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Isolation and characterization of a high molecular weight type IV collagenase isolated from human carcinoma tissue

Tomi T. Tsuda[a], Akira Kodama[b], Masaichi Yamamura[a], Shohei Matsuzaki[c] and Michio Tsuda[a]

[a]*Molecular Life Science,* [c]*Internal Medicine, School of Medicine, Tokai University, Isehara, Kanagawa 259-11, Japan* and [b]*BML Inc., R&D Center, Saitama 350, Japan*

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A proform of high molecular weight type IV collagenase was isolated and purified 1230-fold from human metastatic carcinoma tissue. Like matrix metalloproteinases (MMPs), the enzyme was activated by trypsin and degraded type IV collagen and gelatin at a neutral pH, the activity was inhibited by EDTA and *o*-phenanthroline. However, the molecular weight was much higher than MMPs which degraded type IV collagen, gelatinase A (MMP-2; 72 kDa gelatinase/type IV collagenase) (EC 3.4.24.24), gelatinase B (MMP-9; 92 kDa gelatinase/type IV collagenase) (EC 3.4.24.35), stromelysin-1 (MMP-3; 57 kDa) (EC 3.4.24.17) and stromelysin-2 (MMP-10; 57 kDa) (EC 3.4.24.22). The other significant difference from MMPs was that the enzyme was not activated by 4-aminophenylmercuric acetate nor inhibited by TIMP. Taking together these results, this high molecular weight type IV collagenase might be a newly found enzyme different from MMPs or might have the same configuration as MMPs already reported.

High molecular weight type IV collagenase; Type IV collagenase; Metastatic carcinoma tissue

1. INTRODUCTION

Type IV collagen is one of the major structural proteins in basement membrane of the extracellular matrix (ECM). Since the synthesis, assembly and turnover of ECM seem to be controlled to maintain normal tissue functions, abnormal turnover in collagen matrices would be associated with disease states such as tumor invasion, rheumatoid arthritis and osteoarthritis [1–3]. On the other hand, the matrix metalloproteinases (MMPs) are a group of 8 proteinases degrading the major components of matrices at neutral pH, and it is noted that they play some role in the process of invasion and metastasis of cancer cells [4–6]. Four of 8 MMPs degrading type IV collagen are termed MMP-2 (gelatinase A, 72 kDa collagenase) (EC 3.4.24.24), MMP-9 (gelatinase B, 92 kDa collagenase) (EC 3.4.24.35), MMP-3 (stromelysin-1, EC 3.4.24.17), and MMP-10 (stromelysin-2, EC 3.4.24.22) [7–12]. Correlation between metastatic potential and regulation of MMPs activation, especially MMP-2 and MMP-9 has been elucidated recently [13,14], and almost all MMPs so far characterized have been isolated from culture medium secreted by various cell lines [7–14]. We have found and purified a high molecular weight type IV collagenase from tissue of metastatic liver carcinoma and compared its properties to the previously reported MMPs.

Correspondence address: T.T. Tsuda, Molecular Life Science, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan. Fax: (81) (463) 96-4828.

Abbreviations: APMA, 4-aminophenylmercuric acetate; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; MMPs, matrix metalloproteinases; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinases.

2. MATERIALS AND METHODS

2.1. *Enzyme purification*

The enzyme was purified by a slight modification of the previously described method [15]. 70 g of carcinoma tissue were homogenized with 5-fold volumes of 50 mM Tris-HCl buffer, pH 7.6, containing 520 mM NaCl and 5 mM $CaCl_2$, and sonicated. The preparation was centrifuged at 12,000 rpm ($15,000 \times g$) for 20 min to obtain crude extract. Protein was precipitated from 25% to 55% saturated ammonium sulfate. The precipitate was dissolved at 10 mg/ml in 50 mM Tris-HCl buffer, pH 7.6, and purified further by DEAE-cellulose column and hydroxyapatite column chromatography and finally by Sephacryl S300 HR column chromatography equilibrated with 50 mM Tris-HCl buffer, pH 7.6, containing 20 mM NaCl and 5 mM $CaCl_2$.

