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COLLAGENASE ACTIVITY IN CULTURES OF RAT PROSTATE CARCINOMA *

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Summary

A specific collagenase (EC 3.4.24.3) has been found and purified from serum-free culture medium of 11095 epidermoid carcinoma of rat prostate. The molecular weight of this collagenase was estimated at 71 000 and the pH optimum was approx. 7. At 26°C, the collagenase cleaved collagen at a site 3/4 the length from the N-terminus. At 37°C, this collagenase degraded collagen to smaller peptides. The enzyme activity was inhibited by serum, cysteine and EDTA, but not by protease inhibitors. The presence of collagenase in rat tumor tissue suggests that this enzyme might play a significant role in tissue invasion by cancer cells.

Introduction

Recent studies by several investigators have demonstrated collagenolytic activity in tumors [1—3]. In our previous communications [4,5], we reported that high collagenolytic activity might be related to aggressiveness of malignant tumors of the head and neck. These studies suggest that collagenase might play a significant role in degradation of connective tissue associated with tumor invasion. By using immunofluorescent staining techniques, our studies [5] and others [6] have shown that collagenase is present in the connective tissue stroma and not in tumor cells. These observations raise the question of how tumor cells interact with the connective tissue to stimulate the production of

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active collagenase. The animal models can serve as an effective system to study this problem and the role of collagenase in tumor invasion.

A transplantable prostate carcinoma in rats (called the 11095 squamous cell carcinoma) was used as the animal model for our study and was originally induced with methylcolanthrene in Fisher 344 ratss by Segaloff [7] (Ochsner Foundation, New Orleans, LA). Subsequent transplanations were performed in the rats by subcutaneous injection of tumor suspension in the right flank region. This particular tumor was chosen because it is composed of large cells, containing epithelial pearls similar to human epidermoid carcinoma. The present paper reports the isolation and purification of collagenase from culture medium of rat tumor and its properties.

Methods

Enzyme preparation. The rat tumor tissues, used for isolation of collagenase, were removed two weeks after transplantation. The tumor tissues were first cut into about 1 mm^3 pieces, washed three times in Hank's balanced salt solution containing 100 units penicillin, 100 μg streptomycin, 0.25 mg fugizone/ml and cultured in Dulbecco's modified Eagle medium in Falcon plastic flasks at 37°C under O_2/CO_2 (95 : 5, v/v). The culture medium was changed daily for six days. All media were collected, centrifuged and filtered through glass wool to remove any tissue debris. The filtrates were stored at —20°C before use.

Chromatography. All chromatography was carried out at 4°C. Adsorption chromatography was performed on controlled pore glass beads (CPG-250, 74–125 μm particles, Pierce Chemicals, Rockford, IL). The culture medium (2.55 g protein) of rat tumor was applied to a CPG-250 column (2.6 X 25 cm), which was then eluted stepwise with the following buffers: (a) 0.05 M Tris-HCl (pH 7.6); (6) 1 M NaCl in 0.05 M Tris-HCl (pH 7.6); (c) 1 M Tris-HCl (pH 7.6); (d) 1 M LiBr in 0.05 M Tris-HCl (pH 8.0), and (e) 2 M LiBr in 0.05 M Tris-HCl (pH 8.2). The eluates were monitored for protein and collagenolytic activity and the active fraction concentrated by ultrafiltration with Amicon UM-10 membrane. All buffers contained 0.005 M $CaCl_2$.

The active fraction was dialyzed against 0.05 M Tris-HCl (pH 7.6), 0.2 M NaCl overnight and then applied to a Sephadex G-150 column (1.6 X 100 cm), equilibrated with the same buffer (2.6-ml fractions; flow rate, 15 ml/h). The eluate was assayed for collagenolytic activity and active fractions were pooled, concentrated and rechromatographed on a superfine Sephadex G-100 column (1.5 X 90 cm), equilibrated with the above buffer (1 ml fraction; flow rate, 3.6 ml/h).

Molecular weight determination was conducted by gel filtration on a Sephadex G-150 column (1.5 X 90 cm). The protein standards were γ-globulin (160 000), albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 400).

