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Purification of Progelatinase A (Matrix Metalloproteinase 2) and a Tissue Inhibitor of Metalloproteinase-2(TIMP-2) from T98G Human Glioblastoma Cells

Ho-Jae Lee, Myung-Chul Chung, Choong-Hwan Lee, Hyo-Kon Chun and Yung-Hee Kho*

Microbial Chemistry Research Group, Genetic Engineering Research Institute, Korea Institute of Science and Technology, Taejon 305-600, Korea

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Abstract: The Gelatinases (typeIV collagenases) are metalloproteinases that may play an important role in tumor invasion and metastasis. Progelatinase A was purified from a conditioned medium of T98G human glioblastoma cells. TIMP-2 complexed progelatinase A and free progelatinase A were separated by heparin affinity HPLC. The final product was homogeneous on SDS-PAGE, with a molecular weight of 64,000 daltons without reduction. TIMP-2 and free progelatinase A were separated from TIMP-2 complexed progelatinase A by reverse-phase HPLC in the presence of trifluoroacetic acid. TIMP-2 complexed progelatinase A was resistant to activation by p-aminophenyl mercuric acetate (APMA), and showed less than 20% of the activity of the TIMP-2 free active enzyme. TIMP-2 free progelatinase A was easily activated to the mature form with a molecular weight of 57,000 daltons by APMA, and showed high activity compared to the TIMP-2 complexed enzyme.

Key words: Matrix metalloproteinases, TIMP, glioblastoma.

The matrix metalloproteinases (MMPs) constitute a group of zinc-dependent enzymes which include interstitial collagenases, gelatinases (or typeIV collagenases), and stromelysins. MMPs may be involved in various physiological and pathological processes, such as tissue remodelling, reproduction, morphogenesis, various connective tissue diseases, angiogenesis, cancer cell invasion, and metastasis (Woessner, 1991). The gelatinases which hydrolyze various types of gelatins, as well as typeIV collagen localized in basement membranes, may be involved in the *in vitro* invasion of capillary cells, trophoblasts, and cancer cells (Mignatti *et al.*, 1989; Kliman *et al.*, 1990). Gelatinases are expressed by a number of cancer cell lines (Stetler-Stevenson, 1990; Liotta *et al.*, 1991) as well as by the stromal cells surrounding tumors (Pyke *et al.*, 1992) and, consequently, have been identified as targets for inhibitor theraphy in a number of cancers. Two kinds of gelatinases, a 72-kDa(or 64-kDa under nonreducing conditions) gelatinase (gelatinase A, EC 3.4.24.24) and a 92-kDa (or 90-kDa under nonreducing conditions) gelatinase(gelatinase B, EC 3.4.24.35) have recently been purified from various sources and characterized. Collier *et al.* (1988) purified a 64-kDa gelatinase from a conditioned medium of virally transformed human skin fibroblasts, and determined its primary structure. The enzyme was identical to enzymes secreted by normal skin fibroblasts and transformed epithelial cells. The activities of MMPs are controlled at several levels (Docherty *et al.*, 1992), including proenzyme activation and regulation by the general proteinase inhibitor α_2 macroglobulin, and by the specific tissue inhibitors of metalloproteinases (TIMPs) (Stetler-Stevenson, 1990; Murphy *et al.*, 1991). TIMP-1 and TIMP-2 are secreted from various kinds of cells and form tight complexes with the latent proenzyme forms (zymogens) of gelatinase B and gelatinase A, respectively. Therefore, in most cases these gelatinases are purified in a TIMP-1 or TIMP-2 complexed form from a conditioned medium.

In this study progelatinase A was purified in TIMP-2 free and TIMP-2 complexed forms from a conditioned medium of human T98G glioblastoma cells, then the activities and activation process of the two forms were compared. Also, TIMP-2 and progelatinase A were separated from TIMP-2 complexed progelatinase A by reverse phase HPLC chromatography under acidic conditions.

*To whom correspondence should be addressed.
Tel: (042) 860-4354, Fax: (042) 860-4595

Materials and Methods

Materials

All chemicals were of the highest purity commercially available. Electrophoresis chemicals were purchased from Bio-Rad. Tissue culture plastics were obtained from Falcon, and media and additives were supplied by Gibco. Protease inhibitors were purchased from Boehringer Mannheim.

