Purification of a metalloproteinase inhibitor from human rheumatoid synovial fluid

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A metalloproteinase inhibitor present in human rheumatoid synovial fluid was purified by a combination of heparin–Sephacryl chromatography, concanavalin A–Sephacryl chromatography, ion-exchange chromatography and gel filtration. The M_r of the purified inhibitor was 28000 by SDS/polyacrylamide-gel electrophoresis and 30000 by gel filtration. The inhibitor blocked the activity of the metalloproteinases collagenase, gelatinase and proteoglycanase, but not thermolysin or bacterial collagenase. The serine proteinase trypsin was not inhibited. The inhibitory activity was lost after treatment with trypsin (0.5 μ g/ml) at 37 °C for 30 min, 4-aminophenylmercuric acetate (1 mM) at 37 °C for 3 h, after incubation for 30 min at 90 °C and by reduction and alkylation. These properties suggest that the inhibitor closely resembles the tissue inhibitor of metalloproteinases ('TIMP') recently purified from connective-tissue culture medium.

INTRODUCTION

Collagenase is a metalloproteinase that cleaves all three α -chains of the triple-helical collagen molecule at one point to give characteristic one-quarter and three-quarter products. It is likely that this enzyme is responsible for much of the collagen breakdown and subsequent tissue damage that occurs in diseases such as rheumatoid arthritis.

Although collagenase cannot be directly extracted from connective tissue, it is synthesized and secreted when these tissues are placed in culture medium (Sellers *et al.*, 1978). Collagenase is often found in a latent form, and activity cannot be detected until the medium has been treated with trypsin (Stricklin *et al.*, 1977), chaotropic agents (Sakamoto *et al.*, 1978) or mercurials (Cawston *et al.*, 1981a). Two other metalloproteinases which, after activation, degrade proteoglycan and gelatin are also present in tissue-culture medium (Sellers *et al.*, 1978).

Many workers have detected specific inhibitors of the metalloproteinases in connective-tissue culture medium (Murphy *et al.*, 1977) and in serum (Woolley *et al.*, 1976). A large number of these inhibitors have similar properties, being glycoproteins of M_r 28000. These inhibitors of collagenase have been purified and characterized from human tendon culture medium (Vater *et al.*, 1979), human skin fibroblast medium (Stricklin & Welgus, 1983), rabbit bone culture medium (Cawston *et al.*, 1981b), human amniotic fluid (Murphy *et al.*, 1981), human serum (Woolley *et al.*, 1976; Welgus & Stricklin, 1983), and human articular cartilage (Dean & Woessner, 1984). The purified inhibitor produced by rabbit bone in culture also inhibits proteoglycanase and gelatinase (Cawston *et al.*, 1981b). This inhibitor has been named the 'tissue inhibitor of metalloproteinases' ('TIMP') and it is thought to play a role in the extracellular control of collagenase activity. In a recent study synovial fluids were found to contain three latent metalloproteinases which, after activation, degraded collagen, gelatin and proteo-

glycan (Cawston *et al.*, 1984). In addition to α_2 -macroglobulin, a lower- M_r inhibitor of collagenase was detected that appeared to be similar to TIMP. In the present study we have purified and characterized this inhibitor of metalloproteinases from synovial fluid.

MATERIALS AND METHODS

All materials were of analytical grade or the highest purity available. Hyaluronidase (bovine testis) type IV-S was from Sigma, Poole, Dorset, U.K. DEAE-Sephacryl and concanavalin A–Sephacryl and heparin–Sephacryl were from Pharmacia, Milton Keynes, Bucks., U.K. Ultrogel AcA 44 and TSK-G 3000 SW columns were from LKB Instruments, South Croydon, Surrey, U.K. 14 C-labelled collagen was prepared as described by Cawston & Barrett (1979). Synovial fluid, aspirated as part of a therapeutic procedure from knee joints of patients with rheumatoid arthritis, was centrifuged at 10000 g for 20 min at 4 °C. The cell-free supernatant was stored at –70 °C.

Enzyme and inhibitor assays

[14 C]Acetylated collagen was used to measure collagenase in the diffuse-fibril assay (Cawston & Barrett, 1979). For collagenase-inhibitor assays, a known amount of collagenase was added and assayed in the presence of inhibitor samples. A unit of collagenase degrades 1 μ g of collagen/min at 37 °C and 1 unit of inhibitor inhibits 2 units of collagenase by 50% (Murphy *et al.*, 1977).

Protein determination

Column eluates were monitored for protein by measuring A_{280} . The protein concentration of samples from each stage of the purification procedure was determined with Coomassie Brilliant Blue by the method of Bradford (1976).