Assay procedures. Collagenase activity was determined by the method of Terato et al. [9] in which the substrate is ^{14}C-labelled collagen in solution (20 000 cpm/mg). Collagen was extracted from guinea pig skin with NaCl. For this assay, the substrate contained 50 μl 0.4% radioactive collagen and 20 μl 2 M glucose, all in 0.05 M Tris-HCl (pH 7.6), 0.005 M $CaCl_2$. 100-μl aliquots of

samples were added and the reaction mixtures then incubated at 35°C for 4 h. The reaction was stopped by adding 10 μl 80 mM *o*-phenanthroline in 50% dioxane. The mixtures were kept at the same temperature for 1 h and then cooled to 25°C. The intact collagen molecules were precipitated by addition of 150 μl dioxane. After centrifugation at 10 000 × *g* for 20 min, 0.2 ml supernatant was added to 10 ml 70% Instagel and assayed for radioactivity.

The pH dependence of enzyme action was studied in the range of pH 5.0—9.0 using 0.05 M Tris/maleate and Tris-HCl. Protein was determined by the method of Lowry et al. [10].

Inhibition studies. Inhibitors and their final concentrations used were as follow: fetal calf serum 1 : 10 and 1 : 40 dilution; $5 \cdot 10^{-3}$ M and $5 \cdot 10^{-2}$ M cysteine; $1 \cdot 10^{-2}$ M and $4 \cdot 10^{-2}$ M EDTA; 10^{-3} M *N*-tosyl-L-lysine chloromethylketone; 10^{-3} M L-tosylamido-2-phenylethyl chloromethylketone; 10^{-3} M phenylmethylsulfonyl fluoride and 0.1 mg/ml soybean trypsin inhibitor. All assays were conducted at pH 7.6.

Electrophoresis methods. The purity of enzyme was examined by polyacrylamide gel electrophoresis according to the method of Davis [11] using 7.5% acrylamide. The gels were stained with Coomassie brilliant blue R250. The collagen-degraded products were subjected to electrophoresis in 7.5% polyacrylamide gels according to the method of Nagai et al. [12]. The collagen-degraded products were prepared as follow. The reaction mixtures contained 100 μg collagen and 20 μg purified rat tumor collagenase in 100 μl 0.05 M Tris-HCl (pH 7.6) 0.005 M $CaCl_2$ were incubated at 26°C for 16 h or at 37°C for 4 h and denatured by heating at 45°C for 5 min after adding one drop of acetic acid.

SDS-polyacrylamide gel electrophoresis for determination of molecular weight was performed by the method of Weber and Osborn [13] using 10% polyacrylamide gels. The protein standards were oligomers of lysozyme, from monomer to hexamer (molecular weight range 14 300—85 000, Gallard-Schlesinger Chemicals, Carle Place, NY).

Results

Purification of rat tumor collagenase

A chromatographic pattern of the pooled culture medium through the controlled pore glass beads (CPG-250) is shown in Fig. 1. Most of the collagenolytic activity was retained on the glass beads. Adsorption chromatography on CPG-250 resulted in the separation of collagenolytic activity into two components referred to as fraction III (5% recovery) and fraction V (80% recovery). Only fraction V with high specific activity was further purified by gel filtration.

Gel filtration of the concentrated rat tumor collagenase fraction V was first performed on a Sephadex G-150 column equilibrated with 0.05 M Tris-HCl (pH 7.6), 0.2 M NaCl, 0.005 M $CaCl_2$. As shown in Fig. 2, a single peak of collagenolytic activity was obtained. The active fractions were pooled, concentrated by ultrafiltration and then applied to a superfine Sephadex G-100 column equilibrated with the above buffer. The enzyme eluted in a single symmetrical peak. This enzyme fraction was examined by electrophoresis in 7.5% polyacryl-