Cell and culture conditions

The human glioblastoma cell line T98G was provided by the Japanese Cancer Research Bank, Japan. The cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2. The basal medium (D-MEM/F-12) consisted of a 1 : 1 mixture of Dulbecco's modified Eagle's medium (Gibco) and Ham's F12 medium (Gibco), supplemented with 15 mM HEPES, 1.2 mg/ml $NaHCO_3$, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Preparation of concentrated conditioned media

T98G cells were grown to reach confluence in 850 cm^2 roller bottles containing 200 ml of D-MEM/F-12 medium supplemented with 10% fetal bovine serum. The cells were rinsed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, then placed in 200 ml of serum-free D-MEM/F-12 medium. The serum-free conditioned medium was harvested three times a week, then clarified by sequential centrifugation at 600 x g for 15 min and at 12,000 x g for 30 min. The pooled serum-free conditioned medium was precipitated by adding ammonium sulfate to a final saturation of 80% and allowing the mixture to stand overnight at 4℃. The resultant precipitated proteins were collected by centrifugation at 12,000 x g for 30 min. The precipitated proteins were dissolved in, and dialyzed against, 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.005% Brij-35, and used for the purification of progelatinase.

Purification of progelatinase A

All purification procedures, except heparin affinity HPLC, were carried out at 4℃. The conditioned medium was prepared from a confluent serum-free culture of T98G cells in roller bottles, and concentrated. The concentrated conditioned medium was dialyzed against TNB buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.005% Brij-35) and applied to a gel filtration column (5 cm x 100 cm) of Cellulofine GCL-2000 (Chisso) equilibrated with TNB buffer. A pool of gelatinase active fractions was applied to a column (1.5 cm×8 cm) of gelatin-Cellulofine (Chisso) equilibrated with TNB buffer, and the flow-through was recirculated overnight through the column. Gelatinolytic active fractions were eluted with a linear gradient of 0 to 7.5% (v/v) dimethyl sulfoxide in TNB buffer, and dialyzed against TB buffer (20 mM Tris-HCl, pH 7.5, 0.005% Brij-35). The pooled fractions were applied to a column (1.5 cm×8 cm) of concanavalin A-Sepharose (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and 0.01% Brij-35. Concanavalin A-binding proteins were eluted stepwise with 0.1 M N-acetylglucosamine in TB buffer and 0.5 M α-methylmannoside in TB buffer. Fractions containing gelatinase activity, which did not bind to concanavalin A, were pooled and finally subjected to heparin-affinity HPLC on a Shodex AHR-894 column (8 mm×50 mm, Showa Denko) pre-equilibrated with TB buffer. The active fractions were eluted with a linear gradient of 0 to 0.5 M NaCl in TB buffer at a flow rate of 0.5 ml/min. Fractions from this HPLC step were pooled into either a TIMP-2 complexed progelatinae A pool or a free progelatinase A pool, as determined by SDS-PAGE and gelatin zymography. Each pool was stored at −70℃ before use.

Separation of TIMP-2 and progelatinase A

TIMP-2 complexed progelatinase A purified by heparin affinity HPLC was dialyzed against 0.07% trifluoroacetic acid (TFA) and subjected to reverse-phase HPLC on a Synchropak RP-4 column (4.6 mm×250 mm, SynChrom), preequilibrated with 0.07% TFA, and eluted with a linear gradient of 0 to 80% acetonitrile in 0.07% TFA at a flow rate of 0.5 ml/min. Fractions were pooled into either a TIMP-2 pool or a free progelatinase A pool, and partially dried in a Speed-Vac Concentrator (Savant). Each pool was dissolved in, and dialyzed against, TNB buffer and stored at −70℃ before use.

Activation of progelatinase A

TIMP-2 complexed progelatinase A and free progelatinase A were incubated with 1mM p-aminophenyl mercuric acetate (APMA) in 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 37℃ for periods up to 24 h. The activated gelatinase A was analyzed by SDS-PAGE and gelatin zymography.

Electrophoretic analyses

Unless otherwise noted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on polyacrylamide gel (90×90×0.75 mm) under nonreducing conditions. Resultant gels were stained with Coomasie Brilliant Blue R-250, then destained. Molecular weight standards (Bio-Rad) were routinely

used for estimating molecular weights based on relative mobility.

