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An inhibitor of collagenase from human amniotic fluid

Purification, characterization and action on metalloproteinases

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1. An inhibitor of collagenase of apparent mol.wt. 28000 was isolated from term human amniotic fluid. 2. It is active against mammalian collagenases from a number of species and tissues as well as other mammalian metalloproteinases, but has no activity against bacterial metalloproteinases. 3. Activity is destroyed by treatment with either trypsin or 4-aminophenylmercuric acetate, by heat, and by reduction and carboxymethylation. 4. All the properties observed suggest that it is similar to the synthesized tissue inhibitor of metalloproteinases.

It has been proposed that all connective tissues synthesize collagenase inhibitors to control the local activity of the enzyme (Reynolds *et al.*, 1977; Murphy & Sellers, 1980). Workers in a number of other laboratories have also described such inhibitors in tissue-culture media (Vater *et al.*, 1978; Welgus *et al.*, 1979; Kerwar *et al.*, 1980). Further to this, Sellers *et al.* (1979) have shown that the bone-derived collagenase inhibitors inhibit other metalloproteinases produced by connective tissues in culture. Collagenase inhibitors from human and dog serum have been isolated and characterized by Woolley *et al.* (1976, 1978) but the relationship between these and the tissue-synthesized inhibitors is not yet known. In the present paper we report the presence of a collagenase inhibitor in human amniotic fluid, and on the basis of its behaviour during purification and its subsequent characterization, we compare it with the purified inhibitor ('TIMP') synthesized by rabbit bones in culture and described in the preceding paper (Cawston *et al.*, 1981).

Materials and methods

Materials

All chemicals and biochemicals were described in the preceding paper (Cawston *et al.*, 1981).

Methods

Inhibitor purification. This was performed by the method of Cawston *et al.* (1981), with crude rabbit skin collagenase activated by treatment with 4-aminophenylmercuric acetate (Sellers *et al.*, 1978) as a monitor of inhibitory activity. The subsequent ^{125}I labelling with Bolton & Hunter reagent and SDS/polyacrylamide-gel-electrophoresis techniques were described in the preceding paper (Cawston *et al.*, 1981).

Assays. Collagenase and gelatinase assays were performed as described previously (Sellers *et al.*, 1978, 1979). For comparative purposes the diffuse-fibril assay for collagenase (Cawston & Barrett, 1979) was also used for the final assessment of the purified inhibitor activity (Table 1 below). Neutral metalloproteinase III, thermolysin, plasmin and trypsin were assayed by using either gelatin or [^{14}C]acetylated casein as a substrate. Casein was acetylated with [1-^{14}C]acetic anhydride as described by Cawston *et al.* (1981). Inhibitor activity was assessed either by inclusion with the enzyme in the assay for 15–20h incubations, or by 1h preincubation at 37°C with the enzyme in 1–2h assays.

Sources of enzyme. Purified rabbit bone collagenase and pig synovial collagenase were prepared by the method of Cawston & Tyler (1979), with the respective culture media as starting material. Rabbit skin and human rheumatoid-synovial collagenase were peaks of activity from the fractionation of culture media with Ultrogel AcA44 (Sellers *et al.*, 1978), as were the tissue gelatinases and metalloproteinase III (caseinase). Human leucocyte collagenase and gelatinase were prepared by using the protocol described by Cawston & Tyler (1979). All

Abbreviation used: SDS, sodium dodecyl sulphate.

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Table 1. *Purification of an inhibitor of collagenase from human amniotic fluid*

The purification was performed as described under 'Methods' and by Cawston *et al.* (1981). Inhibitory activity was monitored by using rabbit skin collagenase in the conventional fibril-assay system. Values in parentheses are those obtained by using the diffuse-fibril assay (see 'Methods').

	Total inhibitor (units)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Amniotic fluid [0–90%-satd.-$(NH_4)_2SO_4$ precipitation]	8875	(100)	2	—
Ultrogel AcA 44 eluate	7145	81	227	112
Concanavalin A–Sepharose	3367	38	267	131
Heparin–Sepharose	2270	26	5044	2485
DEAE-Sepharose	2920	33	8343	4111
Zn^{2+}–iminodiacetic acid–Sepharose	1339 (3600)	15	7084 (19000)	3490

activities were activated with 4-aminophenylmercuric acetate (Sellers *et al.*, 1978) after the gel-filtration step and exhaustively dialysed.

