Purification and characterization of a connective-tissue-degrading metalloproteinase from the cytosol of metastatic melanoma cells

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A metalloproteinase with activity against type IV collagen, type I collagen and gelatin has been purified from the cytosol of a highly metastatic mouse melanoma by anion-exchange, zinc-chelated and lectin-affinity column chromatography. The purified enzyme has a molecular mass of approx. 59 kDa and on isoelectric focusing in two-dimensional gels produced three spots with apparent isoelectric points (pI) between 5.7 and 6.1. Enzymic activity with collagen, but not gelatin, substrates was latent, requiring activation by trypsin or organomercurials. Trypsin activation of this metalloproteinase was accompanied by a change in molecular mass, whereas autoactivation after 1 month's storage, was not. The degradation of types I and IV collagen by the melanoma enzyme yielded products of lower molecular masses than those yielded by mammalian collagenases, this characteristic thus differentiating this metalloproteinase from classical collagenases.

INTRODUCTION

Tumour-cell proteinases have been implicated in the pathophysiology of cancer invasion and metastasis (for a review, see Liotta *et al.*, 1983; Nicolson, 1982). Since collagen is the predominant connective-tissue protein through which invading tumour cells must penetrate, there has been considerable interest in identifying collagenolytic proteinases released by tumour cells (McCroskery *et al.*, 1975; Hashimoto *et al.*, 1973). Cancer cells, as well as inflammatory cells and fibroblasts surrounding tumours, have been shown to release different types of collagenases with specificity for either interstitial collagens types I–III or basement-membrane type IV collagen (Liotta *et al.*, 1983; Cawston & Murphy, 1981).

In mammalian cells, with the exception of polymorphonuclear leucocytes, collagenase is not stored intracellularly, but is rapidly secreted after biosynthesis and post-translational processing (Nagase *et al.*, 1983). In contrast, using an assay which is quite sensitive to collagen and gelatin degradation, we have found that highly metastatic mouse B16 melanoma cells have considerable intracellular collagenolytic and gelatinolytic activity, with enzyme enrichment in the plasma membrane (Zucker *et al.*, 1985). Here we describe the purification and characterization of this connective-tissue-degrading enzyme isolated from the cytosol of tumour cells which is able to attack both native types I and IV collagen as well as gelatin.

EXPERIMENTAL PROCEDURES

Materials

Materials were purchased from the following sources: Acrylamide and bisacrylamide, SDS, ammonium persulphate, Coomassie Brilliant Blue-250, Silver Stain Kit were from Bio-Rad; Tris base, Tos-Lys-CH₂Cl treated-trypsin, leupeptin, SBTI, 4-aminophenylmercuric acetate, Tos-Arg-Ome, Bz-Tyr-OEt, azocasein, Suc-(Ala)₃-Nan, Tos-Lys-CH₂Cl, Brij 35, dithiothreitol, iPr₂P-F, Z-Phe-Arg-NHMec, Nonidet and N-ethylmaleimide were from Sigma; Peptide P substrate was from Calbiochem; Ampholines were from Serva. All other chemicals used were of reagent grade.

Preparation and purification of a tumour connective-tissue-degrading enzyme

Intact B16-BL6 melanoma cells were obtained from intraperitoneal tumours 2 weeks after transplantation of 2 × 10⁶ cells into C57BL/6 mice as previously described (Zucker *et al.*, 1985). The tumour was almost totally cellular and was easily stripped from the peritoneum. Tumour-cell suspensions were prepared by mincing the tissue and sieving it through a wire mesh. Macrophage, fibroblast and red-cell contamination amounted to less than 1%, and no collagen fibrils were noted on electron-microscopic examination. All subsequent steps were carried out at 4 °C. Tumour cells, washed repeatedly by centrifugation, were subjected to nitrogen cavitation at 2.4 MPa (350 lbf/in²) for 20 min, followed

Abbreviations used: Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK'); SBTI, soybean trypsin inhibitor; iPr₂P-F, di-isopropyl fluorophosphate; N₂ph, 2,4-dinitrophenyl; Peptide P substrate, N₂ph-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH; APMA, 4-aminophenylmercuric acetate; PAGE, polyacrylamide-gel electrophoresis; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; Z-Phe-Arg-NHMec, benzoyloxycarbonylphenylalanyl-L-arginine-7-(4-methyl)coumarylamide; Suc-(Ala)₃-Nan, 3-carboxypropionyltrialanine *p*-nitroanilide.

by centrifugation at 770 *g* to remove nuclei and intact cells. The supernatant was then centrifuged at 100 000 *g* for 1 h, and the pellet containing the crude organelles and membranes was set aside.

