Biochimica et Biophysica Acta, 384 (1975) 484—492

BBA 67449

PURIFICATION AND CHARACTERIZATION OF COLLAGENASE FROM GUINEA PIG SKIN

CHENG-CHUN HUANG and MAXWELL ABRAMSON

Department of Otolaryngology and Maxillofacial Surgery, University of Iowa College of Medicine, Iowa City, Iowa 52242 (U.S.A.)

(Received August 16th, 1974)

Summary

Guinea pig skin collagenase, isolated from culture medium of whole skin, was separated into two enzymatically active fractions. These two fractions have been purified extensively. Peak II fraction has been purified to homogeneity as examined by polyacrylamide gel electrophoresis. Their molecular weights are approximately 130 000 (peak I) and 40 000 (peak II). Both guinea pig skin collagenase fractions are capable of degrading the native collagen fibrils and are inhibited by serum, cysteine and EDTA. They appear to be glycoproteins.

Guinea pig skin (peak II) and human skin collagenase were compared. They are both glycoproteins and have similar molecular size (M_r = 40 000). Immunodiffusion assay showed that no cross-reactivity was seen between the enzymes, indicating species specificity among collagenases.

Introduction

Since tadpole collagenase was first demonstrated by Gross and Lapiere [1], collagenase have been isolated from a number of animal and human tissues [2]. Most of these enzymes were isolated from culture media. These enzymes are capable of degrading native collagen in similar manner, i.e., cleaving the native collagen molecule at a point three quarters the distance from the amino terminal end at physiological pH and temperature.

At least two collagenases are present in the crude preparations of human skin [3], rabbit skin [4], and possibly rat skin [5] collagenases. These multiple forms of collagenases do not appear to be identical as evidenced by their different molecular weight. The collagenases from the wound rabbit skin also show different responses to inhibitors. However, they all attack the native collagen molecule at the same point, i.e., three quarters of its length from the amino terminal end.

Grillo and Gross [6] and Donoff et al. [4] demonstrated that wounded skin produced higher levels of collagenolytic activity as compared with non-wounded skin. The increase in collagenolytic activity after injury might facilitate cellular detachment from the connective tissue and remodeling of the tissue during repair.

The present paper reports the purification of at least two collagenases from the culture medium of the wounded guinea pig skin, their properties and comparison between guinea pig skin and human skin collagenases.

Methods

Preparation of crude enzyme

Guinea pigs (350 g) were excoriated on the trunk with a wire brush after the animals were anesthetized with Innovar-Vet. The skins were removed under sterile conditions three days later. The fat was removed with a scalpel blade. The skins were then cut into pieces about 2 mm square. After washing twice with Hank's balanced salt solution containing 100 units of penicillin and 100 μg streptomycin per ml, the skins were placed in Dulbecco's modified Eagle's medium and cultured in Falcon plastic flasks at 37°C under an atmosphere of O_2/CO_2 (95 : 5, v/v). Culture medium was changed daily for 10 days. All media except the first day medium were collected, centrifuged, and stored frozen after adding 0.1 vol. of 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$. Media were pooled, condensed with Amicon ultrafilter UM-10, dialyzed at 4°C against distilled water with several changes, lyophilized and stored at −20°C.

Ammonium sulfate precipitation

Crude guinea pig skin collagenase powder was dissolved in 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$ to a concentration of 15 mg protein per ml. The undissolved material was removed by centrifugation. Ammonium sulfate was added slowly to a final saturation of 60% at 4°C. The precipitate was collected by centrifugation at 10 000 × g for 20 min and dissolved in 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$.

Chromatography

Gel filtration of the ammonium sulfate enzyme preparation was carried out on a column (2.6 × 100 cm) of Sephadex G-150 (Pharmacia) equilibrated with 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$ and 0.2 M NaCl. Fractions containing collagenase activity were pooled, concentrated to a small volume with aquacide (Cal-Biochem) and rechromatographed through a column (1.6 × 100 cm) of Sephadex G-150. Fractions having collagenase activity were pooled, concentrated and dialyzed against the same buffer (without NaCl).

