Rapid purification of tissue inhibitor of metalloproteinases from human plasma and identification as a γ -serum protein

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A rapid method is described for the purification of human tissue inhibitor of metalloproteinases (TIMP) from plasma which involves immuno-affinity chromatography and gel filtration. The purified plasma inhibitor is immunologically identical with the TIMP previously purified from human amniotic fluid, human synovial fluid and human fibroblast culture medium. It is proposed that this inhibitor is identical with the plasma inhibitor previously named 'B₁ anticollagenase', although the plasma inhibitor was shown to migrate as a γ -serum component.

INTRODUCTION

Connective-tissue cells when placed in tissue culture secrete a family of metalloproteinases which together can degrade collagens types I, II, III, IV and V, proteoglycan, laminin fibronectin and gelatin (Murphy *et al.*, 1981b). Consequently, these enzymes are thought to be involved in the local turnover of the connective-tissue matrix in both physiological and pathological situations.

A number of control mechanisms exist to ensure that uncontrolled breakdown of connective-tissue matrix does not occur. Firstly, the enzymes are secreted in a proenzyme form which requires activation before the substances can be degraded. Secondly, inhibitors of all these enzymes exist which specifically limit their action in the extracellular space.

We described and purified a specific inhibitor of metalloproteinases which we named the 'tissue inhibitor of metalloproteinases' (TIMP) (Cawston *et al.*, 1981). This protein was subsequently purified from amniotic fluid (Murphy *et al.*, 1981a; Welgus & Stricklin, 1983), human skin fibroblast culture medium (Stricklin & Welgus, 1983), bovine cartilage and body fluids (Bunning *et al.*, 1984), human synovial fluid (Mercer *et al.*, 1985), human tendon (Vater *et al.*, 1979) and chick bone (Yasui *et al.*, 1981), and the amino acid sequence of the human amniotic fluid inhibitor has now been published (Docherty *et al.*, 1985). It is likely that all these proteins share a common identity.

Woolley *et al.* (1976) described a plasma collagenase inhibitor which they named 'B₁ anticollagenase'. Later studies by Welgus & Stricklin (1983) suggested that this protein was identical with the TIMP. In the present study we have developed a rapid purification scheme for the TIMP present in human plasma using immunoaffinity chromatography. The purified protein was characterized and compared with previously purified inhibitors.

EXPERIMENTAL

Materials

Chemicals were obtained from the following suppliers: Ultrogel AcA 44 was obtained from LKB, Croydon, Surrey, U.K., *N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodo-

phenyl)propionate (Bolton and Hunter reagent) from The Radiochemical Centre, Amersham, Bucks., U.K., and CNBr-activated Sepharose from Pharmacia, Milton Keynes, Bucks., U.K.

All other chemicals and biochemicals were commercially available analytical-grade reagents or have been previously described (Cawston *et al.*, 1981).

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 μ g of collagen at 37 °C, and one unit of inhibitor is defined as that giving 50% inhibition of two units of collagenase (Murphy *et al.*, 1977).

Protein determination and column chromatography

Column eluates were monitored for protein by measuring *A*₂₈₀. All columns were run at 4 °C and all buffers contained Brij 35 (0.05%) with NaN₃ (0.02%) as preservative.

Iodination of proteins with ¹²⁵I

Protein samples (80 μ l) with 0.1 M-sodium borate buffer, pH 8.5 (20 μ l), were added to tubes containing 50–100 μ Ci 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 prepared as previously described (Cawston *et al.*, 1981). Gradient polyacrylamide slab gels (15 cm $\times$ 14 cm $\times$ 0.15 cm) were used; the lower gel contained 6–30% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide and the spacer gel contained 4.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide. Polymerization, fixing, staining and all other procedures were as previously described (Cawston *et al.*, 1981).

Plasma

Time-expired plasma was obtained from the Blood Products Laboratory, Elstree, Herts. WD6 3BX, U.K.

Abbreviation used: TIMP, tissue inhibitor of metalloproteinases; DMEM, Dulbecco modified Eagle medium.