The supernatant, designated the 'cytosol', was then subjected to purification for metalloproteinases. $(\text{NH}_4)_2\text{SO}_4$ was added to a final saturation of 25%, and after 1 h the precipitate was collected by centrifugation (13 000 *g* for 30 min). Additional $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to bring saturation to 60%, and the 25–60% -satn. precipitate was redissolved in 5–10 ml of 25 mM-sodium cacodylate buffer, pH 7.2, containing 5 mM- CaCl_2 and 0.05% (v/v) Brij 35, dialysed exhaustively against this buffer and clarified by centrifugation.

The 25–60% $(\text{NH}_4)_2\text{SO}_4$ -satn. enzyme-rich preparation was applied to a column (1.6 cm $\times$ 17 cm) of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and equilibrated with the same buffer. After application of the sample, the cacodylate buffer, pH 7.2, was applied in order to return the absorbance to the baseline value and a 300 ml linear gradient of 0–0.8 M- NaCl in 25 mM-sodium cacodylate/5 mM- CaCl_2 /0.05% Brij 35, pH 7.2, was used to elute the bound proteins. The protein absorbance was measured at 280 nm, and 4 ml fractions were assayed for enzymic activity.

Fractions rich in gelatinolytic and latent collagenolytic activity were combined, dialysed against 25 mM-sodium borate buffer, pH 8.0, containing 150 mM- NaCl , concentrated by ultrafiltration through an Amicon YM-10 membrane and applied to an epoxy-activated Sepharose 6B zinc-chelated column (1.6 cm $\times$ 6.5 cm) prepared and charged with ZnCl_2 (3 mg/ml) according to the manufacturer instructions (Pharmacia Fine Chemicals). The samples were loaded and washed on to the column with borate buffer and then eluted in a stepwise manner with other buffers as described by Cawston & Murphy (1981). Fractions were immediately neutralized to pH 7, dialysed against the Tris buffer, pH 7.8, concentrated by ultrafiltration and kept at 4 °C for assay. Storage at –20 °C or –80 °C resulted in progressive loss of enzymic activity.

The active fractions were combined and passed through a concanavalin A-Sepharose (Pharmacia) column (1 cm $\times$ 6 cm) equilibrated with the enzyme buffer and eluted with 1.0 M- α -methyl D-mannoside as previously described (Liotta *et al.*, 1981).

SDS/PAGE

A discontinuous system for SDS/PAGE was employed using the gel buffer and sample preparation system described by Laemmli (1970) on a slab-gel apparatus. A 12.5% (w/v)-polyacrylamide/SDS separating gel and a 5% -acrylamide stacking gel were used. Reduction of disulphide linkages was carried out by the incorporation of 2.5% (v/v) β -mercaptoethanol into the sample solution. Protein M_r standards (Bio-Rad) were run concurrently. Approximate M_r values for sample proteins were determined by plotting the relative mobilities of the known proteins by the method of Weber & Osborn (1969). Gels were serially stained with Coomassie Blue and silver stains (Merril *et al.*, 1979).

Two-dimensional gel-electrophoresis was performed in an ISODALT II apparatus as described by Anderson & Anderson (1978). Purified cytosol connective-tissue-

degrading enzyme was dissolved in sample buffer containing 8 M-urea, 0.1% Nonidet and 2.5% β -mercaptoethanol, including a carbamoylation train of creatine phosphokinase (Anderson & Hickman, 1979) as an internal isofocusing standard. The first-dimension isoelectric focusing electrophoresis was carried out by using pH 3.5–11 Ampholines and the second-dimension electrophoresis was on a 10%-polyacrylamide slab gel, with a Laemmli (1970) buffer system and M_r standards. Because of the incorporation of urea into the first-dimension gel, isofocusing points are referred to as 'apparent pI' (Malamud & Drysdale, 1978).

Assays for proteinase activity

The degradation of collagen was measured by using soluble lathyritic-rat skin type I collagen labelled *in vitro* with [^3H]formaldehyde as described by Bhatnagar & Decker (1981). All samples were run in duplicate. Latent collagenase was activated by either (a) 5 min pre-incubation with 25 μg of Tos-Phe- CH_2Cl -treated trypsin, followed by neutralization with a 5-fold excess of SBT, or (b) 2 h pretreatment with 0.7 mM-APMA. The activated enzyme was then incubated with 20 μg of [methyl- ^3H]collagen substrate for 18 h at 27 °C, and collagenolytic activity was determined as previously described (Zucker *et al.*, 1985). Enzyme activity was expressed as the degradation (μg) of [methyl- ^3H]collagen.

Fluorography was performed on identically prepared samples as follows: after incubation at 22 °C (rather than 27 °C), samples were thermally denatured at 60 °C for 30 min, after which collagen subunits and degradation products were separated by SDS/PAGE. The gels were processed for fluorography and exposed to Kodak XAR-5 film for 1–2 days as described by Sodek *et al.* (1981).

