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High molecular mass type IV collagen-specific metalloprotease from human carcinoma tissue

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A protease degrading type IV collagen was purified more than 8000-fold from human stomach carcinoma tissue. This protease degraded type IV collagen, while type I, II, III and V collagen, laminin, fibronectin, casein, albumin and hemoglobin were not affected. This enzyme had a pH optimum of pH 7.0–8.0 and was inhibited completely by EDTA and *o*-phenanthroline, but not by seryl, thiol and carboxyl protease inhibitors. Furthermore, the molecular mas of this enzyme was estimated to be 1 MDa by Sepharose 6B column and HPLC-gel filtration. The molecular mass and substrate specificity of this metalloprotease from human carcinoma tissue indicate it to be a new protease.

Metalloprotease; Collagen; Basement membrane; High molecular mass protease; (Human adenocarcinoma)

1. INTRODUCTION

It is supposed that the basement membrane plays an important role as a barrier against tumor invasion and metastasis [1,2]. Type IV collagen is one of the specific components in the basement membrane and reports have appeared concerning a protease which degrades type IV collagen, for example, from human skin fibroblast, human rheumatoid synovial fibroblast, human leukocyte granules, rat mast cell, rabbit bone and metastatic murine tumor cell culture medium [3–12]. However, no investigation of type IV collagen-degrading proteases from human malignant tissue has been published. Here, we report on the purification and characterization of a new metalloprotease from human malignant tissue, which has a high molecular mass and degrades type IV collagen specifically.

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; MCA, methylcoumaryl amide

2. MATERIALS AND METHODS

2.1. *Materials*

DE-52 was purchased from Whatman Paper (Maidstone, England). Hydroxyapatite was from Bio-Rad (Richmond, USA) and Sepharose 6B from Pharmacia (Milwaukee, USA). Trypsin, casein, hemoglobin, bovine serum albumin, soybean trypsin inhibitor and aprotinin were obtained from Sigma (St. Louis, USA). Leupeptin, pepstatin, antipain, E-64, chymostatin, elastatinal and several synthetic peptide substrates were from the Peptide Institute (Osaka). Human plasma fibronectin was obtained from Bethesda Research (Scotland) and murine laminin from Collaborative Research (Lexington, USA). Type I, II, III and V collagens were obtained from Koken (Tokyo). Type IV collagen from human placenta and bovine anterior lens capsule was from Koken or Sigma and from Nitta Gelatin (Osaka).

2.2. *Preparation of isotopically labeled type IV collagen*

^{3}H-labeled type IV collagen was prepared according to Hu et al. [13] and Means and Feeney [14]. The specific count of the labeled collagen was 2100 cpm/μg.

2.3. *Assay of type IV collagen-degrading enzyme activity*

40 μl of enzyme solution was activated by trypsin (17 μg/ml) with 360 μl of 50 mM Tris-HCl buffer (pH 7.6) containing

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20 mM NaCl and 5 mM $CaCl_2$ (basal buffer) at 37°C for 10 min. Activation was stopped by addition of 50 μl of a solution of SBTI (400 μg/ml) and aprotinin (5000 U/ml), followed by 7.5 μg ^{3}H-labeled type IV collagen as a substrate. The reaction was allowed to proceed for 18 h at 37°C and stopped by the addition of 100 μl of a solution of 10% trichloroacetic acid and 5% tannic acid. After 400 μl of the supernatant had been dissolved in 5 ml Aquasol-2, the radioactivity was counted. 1 unit of enzyme activity was expressed as the enzyme degrading 0.5 pg type IV collagen at 37°C for 18 h.