Gelatinolytic activities of secreted proteinases were analyzed by zymography on gelatin-containing gel, as described by Miyazaki *et al.* (1990). Concentrated conditioned media to be analyzed were mixed with equal volumes of concentrated SDS sample buffer (4% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol), then electrophoresed without heating in boiling water. Then, the proteinases separated on the gels were renatured in 2.5% Triton X-100 containing 50 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at room temperature for 1 h, followed by incubation in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.01% NaN_3 at 37℃ for 18 h.

Reverse zymography of gelatinase inhibitors was carried out as described by Miyazaki *et al.* (1993). Briefly, samples were subjected to electrophoresis on polyacrylamide gel containing 0.1% (w/v) SDS and 1 mg/ml gelatin under nonreducing conditions, then renatured in 2.5% Triton X-100. Then, the gel was incubated at 37℃ for 18 h in 4 ml of reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 1 μg/ml human progelatinase A, and 1 mM APMA as the activator of the progelatinase. Gelatinase inhibitors separated 'on the gel formed CBB-stained gelatin bands by blocking the gelatinolytic activity of gelatinase A.

Assay of gelatinase activities

Gelatinase activity was assayed using ^{3}H-labeled human placental typeIV collagen (Du Pont-New England Nuclear; 0.31 mCi/mg protein) as a substrate which had previously been denatured at 60℃ for 30 min. Precursor forms of metalloproteinases were incubated in 55 μl of a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM APMA, and 10 mM $CaCl_2$ at 37℃ for 1 h, then 5 μl of [^{3}H] collagen (0.02 μCi) was added. After further incubation at 37℃ for 18 h, the amount of [^{3}H] collagen fragments produced was determined by the methods of Liotta *et al.* (1980).

Determination of protein concentration

Protein concentration was determined by the dye method using a Bio-Rad protein assay kit, using bovine serum albumin as the standard.

Results and Discussion

Purification of progelatinase A

When analyzed by zymography on gelatin-containing SDS-polyacrylamide gel, the serum-free conditioned medium of T98G cells showed a strong gelatinolytic activity with a molecular weight of 64,000 daltons and a weak activity with a molecular weight of 90,000 daltons. To purify these gelatinases, T98G cells were cultured in serum-free D-MEM/F-12 medium. Approximately 3.6 liters of conditioned medium was concentrated by ammonium sulfate precipitation and subjected to gel filtration chromatography on a Cellulofine GCL-2000 column. The gelatinolytic fractions were combined and applied to a gelatin-Cellulofine affinity column. The flow-through was recirculated, washed with TNB buffer, and eluted with a linear gradient of 0 to 7.5% (v/v) DMSO in TNB buffer (Fig. 1A). The resultant fractions were analyzed by gelatin zymography (Fig. 1B). Most of the protein flowed through the column without adsorption, whereas gelatinolytic activities were adsorbed on the column. The gelatinase activity was eluted with approximately 3% (v/v) of DMSO. The active fractions were pooled and applied to concanavalin A-Sepharose column. The 64-kDa enzyme was recovered in the flow-through fractions, whereas the 90-kDa gela-

