they can digest their substrates, and it is likely that this represents one mechanism of control. The most widely studied latent metalloproteinase, collagenase, can be activated by trypsin and also by mercurials and chaotropic ions, and it has been proposed that latent collagenase consists of the enzyme in combination with inhibitor (Reynolds *et al.*, 1977).

Inhibitors of collagenase have been discovered in serum (Abe & Nagai, 1972; Werb *et al.*, 1974; Woolley *et al.*, 1976), in tissue extracts (McCroskery *et al.*, 1975; Kuettner *et al.*, 1977; Roughley *et al.*, 1978), in media from connective-tissue cells in culture (Nolan *et al.*, 1978; Welgus *et al.*, 1979) and in the media from connective tissues (Murphy *et al.*, 1977; Shinkai *et al.*, 1977; Vater *et al.*, 1979; Nolan *et al.*, 1980). Only the inhibitors synthesized in tissue culture, however, have been

shown to combine with the enzyme to give a complex that is similar to the latent collagenase (Sellers *et al.*, 1977; Vater *et al.*, 1978). In addition, the tissue collagenase inhibitor blocks the action of two other metalloproteinases that degrade gelatin and proteoglycan (Sellers *et al.*, 1979). It therefore seems likely that connective tissues produce this inhibitor to control selectively the extracellular activity of tissue metalloproteinases.

In order to determine if latent collagenase consists of enzyme in combination with this tissue inhibitor, it is necessary to purify both enzyme and inhibitor and study their interaction. In the present paper we describe a purification procedure for the isolation of the rabbit bone inhibitor of collagenase. A preliminary report of part of this work has been published (Cawston *et al.*, 1980b).

Materials and methods

Materials

Chemicals were obtained from the following suppliers: fluorescamine from F. Hoffman–LaRoche, Basel, Switzerland; DEAE-Sepharose and concanavalin A–Sepharose from Pharmacia, Hounslow, Middx., U.K.; *N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate (IM 861, Bolton & Hunter reagent) from The Radiochemical Centre,

Abbreviation used: SDS, sodium dodecyl sulphate.

Amersham, Bucks., U.K.; Coomassie Brilliant Blue G250 from Raymond A. Lamb, London NW10 5JL, U.K.; heparin-Sepharose and the zinc-chelate column were prepared as previously described (Cawston & Tyler, 1979).

All other chemicals and biochemicals were commercially available analytical-grade reagents or have been previously described (Cawston & Tyler, 1979; Tyler & Cawston, 1980; Cawston *et al.*, 1980a).

Methods

Culture techniques. The cranial vaults of newborn (1–2-day-old) rabbits were cultured in a modified form of BGJ medium (Reynolds, 1976) with no added serum. Each dish contained one bone in 5.0 ml of medium and the medium was changed every day for 14 days. Collagenase inhibitor was produced during the first 3–4 days of culture (Sellers & Reynolds, 1977), and media containing inhibitor were pooled, filtered through Whatman no. 54 filter paper and diluted 1:10 with 1M-Tris/HCl buffer, pH 7.6, containing 100 mM-CaCl₂. The pooled medium was stored at –20°C until required for inhibitor purification.

Enzyme assays. [¹⁴C]Acetylated collagen was used to measure collagenolytic activity by using the diffuse-fibril assay (Cawston & Barrett, 1979). For collagenase-inhibitor assays, a known amount of collagenase (0.06 unit) was added and assayed in the presence of inhibitor samples. A unit of collagenase degrades 1 µg of collagen/min at 37°C, and 1 unit of inhibitor is defined as that giving 50% inhibition of 2 units of collagenase (Murphy *et al.*, 1977). Gelatin-degrading activity was assayed using ¹⁴C-labelled gelatin at 37°C as described by Sellers *et al.* (1977). Casein-degrading activity (proteoglycanase; Sellers *et al.*, 1978) was assayed with ¹⁴C-labelled casein prepared by acetylating casein with [1-¹⁴C]acetic anhydride. Casein (20 mg/ml) was treated with [1-¹⁴C]acetic anhydride in the same way as collagen (Cawston & Barrett, 1979), except that the CaCl₂ in the borate buffer was omitted. Incubations were performed at 37°C in a final volume of 250 µl with 100 µg of casein, 100 µl of 100 mM-Tris/HCl buffer, pH 7.6, containing 30 mM-CaCl₂. The reaction was stopped by the addition of 50 µl of 18% (w/v) trichloroacetic acid. After centrifugation at 10000 g for 10 min, a portion (200 µl) of the supernatant was counted for ¹⁴C radioactivity as previously described (Cawston & Barrett, 1979). The release of radioactivity into the supernatant was linear up to 30% lysis of casein. One unit of caseinase activity degrades 1 µg of casein/min at 37°C to peptides soluble in 3% (w/v) trichloroacetic acid.