2.4. *Purification of the protease*

Human stomach adenocarcinoma tissue (58 g) obtained by surgical operation was minced and homogenized in a Waring blender with 5-fold volumes of basal buffer (pH 7.6) containing 500 mM NaCl, and sonicated. The enzyme solution was centrifuged at 12000 rpm for 20 min. The supernatant was precipitated by 25–55% $(NH_4)_2SO_4$ saturation, and the pellet obtained after centrifugation was dissolved and dialyzed vs basal buffer. The dialyzed enzyme solution was applied to a DE-52 column (3.6 × 25 cm) and equilibrated with basal buffer. The column was washed with the same buffer and then eluted with basal buffer (pH 7.6) containing 80 mM NaCl. Active fractions were collected and concentrated by 0–80% $(NH_4)_2SO_4$ saturation extensively, dialyzed vs 50 mM Tris-HCl buffer (pH 7.6) and applied to a hydroxyapatite column (2.6 × 8.0 cm). The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.6) and eluted with the same buffer containing 10 mM sodium phosphate buffer. The enzyme solution was concentrated using $(NH_4)_2SO_4$ and then dialyzed vs basal buffer. This was applied to a Sepharose 6B column (1.6 × 90 cm) equilibrated with basal buffer containing 0.2% polyethylene glycol and eluted with the same buffer. The enzyme activity appeared as a single peak. Active fractions were pooled, concentrated and used for the following studies.

2.5. *pH dependency*

In order to ascertain the pH optimum of the purified enzyme, 50 mM acetate buffer (pH 3.5–6.0) and 50 mM Tris-HCl buffer (pH 6.5–9.0) were used. After trypsin activation of the purified enzyme as described above the assay was performed using each buffer solution instead of basal buffer. The pH of the reaction mixture was confirmed after incubation.

2.6. *Substrate specificity*

The substrate specificity of the purified protease was studied using various synthetic and native substrates. After activation of the protease (600 U) with trypsin, the standard assay was performed using synthetic substrates, for example Bz-Arg-MCA, Boc-Phe-Ser-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Gly-Pro-MCA and Suc-Ala-Ala-Phe-MCA, as substrates instead of ^{3}H-labeled type IV collagen. The reaction was carried out for 6 h at 37°C until the addition of trichloroacetic and tannic acids, and then the fluorescence of the supernatant was measured. To determine the specificity of the protease with respect to native substrate, proteins such as type I, II, III, V collagens, laminin, fibronectin, casein, albumin and hemoglobin were used as substrates. The mixture of activated enzyme and each native substrate was allowed to react for 18 h at 37°C. Subsequently, the mixture was treated with SDS for 5 min at 95°C and applied to SDS-polyacrylamide gel electrophoresis. On comparison with the control (without enzyme), it was established whether or not the bands of substrate had been degraded.

2.7. *Inhibition of enzyme activity*

Inhibition of enzyme activity by various protease inhibitors was studied. Each inhibitor was added to activated enzyme solution and the reaction allowed to proceed for 18 h at 37°C. The following steps were the same as the standard assay described above.

Table 1

Purification of high molecular mass metalloprotease

Procedure	Total protein (mg)	Total activity ($U \times 10^{-3}$)	Specific activity ($U \times 10^{-3}$)	Relative purification (-fold)
25–55% $(NH_4)_2SO_4$ precipitation	507	101	0.2	1
DEAE-cellulose	28	1389	50	250
Hydroxyapatite	6.8	503	74	370
Sepharose 6B	0.26	446	1662	8310

3. RESULTS

3.1. *Purification of type IV collagen-degrading protease*

Representative steps in the purification of type IV collagen-degrading protease from human stomach carcinoma tissue are summarized in table

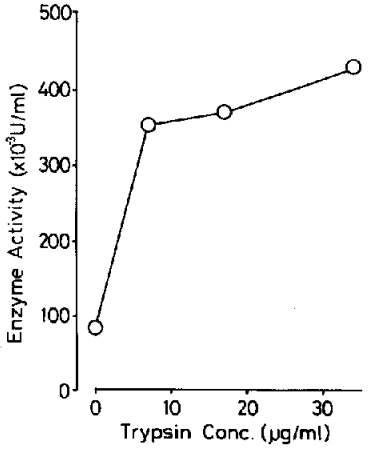


Fig.1. Trypsin activation of purified enzyme. The enzyme solution, containing trypsin added at various concentrations, was activated and assayed. Control was carried out without trypsin.