Preparation of immuno-affinity column

Antibody to human amniotic-fluid TIMP was raised in sheep and characterized (Hembry *et al.*, 1985). The IgG fraction was purified and coupled to CNBr-activated Sepharose according to the manufacturer's instructions.

Agarose electrophoresis

Gels were formed by pouring agarose (1%) dissolved in barbitone buffer, pH 8.8, 56 °C, on to Gel Bond sheets (Miles Laboratories) and 15 μ l wells cut with a well former and the solution allowed to set. Serum or protein samples were diluted 1:1 with 0.1% Bromophenol Blue in barbitone buffer containing glycerol (50%, v/v) and the gel was placed on a LKB Multiphor apparatus and 150 V was applied until the marker dye had moved to the end of the gel plate.

A portion of the gel containing serum standards and human fibroblast TIMP was fixed in 1.17% (v/v) picric acid dissolved in 16.7% (v/v) acetic acid for 15 min. The gel was pressed, dried and stained in Coomassie Brilliant Blue [5%, w/v, dissolved in destaining solution (methanol/acetic acid/water, 4:4:1, by vol.)] for 30 min, then destained and dried.

A second portion of the gel, also containing standards, human fibroblast TIMP and plasma TIMP, was immunofixed by using sheep anti-(human amniotic-fluid TIMP) IgG. Cellulose acetate paper was soaked in sheep anti-TIMP antiserum then laid on to the gel for 30 min in a humidity chamber. The gel was pressed and washed in 0.9% saline (NaCl). After pressing and drying, the gel was stained in Coomassie Brilliant Blue as described above.

Double immunodiffusion was carried out by using standard methods (Ouchterlony & Nilsson, 1978). After being washed, plates were stained in Coomassie Brilliant

Fig. 1. Sepharose immuno-affinity chromatography of plasma TIMP

Plasma (400 ml) was applied to an IgG-Sepharose column (0.8 cm $\times$ 10 cm) after it had passed through a Sepharose 6B column (1.6 cm $\times$ 30 cm). Fractions (2.0 ml) were collected and, after washing the column extensively with 25 mM-sodium cacodylate buffer, pH 7.6, containing 10 mM-CaCl₂, 0.05% Brij 35, 0.02% NaN₃ and 0.5 mM-NaCl, the column was eluted with 50 mM-glycine/HCl buffer, pH 3.0, containing 0.5 M-NaCl. The column eluate was monitored for protein (A_{280} , $\blacklozenge$) and collagenase inhibitor (—). (The inhibitory activity due to α_2 -macroglobulin is not shown.)

Fig. 2. Gel filtration of human plasma TIMP

Pooled fractions from the IgG-Sepharose column were concentrated (2 ml) and applied to an Ultrogel AcA 44 column (1.6 cm $\times$ 87 cm) equilibrated with 25 mM-sodium cacodylate, pH 7.2, containing 0.05% Brij 35, 0.02% NaN₃, 1.0 M-NaCl and 10 mM-CaCl₂. The column was eluted with this buffer at a flow rate of 12 ml/h and the fractions (1.8 ml) monitored for protein ($\blacktriangle$) and collagenase inhibitor ($\bullet$).

Blue [1 mg/ml in ethanol/formic acid/water solvent (1:1:1), by vol.] and destained in solvent alone.

Human fibroblast TIMP

Human foetal lung fibroblasts (FL2002; Flow Laboratories) were grown in culture in DMEM containing 1% acid-treated foetal bovine serum and the fibroblast inhibitor purified essentially by the method of Cawston *et al.* (1981).

RESULTS

In the present study we employed immunoaffinity chromatography as a rapid purification method for TIMP.