Inactivation of inhibitor. Inhibitor was treated at a concentratin of 15 μg of protein/ml in 50 mM-Tris/HCl, pH 7.5, containing 200 mM-NaCl, 5 mM-$CaCl_2$, 0.05% (w/v) Brij 35 and 0.02% (w/v) NaN_3 with trypsin and 4-aminophenylmercuric acetate under various conditions. Trypsin activity was terminated with a fivefold (w/w) excess of soya-bean trypsin inhibitor. The conditions for reduction and alkylation are given in Table 3 below; the inhibitor was exhaustively dialysed against the above buffer before assay. Heat treatment was performed at the same concentration and in the same buffer as described for trypsin treatment.

Results

The purification of the collagenase-inhibitory activity of amniotic fluid could be effected by using the protocol for the rabbit bone collagenase inhibitor derived from tissue culture, as described by Cawston *et al.* (1981) (present Table 1). Most of the activity bound to concanavalin A–Sepharose and was eluted with 5% (w/v) α-D-methyl mannoside, indicating that it is a glycoprotein. Subsequent purification on heparin–Sepharose, DEAE-Sepharose and Zn^{2+}–iminodiacetic acid–Sepharose gave a preparation of specific activity 19000 units/mg of protein. This compares with a value of 26000 units/mg for the rabbit bone inhibitor. The inhibitor was labelled with ^{125}I by using the Bolton & Hunter reagent and subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 1). It ran as a single band of apparent mol.wt. 28000, as did the rabbit bone inhibitor. On Ultrogel AcA 44 filtration the amniotic-fluid inhibitor was eluted as a single peak corresponding to an apparent mol.wt. of 33000.

Between 10 and 40% of the original amniotic-fluid inhibitory activity (after the first-gel filtration step; Table 1) did not bind to concanavalin A–Sepharose, nor did it bind on subsequent reapplication. This inhibitory activity behaved identically with the concanavalin A-binding inhibitor on subsequent purification with the matrices described in Table 1. The final preparation had a specific activity of 7000 units/mg and was not pure when analysed by SDS/polyacrylamide-gel electrophoresis. This was expected, since it was associated with a large number of other proteins that did not bind to concanavalin A–Sepharose. Subsequent characterization showed that the non-binding inhibitor had properties essentially similar to those of the purified concanavalin

Table 2. *Activity of the concanavalin A-binding inhibitor from amniotic fluid against mammalian metalloproteinases*

The effect of the purified inhibitor on various metalloproteinases was studied as described under 'Methods'. (C) denotes that the enzyme was only partially purified from culture media, and (P) that it was more rigorously purified, as outlined under 'Methods'. The results are expressed as units of inhibitor/ml, where 1 unit of inhibitor gives 50% inhibition of 2 units of enzyme.

Proteinase	Activity (units/ml)
Collagenases	
Rabbit skin (C)	219
Rabbit bone (P)	189
Pig synovial (P)	240
Human rheumatoid synovial (C)	32
Human leucocyte (P)	5
Gelatinases	
Rabbit skin (C)	91
Rabbit bone (C)	28
Human rheumatoid synovial (C)	13
Human leucocyte (P)	92
Metalloproteinase III	
Rabbit bone (P) (casein as substrate)	50
Pig synovial (P)	44
Human rheumatoid synovial caseinase (C)	10

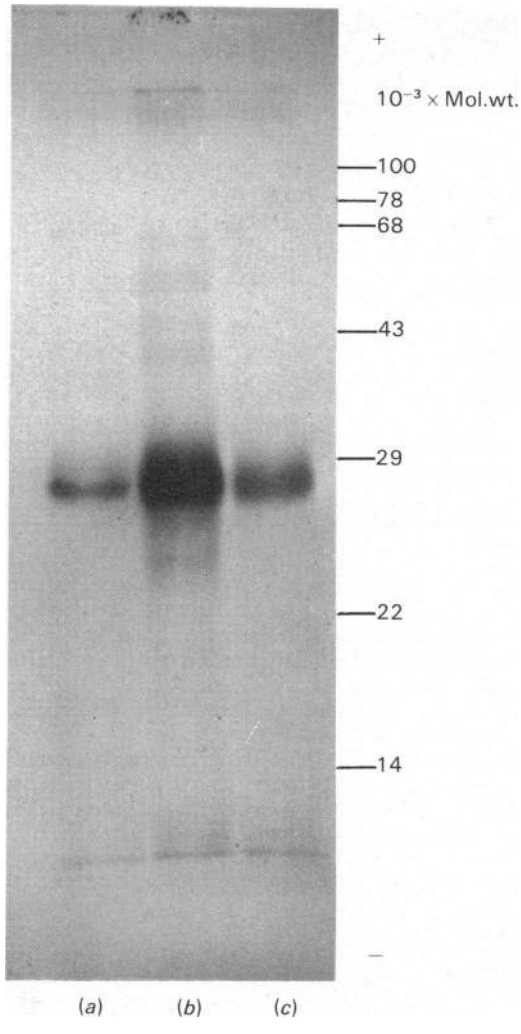

Fig. 1. *Radioautograph of SDS/polyacrylamide-gel electrophoresis of ^{125}I-labelled human amniotic-fluid inhibitor and rabbit bone inhibitor*