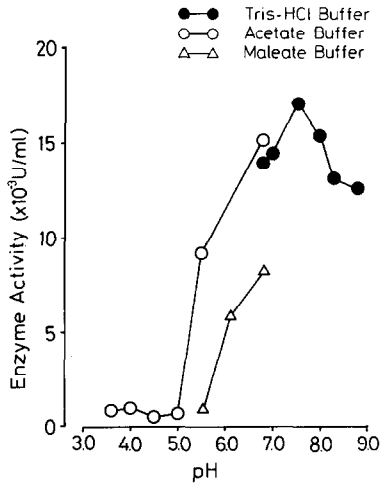


Fig.2. pH dependency. 50 mM acetate buffer (pH 3.5–6.0) and 50 mM Tris-HCl buffer (pH 6.5–9.0) were used instead of basal buffer during assay.

1. By means of this procedure, approx. 8300-fold purification of the protease was achieved. After DEAE-cellulose column chromatography, the total activity of the protease was increased. In Sepharose 6B column chromatography, the peak of enzyme activity was eluted as a single peak between those of blue dextran and ferritin. When this final enzyme preparation was applied to HPLC gel filtration (TSK G4000SW column), the enzyme activity was also eluted as a single peak and its molecular mass was estimated to be 1 MDa using blue dextran, IgM, ferritin and bovine serum albumin as standards. This value remained unchanged in the presence of 0.1% Triton X-100, Brij and 1 M NaCl. The final enzyme preparation was inactive and was activated by trypsin (fig.1).

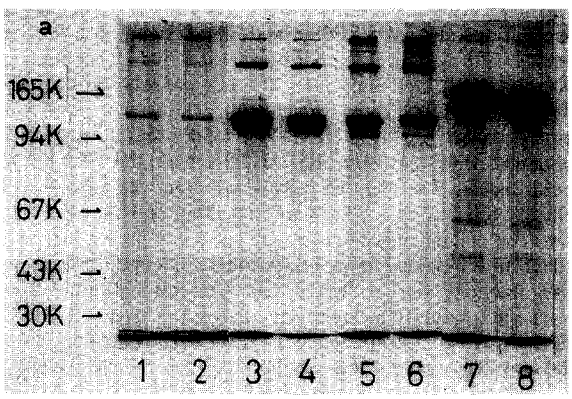
Table 2

Inhibitor sensitivity

Compounds (final concentration)	% inhibition
Leupeptin (10 μg/ml)	0
Antipain (10 μg/ml)	11
PMSF (5 mM)	10
Elastatinal (1 μg/ml)	0
(10 μg/ml)	36
Chymostatin (10 μg/ml)	10
E-64 (10 μg/ml)	0
Pepstatin (10 μg/ml)	0
Phosphoramidon (0.5 mM)	51
EDTA (5 mM)	97
(10 mM)	100
o-Phenanthroline (10 mM)	100

3.2. *pH dependency*

Fig.2 shows the pH dependence of the purified enzyme. The pH optimum of this enzyme was between pH 7.0 and 8.0, the maximum activity being around pH 7.6.