Abbreviation used: TIMP, tissue inhibitor of metalloproteinases.

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Fig. 1. Heparin-Sepharose chromatography of synovial-fluid inhibitor of metalloproteinases

Synovial fluid was dialysed against 25 mM-sodium cacodylate buffer, pH 7.5, containing 10 mM- CaCl_2 , 0.05% Brij 35 and 0.02% NaN_3 , and centrifuged at 2840 *g* for 1 h at 4 °C. Heparin-Sepharose was added by a batchwise technique and then poured into a column (1.6 cm $\times$ 15 cm) and washed with the above buffer at 20 ml/h. Adsorbed protein was removed by washing the column with a linear salt gradient (0–0.8 M-NaCl;—). Fractions (4 ml) were monitored for protein (A_{280} ; —), collagenase (----) and collagenase inhibitor (-·-·-).

Iodination of proteins with ^{125}I

Protein samples (80 μl , containing 0.5–10 μg of protein) mixed 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- ^{125}I)iodophenyl-propionate by using the method of Bolton & Hunter (1973).

Samples were mixed with 50 mM-Tris/HCl, pH 6.8, containing 1% (w/v) SDS, 40% (v/v) glycerol and 0.0002% Bromophenol Blue and heated at 100 °C for 10 min. Linear gradient gels (190 mm $\times$ 160 mm $\times$ 1 mm) were poured by the use of a gradient former (LKB Instruments) with 10–22% (w/v) total acrylamide and 2.5% (w/w) bisacrylamide. Samples were applied to a 4.5% (w/v) total acrylamide plus 2.5% (w/w) bisacrylamide spacer. The gels were polymerized in the presence of 0.01% (w/v) ammonium persulphate and 0.013 (v/v) *NNN'*-tetramethylethylenediamine. The gel was run at 150 V for 5 h, the buffer system of Laemmli & Favre (1973) being used.

The gel was fixed, stained with Coomassie Brilliant Blue, dried and radioautographed (Cawston *et al.*, 1981b).

RESULTS

Cell-free supernatants of synovial fluid aspirated from rheumatoid joints were thawed, pooled (2.5 litres) and treated with 100 μg of hyaluronidase/ml at room temperature for 1 h to depolymerize hyaluronic acid and so decrease fluid viscosity. After dialysis overnight at 4 °C against 25 mM-sodium cacodylate buffer, pH 7.5, containing 10 mM- CaCl_2 , 0.05% Brij 35 and 0.02% NaN_3 , the fluid was centrifuged at 2840 *g* for 1 h at 4 °C to

remove precipitated protein. Heparin-Sepharose (50 ml) was added to the supernatant, stirred for 2 h, allowed to settle and the supernatant aspirated. The heparin-Sepharose was poured into a column and eluted with the sodium cacodylate buffer, followed by an NaCl gradient to remove adsorbed protein (Fig. 1). The bound inhibitor was eluted from the column at 0.2–0.4 M-NaCl. Two peaks of collagenase were detected either side of the eluted inhibitor. Much of the synovial-fluid protein did not bind to the heparin-Sepharose under these conditions and remained in the supernatant.

The fractions containing inhibitor were combined and dialysed against 25 mM-sodium cacodylate buffer, pH 7.5, containing 0.05% Brij 35, 0.02% NaN_3 , 1 mM- MgCl_2 , 1 mM- MnCl_2 , and 1 M-NaCl, and then applied to a concanavalin A-Sepharose column (1.6 cm $\times$ 8 cm) pre-equilibrated with this buffer (Fig. 2). The column was washed until the absorbance of the eluant returned to background and then eluted with the same buffer containing 2% (w/v) 1-*O*-methyl α -D-glycopyranoside to remove the adsorbed glycoproteins.

Two peaks of inhibitor were detected: one main peak, which bound to concanavalin A-Sepharose, and a non-binding peak that was eluted with the column buffer alone. Collagenase did not bind to the concanavalin A-Sepharose and was eluted with the non-binding inhibitor. The fractions containing the concanavalin A-Sepharose 'binding' inhibitor were pooled and concentrated by ultrafiltration with an Amicon PM10 membrane and dialysed against 40 mM-sodium barbitone buffer, pH 7.6, containing 40 mM-NaCl, 0.05% Brij 35 and 0.02% NaN_3 . The concentrated fractions were applied to a DEAE-Sepharose column (1.6 cm $\times$ 40 cm) pre-equilibrated with barbitone buffer (Fig. 3). The inhibitor was retarded but was not bound by the column.