Ion exchange chromatography was carried out on a column (2.6 × 25 cm) of DEAE-Sephadex A-50 (Pharmacia) equilibrated with 0.01 M Tris · HCl (pH 7.2) containing 0.01 M calcium acetate (starting buffer) [7]. The enzyme solution was dialyzed against the starting buffer for 2 h before applying on the column. The column was eluted stepwise with the following buffers: (a) the starting buffer, (b) 0.02 M Tris/acetate (pH 6.2) containing 0.02 M calcium

acetate, and (c) 0.06 M sodium acetate (pH 5.6) containing 0.1 M calcium acetate. Fractions containing collagenase activity were pooled, concentrated and dialyzed against 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$.

Affinity chromatography

Collagen was coupled to Sepharose 4B (Pharmacia) as described by Bauer et al. [8] according to the method of Cuatrecasas [9]. The enzyme preparation obtained from Sephadex G-150 was applied on a column (1.2 × 12 cm) of collagen-Sepharose and eluted with 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$ followed by 1.0 M NaCl in the same buffer.

Assay procedures

Collagenase activity was determined by release of soluble radioactivity from ^{14}C-labelled reconstituted collagen fibrils [10]. Collagen was extracted from guinea pig skin with sodium chloride and purified by the method of Gross [11]. The reconstituted collagen fibrils were prepared from the solution of ^{14}C-labelled collagen by mixing 50 µl of 0.4% ^{14}C-labelled collagen (approx. 4000 cpm) and 50 µl of 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$ and allowed to gel at 37°C for at least 2 h. An equal volume of enzyme was added to the collagen gel for assay.

Viscometry was also used to monitor collagenase activity at 26°C using Ostwald viscometers having a flow time for water of 65 s at 26°C. The reaction mixture contained 0.2 ml of 0.4% collagen, 0.2 ml of enzyme solution and 0.15 ml of 1 M NaCl in 0.05 M Tris · HCl (pH 7.6) containing 0.005 M $CaCl_2$. A control without enzyme was run at the same time. Protein was determined by the method of Lowry et al. [12].

Disc gel electrophoresis

Collagen in reaction mixtures was denatured by heating at 45°C for 5 min and subjected to electrophoresis in polyacrylamide gels according to the method of Nagai et al. [13].

The purity of enzyme was examined by disc gel electrophoresis according to the method of Davis [14], using a 15% concentration of acrylamide. The gels were stained with Coomassie blue for protein and with periodic acid-Schiff reagent for carbohydrate [15].

Preparation of anti-enzyme antisera and immunodiffusion

White rabbits weighing 4—5 kg were injected with purified guinea pig skin collagenase (peak II). The first injection was given subcutaneously as an emulsion (0.5 mg protein) with complete Freund's adjuvant while the next two booster injections in two week intervals were given into the ear vein. Blood was taken from the ear vein two weeks after the last injection. Sera were isolated, collected and stored at −40°C in small portions. Immunodiffusion analysis on agar gel was carried out according to the method of Ouchterlony [16].