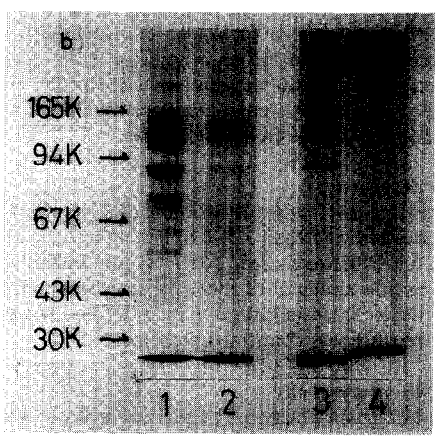


Fig.3. (a) Substrate specificity (1). Substrate specificity was studied using SDS-PAGE. Type I, II, III and V collagens were used as substrates and were incubated with enzyme. The mixture was then applied to a 7% acrylamide gel. Lanes: 1, type I collagen alone; 2, type I + enzyme; 3, type II alone; 4, type II + enzyme; 5, type III alone; 6, type III + enzyme; 7, type V alone; 8, type V + enzyme. (b) Substrate specificity (2). Type IV collagen (from bovine placenta and bovine anterior lens capsule) was used as substrate, and SDS-PAGE was carried out using 7% acrylamide gel. Lanes: 1, type IV collagen from bovine placenta alone; 2, type IV from bovine placenta + enzyme; 3, type IV from bovine anterior lens capsule alone; 4, type IV from bovine anterior lens capsule + enzyme.

3.3. *Inhibition of enzyme activity*

Table 2 lists the effects of several protease inhibitors on the activity of the protease. The purified enzyme was inhibited by only EDTA, *o*-phenanthroline and phosphoramidon which are effective inhibitors of metalloprotease, but was insensitive to leupeptin, antipain, chymostatin, E-64, pepstatin and PMSF. Elastatinal (10 μg/ml) inhibited 36% of the protease activity.

3.4. *Substrate specificity*

Specific degradation of various kinds of native substrates by the purified enzyme was determined through SDS-polyacrylamide gel electrophoresis. Type I, II, III and V collagens, fibronectin, laminin, casein, albumin and hemoglobin were incubated with activated enzyme for 18 h at 37°C, however the main bands of all substrates did not vary. On the other hand, type IV collagen (from bovine placenta and bovine anterior lens capsule) was also incubated with enzyme. The 100 and 90 kDa bands of each type IV collagen disappeared, and other chains were also partially degraded (fig.3).

The enzyme did not cleave any synthetic peptide substrates used as substrate.

4. DISCUSSION

The purification of a protease from human malignant tumor has been described. The protease has a high molecular mass (1 MDa) and degrades type IV collagen specifically. During purification, especially after DEAE-cellulose column chromatography, the total activity increased. This phenomenon shows that one or more native inhibitors against this enzyme might exist in tumor tissue, and were removed by the purification step. We also found that this enzyme existed as a latent form and was activated by trypsin. Inhibition by EDTA, *o*-phenanthroline and phosphoramidon suggests that this enzyme is a metalloprotease. These characteristics are similar to those of other collagenase type proteases reported. From the specificity of the purified protease with respect to native substrates, it is inferred that this enzyme differs from gelatinase, proteoglycanase and collagenase type proteases which degrade basement membrane components. Gelatinase cleaves gelatin, casein, elastin, and type IV and V collagens [15]. Proteoglycanase cleaves proteoglycan, laminin, fibronectin and type I and IV collagens [9]. The protease from rabbit bone culture medium degrades type IV and V collagens, gelatin, laminin and fibronectin [8,9], and that from human rheumatoid synovial fibroblast culture medium gives rise to degradation of proteoglycan, laminin, gelatin, fibronectin and type IV collagen [4]. The proteases from human polymorphonuclear leukocyte granules and rat mast cells also degraded type IV collagen, however these are serine proteases [5–7]. Furthermore, all other type IV collagen-degrading proteases except our enzyme have molecular masses below 100 kDa. Based on these results, our enzyme seems to be a new protease derived from human carcinoma tissue. In addition, it is believed that this protease degrades type IV collagen and destroys the function of the basement membrane, resulting in susceptibility to tumor invasion and metastasis. It is very interesting that the type IV collagen-degrading protease exists in malignant tumor tissue, since the first step in the mechanism of tumor invasion and metastasis involves destruction of the basement membrane.

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