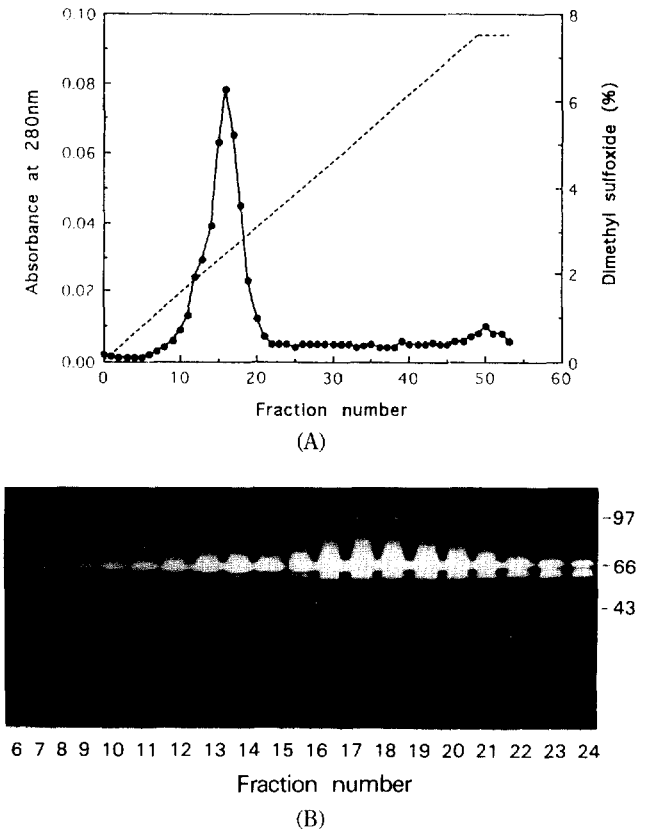

Fig. 1. Gelatin-affinity Cellulofine chromatography of progelatinase A obtained from gel filtration chromatography. The proteinase pool from the Cellulofine GCL-2000 column was applied to the column and then eluted with a linear gradient of 0 to 7.5% dimethyl sulfoxide. (A), elution pattern of proteins in chromatography —, A_{280}; ----, DMSO concentration. (B), proteinase zymography on gelatin-containing gel of the fractions obtained from gelatin affinity chromatography. *Ordinate*, molecular size in kilodaltons.

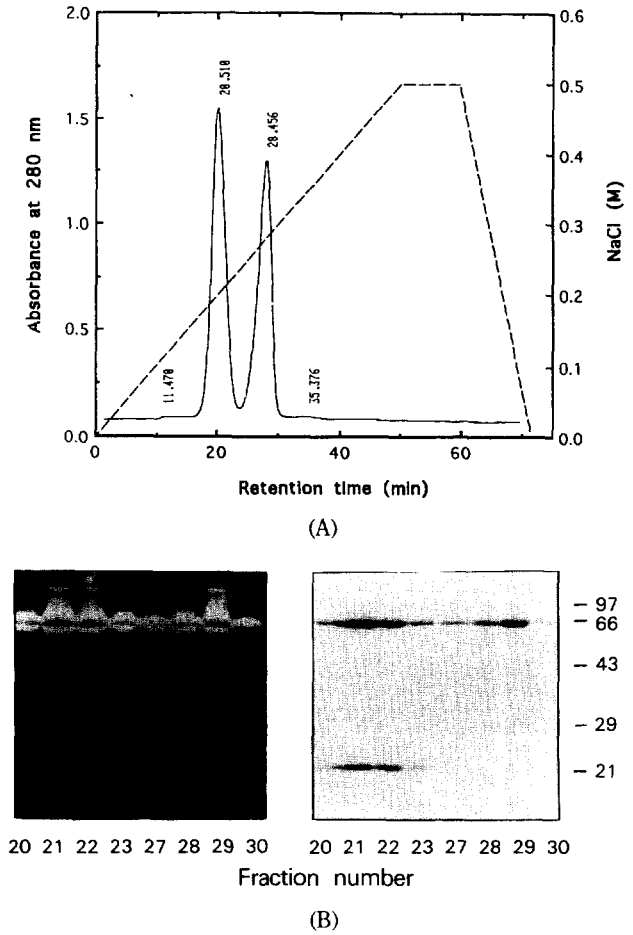

Fig. 2. Heparin-affinity HPLC of progelatinae A obtained from LCA-agarose column. The flow-through fraction from LCA-agarose column was applied to heparin-affinity column, and then the bound proteins were eluted with a linear gradient of 0 to 0.5 M NaCl. (A), elution pattern of proteins in chromatography —, A_{280}; ----, NaCl concentration. (B), proteinase zymography (*left*) on gelatin-containing gel and SDS-PAGE (*right*) of the fractions obtained from heparin affinity HPLC. *Ordinate*, molecular size in kilodaltons.