Samples of (*a*) rabbit bone inhibitor (64ng), (*b*) human amniotic-fluid inhibitor, DEAE-Sepharose eluate (320ng), and (*c*) human amniotic-fluid inhibitor, Zn-chelate–Sepharose eluate (200ng), were labelled with ^{125}I by using Bolton & Hunter reagent, electrophoresed through an SDS/12% (w/v)-Polyacrylamide gel under the conditions described by Laemmli & Favre (1973) and radioautographed.

A-binding activity in terms of its ability to inhibit metalloproteinases. It was, however, more stable to heat and trypsin or 4-aminophenylmercuric acetate treatment, probably owing to the presence of other proteins (results not shown).

The inhibitory activity of the amniotic fluid was routinely monitored with rabbit skin collagenase, as described under 'Methods'. The activities of the purified major inhibitor on different preparations of collagenase are given in Table 2. It appeared to act most efficiently against rabbit or pig tissue enzymes and was less active against the human synovial collagenase preparation available. It is possible that this reflects the amount of catalytically inactive collagenase in human tissue-culture media. The amniotic-fluid inhibitor acted only weakly against human leucocyte collagenase, in a manner analogous to both the rabbit bone inhibitor and β_1-anticollagenase (Woolley *et al.*, 1978).

The fully purified amniotic-fluid inhibitor was active against other mammalian metalloproteinases, as shown in Table 2. However, it had no activity against either bacterial collagenase (with collagen as substrate) or thermolysin (either gelatin or casein as substrates). It also had no activity against serine proteinases such as plasmin and trypsin.

Table 3 shows the destructive effect of both trypsin and 4-aminophenylmercuric acetate treatment on the purified inhibitor. Reduction and alkylation also removed the inhibitory activity. The inhibitor was relatively heat-stable, activity being destroyed after 30min at 90°C.

Discussion

The presence of collagenase inhibitors in connective tissues has been described by workers in a number of laboratories (Reynolds *et al.*, 1977; Nolan *et al.*, 1978; Woolley *et al.*, 1978; Roughley *et al.*, 1978; Vater *et al.*, 1979; Welgus *et al.*, 1979), indicating that mechanisms for the control of extracellular enzyme activity are an important feature in determining rates of collagen degradation. Of most apparent significance are the inhibitors produced by connective tissues in culture (Reynolds *et al.*, 1977), although these inhibitors do not appear to be related to those extracted from tissue (Roughley *et al.*, 1978). The finding that amniotic fluid contains a collagenase inhibitor with similar size and charge, i.e. similar behaviour on purification, to a synthesized inhibitor from rabbit bone cultures suggests that there is a relationship between inhibitors produced *in vitro* and those found *in vivo*. Further studies are required to characterize both inhibitors more fully, in terms of their interaction with collagenase and other metalloproteinases. We have also found that rabbit amniotic fluid, human cerebrospinal fluid and human synovial fluid contain collagenase inhibitors. Although the precise role of these inhibitors is uncertain, their occurrence *in vivo* suggests that they play a part in the regulation of metalloproteinase activity. It is likely that the second inhibitor found in human amniotic fluid, which did not bind to concanavalin A–Sepharose, was the same as the binding activity, but had lost

Table 3. *Effect of various treatments on the activity of human amniotic-fluid inhibitor of collagenase*
The effect of trypsin, 4-aminophenylmercuric acetate, reduction and alkylation, and heat, on the activity of the final preparations of purified concanavalin A-binding inhibitor were studied as described under 'Methods'. The results are expressed as percentage inactivations relative to inhibitor held under the same incubation conditions (A, B, C) or untreated material (D).

Effector	Conditions of treatment	Collagenase-inhibitor inactivation (%)
(A) Trypsin	10 μg/ml, 37°C, 30 min	60
	100 μg/ml, 37°C, 30 min	97
(B) 4-Aminophenylmercuric acetate	1 mM, 37°C, 3 h	100
(C) 2-Mercaptoethanol (10 mM) and iodoacetic acid (20 mM)	4°C, 16 h 20°C, 1 h	100
(D) Heat	50°C, 30 min	15
	60°C, 30 min	21
	70°C, 30 min	47
	80°C, 30 min	69
	90°C, 30 min	100

mannose or glucose residues by enzymic processing in the amniotic cavity.

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