Protein determination. Column eluates were monitored for protein by measuring *A*₂₈₀. The protein content of samples from each stage of the

purification was determined with fluorescamine as previously described (Cawston & Tyler, 1979).

Iodination of proteins with ¹²⁵I. Protein samples (80 µl) with 0.1 M-sodium borate buffer, pH 8.5 (20 µl), were added to tubes containing 50–100 µCi of dried *N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]-iodophenyl)propionate and agitated for 15 min at 0°C.

SDS/polyacrylamide-gel electrophoresis. Protein samples were mixed with 50 mM-Tris/HCl, pH 6.8, containing 1% (w/v) SDS, 1% (v/v) glycerol and 0.0002% Bromophenol Blue and heated at 100°C for 5–10 min. Polyacrylamide slab gels (17.4 cm × 13.6 cm × 0.014 cm) were poured, the lower gel containing 12.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide and the spacer gel containing 4.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide. Polymerization and all other procedures were as previously described (Cawston & Tyler, 1979), and the gels were run in a Shandon (model 200) vertical polyacrylamide-slab-gel electrophoresis apparatus.

Gel slabs were fixed for 5 min in 40% (w/v) trichloroacetic acid, washed in water and stained for 30–60 min in a solution of 0.25% Coomassie Brilliant Blue G250 in methanol/acetic acid/water (5:1:4, by vol.), and destained in methanol/acetic acid/water (30:1:69, by vol.). The polyacrylamide gels were photographed, dried and radioautographed as described previously (Cawston & Tyler, 1979).

Column chromatography. All columns were run at 4°C and all buffers contained 0.05% Brij 35, with NaN₃ (0.02%) as a preservative.

Purification of rabbit bone collagenase. Rabbit bones were cultured for 14 days, and the daily changes of medium from day 4 onwards were combined. The pooled medium was concentrated by ultrafiltration with a Millipore Pellicon membrane (type PTGC), and purified by the method of Cawston & Tyler (1979).

Results

Scheme 1 summarizes the purification procedure that we used for the isolation of the rabbit bone tissue inhibitor of metalloproteinases.

Concentration of medium

The pooled medium was thawed and concentrated either by ultrafiltration through a Millipore Pellicon PTGC membrane, or dialysed against water to remove most of the salt, freeze-dried, and then resuspended in 25 mM-sodium cacodylate buffer, pH 7.2, containing 10 mM-CaCl₂, 0.05% Brij 35, 1 M-NaCl and 0.02% NaN₃ (column buffer). Medium concentrated by either method was dialysed against this buffer overnight and then centrifuged at 40000 g for 1 h at 4°C to remove any insoluble protein.

Gel filtration on Ultrogel AcA 44. A column of Ultrogel AcA 44 was equilibrated with column buffer, and the concentrated medium was applied and eluted by reverse flow. Fig. 1 illustrates the

Scheme 1. *Purification of rabbit bone inhibitor*

elution profile that was obtained. The tissue inhibitor was eluted in a broad peak relatively free of the major protein and of the three latent metalloproteinases. A large peak of latent collagenase was eluted before the inhibitor. The fractions containing inhibitor were combined and MgCl_2 (1 mM) and MnCl_2 (1 mM) added. In all, four such gel-filtration columns were run, and the fractions containing inhibitor from each were combined.

Concanavalin A-Sephacel chromatography

The combined fractions from the Ultrogel AcA 44 columns were applied to a column of concanavalin A-Sephacel equilibrated with column buffer containing MgCl_2 (1 mM) and MnCl_2 (1 mM) and then eluted with this buffer until the absorbance of the fractions was equal to that of the buffer. The adsorbed glycoproteins were removed from the column by washing with column buffer containing 2% (w/v) 1-*O*-methyl α -D-glucopyranoside. A small protein peak was eluted that contained the tissue inhibitor (Fig. 2). The fractions containing the inhibitor were pooled and concentrated by ultrafiltration with an Amicon PM10 membrane and dialysed against 40 mM-sodium barbitone buffer containing 40 mM-NaCl, 0.05% Brij 35 and 0.02% NaN_3 .