Fig. 2. Concanavalin A-Sepharose chromatography of synovial-fluid inhibitor of metalloproteinases

Pooled fractions from the heparin-Sepharose column containing the synovial-fluid inhibitor in 25 mM-sodium cacodylate buffer, pH 7.5, containing 1 mM-MgCl₂, 1 mM-MnCl₂, 1 M-NaCl, 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ were applied to a pre-equilibrated column (1.6 cm × 8 cm) of concanavalin A-Sepharose and washed with this buffer at 15 ml/h. Adsorbed protein was removed by washing with buffer containing 2% (w/v) 1-O-methyl α -D-glycopyranoside (MDGP), and the fractions (5 ml) were monitored for protein (A_{280} ; —), collagenase (---) and collagenase inhibitor (-·-·).

Fig. 3. DEAE-Sepharose chromatography of synovial-fluid inhibitor of metalloproteinases

Pooled and concentrated fractions from concanavalin A-Sepharose chromatography containing the synovial-fluid inhibitor were applied to a column of DEAE-Sepharose (1.6 cm × 40 cm) equilibrated with 40 mM-sodium barbitone buffer, pH 7.6, containing 40 mM-NaCl, 0.05% Brij 35 and 0.02% NaN₃, and washed with this buffer at 12 ml/h. Adsorbed protein was removed by washing with 1 M-NaCl and the fractions (2 ml) were monitored for protein (A_{280} ; —) and collagenase inhibitor (-·-·).

Adsorbed protein was removed by 1 M-NaCl in barbitone buffer (result not shown).

The combined fractions containing inhibitor from the DEAE-Sepharose column were concentrated by ultrafiltration on an Amicon PM10 membrane and applied to a gel-filtration column of Ultrogel AcA 44 (1.6 cm × 90 cm) pre-equilibrated with 25 mM-sodium cacodylate buffer, pH 7.5, containing 1 M-NaCl, 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ (Fig. 4).

The fractions containing inhibitor were pooled and dialysed against 25 mM-sodium cacodylate buffer, pH 7.5, containing 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃. The pool was applied to a pre-equilibrated heparin-Sepharose column (1.6 cm × 10 cm) and the column washed with this buffer, and then eluted with

Fig. 4. Gel filtration of synovial-fluid inhibitor of metalloproteinases

Concentrated fractions from DEAE-Sepharose containing the synovial-fluid inhibitor were applied to a column (1.6 cm × 90 cm) of Ultrogel AcA 44 equilibrated with 25 mM-sodium cacodylate buffer, pH 7.5, containing 1 M-NaCl, 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃. The column was eluted with this buffer at 12 ml/h and the fractions (1.5 ml) monitored for protein (A_{280} ; —) and collagenase inhibitor (-·-·).

buffer containing 0.2 M-NaCl (Fig. 5). A small amount of protein did not bind to the column, whereas the bound inhibitor was eluted by the salt buffer. Any further bound protein was eluted from the column by a 1 M-NaCl wash (results not shown). The fractions containing inhibitor were pooled and concentrated by ultrafiltration on an Amicon PM10 membrane and applied to a gel-filtration column of TSK-G 3000 SW (7.5 mm × 600 mm) pre-equilibrated with 25 mM-sodium cacodylate buffer, pH 7.2, containing 100 mM-NaCl, 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ and eluted with this buffer (Fig. 6). Two gel-filtration columns were run, each giving a single peak of inhibitor. The fractions containing inhibitor were pooled and were concentrated by ultrafiltration on an Amicon PM10 membrane and stored at 4 °C.

At each stage of the purification procedure samples

Fig. 5. Heparin-Sephadex chromatography of synovial-fluid inhibitor of metalloproteinases

Pooled fractions from the gel-filtration column containing the synovial-fluid inhibitor were applied to a heparin-Sephadex column (1.6 cm $\times$ 10 cm) pre-equilibrated with 25 mM-sodium cacodylate buffer, pH 7.5, containing 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ and eluted at 15 ml/h with buffer, followed by a 0.02 M-NaCl wash (where shown). The fractions (1 ml) were monitored for protein (A_{280} ; —) and collagenase inhibitor (---).