The suitability of the [^3H]methylated type I collagen as a substrate in the collagenase assay was assessed by measuring the conformation of formaldehyde-treated collagen and random-coiled gelatin substrates using c.d. according to a modification (Zucker *et al.*, 1987) of the method of Piez & Sherman (1970). The helical form of collagen at 4, 24 and 29 °C showed a positive band at 221 nm, with a $[\theta]$ of 6700 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. Spectra of the random-coiled form (heated to 75 °C for 20 min) studied at 40 °C showed a small negative plateau at about 225 nm with a $[\theta]$ of –1420 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. These data confirm the native triple-helical configuration of the type I collagen substrate.

Degradation of thermally reconstituted 'in-vivo' glycine- ^{14}C -labelled type I collagen fibrils was performed as described by Ramamurthy & Golub (1983). Acid-soluble [glycine- ^{14}C]collagen (200 μg ; 15000 d.p.m. per assay) was incubated at 35 °C for 6 h to allow collagen fibrils to form. Trypsin-activated enzyme was incubated with the collagen fibrils for 3, 6, 12, and 24 h at 35 °C, at which times collagen degradation was assayed as described above. One unit of enzyme activity reflected the digestion of 1 μg of collagen/min. The trypsin/SBTI blank produced 3% digestion of the substrate in 24 h.

Assay for type IV collagenolytic activity was performed with proline- ^3H -labelled type IV procollagen at 35 °C as described by Liotta *et al.* (1981).

Gelatinolytic activity was assessed during 2 h incubation at 37 °C using a methyl- ^3H -labelled type I collagen substrate (20 μg /sample) heated to 45 °C for 20 min by

Table 1. Purification of collagen-type-I-degrading enzyme from the cytosol of B16-BL6 melanoma cells

Collagenolytic activity was assayed by using soluble [*methyl*-³H]collagen type I as substrate. Samples were pre-activated with trypsin and then treated with SBTI.

Sample	Total protein (mg)	Total activity (μg of collagen degraded)	Recovery (%)	Specific activity (μg degraded/mg of protein)	Purification (fold)
Crude supernatant	105.0	46.5	100	0.4	1
25–60% (NH ₄) ₂ SO ₄	20.6	47.4	102	2.3	5
DEAE-Sephadex active fractions	8.9	114.0	246	12.8	29
Zinc-chelated column eluted by:					
Cacodylate buffer, pH 6.5	1.4	38.0	82	28.1	64
Acetate buffer, pH 4.7	0.7	23.4	50	34.4	78
Concanavalin A-Sepharose	0.9	82.5	177	90.5	206

a modification (Zucker *et al.*, 1985) of the method of Harris & Krane (1972). No activation of enzyme was required to demonstrate activity. One unit of gelatinolytic activity reflected the digestion of 1 μg of gelatin/min at 37 °C.

The pH-dependence of enzyme action was studied over the pH range 5.0–9.0, 25 mM-sodium cacodylate and Tris/HCl buffers being used.

To characterize the class of proteinase responsible for substrate degradation, inhibitors were pre-incubated with activated enzyme preparations for 2 h at 22 °C before initiating the proteinase assays. Final drug concentrations in the assay mixture are listed.

The synthetic Peptide P substrate, which possesses a peptide sequence similar to that in the region of the vertebrate-collagenase-sensitive Gly-Ile bond in the α1 chain of type I collagen, was used as a sensitive screening assay for connective-tissue-degrading enzymes as described by Masui *et al.* (1977). Trypsin- and chymotrypsin-like proteolytic activities were assayed using the synthetic substrates, Tos-Arg-OMe and Bz-Tyr-OEt respectively, as previously described (Zucker *et al.*, 1985). Hydrolysis of azocasein during an 18 h incubation was assayed by a standard procedure (Zucker *et al.*, 1985). Elastase activity was measured by using the synthetic peptide substrate Suc-(Ala)₃- (Bieth *et al.*, 1974). Cysteine proteinase activity was determined using the sensitive substrate Bz-Phe-Arg-NHMec (Zucker *et al.*, 1985).

Partial purification and characterization of human neutrophil gelatinase and collagenase

Human neutrophils were isolated from peripheral blood and disrupted by nitrogen cavitation with recovery of the postnuclear granular pellet. Collagenase and gelatinase were separated by Ultrogel AcA44 filtration chromatography and further purified by Mono Q (Pharmacia) anion-exchange chromatography by a modification of the methods described by Sopata & Dancewicz (1974) and Murphy *et al.* (1980). Using these enzymes we were able to demonstrate that the [*methyl*-³H]collagen substrate employed was not attacked by neutrophil gelatinase and the gelatin substrate was likewise resistant to digestion by 'classical' neutrophil collagenase.

Miscellaneous

Protein determinations were made by the method of Markwell *et al.* (1978), with bovine serum albumin as a standard.

H.p.l.c. on a TS 3000 SW column was performed at ambient temperatures as previously described (LaBombardi *et al.*, 1983).