DEAE-Sephacel chromatography

The concentrated fractions from step 3 in the sodium barbitone buffer were loaded on to a column

Fig. 1. *Gel filtration of rabbit bone inhibitor of collagenase*

Concentrated medium (55 ml) was applied to a column (4.4 cm $\times$ 115 cm) of Ultrogel AcA 44 equilibrated with 25 mM-sodium cacodylate, pH 7.2, containing 1 M-NaCl, 10 mM- CaCl_2 , 0.05% Brij 35 and 0.02% NaN_3 (column buffer). The column was eluted with this buffer at a flow rate of 30 ml/h and the fractions (11.6 ml) monitored for protein (A_{280} , —), collagenase ($\bullet$) and metalloproteinases (^{14}C gelatin-degrading activity, $\circ$; ^{14}C casein-degrading activity, $\square$) and collagenase inhibitor (----).

Fig. 2. Concanavalin A-Sepharose chromatography of rabbit bone inhibitor of collagenase

Pooled fractions (790 ml) from the Ultrogel AcA 44 columns in column buffer containing 1 mM-MgCl₂ and 1 mM-MnCl₂ were loaded on to a pre-equilibrated column (1.6 cm × 11.5 cm) of concanavalin A-Sepharose at a flow rate of 30 ml/h and eluted with this buffer. Adsorbed protein was removed by washing with column buffer containing 2% (w/v) 1-O-methyl α-D-glucopyranoside and the fractions (11.5 ml) were monitored for protein (A_{280} ; —) and collagenase inhibitor (----).

of DEAE-Sepharose pre-equilibrated with this buffer. The column was eluted with a large volume of sodium barbitone buffer, and Fig. 3 illustrates the elution profile that was obtained. The tissue inhibitor was retarded by the column under these conditions, but not bound, and the fractions containing inhibitor were relatively free of contaminating protein, as determined by the A_{280} elution profile. The adsorbed protein was removed with 1 M-NaCl. Those fractions containing inhibitory activity were combined.

Heparin-Sepharose chromatography

The combined fractions from DEAE-Sepharose chromatography were dialysed for 6 h against 25 mM-sodium cacodylate buffer containing 0.05% Brij 35, 0.02% NaN₃ and 10 mM-CaCl₂ and applied to a column of heparin-Sepharose equilibrated with this buffer. The column was eluted with this buffer, and then with a salt gradient to remove the adsorbed protein. The inhibitor was eluted from the column with 0.2–0.4 M-NaCl, whereas most of the protein applied passed straight through the column (Fig. 4).

Zinc-chelate affinity chromatography

The heparin-Sepharose fractions containing inhibitor were combined, and were dialysed overnight against 25 mM-sodium borate buffer, pH 8.0, containing 0.15 M-NaCl, 0.05% Brij 35 and 0.02% NaN₃, and loaded on to a column of iminodiacetic acid-Sepharose saturated with ZnCl₂ and equilibrated with the borate buffer. The column was

Fig. 3. DEAE-Sepharose chromatography of rabbit bone inhibitor of collagenase

Fractions containing rabbit bone inhibitor of collagenase from concanavalin A-Sepharose chromatography were concentrated by ultrafiltration and dialysed overnight against 40 mM-sodium barbitone, pH 7.6, containing 40 mM-NaCl, 0.05% Brij 35, and 0.02% NaN₃. The concentrated fractions (5 ml) were loaded on to a pre-equilibrated column of DEAE-Sepharose (0.9 cm × 28 cm) and eluted at a flow rate of 10 ml/h with a large volume of the above buffer. Adsorbed protein was removed by washing with 1 M-NaCl and the fractions (1 ml) were monitored for protein (A_{280} ; —) and collagenase inhibitor (----).

Fig. 4. Heparin-Sepharose chromatography of rabbit bone inhibitor of collagenase

Pooled fractions (45 ml) from DEAE-Sepharose chromatography in 25 mM-sodium cacodylate buffer, pH 7.6, containing 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ were applied to a column (0.9 cm × 5.0 cm) of heparin-Sepharose and eluted with this buffer. Adsorbed protein was removed by washing the column with a linear salt gradient (0–0.7 M-NaCl); —, A_{280} ; ----, collagenase inhibitor; ····, conductivity. Further protein was eluted from the column after washing with 1 M-NaCl (not shown).

washed, and eluted in a stepwise manner, as described in the legend to Fig. 5, which illustrates the elution profile, and the inhibitor was eluted from the column at pH 6.5. These fractions were quickly pooled and concentrated by ultrafiltration with a PM10 membrane and stored at 4°C.