Results

Purification of collagenase

The ammonium sulfate enzyme preparation was separated into two frac-

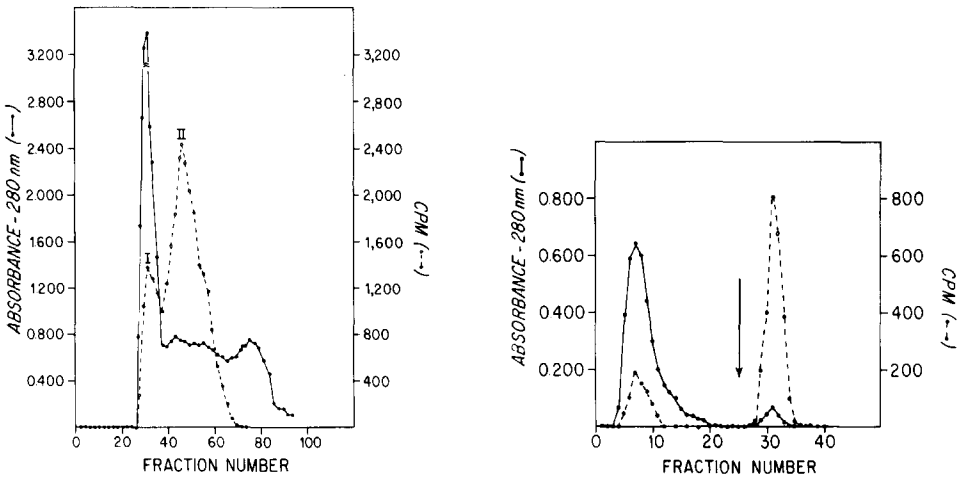

Fig. 1. Gel filtration of guinea pig skin collagenase separated on Sephadex G-150 with 0.05 M Tris · HCl (pH 7.6) containing 0.2 M NaCl and 0.005 M $CaCl_2$. A sample of 314 mg was applied to the column (2.6 × 100 cm). Effluent fractions of 5 ml were collected at a rate of 60 ml/h. Collagenase activity was measured by incubating 100 μl of the eluant fractions for 4 h in a shaking water bath at 37°C with ^{14}C-labelled collagen fibrils.

Fig. 2. Affinity chromatography of guinea pig skin collagenase (peak I) on collagen-Sepharose. A sample of 30 mg of partially purified collagenase peak I was applied to the column (1.2 × 12 cm) and effluent fractions of 4 ml were collected at a rate of 36 ml/h. Elution was accomplished by the addition of 1 M NaCl to the eluant buffer (see arrow). Collagenase activity was measured by incubating 100 μl of the eluant fractions for 4 h in a shaking water bath at 37°C with ^{14}C-labelled collagen fibrils.

tions designated as peaks I and II by gel filtration through the Sephadex G-150 column (2.6 × 100 cm) as shown in Fig. 1. Peaks I and II were separately re-chromatographed on the Sephadex G-150 column (1.6 × 100 cm). Both peaks I and II were then re-purified separately.

Peak I was applied to a collagen-Sepharose column and its chromatogram is demonstrated in Fig. 2. Most of the collagenase activity bound to collagen-Sepharose can be eluted with a buffered solution at 1 M NaCl. The fractions having collagenase activity appear as two protein bands on polyacrylamide gel electrophoresis (Fig. 3). Both these two bands showed a positive-periodic Schiff reaction.

Purification of Peak II was first carried out by ion-exchange chromatography on a column (2.6 × 25 cm) of DEAE-Sephadex A-50. The column was eluted stepwise with the three buffers as shown in Fig. 4. Collagenase activity was eluted with 0.06 M sodium acetate (pH 5.6) containing 0.1 M calcium acetate. This fraction still contained contaminating proteins which were removed by polyacrylamide gel electrophoresis of Davis' method [14]. One gel was stained with Coomassie blue for protein. The corresponding protein bands on the unstained gels were removed by using the stained gel as a reference. The sections of the removed gels were crushed into small pieces. The resultant pieces were applied into a disposable pipette and eluted with 0.05 M Tris · HCl (pH 7.6) containing 1 M NaCl and 0.005 M $CaCl_2$. The eluants having enzyme activity were pooled. Only one protein band was shown on polyacrylamide gel (Fig. 3). The protein can be stained with the periodic Schiff reagent. The yield and purification of a typical enzyme preparation are summarized in Table I.