Fig. 6. H.p.l.c. gel filtration of synovial-fluid inhibitor of metalloproteinases

Concentrated pooled fractions from heparin-Sephadex containing the synovial-fluid inhibitor were applied to a TSK-G 3000 SW (7.5 mm $\times$ 600 mm) gel-filtration column pre-equilibrated with 25 mM-sodium cacodylate buffer, pH 7.2, containing 100 mM-NaCl, 10 mM-CaCl₂, 0.05% Brij 35 and 0.02% NaN₃ and eluted with this buffer at 6 ml/h. The column was monitored for protein (A_{280} ; —) and collagenase inhibitor (---).

were taken and assayed for inhibitory activity against collagenase and for protein content. The results are shown in Table 1. The purified inhibitor had a final specific activity of 909 units/mg. Only 0.05% of the original inhibitory activity was recovered by the purification procedure. The original estimates of in-

hibitory activity include activity from large amounts of α_2 -macroglobulin present in synovial fluid. α_2 -Macroglobulin did not bind to heparin-Sephadex, causing a decrease of inhibitory activity after the heparin-Sephadex chromatography. Further loss of inhibitory activity during purification is due to the inhibitor rapidly losing activity in dilute solution.

Protein samples from each stage of the purification were labelled with ¹²⁵I, denatured with SDS, reduced with 2-mercaptoethanol and applied to an SDS/polyacrylamide gradient gel.

The purified inhibitor when run gave two main bands corresponding to apparent M_r values of 5600 and 28000. The autoradiograph is shown in Fig. 7.

No alteration in these two bands was observed after prolonged treatment (16 h) with 10 mM-dithiothreitol in 6 M-urea at 60 °C.

Interaction with collagenase

Increasing amounts of purified inhibitor were added to known amounts of purified collagenase in the assay and the percentage inhibition was calculated at each concentration of inhibitor. The results are shown in Fig. 8.

With purified collagenase the inhibitor formed a complex which, by the use of gel-filtration methods previously described (Cawston *et al.*, 1983), was found to have an M_r of 56500.

Specificity

The purified inhibitor blocked the activities of collagenase, gelatinase and proteoglycanase. It did not inhibit either thermolysin or bacterial collagenase or the serine proteinase trypsin.

Stability

Complete loss of inhibitory activity occurred after incubation with trypsin (0.5 μ g/ml) for 30 min at 37 °C and 1 mM-4-aminophenylmercuric acetate for 3 h at 37 °C and by reduction (5 mM-dithiothreitol for 2 h at 20 °C) followed by alkylation (20 mM-iodoacetic acid for 2 h at 20 °C). The inhibitor was relatively heat-stable, activity being destroyed after 30 min at 90 °C.

DISCUSSION

In the present study we have isolated a metalloproteinase inhibitor from rheumatoid synovial fluid that closely resembles inhibitors previously purified from rabbit bone medium (Cawston *et al.*, 1981b), human amniotic fluid

Table 1. Purification of synovial-fluid inhibitor of metalloproteinases

Stage	Inhibitor (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
1. Hyaluronidase treated synovial fluid	416075	99895	4.17	100	1
2. Heparin-Sephadex	6792	341	20	1.6	5
3. Concanavalin A-Sephadex	2639	49	54	0.63	13
4. DEAE-Sephadex	1674	3.50	480	0.40	115
5. Gel filtration	1218	0.50	2471	0.29	593
6. Heparin-Sephadex	723	0.50	1443	0.17	346
7. TSK-G 3000 SW	200	0.22	909	0.05	218

Fig. 7. SDS/polyacrylamide-gel electrophoresis of synovial-fluid inhibitor of metalloproteinases

A portion of the inhibitor peak eluted from the heparin-Sepharose column (Fig. 6) was iodinated with ^{125}I and applied to a gradient polyacrylamide gel containing 0.1% SDS. Labelled rabbit bone inhibitor and M_r standards were treated in the same way. After electrophoresis the gel was stained, dried and radioautographed. The synovial-fluid inhibitor had major bands corresponding to M_r 28000 and 56000. M, M_r standards; 1, synovial-fluid inhibitor; 2, rabbit bone inhibitor.

(Murphy *et al.*, 1981), human articular cartilage (Dean & Woessner, 1984), human skin fibroblast medium (Stricklin & Welgus, 1983), human tendon culture medium (Vater *et al.*, 1979), chick bone culture medium (Sakamoto *et al.*, 1981) and bovine cartilage and body fluids (Bunning *et al.*, 1984). All these inhibitors have similar properties and they have been named the 'tissue inhibitor of metalloproteinases' (TIMP) (Cawston *et al.*, 1981b). The synovial-fluid inhibitor is also a glycoprotein, has an M_r of 28000, and specifically inhibits metalloproteinases. It binds to collagenase forming a characteristic complex of M_r 56500

Fig. 8. Interaction of purified synovial-fluid inhibitor of metalloproteinases with purified pig synovial collagenase

Increasing amounts of a solution containing purified inhibitor (0.5 unit/ml) were added to assays containing 0.07 unit of purified pig synovial collagenase/ml. Progressive inhibition of the enzyme was seen with increasing amounts of inhibitor.

and shows similar properties in terms of its stability and sensitivity to heat, reduction and alkylation.