RESULTS

Melanoma metalloproteinase

Cell fractions obtained from melanoma cells isolated from intraperitoneal tumours had specific collagenolytic and gelatinolytic activities comparable with those from tumour cells cultivated in plastic dishes (results not shown). On the basis of the higher cell yield and lesser expense, enzyme purification was carried out by using tumours propagated *in vivo*. B16-BL6 melanoma connective-tissue-degrading enzyme was purified from the tumour cytosol with total enzyme recovery and enrichment as shown in Table 1. Recovery of collagenolytic and gelatinolytic activity greater than 100% probably reflects the removal of enzyme inhibitors by the chromatographic procedures. The presence of Ca²⁺ in the collagenase assay was an absolute requirement to demonstrate enzyme activity. Material enriched in collagenolytic and gelatinolytic activity could be precipitated between 25 and 60% saturation of (NH₄)₂SO₄. Collagenolytic activity without trypsin or APMA activation represented 0–20% of the activity noted after activation.

Anion-exchange chromatography with DEAE-Sephadex was an essential step in the purification of the melanoma connective-tissue-degrading enzyme. A protein peak eluted at a conductivity of 22 mmho contained most of the collagenolytic and gelatinolytic activity (Fig. 1). A 5-fold purification of collagenolytic activity was achieved at this step.

These active fractions were dialysed, concentrated, and applied to a zinc-chelated Sepharose column. Elution with 25 mM-sodium cacodylate, pH 6.5, followed by 50 mM-sodium acetate buffer, pH 4.7, produced absorbance peaks which, when activated by trypsin, had a specific collagenolytic activity 2–3-fold higher than the DEAE-Sephadex active fractions. These two active

Fig. 1. DEAE-Sephadex anion-exchange chromatography of the 25–60%-(NH₄)₂SO₄-precipitated B16-BL6 melanoma cytosolic proteins

The precipitated proteins were dissolved in 25 mM-sodium cacodylate buffer, pH 7.2, and applied to a 1.6 cm × 17 cm column of DEAE-Sephadex A-50. A 300 ml linear gradient of 0–0.8 M-NaCl in 25 mM-sodium cacodylate/5 mM-CaCl₂/0.05% Brij 35, pH 7.2, was used to elute the bound proteins. To exclude the affects of cysteine proteinase and trypsin-like serine proteinase, samples were pretreated with 50 µg of Tos-Lys-CH₂Cl/ml before the gelatinase assay and after trypsin/SBT1 treatment in the collagenase assay. —, A_{280} ; ●---●, collagenolytic activity (µg of [*methyl*-³H]collagen degraded over 18 h); ○---○, gelatinolytic activity; , conductivity (mmho). In this experiment, fractions eluted between 208 and 260 ml were pooled for further purification.

Fig. 2. SDS/PAGE (12.5% gel) of the connective-tissue-degrading enzyme purified from B16 melanoma cytosol

(a) Lanes 7 and 8 contain the proteins precipitated by 25–60% - and 0–25% -(satd.)-(NH₄)₂SO₄ respectively. Lane 6 contains the collagenolytic/gelatinolytic fractions isolated by DEAE-Sephadex column chromatography. Lanes 2–5 contain the active enzyme fractions prepared by zinc-chelated column chromatography eluted at pH 4.7 (lanes 2–3) and pH 6.5 (lanes 4–5). Lanes 2 and 4 were activated with APMA. Lane 1 contains the Bio-Rad molecular-mass standard proteins: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa). The gel was subjected to Coomassie Blue staining followed by silver staining and photographed between each step. Lanes 1, 7 and 8 represent the Coomassie Blue-stained photograph and lanes 2–6 were silver-stained. (b) SDS/PAGE (12.5% gel) of purified enzyme (after DEAE-Sephadex, zinc-chelated column and concanavalin A-Sepharose column chromatography) under non-reducing (lane 1) and reducing conditions (lane 2). The gel was silver-stained. Molecular-mass markers are identified in the left-hand margin. Reduction was performed with 2.5% (v/v) β-mercaptoethanol in the sample solution.

Fig. 3. Two-dimensional electrophoresis of purified melanoma metalloproteinase using the ISODALT-2 system

Under non-reducing conditions the purified enzyme was separated by size along the vertical axis and by charge on the horizontal axis. The arrows identify three spots after silver staining. A carbamoylation train of creatine phosphokinase as an internal standard is given at the bottom and the molecular-mass standards are noted on the vertical axis. The numbering system (0–30) refers to the individual carbamoylation spots of creatine phosphokinase according to the nomenclature of Anderson & Hickman (1979).

fractions were combined and applied to a concanavalin A–Sephacrose column and eluted with 0.5 M- α -methyl D-mannoside. Virtually all of the collagenolytic and gelatinolytic activity was found in the unbound protein peak (A_{280}) with no activity in the bound protein peak. This purification scheme resulted in a 206-fold purification of collagenolytic activity as compared with the starting melanoma cytosol (Table 1).