Purification of the tissue inhibitor of metalloproteinases

Samples were taken from each stage of the purification (labelled 1–6, Scheme 1) and assayed for inhibitory activity against rabbit bone collagenase and for protein content. The results of these assays are shown in Table 1. The purified inhibitor had a final specific activity of 26 000 units/mg, and was purified more than 1300-fold when compared with the starting concentrated medium. Only 9% of the original activity was recovered by this procedure. The purified inhibitor in dilute solutions rapidly lost activity, and a decrease in specific activity occurred during the final purification step, from 41 000 to 26 000 units/mg. The zinc-chelate column did not necessarily cause this loss of activity, since when this step was replaced with a further Ultrogel AcA 44 column, larger losses of activity were observed. A further purification step was necessary after the

heparin–Sephacrose column to remove small amounts of contaminating protein. No suitable method was found to prevent this loss of activity. It is likely that the potential specific activity is greater than 41 000 units/mg, and probably approaches the values reported for pig synovial collagenase by Cawston & Tyler (1979).

Fig. 5. Zinc-chelate affinity chromatography of rabbit bone inhibitor of collagenase

Fractions containing collagenase inhibitor from heparin–Sephacrose chromatography (39 ml) in 25 mM-sodium borate buffer, pH 8.0, containing 0.15 M-NaCl, 0.05% Brij 35 and 0.02% NaN₃ were loaded on to a column (0.8 cm × 6.0 cm) of iminodiacetic acid–Sephacrose saturated with ZnCl₂ and equilibrated with the above buffer. The column was eluted at 18 ml/h with the following buffers: (1) 25 mM-sodium cacodylate, pH 6.5; (2) 25 mM-sodium cacodylate, pH 6.5, containing 0.8 M-NaCl; (3) 50 mM-sodium acetate, pH 4.7, containing 0.8 M-NaCl. All buffers contained Brij 35 (0.05%), NaN₃ (0.02%) and CaCl₂ (1 mM). The column eluate was monitored for protein (A₂₈₀, —) and collagenase (----).

Fig. 6. SDS/polyacrylamide-gel electrophoresis of samples from each stage of the inhibitor purification: comparison of Coomassie Brilliant Blue staining (a) with radioautography (b)

Protein samples from each stage of the inhibitor purification (1–6, Scheme 1) were labelled with ¹²⁵I and boiled in SDS under reducing conditions. After loading on to an SDS/polyacrylamide gel, electrophoresis was continued for 4.5 h at 150 V. The gel was fixed and stained with Coomassie Brilliant Blue and then dried on to Whatman 3MM filter paper and radioautographed for 4 h.

Table 1. Purification of rabbit bone inhibitor of collagenase

	Inhibitor (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
Concentrated medium	45 400	2452	18.5	100	1
Gel filtration (Ultrogel AcA 44)	22 100	96	230	49	12
Concanavalin A–Sephacrose	22 000	8.08	2723	48	147
DEAE–Sephacrose	14 000	1.48	9459	31	511
Heparin–Sephacrose	7700	0.19	40 526	17	2170
Zinc-chelate–Sephacrose	4100	0.16	25 625	9	1348

Purity of the inhibitor preparation

The fractions from each stage of the purification procedure were labelled with ^{125}I and denatured and reduced with SDS and 2-mercaptoethanol before SDS/polyacrylamide-gel electrophoresis. Fig. 6 shows the proteins present in each fraction after staining (a) and radioautography (b) of the SDS/polyacrylamide gel. Trace contaminants could be seen in the radioautograph in the final zinc-chelate-column fraction.

To assess further the purity of the final preparation of rabbit bone inhibitor, the dried gel was cut vertically and then into 0.5 cm horizontal strips, and each slice counted for radioactivity in a Packard PGD autogamma counter. The results demonstrated that 95% of the radioactivity was associated with the major protein band.

Interaction of purified rabbit bone inhibitor and purified rabbit bone collagenase

Increasing amounts of the purified inhibitor were added to a known amount of purified collagenase in the assay, and the percentage inhibition was calculated at each concentration of inhibitor. These results are shown in Fig. 7. It is possible to calculate the amount of inhibitor that interacts with the collagenase to give exactly 100% inhibition. From the graph obtained and by using the information given in the legend to Fig. 7, it was calculated that 1

Fig. 7. Interaction of purified rabbit bone inhibitor with purified rabbit bone collagenase

Increasing amounts of a solution containing purified inhibitor (1.9 units/ml; 70 ng/ml) were added to assays containing 30 μl of purified collagenase (2.93 units/ml; 160 ng/ml). The enzyme was inhibited linearly with increasing concentration of inhibitor, and from the graph it was calculated that 1 mol of inhibitor interacted with 0.95 mol of collagenase (molecular weights from SDS/polyacrylamide-gel electrophoresis: inhibitor, 28 000; collagenase, 44 000).

mol of inhibitor interacted with 0.95 mol of collagenase.