We have used the purification scheme developed for the isolation of the rabbit bone TIMP (Cawston *et al.*, 1981b) with the modifications introduced by Bunning *et al.* (1984). Large volumes (2.5 litres) of rheumatoid synovial fluid were required for the present study, with only 0.2–0.5 mg of protein being finally recovered. Heparin-Sepharose used in a batch method proved to be a very powerful technique for rapidly purifying synovial fluid TIMP free from α_2 -macroglobulin and most of the proteins present in the original fluid. Only 350 mg of protein bound to the heparin-Sepharose out of 100 g of protein originally present and this technique has subsequently proved to be useful in purifying TIMP from human plasma (T. E. Cawston, unpublished work).

The synovial-fluid TIMP was difficult to purify after the five steps of the conventional purification scheme. The inhibitor appeared to be relatively pure, but was contaminated by a band of higher (56000) M_r . Attempts at further purification were, however, unsuccessful, and after the next two steps the contaminating band was still present, even though the h.p.l.c. gel filtration was a size separation. Subsequent work with the synovial-fluid inhibitor, a similar human plasma inhibitor and rabbit bone inhibitor suggests that the higher- M_r band represents an aggregate of the M_r -28000 protein (T. E. Cawston, unpublished work), which was not denatured in the reducing SDS/polyacrylamide gel. The presence of aggregated material in the purified inhibitor preparation was confirmed by gel filtration, where high- M_r inactive aggregates were eluted at the void volume. Subsequent SDS/polyacrylamide-gel analysis of

the void-volume peak confirmed that both the 28000- M_r and the 56000- M_r protein were present in this peak.

The specific activity of the purified inhibitor was lower than that reported for the rabbit bone inhibitor, and there are a number of reasons for this. Firstly, only a small amount of inhibitor is present in synovial fluid, and the inhibitor appears to be quite labile, especially towards the end of the purification scheme. Also, there are seven steps in the purification, and although the final two stages were included in an attempt to remove the higher- M_r band, the stages were associated with the decrease in specific activity from 2500 units/mg to 900 units/mg, since the purified inhibitor is unstable in dilute solution. It is expected that the final specific activity of the human inhibitor will be lower than that of the rabbit inhibitor, since the specific-activity values for the human collagenase is correspondingly lower than that of either the rabbit or the pig collagenase (Cawston & Tyler, 1979).

A proportion of the inhibitor did not bind to concanavalin A-Sepharose, and this inhibitor activity has not been examined or purified further. Bunning *et al.* (1984) investigated bovine body fluids and showed that a large proportion of the collagenase inhibitor from bovine amniotic fluid and cartilage extracts were non-glycosylated, but appeared to be similar to the glycosylated protein, although it had a lower M_r . Also, Murphy *et al.* (1985) have described the occurrence, in human fibroblast culture medium, of non-glycosylated forms of collagenase inhibitor that are immunologically identical with TIMP.

Synovial-fluid collagenase also bound to the heparin-Sepharose column (Fig. 1) and was eluted from the column in an apparent double peak on both sides of the inhibitor. Collagenase is co-eluted with inhibitor on this matrix, and the inability to detect collagenase when excess inhibitor is present illustrates the problem of accurately determining enzyme activities in the presence of inhibitors (Cawston *et al.*, 1984).

Synovial-fluid contains higher activities of collagenase inhibitor than corresponding plasma samples (E. Mercer, P. McLoughlin & T. E. Cawston unpublished work), which suggests that it is produced locally by the tissues surrounding the joint, possibly as a response to the raised metalloproteinase activities. It will be important to examine synovial fluids taken from patients where active cartilage breakdown is occurring to see if any correlation exists between the amount of enzyme, inhibitor or enzyme-inhibitor complex and cartilage loss.

This is the first reported isolation of TIMP in synovial fluid and suggests that there is a relationship between those inhibitors produced *in vitro* and those found *in vivo*.

Although the precise role of this inhibitor is not yet clear, its occurrence *in vivo* and its ability to inhibit not only collagenase but other metalloproteinases suggests that it may play an important part in the regulation of metalloproteinase activity, effectively controlling the amount of connective-tissue breakdown.

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