2.2. *Enzyme assay*

2.2.1. ^{3}H-labeled type IV collagenolytic activity

Type IV collagen from human placenta was purchased from Sigma (St. Louis, USA) and ^{3}H-labeled type IV collagen was prepared as described previously by Tsuda et al. [15]. Specific activity of ^{3}H-labeled type IV collagen was 330 kBq/mg protein. Enzyme was activated with trypsin (17 μg/ml) treatment at 37°C for 10 min, trypsin digestion was stopped with aprotinin and soybean trypsin inhibitor prior to the addition of substrate. Then 6.0 μg of ^{3}H-labeled type IV collagen (2 kBq) was added as a substrate. The reaction mixture (500 μl) was incubated for 17 h at 37°C. The reaction was stopped and the residual substrate was precipitated by the addition of 100 μl of 10% trichloroacetic acid (TCA) and 5% tannic acid. Collagenolytic activity was measured as radioactivity of the TCA supernatant.

One unit was expressed as the amount of enzyme that was capable

of solubilizing 1000 cpm of radioactivity into the TCA soluble fraction for 17 h at 37°C.

2.2.2. ^{3}H-labeled gelatinolytic activity

^{3}H-labeled gelatin was obtained by heat denaturing of ^{3}H-labeled type IV collagen at 60°C for 30 min. The assay procedure was the same as that described above.

2.3. *Inhibition test*

Assays were performed at pH 7.6 in 50 mM Tris-HCl buffer containing 20 mM NaCl and 5 mM $CaCl_2$ using trypsin activated enzyme. All commercial inhibitors were purchased from Sigma (St. Louis, MO, USA), except for leupeptin, pepstatin and E64, which were from the Peptide Institute (Osaka, Japan). Tissue inhibitor of metalloproteinases (TIMP) was kindly supplied from Dr. Gen Kawano, Toray Inc. (Tokyo, Japan).

2.4. *4-Aminophenylmercuric acetate (APMA) activation*

The enzyme preparation was incubated for 2 h at 37°C in the presence of 1 mM APMA prior to the addition of ^{3}H-labeled type IV collagen as a substrate. After 17 h incubation collagenolytic activity of enzyme was measured as radioactivity (cpm) of the TCA supernatant.

2.5. *Zymography*

Substrate gels (zymogram) were used to examine the gelatin degrading species. 1.0 mg/ml of gelatin (Merck, Darmstadt, Germany) was incorporated into non-reducing SDS-polyacrylamide 7.5% gels. After electrophoresis at 0.8 mA/cm for 3 h at 6°C, SDS was removed with 2.5% Triton X-100 prior to incubation for 17 h at 37°C in 50 mM Tris-HCl, pH 7.6, buffer containing 20 mM NaCl and 5 mM $CaCl_2$. After enzyme reaction the gels were stained with Coomassie blue and destained with 30% methanol and 10% acetic acid in distilled water. Gelatinolytic enzyme was detected as transparent bands on the blue background of stained gel containing gelatin.

2.6. *Protein determination*

The protein concentration was estimated by Lowry method [16].

2.7. *Gel filtration HPLC*

Approximately 2 mg of partially purified enzyme were applied to a 10 mm × 30 cm prepacked column of Superose 6 HR (Pharmacia, Milwaukee, USA) equilibrated with 50 mM Tris-HCl, pH 7.6, buffer containing 20 mM NaCl, 5 mM $CaCl_2$ and 0.05% Brij-35. The flow rate was maintained at 0.3 ml/min, and the fractions (0.3 ml) were collected. Absorbance was recorded at 280 nm. Enzyme activity was assayed using 100 μl of each fraction. Molecular weight markers were: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.35 kDa).

2.8. *Estimation of the molecular weight after trypsin treatment*

Three mg of purified enzyme were treated with trypsin at 37°C for 10 min and applied to a Sephacryl S300 HR column equilibrated with 50 mM Tris-HCl buffer, pH 7.6, containing 20 mM NaCl and 5 mM $CaCl_2$. Absorbance at 280 nm and type IV collagenolytic activity were measured in each tube.

3. RESULTS

3.1. *Enzyme purification*

A latent form of high molecular weight type IV collagenase was purified from solid metastatic liver carcinoma from rectal adenocarcinoma. A typical purification procedure was summarized in Table I, with final purification by Sephacryl S300 column chromatography calculated to be 1,230-fold (Table I). High molecular weight type IV collagenase was eluted in the 1st peak that was fraction 1 in Fig. 1A.

Eight cases of metastatic liver carcinoma were examined by the same procedure described here, and the enzyme was detected in 3 cases in which primary lesion was rectal adenocarcinoma. The enzyme was purified from them by the same procedure as described above.