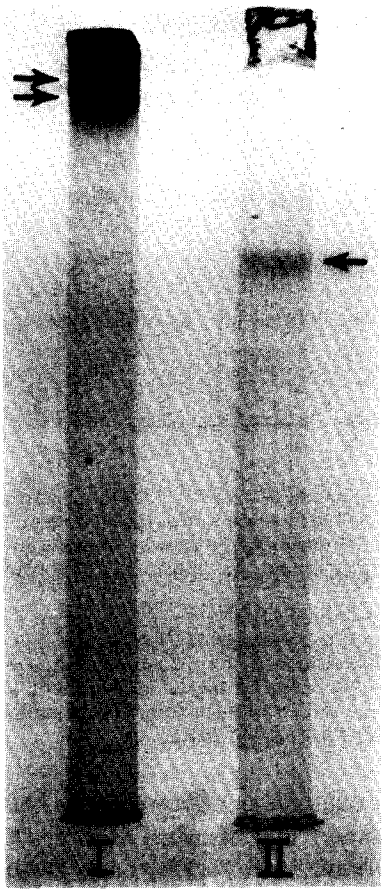

Fig. 3. Polyacrylamide gel electrophoresis of guinea pig skin collagenases. Peak I (80 μg of protein) and Peak II (20 μg of protein) were applied to the 15% polyacrylamide gels. The gels were stained with Coomassie blue.

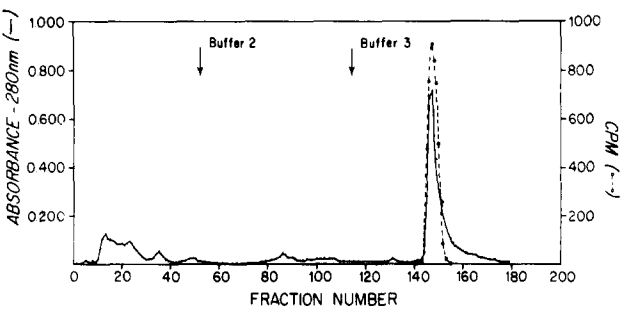

Fig. 4. DEAE-Sephadex A-50 chromatography of partially purified collagenase peak II from gel filtration. Approximately 88 mg of enzyme protein in 13 ml of 0.01 M Tris · HCl (pH 7.2) containing 0.01 M calcium acetate (starting buffer) was applied to a column (2.6 × 25 cm) and effluent fractions of 4.5 ml were collected at a rate of 14 ml/h. The column was eluted stepwise with the buffers (a) starting buffer, (b) buffer 2: 0.02 M Tris/acetate (pH 6.2) containing 0.02 M calcium acetate, and (c) buffer 3: 0.06 M sodium acetate (pH 5.6) containing 0.1 M calcium acetate (see arrows). Collagenase activity was measured by incubating 100 μl of the eluant fractions for 4 h in a shaking water bath at 37°C with ^{14}C-labelled collagen fibrils.

TABLE I

PURIFICATION OF GUINEA PIG SKIN COLLAGENASE

Purification step		Total protein (mg)	Specific activity* (units/mg protein)	Total activity (units)	Recovery (%)
Crude enzyme		1311	36	47196	100
$(NH_4)_2SO_4$ ppt		314	167	52438	111
Sephadex G-150	I	54.6	146	7971	17
	II	88.2	328	28929	61
Collagen-Sepharose	I	4.6	696	3201	6.7
DEAE-Sephadex	II	11.5	716	8234	17
Polyacrylamide gel	II	0.8	3884	3107	6.5

* Specific activity refers to the mg collagen solubilized per mg protein.

Properties of guinea pig collagenases

The molecular weights of the guinea pig collagenases were estimated approximately 130 000 (peak I) and 40 000 (peak II) by gel filtration through a column (1.6 × 100 cm) of Sephadex G-150 according to Andrew's method [17].