Loss of activity of purified inhibitor after incubation with either trypsin or at high temperatures

Complete loss of inhibitor activity was found only after incubation with 250 μg of trypsin/ml for 30 min at 37°C. The purified inhibitor was stable to high temperatures, and complete loss of activity was only achieved after incubation for 1 h at 90°C.

Specificity of rabbit bone inhibitor

The purified inhibitor blocked the activities of rabbit bone collagenase, gelatinase and proteoglycanase, and of human leucocyte collagenase and gelatinase. It did not inhibit either thermolysin or bacterial collagenase, or the serine proteinases trypsin and plasmin. The inhibitor also blocked the activity of pig synovial collagenase.

Discussion

In the present paper we describe a purification procedure for the collagenase inhibitor synthesized by newborn-rabbit bones in tissue culture. Although large numbers of bones were cultured (total of 12 litres of culture medium), only a small amount of purified inhibitor protein (180 μg) was isolated. A number of factors are responsible for this low yield, which include the relatively small amounts of inhibitor present in the culture medium during the first 4 days and the overall low recovery of inhibitor activity by the purification procedure (9%). The isolated inhibitor was 95% pure. Two steps proved to be of particular value in the purification procedure. The concanavalin A-Sepharose column gave a good purification and selectively bound carbohydrate-containing protein. The DEAE-Sepharose column retarded the inhibitor under the conditions used.

The purified rabbit bone inhibitor was a potent inhibitor of collagenase. It was relatively heat-stable, resistant to low concentrations of trypsin, but destroyed by high concentrations of trypsin and also by reduction and alkylation. It inhibited the three rabbit bone metalloproteinases and human leucocyte collagenase and gelatinase, but neither bacterial collagenase nor thermolysin, nor the serine proteinases trypsin and plasmin. These properties are similar to those reported for inhibitors purified from tendon cultures (Vater *et al.*, 1979) and human-skin-fibroblast culture medium (Welgus *et al.*, 1979), and partially purified from bovine aorta cultures (Nolan *et al.*, 1980) and pig smooth-muscle-cell culture medium (Nolan *et al.*, 1978). It is not certain, however, if these inhibitors are similar proteins, because differences do exist in their properties and also in the way they interact with collagenase. No

enzyme-inhibitor complex was reported for the human tendon inhibitor (Vater *et al.*, 1979), and although the human fibroblast inhibitor (Welgus *et al.*, 1979) interacted with collagenase on a mol/mol basis, the binding appeared to be weak, since no complex of enzyme and inhibitor could be demonstrated. The rabbit bone inhibitor bound to purified collagenase with a 1:1 stoichiometry. The inhibition was not reversible either by trypsin treatment or by addition of mercurials, as also found for the pig aorta inhibitor (Kerwar *et al.*, 1980). The rabbit bone inhibitor binds tightly to the enzyme, and a complex is formed of higher molecular weight (T. E. Cawston, unpublished work). A detailed study of the interaction between purified enzyme and purified inhibitor will therefore be necessary to determine how the inhibitor interacts with the enzyme and the precise relationship of such an enzyme-inhibitor complex to the latent enzyme.

The physiological role of the rabbit bone inhibitor of collagenase remains unclear. Because the inhibitor specifically blocks the activity of mammalian metalloproteinases other than collagenase, we have tentatively named the inhibitor 'TIMP' (Tissue Inhibitor of MetalloProteinases) so that it can be distinguished from other inhibitors that are not synthesized by tissues in culture. Much interest has focused on the amount of this inhibitor ('TIMP') relative to enzyme found in the culture media of connective tissue that is actively resorbing. Cambray *et al.* (1980) showed that amounts of inhibitor were lower in rabbit synovium cultures when the tissue was taken from experimentally arthritic rabbits, as compared with normal animals. Sellers *et al.* (1980) reported lower amounts of inhibitor and increased active collagenase activities in bone cultures where agents were added to induce resorption. Although it is not yet clear if the inhibitor isolated from rabbit bones reacts with collagenase to form the latent complex, it seems likely from these studies that the potent inhibitor of collagenase that we have purified plays a vital role in controlling the extracellular activity of metalloproteinases *in vivo*.

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