3.2. *Activation*

The purified high molecular weight collagenase was inactive. It was activated only by trypsin and not by 1 mM or 2 mM APMA (Table II), indicating that the enzyme was present as a latent form and that it required activation.

3.3. *Molecular weight*

The molecular weight was estimated to be over 2,000 kDa given by Sephacryl S300 HR column chromatography (Fig. 1A) and confirmed by Superose 6 HR HPLC. Addition of non-ionic detergent such as Brij-35 caused no change in molecular weight. The purified enzyme obtained as the 1st peak of Sephacryl S300 column chromatography (Fig. 1A) was re-chromatographed and eluted again at the same position as eluted before. Trypsin treatment gave no difference in elution pattern of Sephacryl S300 HR column chromatography (Fig. 2) nor produced any smaller enzyme including 95 kDa gelatinase.

Table I

Purification of high molecular weight type IV collagenase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg prot.)	Relative purification (fold)	Recovery (%)
Crude extract	3196	11,550	3.6	1	100
Amm. Sul. ppt.	1009	3196	3.2	0.9	28
DEAE-cellulose	99	9884	100	28	86
Hydroxyapatite	29	5140	176	49	45
Sephacryl S300 HR column chromatography	0.45	1955	4433	1231	17

Starting material was 70 g of metastatic liver carcinoma tissue. One unit was expressed as ^{3}H-labeled type IV collagenolytic activity that was capable of solubilizing 1000 cpm of the radioactivity into TCA soluble fraction.

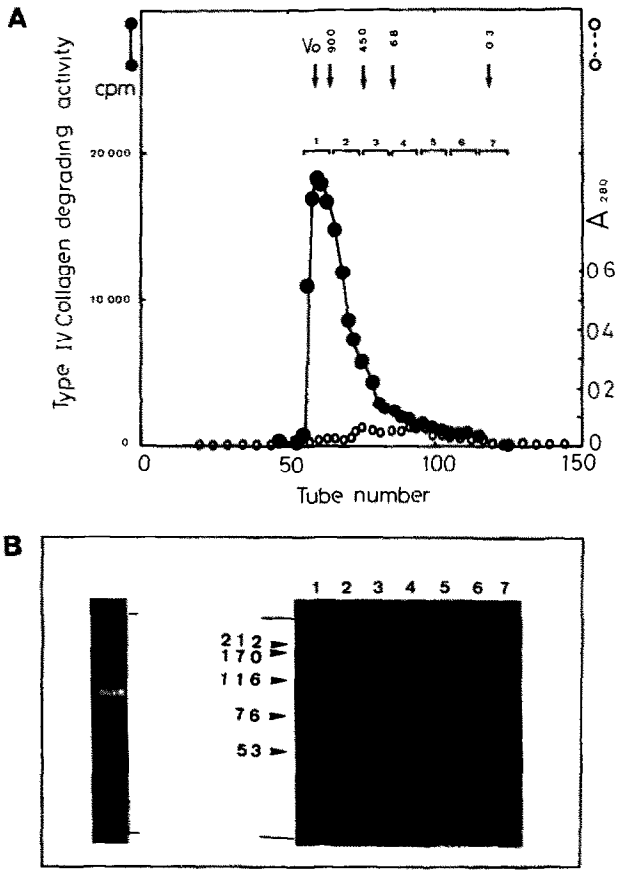

Fig. 1. (A) Sephacryl S300 column chromatography of high molecular weight type IV collagenase. 14 mg of partially purified enzyme in 2 ml were applied to a column of Sephacryl S300 HR equilibrated with 50 mM Tris-HCl, pH 7.6, buffer containing 20 mM NaCl and 5 mM $CaCl_2$. Column size was 2.5 × 90 cm, flow rate was 45 ml/60 min and 2.4 ml of tubes were collected. Absorbance at 280 nm (○) and ^{3}H-labeled type IV collagenolytic activity (●) was measured. Each 10 tubes (30 ml) was concentrated by Centricut 10 (Kurabou, Osaka, Japan) to 2 ml, cited fraction number from 1 to 7. Molecular weight markers were blue dextran (void volume), immunoglobulin M (900 kDa), ferritin (450 kDa), bovine serum albumin (68 kDa) and pyridoxal phosphate (0.3 kDa). (B) Pattern of zymography, before and after Sephacryl S300 HR column chromatography. Left: before column chromatography. Applied volume was 4 μl per lane. Molecular weight of the gelatinolytic enzyme observed as a transparent band was 95 kDa. Right: after column chromatography. Fractions 1 to 7 of column chromatography, shown in Fig. 1A, were applied onto lane 1 to 7. Applied volume was 4 μl per lane. Procedure of zymography was described in section 2. Molecular weight markers were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). Note that the transparent band was not detected in the lane 1 (fraction 1 in Fig. 1A) or 2 (fraction 2 in Fig. 1A), though a significant band was observed in lane 3 and 4. The molecular weight of the gelatinolytic enzyme was 95 kDa. The gelatinolytic activity can be compared between left and right quantitatively, since 2 ml of sample was fractionated into 7 fractions from 1 to 7, cited in Fig. 1A, then the volume of each fraction was concentrated to 2 ml.