PAGE and silver staining of these fractions under non-reducing conditions demonstrated the progressive protein purification (Fig. 2a). The active fractions isolated on the zinc-chelated column and the concanavalin A–Sephacrose column ran as a major protein band of approx. 59 kDa, with two minor contaminating bands of 112–116 kDa noted on storage of the enzyme at -80°C . Reduction of the melanoma connective-tissue-degrading enzyme by β -mercaptoethanol before SDS/PAGE, resulted in a single band of approx. 63 kDa (Fig. 2b).

Two-dimensional gel electrophoresis of the purified enzyme revealed three closely related spots of identical molecular mass with pI values of approx. 5.7–6.1 (Fig. 3).

SDS/PAGE of the purified connective-tissue-degrading enzyme before and after treatment with trypsin showed conversion of the 59 kDa polypeptide into components of 48, 44 and 40 kDa within 5 min, with a predominance of the 40 kDa component noted after 15 min of incubation at 37°C (Fig. 4). The 59 kDa latent collagenolytic enzyme could be activated non-enzymically by 0.7 mM-APMA without change in molecular

Fig. 4. SDS/PAGE (12.5%) of melanoma connective-tissue-degrading enzyme under non-reducing conditions after incubation with trypsin

Purified enzyme isolated by zinc-chelated column chromatography (lane 1) was incubated with 25 μg of trypsin (T) for 5 min (lane 2) and 15 min (lane 3), followed by boiling without reduction. Molecular-mass markers are listed on the left. The arrows refer to enzyme activation products produced by the incubation.

mass (Fig. 2a). After 1 month of storage at 4°C , the latent collagenolytic enzyme became autoactivated without a detectable change in molecular mass. Treatment of the autoactivated enzyme with trypsin or APMA resulted in no enhancement of collagenolytic or gelatinolytic activity.

H.p.l.c. of the purified latent enzyme revealed a single symmetrical peak of approx. 50–60 kDa.

Assessment of gelatinolytic activity during enzyme purification revealed that gelatinolytic and collagenolytic activities appeared in the same active fractions on anion exchange (Fig. 1), zinc-chelated column and concanavalin A–Sephacrose column chromatography (results not shown).

Substrate specificity

The activated crude and purified enzyme preparations degraded γ , β , α_1 and α_2 chains of soluble type I collagen with some activity noted at 4 h, but with progressive degradation noted by 18 h at 22°C (Figs. 5a and 5b). The earliest observable collagen degradation products consisted of a diffuse band of proteins extending between 65 and 95 kDa. TC^A ($\alpha^A = 75$ kDa) products were not revealed, even with lower melanoma enzyme concentrations or at shorter time intervals. In contrast, neutrophil collagenase produced the typical α_1^A and α_2^A degradation products of collagen (Fig. 5a).

Purified autoactivated melanoma enzyme (0.5 μg) was able to degrade reconstituted [^{14}C]collagen fibrils with 1.8, 3.5, 6.9 and 18.8% degradation noted after 3, 6, 12 and 24 h of incubation respectively, which is equivalent to 50.5 collagenase units per mg of enzyme.

The crude and purified enzyme preparations were also active in the hydrolysis of the synthetic Peptide P substrate. An 80-fold enrichment factor was noted during the purification of the enzyme.

Crude and purified preparations of the melanoma connective-tissue-degrading enzyme (without trypsin or APMA activation) were able to rapidly degrade [^3H]gelatin. The purified enzyme had a specific activity of 116 gelatinolytic units per mg of enzyme. Within 15 min of incubation at 37°C , the purified enzyme degraded the α_1 and α_2 chains of gelatin into products of 75 and

Fig. 5. Fluorograms showing the digestion of type I collagen by (a) trypsin-activated B16 melanoma connective-tissue-degrading enzyme after 4 h of incubation at 22 °C and (b) APMA-activated B16 melanoma connective-tissue-degrading enzyme after 6 h (lanes 2–3) and 18 h (lanes 4–5) of incubation at 22 °C

(a) All incubations contained 10 μ g of [*methyl*- 3 H]-collagen type I. Lanes 2–5 were pre-activated with trypsin and then inactivated with SBTI. Lanes 1 and 5 had no tumour added. Lane 2 contained 2 μ g of neutrophil collagenase. Lane 3 contained 9 μ g of partially purified tumour connective-tissue-degrading enzyme eluted from a zinc-chelated Sepharose column. Lane 4 contains 3 μ g of the purified tumour enzyme (fraction not binding to a concanavalin A column). The γ , β , α_1 and α_2 chains of type I collagen are identified along with the α_1^* and α_2^* (TCA^*) products produced by neutrophil collagenase. The purified tumour enzyme produced a diffuse band of collagen degradation products of less than 95 kDa. (b). All incubations contained 10 μ g of [*methyl*- 3 H]-collagen type I. Lane 1 had no enzyme added. Lanes 2 and 4 contained 4 μ g of purified enzyme eluted from a zinc-chelated column at pH 4.7. Lanes 3 and 5 contained 5 μ g of purified enzyme eluted from the concanavalin A-Sepharose column. The γ , β , α_1 and α_2 chains of collagen type I are identified along with the large digestion products (indicated by arrows) and the buffer front (B.F.). The approximate molecular mass of the 6 h digestion products of collagen are 65 kDa and 50 kDa.