Plasma treatment

Time-expired human plasma (400 ml) was thawed and dialysed against 25 mM-sodium cacodylate (pH 7.6)/0.05% Brij 35/10 mM-CaCl₂/0.02% NaN₃/0.5 M-NaCl and then centrifuged at 40 000 *g* for 1 h. The supernatant was passed through a column of Sepharose 6B (1.6 cm $\times$ 30 cm) to remove any plasma protein (which binds non-specifically to Sepharose) and was then allowed to flow through a column (5 cm $\times$ 0.6 cm) of IgG [anti-(human amniotic-fluid TIMP)]-Sepharose immuno-affinity column. Fractions (2 ml) were collected and, after the column had been washed extensively with the above buffer, it was eluted with 50 mM-glycine/HCl buffer (pH 3.0)/0.5 M-NaCl. The elution profile is shown in Fig. 1. Two peaks of collagenase-inhibitory activity were identified. The first, unbound, peak corresponds to the major plasma proteinase inhibitor α_2 -macroglobulin (not shown in Fig. 1) and the second, which bound specifically to the IgG (anti-TIMP)-Sepharose column, corresponds to plasma TIMP.

This peak was pooled, concentrated, and applied to an Ultrogel AcA 44 column (1.6 cm $\times$ 88 cm) equilibrated with 25 mM-sodium cacodylate (pH 7.2)/0.05% Brij/

Table 1. Purification of TIMP from human plasma

Fraction	Protein (mg)	Inhibitor (units)	Specific activity (units/mg)	Purification factor
Plasma	4560	*5040	1.11	1
IgG-Sepharose	264	4000	149	135
Ultrogel AcA 44	0.16	760	4750	4279

* Treated with methylamine.

0.02% NaN_3 /1 M-NaCl and eluted at 12.0 ml/h. The fractions (1.8 ml) were assayed for collagenase inhibitor activity and the elution profile is shown in Fig. 2. A single peak of inhibitory activity was eluted at a position corresponding to an estimated M_r 30 500. However, insufficient protein was present in the column fractions to be detected by measuring the A_{280} . The fractions containing inhibitory activity were pooled and concentrated. The details of the purification are given in Table 1, which shows that 160 μg of TIMP was purified from plasma (400 ml) and that the final specific activity was 4750 units/mg, giving a purification factor of 4280-fold. It is difficult to determine the exact content of plasma inhibitor, since α_2 -macroglobulin causes considerable interference. However, in the present study we treated plasma with methylamine and destroyed α_2 -macroglobulin, and a value of approx. 5040 units of inhibitor was calculated for the plasma sample.

SDS/polyacrylamide-gel electrophoresis

Samples of the IgG-Sepharose column pool and the AcA 44 column pool were iodinated with Bolton and Hunter reagent and applied to an SDS/polyacrylamide gel to determine the purity of the isolated protein. Several protein bands were identified after fixing, staining and autoradiography of the gel in both samples. The IgG-Sepharose column pool contained predominantly a protein band of M_r 27 500, but also 53 000- and 82 000- M_r proteins, with other minor proteins present (Fig. 3). The gel-filtration pool contained low levels of a 28 000- M_r protein and proteins of M_r 56 000 and 70 000 (Fig. 3), even though these latter proteins had been eluted from the gel-filtration column at a position corresponding to an apparent M_r of 30 000 (Fig. 2).

Interaction with collagenase

Purified pig collagenase (8 μg) was iodinated and chromatographed on Ultrogel AcA 44 as previously described (Cawston *et al.*, 1983). The 30 000- M_r peak was pooled, and one-twentieth of this pool was reappplied to the column in both the presence or absence of the purified plasma TIMP and the fractions counted for ^{125}I radioactivity in a counter (Fig. 4). When no inhibitor was present the enzyme was eluted with an apparent M_r of 30 000, whereas a higher- M_r (55 000) enzyme-inhibitor complex was formed when the purified plasma inhibitor was present. These results indicate that the plasma TIMP binds to collagenase in a fashion identical with that previously described for the rabbit bone inhibitor (Cawston *et al.*, 1983) and subsequently shown for the human fibroblast inhibitor (T. E. Cawston, unpublished work).

Fig. 3. SDS/polyacrylamide-gel electrophoresis of samples from the plasma TIMP purification

Protein samples of the IgG-Sepharose column pool (b) and the Ultrogel AcA 44 column pool (a) were labelled with ^{125}I 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 12 h.