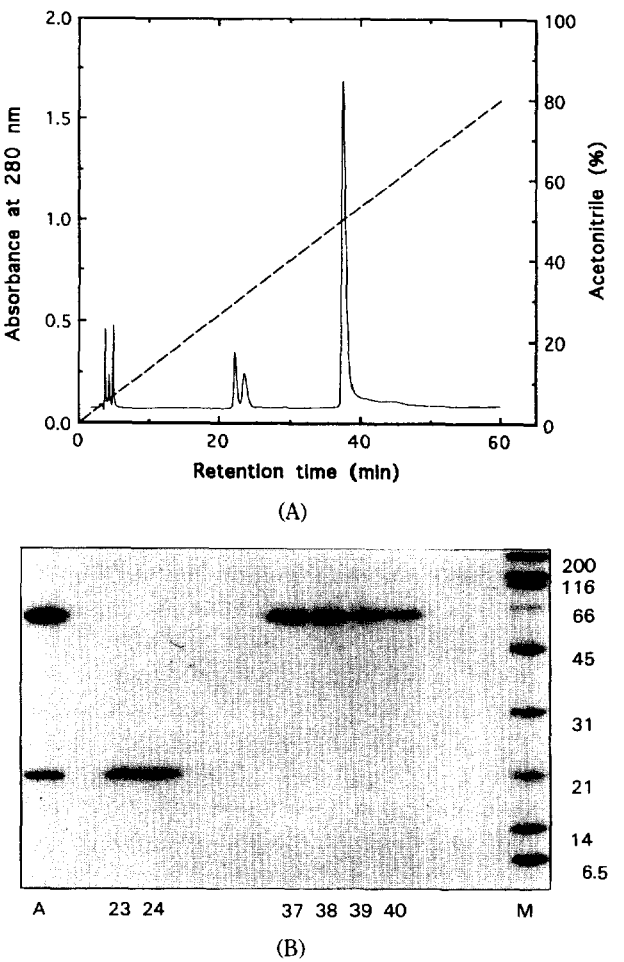

Fig. 3. Separation of TIMP-2 and progelatinase A from TIMP-2 complexed progelatinase A. The purified preparation of TIMP-2 complexed progelatinase A was pooled and applied to reverse-phase HPLC on a SynChropak RP-4 column, and the bound proteins were eluted with a linear gradient of 0 to 80% acetonitrile in 0.07% trifluoroacetic acid. (A), elution pattern of proteins in chromatography —, A_{280}; ----, acetonitrile concentration. (B), SDS-PAGE of fractions obtained from RP-4 HPLC. A, TIMP-2 complexed progelatinase A; M, molecular mass standards (kDa).

tinase, and a small amount of the 64-kDa gelatinase were adsorbed on the column and eluted with 0.5 M α-methylmannoside (data not shown). The concanavalin A-Sepharose flow-through was further purified by heparin affinity HPLC. The 64-kDa progelatinase was adsorbed on the heparin affinity column and eluted with a linear gradient of 0 to 0.5 M NaCl in TB buffer (Fig. 2A). The resultant protein peaks were analyzed by SDS-PAGE and gelatin zymography (Fig. 2B). The 64-kDa progelatinase eluted with 0.2 M NaCl (fractions 20 to 23) contained a low molecular weight protein with an apparent molecular weight of 21,000 daltons without reduction. The electrophoretic mobilitiy of the 21-kDa protein was consistent with that of TIMP-2, which was reported by Murphy *et al.* (1990). The 64-kDa progelatinase eluted with 0.3 M NaCl (fractions 27 to 30) was the TIMP-2 free form. TIMP-2 complexed progelatinase A (810 μg) and free progelatinase A (600 μg) were purified from the culture medium (3.6 l) of human T98G glioblastoma cells.

The existence of complexes between progelatinase A and TIMP-2 has been identified in a number of normal and tumorigenic cell culture systems (De Clerck *et al.*, 1989; Wilhelm *et al.*, 1989). This study investigates the purification of TIMP-2 free progelatinase A and TIMP-2 complexed progelatinase A from a conditioned medium of human T98G glioblastoma cells. T98 G glioblastoma cells produce an excess of the 64-kDa enzyme over the inhibitor, thereby allowing separation of the uncomplexed progelatinase A from the complex.

Separation of TIMP-2 and progelatinase A

Human progelatinase A is eluted from gelatin affinity

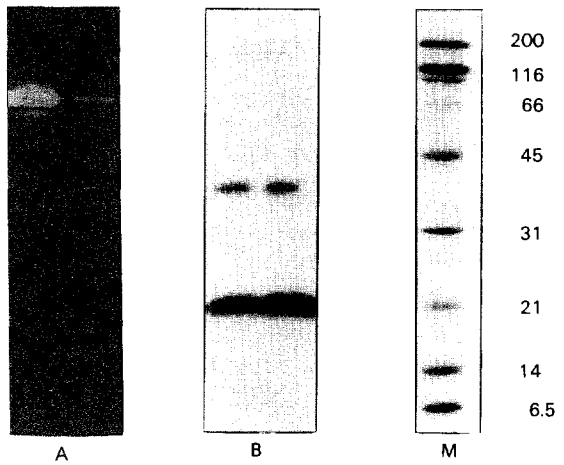

Fig. 4. Detection of gelatinolytic activity and TIMP-2 inhibitory activity. Experimental conditions are described in the text. A, Proteinase zymography on gelatin-containing gel of progelatinase A separated from RP-HPLC. B, Reverse-zymography on gelatin-containing gel of TIMP-2 separated from RP-HPLC in the presence of active gelatinase A. M, Molecular mass standards(kDa).