Fig. 6. Fluorogram showing the digestion of type I gelatin by non-activated B16 melanoma connective-tissue-degrading enzyme after 15 min of incubation at 37 °C

All incubations contained 10 μ g of [*methyl*- 3 H]-collagen preheated to 45 °C for 20 min to produce the gelatin substrate. Lane 1 contains no added enzyme. Lane 2 contains the active enzyme fractions isolated by the DEAE-Sephadex chromatography and not binding to a zinc-chelated column. Lanes 3 and 4 contain the purified fractions eluted from a zinc-chelated column (pH 6.5) and concanavalin A-Sepharose column respectively. The larger digestion products of approx. 75 and 67 kDa are depicted by the arrows. 3 H-labelled molecular-mass markers are noted in lane 5 (phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; lactalbumin, 18 kDa).

67 kDa, as well as several lower-molecular-mass fragments (Fig. 6).

Maximal enzyme activity for both collagen and gelatin substrates was present over a broad neutral pH range between pH 7 and 9, with an optimum at pH 7.5 (results not shown).

Inhibition of type I collagenolytic and gelatinolytic activity

Table 2 shows comparative inhibition of purified melanoma connective-tissue-degrading enzyme by chelating agents, serine- and cysteine-proteinase inhibitors and human serum. The chelating agents EDTA and 1,10-phenanthroline, and dilute human serum, had a marked dose-dependent inhibitory effect on trypsin-activated collagenolytic and gelatinolytic activity. Dithiothreitol and inhibitors of serine and cysteine proteinases were ineffective. The addition of 25 mM- CaCl_2 to EDTA-, EGTA- or 1,10-phenanthroline-inhibited enzyme restored 80% of the collagenolytic activity.

Incubation of purified enzyme with [*proline*- 3 H]-collagen type IV resulted in partial digestion of the α_1 and α_2 chains, yielding products of 100, 80, 30 and 20 kDa (Fig. 7a). The addition of 30 mM-EDTA markedly inhibited the digestion of collagen type IV. SBTI had a small inhibitory effect on the digestion of collagen type IV, whereas *N*-ethylmaleimide was inactive (Fig. 7b). The digestion of collagen type IV was progressive during a

Table 2. Inhibition of purified melanoma collagen- and gelatin-degrading enzyme activity by proteinase inhibitors

Inhibitor	Concn. (mM)	Inhibition of enzyme activity (%)	
		Collagenolytic	Gelatinolytic
EDTA	16.0	100	100
1,10-Phenanthroline	1.0	80	96
iPr ₂ P-F	10.0	0	0
Tos-Lys-CH ₂ Cl	0.14	0	0
Dithiothreitol	1.0	0	13
Leupeptin	0.4	11	0
Iodacetate	1.0	0	7
Human serum	1:10 dilution	100	75

24 h incubation at 35 °C, with preferential digestion of the α_2 chain. Calculation of cleavage percentages from densitometric scans (Liotta *et al.*, 1981) for the film exposed overnight revealed that 91% of the α_2 chains and 66% of the α_1 chains were degraded over 24 h.

Since the crude B16 melanoma cytosol contains serine and cysteine proteinases as well as a connective-tissue-degrading metalloproteinase (Zucker *et al.*, 1985), it was necessary to assay the purified enzyme with other substrates. Purified melanoma metalloproteinase exhibited no activity against various sensitive substrates used to demonstrate trypsin-like (Tos-Arg-OMe), chymotrypsin-like (Bz-Tyr-OEt), elastase-like [Suc-(Ala)₃-Nan] and general serine-proteinase (azocasein) activities, nor against a synthetic substrate used to detect cysteine proteinases (Z-Phe-Arg-NHMec).