Immunological identity with human fibroblast inhibitor

Antiserum raised in sheep to human amniotic-fluid TIMP (Hembry *et al.*, 1985) was used to establish the co-identity of the plasma TIMP with human fibroblast inhibitor. Alternate wells were filled with purified plasma TIMP (1) and purified fibroblast inhibitor (2), and the centre well filled with IgG [anti-(human TIMP)]. Fig. 5

Fig. 4. Gel filtration of ^{125}I -labelled collagenase with and without plasma inhibitor

A sample of ^{125}I -labelled pig collagenase (0.8 mg) was divided into two equal portions. One was mixed with plasma inhibitor and immediately loaded on to a column of Ultrogel AcA 44 (1.6 cm $\times$ 88 cm) equilibrated with 25 mM-sodium cacodylate buffer containing 1 M-NaCl. The column was eluted with this buffer at a flow rate of 12 ml/h. Fractions (1.8 ml) were collected and counted for ^{125}I radioactivity. The other portion was subsequently loaded on to the same column and eluted in an identical manner. The elution profile is shown for collagenase chromatographed with (—) and without (---) inhibitor.

shows that a line of identity was present, clearly indicating that the plasma TIMP was immunologically identical with the fibroblast and amniotic-fluid inhibitor.

Mobility of plasma TIMP on agarose electrophoresis

Samples of fibroblast and plasma TIMP were applied to agarose gels, pH 8.5, and a voltage of 150 V was applied. Fig. 6 shows that, after staining, the fibroblast TIMP moves with a mobility corresponding to the serum proteins (track a). However, insufficient protein was present in the plasma TIMP sample to be detected by staining (track c). Immunofixation of the gels after electrophoresis (tracks b and d) confirmed the presence of plasma TIMP and fibroblast TIMP in the γ -serum-protein region.

DISCUSSION

A collagenase inhibitor separate from α_2 -macroglobulin was first detected in human serum by Woolley *et al.* (1976), who partially purified and characterized the protein which they named 'B₁ anticollagenase,' since it moved with B₁ serum proteins on agarose electrophoresis. It inhibited collagenases purified from several human tissues. Serine, thiol and aspartate proteinases were not inhibited, and the bacterial metalloproteinases, clostridial collagenase and thermolysin were not inhibited.

Fig. 5. Double immunodiffusion of plasma TIMP and human fibroblast TIMP against sheep anti-(human TIMP) IgG

The centre well was filled with sheep anti-(human TIMP) IgG and alternate outer wells were filled with purified human plasma TIMP (1) and purified human fibroblast TIMP (2). The plates were incubated for 2 days at 4 °C and then washed and stained. A line of identity was present, clearly indicating that the plasma TIMP was immunologically identical with the fibroblast TIMP.

A later-improved purification scheme was published (Mooney & Woolley, 1983), and the purified inhibitor was shown to be a cationic glycoprotein of M_r 40000 (by gel filtration) and of M_r 33000 by SDS/polyacrylamide-gel electrophoresis (Woolley *et al.*, 1980).

Subsequently other groups have reported that serum contains a collagenase inhibitor distinct from α_2 -macroglobulin. Welgus *et al.* (1983) and Cooper *et al.* (1985) used a specific antibody to human skin fibroblast TIMP in an enzyme-linked immunosorbent assay and identified immunoreactive material in human serum ($1.03 \pm 0.27 \mu\text{g/ml}$) and human plasma ($0.48 \pm 0.16 \mu\text{g/ml}$) whilst human amniotic fluid ($3.88 \pm 1.36 \mu\text{g/ml}$) contained higher levels.