Table 1. Effects of proteinase inhibitors on gelatinolytic activity of purified TIMP-2 free gelatinase A. Each proteinase inhibitor was incubated at 37℃ for 3 h with purified TIMP-2 free gelatinase A in reaction mixture for the assay of gelatinolytic activity

Inhibitors	Specificity	Effect on gelatinase A
1,10-Phenanthroline (5 mM)	Metalloproteinases	Complete inhibition
EDTA (5 mM)		Complete inhibition
EGTA (5 mM)		Complete inhibition
Actinonin (10 μg/ml)		Half inhibition
Leupeptin (10 μg/ml)	Cystein proteinases	No effect
Aprotinin (10 μg/ml)		No effect
Antipain (10 μg/ml)		No effect
N-Ethyl maleimide (5 mM)		No effect
PMSF (5 mM)	Serine proteinases	No effect
Soybean trypsin inhibitor (10 μg/ml)		No effect
Chymostatin (10 μg/ml)		No effect
Pepstatin (10 μg/ml)	Aspartic proteinases	No effect
Bestatin (10 μg/ml)	Aminopeptidases	No effect
Amastatin (10 μg/ml)		No effect

resin with TIMP-2. TIMP-2 and progelatinase A were separated from TIMP-2 complexed progelatinase A which had been purified by heparin HPLC affinity chromatography. Separation of the inhibitor from the proteinase can be accomplished by RP-4 HPLC with 0.07% trifluoroacetic acid. The results in Fig. 3A show dissociation of the complex after acidification of the sample with trifluoroacetic acid. On analysis by SDS-PAGE (Fig. 3B), two small peaks (fractions 23, 24) showed an apparently single band with a molecular weight of 21,000 daltons under nonreducing conditions, whereas a following single peak (fractions 37 to 40) showed a single band with a molecular weight of 64,000 daltons. TIMP-2 free progelatinase A (fractions 36 to 40) separated from RP-HPLC showed gelatinolytic activity on gelatin zymography (Fig. 4A). When the two peaks (fractions 23, 24) were analyzed by reverse zymography, two gelatinase inhibitor bands of approximately 21 and 41-kDa were detected (Fig. 4B). The major 21-kDa protein was identified as TIMP-2 and the 41-kDa gelatinase inhibitor band was probably a dimer form of TIMP-2. Since reverse zymography is a very sensitive method, a small amount of inhibitor protein can be detected on the gel. The 41-kDa protein was not detected on SDS-PAGE.

Table 2. [^{3}H] Gelatin degradation by activated and latent progelatinase A. Purified free progelatinase A and TIMP-2 complexed progelatinase A were treated or not with APMA (1 h, 37℃) and then incubated (1 μg of enzyme/reaction) with 0.06 μg of [^{3}H] gelatin for 18 h at 37℃. Solubilized products were counted for radioactivity in a liquid scintillation spectrometer

Enzyme	^{3}H-labelled products (d.p.m)
Progelatinase A	
−APMA	802
+APMA	6652
TIMP-2 complexed progelatinase A	
−APMA	660
−APMA	1632
Blank	634

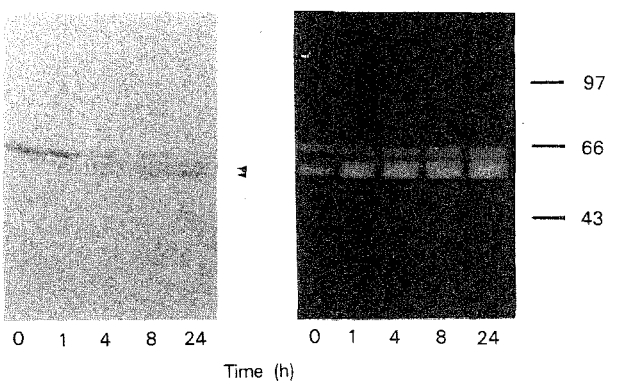

Fig. 5. Time course of activation of TIMP-2 complexed progelatinase A by APMA. TIMP-2 complexed progelatinase A (1 μg) was incubated with 1 mM APMA at 37℃ in 50 μl of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ for the indicated lengths of time, and then analyzed by SDS-PAGE (*left*) and gelatin zymography (*right*). *Arrows*, activated gelatinase A. *Ordinate*, molecular size in kilodaltons.