DISCUSSION

The goal of this discussion will be to show how the mouse melanoma connective-tissue-degrading enzyme compares with previously described metalloproteinases. Mammalian collagenases have been reported to be similar in terms of their neutral pH optimum and inhibition by metal chelators, but have considerable diversity in terms of molecular size, state of enzyme activation, cellular localization, range of specificity for different types of collagen substrates and capacity to degrade denatured collagen (gelatin) (Liotta *et al.*, 1983; Harris & Cartwright, 1977). Both latent and active collagenases with specificity toward either interstitial collagens types I–III or basement-membrane collagen type IV have been demonstrated to be released in large amounts by different types of cancer cells (Yamanishi *et al.*, 1973; McCroskery *et al.*, 1975; Steven & Itzhaki, 1977; Tane *et al.*, 1978; Liotta *et al.*, 1981). A correlation between metastatic behaviour and the secretion of type IV collagenase (Liotta *et al.*, 1980) or type I collagenase (Tarin *et al.*, 1982) by animal tumour-cell lines has been independently claimed. Gelatinases are a separate category of metalloproteinases which preferentially degrade gelatin rather than interstitial-collagen substrates (Harris & Krane, 1972; Sopata & Danciewicz, 1974; Sellers *et al.*, 1978; Murphy *et al.*, 1985). Proteoglycanase (termed 'stromelysin' by Chin *et al.*, 1985) refers to yet another type of metal-dependent neutral

Fig. 7. (a) Fluorogram showing the digestion of type IV collagen by autoactivated (after 4 °C storage for 1 month) melanoma connective-tissue-degrading enzyme and (b) degradation of type IV collagen by purified autoactivated melanoma connective-tissue-degrading enzyme in the presence of various proteinase inhibitors

(a) The reaction mixture contained 20 mM-CaCl₂, 100 μ g of bovine serum albumin/ml, 160 μ g of SBTI/ml and 12 mM-*N*-ethylmaleimide and incubation was at 35 °C for 16 h. Lane 1 contained type IV [³H]collagen alone (4.8 μ g; 80000 c.p.m.); lane 2 contained purified enzyme (2 μ g) + substrate + 20 mM-EDTA; lane 3 contained the enzyme and the substrate. The arrows on the left at 100, 80, 30 and 20 kDa identify the digestion products. A 60 kDa substrate contaminant (C) is also noted. For comparison, crude human type IV collagenase (A2058) was incubated with type IV collagen substrate (lane 4). Cleavage products of 75% and 25% the length of pro- α_1 and pro- α_2 chains of type IV collagen are as noted previously and are depicted by the arrows of the right (Salo *et al.*, 1983). Lane 5 contains substrate alone, which was run at the same time as lane 4. (b) The incubations were performed at 35 °C for 16 h. Lane 1 contains substrate (S) only; lane 2 contains S + 1.2 μ g of purified enzyme (E) + 160 μ g of SBTI; lane 3 contains S + E + 12 mM-*N*-ethylmaleimide; lane 4 contains S + E + 30 mM-EDTA; lanes 5 and 6 contain S + E without inhibitor.

proteinase that degrades many connective-tissue substrates, with a preference for proteoglycans (Galloway *et al.*, 1983; Okada *et al.*, 1986).

In the present study we have used $(\text{NH}_4)_2\text{SO}_4$ precipitation and anion-exchange, zinc-chelated column and lectin-affinity column chromatography to purify a single-chain 59 kDa protein from the cytosol of highly metastatic B16-BL6 melanoma cells which has both active gelatinolytic activity and latent types I and IV collagenolytic activity. In further discussion we will refer to this enzyme as a 'connective-tissue-degrading metalloproteinase'. On two-dimensional gels this purified enzyme gave three closely related spots with apparent pI values of 5.7–6.1. This configuration is compatible with the presence of a single species of molecule with charge microheterogeneity, the significance of which is still unclear. Similar to classical mammalian collagenases and gelatinases, the melanoma metalloproteinase required the presence of Ca^{2+} for activity and was inhibited by metal chelators and serum (Harris & Krane, 1972; Cawston & Murphy, 1981; Liotta *et al.*, 1981).

The degradation of the triple-helical structure of collagen at 22 °C by the melanoma metalloproteinase to products smaller than the 75 kDa TC^A fragments differentiates this enzyme from classical mammalian collagenases, which are limited to attacking the single locus at three-quarters of the distance from the *N*-terminal end of the molecule (McCroskery *et al.*, 1975; Cawston & Murphy, 1981; Welgus *et al.*, 1982). The initial cleavage of gelatin by the melanoma metalloproteinase, however, produced a 75 kDa fragment. Using the synthetic model collagen N_{25}ph -octapeptide substrate (Peptide P) and monitoring its degradation by reverse-phase liquid chromatography, we have shown that the melanoma proteinase attacks this substrate exclusively at the Gly-Ile bond (procedure of Gray & Sanei, 1982; results not shown). Welgus *et al.* (1982) reported that purified skin fibroblast collagenase is capable of cleaving denatured collagen with the earliest major cleavage of types I–III occurring at a locus three-quarters the distance from the *N*-terminal end. Of relevance to our study, Welgus *et al.* (1985) recently purified a classical collagenase from rat uterine cells that not only was able to cleave denatured collagen chains with greater catalytic efficiency than native collagen, but also attacked a wider spectrum of peptide bonds in gelatin than in collagen.