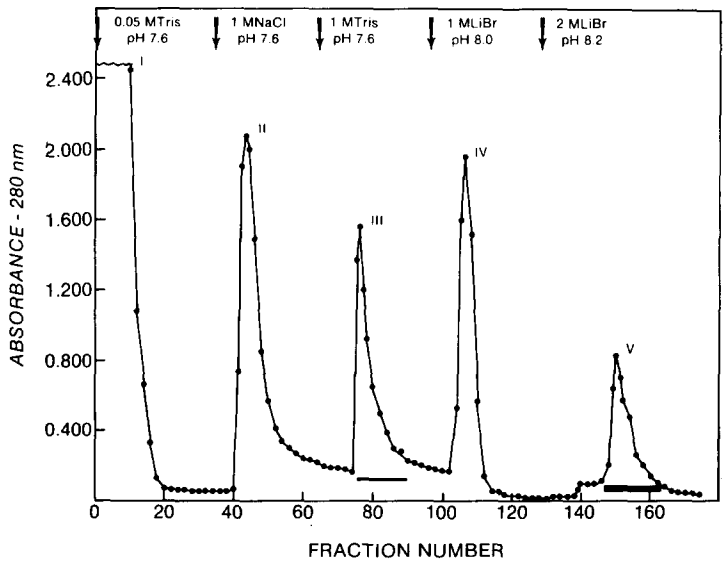

Fig. 1. Adsorption chromatography on controlled pore glass beads (CPG-250). Culture medium (2.55 g protein) of epidermoid carcinoma of prostate in rats was applied to a CPG-250 column (2.6 X 25 cm). The column was then eluted stepwise with the buffers as shown (10-ml fractions; flow rate, 40 ml/h). The bars on the figure indicate fractions having collagenase activity.

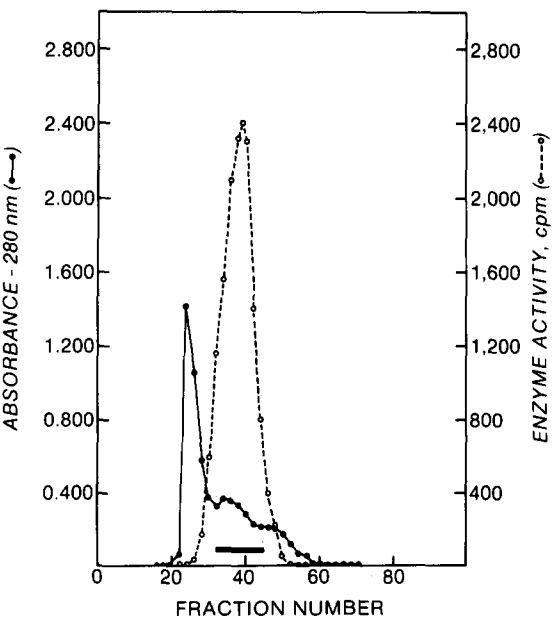

Fig. 2. Gel filtration in Sephadex G-150 of the concentrated rat tumor collagenase fraction V from adsorption chromatography. The sample (113 mg protein) was applied to a Sephadex G-150 column (1.6 X 100 cm) and the column was eluted with 0.05 M Tris-HCl (pH 7.6) o.2 M NaCl, 0.005 M $CaCl_2$ (2.6-ml fractions; flow rate, 15 ml/h). 100 μl aliquot from each fraction was used for collagenase activity assay.

TABLE I

PURIFICATION OF RAT TUMOR COLLAGENASE

Specific activity refers to μg collagen degraded/min per mg protein at 35°C.

Purification procedure	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Recovery (%)
Tissue culture medium	2550	0.12	306.0	100
CPG	113	2.1	237.3	77.5
Sephadex G-150	10.1	16.6	167.7	54.8
Sephadex G-100 (superfine)	2.99	34.9	104.3	34.1

amide gels and one protein band was observed. SDS-polyacrylamide electrophoresis in 10% gels also showed a single sharp protein band. This highly purified enzyme preparations has been used in all experiments described in the present study. The yield and purification of a typical enzyme preparation are summarized in Table I.

Molecular weight of rat tumor collagenase

The molecular weight of rat tumor collagenase was estimated at approx. 63 000 or 71 000 by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively. The molecular weight for this enzyme derived from SDS-polyacrylamide gel electrophoresis seems to be more reliable than that derived from gel filtration.

Action of collagenase on soluble collagen

At 26°C, the purified rat tumor collagenase degraded the collagen molecule and produced the fragments corresponding to the size of 3/4 of the original molecule. The denatured products of enzyme/collagen reaction mixture on polyacrylamide gel demonstrated one new band below the original β band and another two new bands below the original α bands.

Inhibition of collagenase

The rat tumor collagenase activity was inhibited by common mammalian collagenase inhibitors as shown in Table II. Thus, serum, cysteine, and EDTA

TABLE II

EFFECT OF INHIBITORS ON RAT TUMOR COLLAGENASE

Inhibitor	Concentration	Activity (cpm)	Inhibition (%)
None		1555	
Serum	1 : 10	303	81
	1 : 40	526	66
Cysteine	50 mM	130	92
	5 mM	533	66
EDTA	40 mM	51	97
	10 mM	256	84

were shown as strong inhibitors of this collagenase. The protease inhibitors such as *N*-tosyl-L-lysine chloromethylketone, L-tosylamido-2-phenylethyl chloromethylketone, phenylmethylsulfonyl fluoride and soybean trypsin inhibitor had very little inhibitory effect on this collagenase activity.

pH dependence of the enzyme activity

The optimal pH for collagenase activity appeared near neutrality (pH 7.0). This value is quite similar to those of most mammalian collagenases.