Properties of purified progelatinase A

The effects of several kinds of proteinase inhibitors on the activity of the purified 64-kDa gelatinase were

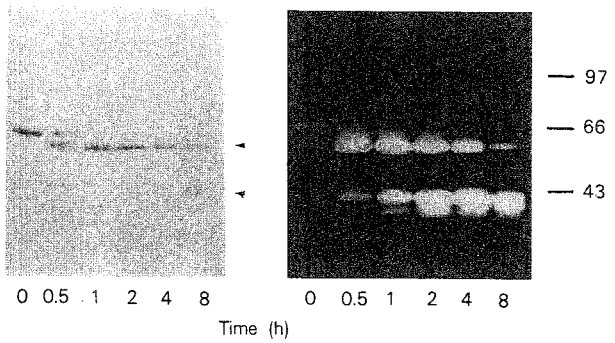

Fig. 6. Time course of activation of TIMP-2 free progelatinase A by APMA. TIMP-2 free progelatinase A (1 μg) was incubated with 1 mM APMA at 37℃ in 50 μl of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ for the indicated lengths of time, and then analyzed by SDS-PAGE (*left*) and gelatin zymography (*right*). *Arrows*, activated gelatinase A, *Ordinate*, molecular size in kilodaltons.

examined using a [^{3}H] gelatin degradation assay (Table 1). The activity of progelatinase A was completely inhibited by metalloproteinase inhibitors, such as 1,10-phenanthroline, EDTA, and EGTA, and was partially inhibited by actinonin. These results indicate that the purified gelatinase is a neutral metalloproteinase.

Purified TIMP-2 complexed progelatinase A and free progelatinase A showed little gelatinolytic activity under the assay conditions. Matrix metalloproteinases are usually secreted as latent proenzymes (zymogens) and require activation for their enzymatic activity. *In vitro*, the zymogen forms of metalloproteinases can usually be activated by proteolytic cleavage either by serine proteases, such as trypsin and plasmin, or by autocatalytic cleavage induced by organomercurial compounds, such as APMA. To test the effects of such activators, progelatinase A was incubated in the presence and absence of 1 mM APMA at 37℃ for 1 h. The gelatinolytic activity was measured by a soluble gelatin degradation assay. As shown in Table 2, exposure of free progelatinase A to APMA resulted in high enzymatic activity. In contrast, TIMP-2 complexed progelatinase A showed less than 20% of the activity of the TIMP-2 free active enzyme.

The change in molecular size of TIMP-2 complexed and free progelatinase A by APMA treatment was analyzed by SDS-PAGE and gelatin zymography. When the TIMP-2 complexed progelatinase A was incubated with APMA, gelatinolytic bands of 60- and 57-kDa appeared on the zymogram after 1 h of incubation (Fig. 5 *right*). The 60- and 57-kDa forms were considered to be the intermediate and mature forms of gelatinase A, respectively (Collier *et al.*, 1988). On SDS-PAGE the 64-kDa proenzyme was converted slowly to the 60-kDa form and the 57-kDa form (Fig. 5 *left*). When TIMP-2 free progelatinase A was incubated with APMA, the 64-kDa activity was completely converted to the activities of the 57- and 41-kDa forms within 4 h on zymography (Fig. 6 *right*). SDS-PAGE revealed that the majority of the proenzyme was degraded after 1 h of incubation (Fig. 6 *left*). This comparison of the intensities of the 57- and 41-kDa bands on SDS-PAGE and zymography suggests that the latter has a higher specific activity than the former.

The proenzymes of MMPs, including TIMP-2 free and latent progelatinase A, are rapidly converted to their mature forms by incubation with APMA (Collier *et al.*, 1988; Okada *et al.*, 1990). TIMP-2 complexed progelatinase A is resistant to activation by APMA and shows little enzymatic activity in a solution assay. In contrast, APMA activation of TIMP-2 free progelatinase A generates a potent enzyme and yields the previously reported 57-kDa form (Stetler-Stevenson *et al.*, 1989). The free enzyme fraction also generates additional fragments of 41-kDa. The 41-kDa protein shows high gelatinolytic activity on zymogram. The generation of low molecular mass forms with enzymatic activity from the zymogen has been reported from other metalloproteinases (Matrisian, 1990), and with gelatinases (Howard *et al.*, 1991).

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