Anti-guinea pig skin collagenase antiserum gave a single precipitin band with its antigen as shown in Fig. 5. This figure also indicates that no reaction was seen on immunodiffusion between anti-guinea pig skin collagenase antiserum and human skin collagenase.

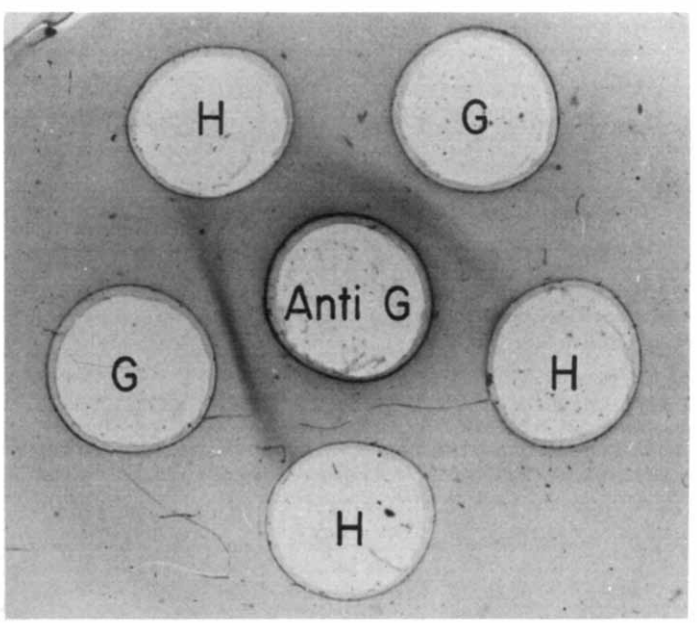

Fig. 5. Immunodiffusion analysis of antiserum against guinea pig skin collagenase (peak II). H, human skin collagenase; G, guinea pig skin collagenase (peak II).

TABLE II

EFFECT OF INHIBITORS ON GUINA PIG COLLAGENASE

	Peak I		Peak II	
	cpm* (above blank)	% inhibition	cpm* (above blank)	% inhibition
Enzyme control	918		2070	
with serum (1 : 10)	490	46.7	460	78
with serum (1 : 40)	630	31.2	772	63
with serum (1 : 100)	866	5.5	1424	31
with cysteine (0.05 M)	310	66.3	572	72
with cysteine (0.005 M)	818	11.0	1682	19
with EDTA (0.04 M)	82	91.1	362	83
with EDTA (0.01 M)	176	81.0	430	80

* Total radioactivity per incubation mixture was 4000 cpm.

Effect of inhibitors

Enzymatic activities of both guinea pig skin collagenase fractions were inhibited by fetal calf serum, cysteine, and EDTA (Table II). The data indicate that serum has less inhibitory effect on peak I than on peak II collagenase.

Viscosity and disc electrophoresis

Both guinea pig skin collagenase fractions are capable of reducing the initial specific viscosity at 26°C by approx. 30% and 50% respectively in six hours of incubation (Fig. 6). When the denatured products of enzyme-collagen reaction mixtures were examined by polyacrylamide gel electrophoresis, one new band below the original β band and another two new bands below the original α bands appeared on the gel (Fig. 7).

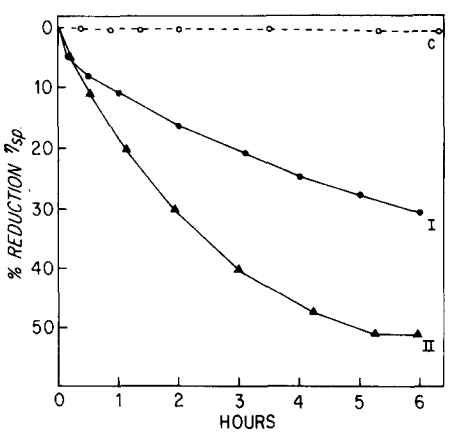

Fig. 6. Effect of guinea pig skin collagenases (peaks I and II) on viscosity at 26°C and pH 7.6. Reaction mixtures contained 0.2 ml of 0.4% collagen, 0.2 ml of enzyme solution and 0.15 ml of 1 M NaCl in 0.05 M Tris · HCl (pH 7.6). Starting viscosity, η_{sp} = 2.40; C, control.