Discussion

Our present studies have demonstrated the release of collagenolytic activity in cultures of minced transplantable rat prostate epidermoid carcinoma tissue incubated in serum-free medium. These cultures provide plentiful supply of crude tumor enzyme for purification allowing us to study the original site of enzyme synthesis and mechanisms of collagen breakdown in tumors.

Collagenases have been shown to have a high affinity to glass surface [14] or insoluble particulate materials [15]. The high affinity of collagenase to glass allows rapid separation of collagenase from other proteins. In the present studies, chromatography on controlled pore glass beads (CPG-250) is shown to be very useful for the purification and concentration of collagenase from a large volume of culture medium. 3 l culture medium (2.55 g protein) can be passed through a CPG-250 column (2.6 X 25 cm). Most collagenolytic activity in the culture medium was retained on the glass beads. The column was then eluted by different chaotropic eluting agents [8] by varying the pH and changing the ionic strength as shown in Fig. 1. The collagenolytic activity was eluted into two peaks with 1 M Tris, pH 7.6 (fraction III) and with 2 M LiBr, pH 8.2 (fraction V). Purification of the rat tumor collagenase up to 20-fold was achieved during adsorption chromatography. Fraction V with most enzyme activity was further purified to homogeneity by gel filtrations through Sephadex G-150 and superfine Sephadex G-100. 290-fold increase in specific activity was obtained. The relationship between fractions III and V was not studied due to the small amount of fraction III.

The purified rat tumor collagenase has a specific activity of 34.9 units/mg. We have attempted further to purify the enzyme in order to increase its specific activity as high as those reported recently for other tissue collagenases [16—18]. Further purification of the enzyme by means of ion-exchange chromatography using DEAE-cellulose or QAE-Sephadex A-50 and affinity chromatography with collagen-Sepharose 4B did not increase specific activity significantly. Although we were able to purifiy guinea-pig skin collagenase [19] by means of extraction after polyacrylamide gel electrophoresis, the rat tumor collagenase completely lost its activity in polyacrylamide gel electrophoresis. The difference in stability of enzyme activity in polyacrylamide gel electrophoresis from other collagenases may be due to species difference.

The rat tumor collagenase appears to be similar in many respects to the enzyme from human tumor tissues as well as the normal mammalian enzymes. These enzymes are similar in their action on soluble collagen substrate, in their pH optimum and in their response to collagenase inhibitors. When compared

with rabbit VX-2 carcinoma collagenase either from culture medium [1] or from homogenate [16], the rat tumor collagenase showed some differences in response to cysteine and in molecular weight. The rat tumor enzyme was more sensitive to inhibition by cysteine and was larger than the rabbit tumor enzyme in molecular size. The rat tumor collagenase has a rather high molecular weight suggesting that the purified enzyme may contain a proenzyme or an enzyme-inhibitor complex or other impurities. The presence of an enzyme-inhibitor complex or other impurities is not likely since polyacrylamide gel electrophoresis with or without SDS showed one protein band. Obtained low specific activity reveals that the purified rat tumor collagenase may contain a proenzyme in similar molecular size as the active one. However, we did not see any increase in specific activity when the purified enzyme was treated with trypsin according to the method of Vaes [20]. Activation of proenzyme may require a specific protease. The purified rat tumor collagenase similar to the rheumatoid synovial collagenase [21], in addition to producing α^A and β^A, is capable of degrading collagen into smaller fragments at 37°C.

Collagenase found in the human tumor connective tissue stroma [5,6] suggests that the enzyme may be a wound response of host to the presence of the tumor cells. Tumors can be considered as non-healing wounds in that the high production of collagenase may occur resulting destruction of connective tissue at the margin of tumors. This has not ruled out the possibility that the collagenase could be derived from macrophages or leucocytes of tumors. Macrophages have been shown to produce collagenase by incubation with endotoxin [22] and with lymphokins [23]. This is not likely since our rat tumors were subcutaneous and showed no histologic evidence of infection. Neutrophils were not present and macrophages were rarely seen. The production of a large amount of active collagenase from tumor tissues may be related to activation of inactive form by proteolytic enzymes which are present in tumor tissues with high quantities. The mechanisms by which the tumor cells interact with the connective tissue stroma to produce active collagenase still remains an unsolved problem.

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