Stricklin & Welgus (1983) used the human-skin-fibroblast-inhibitor purification procedure to partially purify an inhibitor from human serum that appeared to be identical in size with the fibroblast inhibitor in terms of its size by gel filtration, its behaviour on ion-exchange columns and its immunological co-identity. They suggested that this protein was identical with the human

Fig. 6. Mobility of human plasma TIMP on agarose electrophoresis

Agarose gels were poured and run as described in the Experimental section. Fibroblast TIMP moved with a mobility corresponding to the γ -serum proteins. Purified plasma TIMP could not be detected after electrophoresis and staining (c), but was detected in the serum protein region after immunofixation (d) with anti-(human TIMP) antibody. Human serum was run in the first track (serum). Fibroblast TIMP stained (a) and immunofixed (b), and human plasma TIMP stained (c) and immunofixed (d) were loaded in the remaining tracks.

fibroblast TIMP and was the same protein previously named 'B₁ anticollagenase' by Woolley *et al.* (1976). This conclusion was confirmed by Bunning *et al.* (1984), who showed that bovine cells in culture produced a protein identical with that found in bovine serum and amniotic fluid.

In the present study we have developed a rapid and specific purification method for TIMP from human plasma. One of the problems we encountered was to find a way to isolate plasma TIMP in the presence of such large amounts of α_2 -macroglobulin. The immuno-affinity column achieved this, but only small amounts of protein were recovered from the plasma. In addition, the protein once recovered was relatively unstable and this resembled the TIMP recently purified from human synovial fluid (Mercer *et al.*, 1985). Multiple bands on SDS/polyacrylamide gels were also seen in the preparation. After gel filtration of the plasma TIMP a peak of inhibitory activity was eluted at a position that corresponded with an apparent M_r of 30000, but by the time this peak was concentrated and loaded on to an SDS/polyacrylamide gel, higher- M_r aggregates had formed (M_r 56000 and 70000) and only minor traces of the 27500- M_r band remained, even though large amounts of this were present in the original IgG-Sepharose column inhibitor pool. This behaviour was very similar to that of the human synovial-fluid TIMP (Mercer *et al.*, 1985), which also formed higher- M_r aggregates.

An interesting observation in the present study concerns the behaviour of the purified TIMPs on

agarose-gel electrophoresis. Both the fibroblast and plasma protein move as γ -serum-protein components. This conflicts with the previous description by Woolley *et al.* (1976) of B₁ anticollagenase as a B₁ serum component. We would confirm that TIMP is identical with B₁ anticollagenase, since we have shown immunological identity between human amniotic-fluid TIMP, fibroblast TIMP and B₁ anticollagenase purified by using the revised method of Mooney & Woolley (1983) (G. Murphy, D. E. Woolley & T. E. Cawston, unpublished work). It is possible that differences exist in the extent of glycosylation which could alter the mobility on agarose electrophoresis and that different purification methods select different populations of glycosylated proteins. The recent studies of Murphy *et al.* (1985) clearly show that the carbohydrate is not essential for the inhibition of the metalloproteinases, and so it would appear that classification of these inhibitors on the basis of their mobility on agarose electrophoretic gels is unreliable.

A recent series of papers by McCartney & Tschesche (1983a,b,c) have described the purification and properties of an inhibitor of leucocyte collagenase both from polymorphonuclear leucocytes and from human plasma by the method of Woolley *et al.* (1976). Both the leucocytes and their plasma inhibitor have a free thiol group, which they report is necessary for inhibition of the leucocyte enzyme to take place. Both inhibitors can bind to leucocyte collagenase and other mammalian collagenases and can then be subsequently re-activated by reaction with cystine or other disulphide-bonded molecules.

Although these proteins described by McCartney & Tschesche (1983a,b,c) are of similar size to the TIMP molecules, a number of differences exist. They have an inhibition mechanism different from that shown with TIMP-collagenase complexes, they have a free thiol group and a different compositional analysis (see Stricklin & Welgus, 1983). McCartney & Tschesche (1983b) claim that the leucocyte inhibitor is distinct from the plasma inhibitor that they purified and suggest that the latter is identical with B₁ anticollagenase. However, in view of the reported differences between these studies it is possible that, in addition to α_2 -macroglobulin and TIMP/B₁ anticollagenase, a further collagenase inhibitor is present in human plasma.

The origin of the TIMP in human plasma is not certain, but we consider it likely that, since this protein is synthesized by a wide range of connective-tissue cells, its presence in plasma represents a non-specific removal of the inhibitor from connective tissues rather than its specific synthesis as a plasma component.

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