3.4. *Detection of activity of high molecular weight type IV collagenase*

^{3}H-labeled substance degrading activity of both type IV collagen and gelatin were apparently found in this enzyme. Zymography was not adequate for this detection, because no transparent band was obtained even with the activated enzyme.

3.5. *Separation of high molecular weight type IV collagenase from 95 kDa gelatinase*

The radiological and zymographic analyses revealed that the partially purified enzyme before applied to Sephacryl S300 column chromatography contained type IV collagenase activity as well as a low molecular weight enzyme (95 kDa gelatinase) which was shown on zymography (Fig. 1B, left column). The high molecular weight type IV collagenase was separated from 95 kDa gelatinase by Sephacryl S300 column chromatography. The purified enzyme (fraction 1 in Fig. 1A), showed high ^{3}H-labeled type IV collagen degrading activity, but no gelatinolytic activity was shown on zymography (Fig. 1B, lane 1 in right column).

3.6. *Inhibition by TIMP and others*

As shown in Table III, the activity of the high molecular weight type IV collagenase was inhibited by metal chelators such as EDTA and *o*-phenanthroline, but not other seryl or thiol protease inhibitors, indicating that the enzyme was a metalloproteinase. In addition the enzyme activity was inhibited by reducing agents.

It was noted that TIMP, an endogeneous inhibitor of MMPs, was totally ineffective.

4. DISCUSSION

Following the previous reports on a type IV collagenase with high molecular mass which was isolated from human stomach carcinoma [15,17], the present study indicates that a high molecular weight type IV collagenase activity has been observed in 3 out of 8 different cases of metastatic liver carcinoma examined. Also, this enzyme activity was found in 2 cases of 5 hepatocellular carcinoma, but not in noncancerous liver tissues.

We have described further characterization of this purified high molecular weight type IV collagenase and compared its properties with the previously reported MMPs, which are to some extent in common; the en-

Table II

Activation by trypsin and APMA

	High M.W. type IV collagenase activity (cpm)	95 kDa gelatinase activity (cpm)
No addition	486	177
APMA	809	2142
Trypsin	5195	2403

High molecular weight type IV collagenase (1.1 μg) and 95 kDa enzyme (3 μg) were incubated with APMA (1 mM) at 37°C for 2 h or with trypsin (17 μg/ml) at 37°C for 10 min, respectively. ^{3}H-labeled type IV collagenolytic activity was measured.

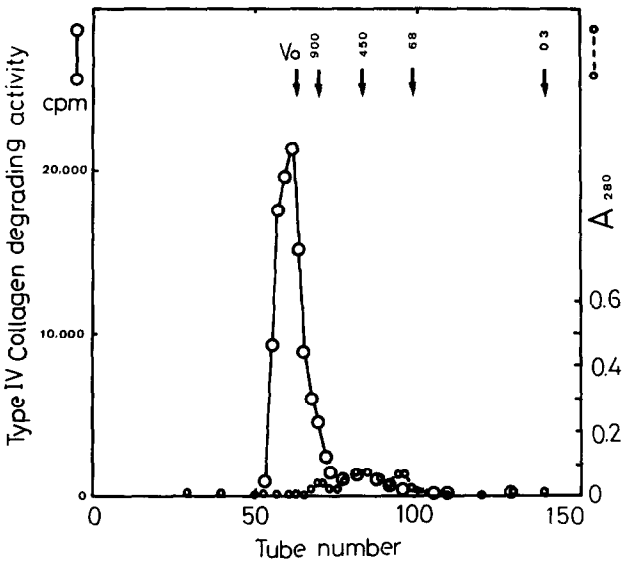

Fig. 2. Sephacryl S300 column chromatography of trypsin treated high molecular weight type IV collagenase. Purified high molecular weight type IV collagenase, shown as fr.1 in Fig. 1A, was treated by trypsin at 37°C for 10 min and applied to a Sephacryl S300 HR column. The running conditions were the same as in Fig. 1A.