Marked similarities in terms of enzyme latency and response to inhibitors have been reported for both collagenases and gelatinases derived from different mammalian cells (Roswit *et al.*, 1983; Williams & Lin, 1984; Otsuka *et al.*, 1984). Both metalloproteinases recognize the same peptide linkages for cleavage, yet each differs distinctly in the effect caused by the secondary binding sites of the substrate molecule (Sellers *et al.*, 1978). An essential feature in the degradation of the collagen molecule *in vivo* appears to involve a well-organized transition from collagenase substrate to gelatinase substrate, depending on the denaturation temperature of intermediately cleaved products (Sunadi & Nagai, 1983). Although similarities in chromatographic and electrophoretic mobility have also been reported, gel-filtration and ion-exchange chromatography have been used successfully in separating collagenases and gelatinases released by different cell types (McCroskery *et al.*, 1975; Sellers *et al.*, 1978; Nagase *et al.*, 1983; Welgus *et al.*, 1985). In our study, using

anion and cation exchangers (Mono Q and S Beads; Pharmacia), gel-filtration chromatography (Sephadex G-200 and Bio-Gel A 1.5m) and affinity chromatography (Heparin-Sepharose and Red Sepharose CL-6B; Pharmacia), we have been unable to separate tumour collagenolytic and gelatinolytic activities (results not shown) and therefore conclude that a single protein species of 59 kDa appears to be responsible for the activities against collagen and gelatin type I substrates. An alternative explanation is that the 59 kDa protein that we isolated consists of two distinct enzymes of identical molecular mass and similar pI, leading to difficulty in separation. To further substantiate our claim of purification from tumour cells of a single metalloproteinase with both collagenolytic and gelatinolytic activities, we used similar chromatographic procedures to completely separate human neutrophil collagenase and gelatinase.

In our studies, the activation of melanoma metalloproteinase by organomercurials or autoactivation occurring during 1 month of incubation at 4 °C was accompanied by no change in molecular mass. Stricklin *et al.* (1983) described a similar process of activation of human skin procollagenase by organomercurials and trypsin. They proposed that procollagenase has an autocatalytic property that is enhanced by treatment with agents such as mercurials and trypsin that function by perturbation of the zymogen conformation in an intramolecular fashion. In contrast with most other reports of gelatinolytic metalloproteinases (Welgus *et al.*, 1985; Murphy *et al.*, 1985; Okada *et al.*, 1986), the melanoma metalloproteinase did not require activation in order to degrade type I gelatin.

Contrary to previous reports of minimal amounts of collagenolytic and gelatinolytic activities within tumour cells (McCroskery *et al.*, 1975), we have demonstrated appreciable amounts of both proteinase activities in the cytosol of nitrogen-cavitated mouse melanoma cells (Zucker *et al.*, 1985). In fact, the highly metastatic BL-6 subclone of the B16 melanoma releases relatively small amounts of enzyme *in vitro* (S. Zucker, J. Wieman, R. Lysik, D. P. Wilkie, N. Ramamurthy & B. Lane, unpublished work) as compared with the intracellular concentration of enzyme. The localization of metalloproteinase within the tumour cell and its ultimate distribution *in vivo* remain uncertain. On the basis of our demonstration that the melanoma plasma membrane is highly enriched in metalloproteinase activity (Zucker *et al.*, 1985), it is proposed that the enzyme isolated from cytosol may be *en route* to becoming incorporated within the plasma membrane, as opposed to being a precursor to a secretory molecule, which has been demonstrated with normal mesenchymal cells (Harris & Cartwright, 1977; Nagase *et al.*, 1981).

Another important observation of the present study was that the melanoma connective-tissue-degrading enzyme was able to digest type IV, as well as type I, collagen and gelatin. Basement-membrane-associated collagen type IV is not susceptible to attack by classical tumour collagenase, which preferentially degrades interstitial collagen types I–III (Liotta *et al.*, 1983). Liotta *et al.* (1981) and Salo *et al.* (1983) have described the purification of a type IV-collagen-degrading enzyme (68 and 62 kDa) that is secreted by metastatic mouse sarcoma cells. The enzyme degraded the type IV procollagen substrate containing the pro- $\alpha_1(\text{IV})$ and

pro- α_2 (IV) chains (185 and 170 kDa respectively) at the same rate to form two pairs of degradation fragments corresponding in molecular mass to approx. 135, 120, 60 and 50 kDa. In contrast, the melanoma connective-tissue-degrading enzyme that we have described degrades the pro- α_1 (IV) and pro- α_2 (IV) chains to somewhat-lower-molecular-mass fragments. Furthermore, the enzyme exhibits a preference for degradation of the pro- α_2 (IV) chain.

Although the pathophysiological role for the metalloproteinase that we have isolated from melanoma cells is uncertain, its capacity to degrade basement membrane as well as interstitial collagen and gelatin provides the enzyme with a spectrum of relevant collagen substrates that are encountered by tumour cells during the process of cancer invasion.

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