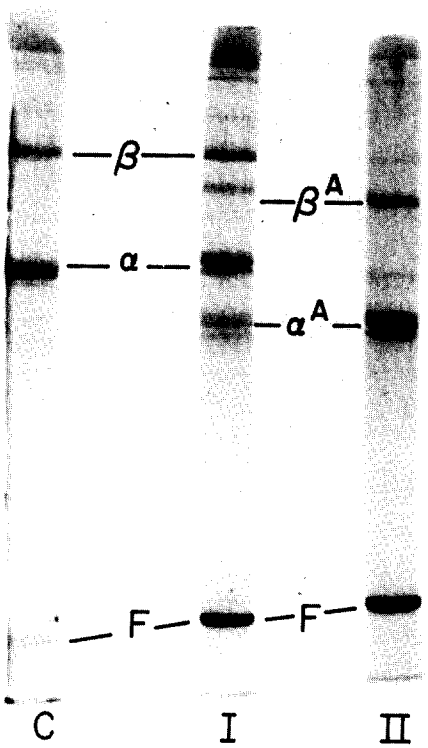

Fig. 7. Acrylamide gel electrophoresis patterns of denatured enzyme-collagen reaction mixtures at 26° C. Left to right: control, peak I and peak II. F is the buffer front marker.

Discussion

Guinea pig skin collagenase, prepared from tissue culture of whole skin, can be separated into two enzymatically active fraction (peaks I and II) by using gel filtration through Sephadex G-150. These two enzyme fractions are possibly derived from different parts of skin, i.e., epithelial and granulation tissue as described by Grillo and Gross [6] and Donoff et al. [4]. They are different in molecular size. Both enzyme fractions were further purified by the procedures of affinity chromatography with collagen-Sepharose or DEAE-Sephadex A-50 chromatography followed with polyacrylamide gel electrophoresis. The specific activities increased 20-fold and 110-fold, respectively. Peak II has been purified to homogeneity as examined by polyacrylamide gel electrophoresis, however, peak I contains two protein bands.

These enzyme fractions are similar to other human and animal collagenases in characteristics. They require Ca^{2+} for enzyme activity which is inhibited by serum, cysteine and EDTA. Peak I is possibly less sensitive to serum than peak II. Viscometry determination shows that these two fractions are capable of decreasing the initial specific viscosity of collagen solution 30% and 50% respectively at 26° C in 6 h. Polyacrylamide gel patterns of enzyme-collagen

reaction mixtures are identical as shown in Fig. 7. These enzymes appear to be glycoproteins as their bands on polyacrylamide gels can be stained with periodic acid-Schiff reagent. More extensive studies will be required in order to characterize and compare both guinea pig skin collagenases, including amino acid and carbohydrate analyses and immunological study.

Guinea pig skin (peak II) and human skin collagenases were compared. Their molecular weights are about the same, approx. 40 000. The preliminary results show that there are slight differences in their amino acid composition and that both enzymes contain glucosamine and galactosamine. The staining of polyacrylamide gels with periodic acid-Schiff reagent shows that both enzymes are glycoproteins. No cross-reactivity was found between guinea pig and human enzymes. The fact that immunological species specificity exists among collagenases in the present study is consistent with the results reported by Bauer et al. [3]. Further studies, such as amino acid sequence and carbohydrate composition, will be required to demonstrate the difference between guinea pig and human enzymes.

Acknowledgements

We wish to thank Mr Ronald Salome for his excellent technical assistance. This investigation was supported by the Deafness Research Foundation and Public Health Service Research Grant NS 10608.

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