zyme is activated by trypsin, degrading type IV collagen and gelatin, and is inhibited by metal chelators such as EDTA and *o*-phenanthroline as well as reducing agents [18]. However, in view of the molecular size and mode of activation and inhibition, the enzyme described here is definitely different from the MMPs. The molecular weight of this enzyme is estimated to be over 2,000 kDa, which is not identical to any one of the MMPs family nor some other similar enzymes such as heterodimer of MMP-9 (215 kDa) [19] and a matrix metalloproteinase (700 kDa) [20]. In addition, the high molecular weight type IV collagenase is neither activated by APMA, a general activator of MMPs, nor inhibited by TIMP which will form a stable stoichiometric complex with MMP-9 and MMP-2, namely, TIMP and TIMP-2, respectively [21,22], although a few reports are available as to TIMP resistant matrix metalloproteinase having the molecular weight of 700 kDa [20].

It should be noted that the MMPs isolated and characterized are mostly from the cultured medium of various cell lines, and in this experiment, the enzyme purification has been carried out by using tissues from solid carcinoma. Despite the sensitive zymographic analysis, on which MMPs can be detected, neither the active nor latent form of the high molecular weight type IV collagenase was found, even though ^{3}H-labeled collagen and gelatin degrading activities were noted. This may be due to the immobility of the high molecular weight enzyme on SDS-PAGE.

On zymography, 95 kDa gelatinase has been found in all the samples examined including 8 cases of metastatic liver carcinoma, 5 cases of hepatocellular carcinoma and 4 cases of noncancerous liver tissues. The properties of this 95 kDa enzyme partially purified are very similar to those of the ordinary MMPs. This 95 kDa enzyme is activated by both APMA and trypsin with a fall in molecular weight. There is a possibility that the 95 kDa enzyme forms complexes with TIMP-1 and that the complex can be activated to give active enzyme. The activity of this enzyme is inhibited by EDTA and *o*-phenanthroline, but not by seryl or thiol proteinase inhibitors. From these results and its molecular weight, 95 kDa gelatinase detected here would be gelatinase B (MMP-9, 92 kDa type IV collagenase, EC 3.4.24.35).

It has been reported that high expression of gelatinase B (MMP-9) is associated with oncogene transformation [8,23], and a good correlation is found between this enzyme activity and the metastatic potential of several types of tumors [23–28]. Although the physiological significance of this high molecular weight type IV collagenase remains unclear, the enzyme may play some role

Table III

Effect of inhibitors on high molecular weight type IV collagenase activity

Inhibitor	Concentration	Inhibition (%)
EDTA	5 mM	99
	10 mM	100
o-Phenanthroline	5 mM	99
	10 mM	99
Leupeptin	5 μg/ml	0
	10 μg/ml	0
Pepstatin	5 μg/ml	0
	10 μg/ml	0
E-64	5 μg/ml	0
	10 μg/ml	0
PMSF	5 mM	0
	10 mM	0
TIMP	1.1 μg/ml	0
	5.3 μg/ml	0
	10.6 μg/ml	0
	21.3 μg/ml	0
2-Mercaptoethanol	5 mM	88
	10 mM	95
DTT	5 mM	93
	10 mM	94
Human serum	4%	76
	8%	92

High molecular weight type IV collagenase was activated by trypsin prior to addition of inhibitors and ^{3}H-labeled substrate. Then enzyme reaction was performed at 37°C for 17 h.

in cancer metastasis, because significantly high activity has been noted in 3 out of 8 metastatic cases (601 units/g tissue, 523 units/g tissue, 323 units/g tissue, respectively), and no activity has been detected in noncancerous liver tissue ($n = 4$). Further studies on the relative quantity of this enzyme and ubiquitous inhibitors and histological localization in various carcinoma tissues will be needed to elucidate the pathophysiological significance as well as the metastatic ability of tumor cells.

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