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Gerald S. Lazarus, … , Howard A. Bladen, Harold M. Fullmer

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Research Article

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Degradation of Collagen by a Human Granulocyte Collagenolytic System

GERALD S. LAZARUS, JOHN R. DANIELS, ROBERT S. BROWN, HOWARD A. BLADEN, and HAROLD M. FULLMER

From the Laboratory of Histology and Pathology and the Laboratory of Biochemistry of the National Institute of Dental Research, and the Medicine Branch of the National Cancer Institute, Bethesda, Maryland 20014

ABSTRACT This report suggests a mechanism for collagen degradation mediated by human granulocytic leukocytes. A specific collagenase, which is extractable from human granulocytes, has been partially purified by DEAE chromatography. This collagenolytic enzyme is operative at physiological pH and is inhibited by EDTA, cysteine, and reduced glutathione but not by human serum. The enzyme cleaves the collagen molecule into two specific products, without loss of helical conformation. Electron micrographs of segment long spacing aggregates indicate that the cleavage occurs one-quarter of the length from the carboxy terminal end of the molecule. Experiments with crude extracts from granulocytes suggest that the specific products of granulocyte collagenase activity are then degraded by other proteases present in the human granulocyte.

INTRODUCTION

Degradation of collagen fibrils and collagen-containing structures such as basement membrane occurs in inflammatory responses concomitant with leukocyte infiltration (1). The precise mechanisms involved have required clarification since native collagen is resistant to hydrolysis by many proteolytic enzymes. Recently we have detected a collagenolytic enzyme which is directly extractable from the granule fraction of human granulocytic leukocytes (2). This enzyme cleaves the collagen molecule into two distinctive products, which are similar to those produced by collagenases derived from tadpole skin (3–7), human skin (8–10), and synovium (11, 12), and the postpartum rat uterus (13). Unlike these and similar collagenolytic activities released from human gingiva (14–16) and bone (17, 18), granulocytes yield collagenase on extraction and do not require tissue culture to produce detectable enzyme. The only other extractable collagenolytic factor described has been an acid hydrolase found in rat bone (19). In this report we detail the partial purification of the enzyme, describe some of its properties, and suggest a mechanism by which human granulocytes mediate collagen degradation.

METHODS

Enzyme preparation. The white cells were obtained from 500 ml units of fresh whole blood which were drawn from normal adult donors. The units of blood were centrifuged at 1500 *g* for 3 min and the 60 ml white cell–rich interface between the plasma and packed red blood cells was collected. Six of these white cell-rich units were combined and added to an equal volume of solution containing 0.45% of sodium chloride and 1.5% of dextran (mol wt 186,000). After allowing the red cells to settle for approximately 1/2 hr at room temperature the white cell–rich supernatant was decanted and centrifuged at 1500 *g* for 8 min. All subsequent steps were carried out at 4°C. The cell button was resuspended in saline and the remaining red cells removed by brief hypotonic hemolysis (20). The white cells were then washed three times with saline and collected by centri-

Dr. Lazarus' present address is Department of Dermatology, Massachusetts General Hospital, Boston, Mass. Dr. Brown's present address is Yale New Haven Medical Center, New Haven, Conn.

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fugation at 400 *g* for 10 min. This preparation was evaluated by cell counts and differential smears. The cells were then homogenized by hand in Tyrodes solution using a ground glass homogenizer. After the homogenate was repeatedly frozen and thawed it was centrifuged at 25,000 *g* for 15 min. The supernatant was dialyzed for 1 hr against 0.002 M calcium chloride in 0.01 M Tris buffer, pH 8.5, and then clarified by centrifugation.

The crude extract was purified by chromatography on diethylaminoethyl cellulose [1] in a water-jacketed column (1.7 cm diameter by 7 cm length) at 1°C. The eluting buffer was 0.01 M Tris, pH 8.5, containing 0.002 M calcium chloride on which was superimposed a linear gradient from 0.0 to 0.15 M sodium chloride over a total volume of 400 ml. The flow rate was 50 ml/hr and 5-ml aliquots were collected. Optical density at 280 mμ was continuously monitored. Activity was located by acrylamide gel electrophoresis of the products of incubations of eluate with collagen at 25°C for 24 hr.

Protein determinations were done using the Miller modification of the Lowry technique (21) and caseinolytic activity was assayed using the method of Nagai, Lapiere, and Gross (5).

Substrate preparation. The acid-extracted radioactive collagen was prepared after injecting 40-day-old Sprague Dawley rats intraperitoneally with uniformly labeled ^{14}C-glycine. Each rat received 50 μc of the isotope 72, 60, 48, 24, and 12 hr before sacrifice. The acid-extractable collagen was purified as described by Kang, Nagai, Piez, and Gross (6). Resistance to nonspecific degradation was evaluated by incubating it with a number of different proteolytic enzymes in the ^{14}C-labeled collagen fibril assay. The collagen was found to be almost completely resistant to solubilization by enzymes other than clostridial collagenase. Indeed, when collagen fibrils were incubated with trypsin (50% w/w) only 5% of the substrate was solubilized.

Collagenase assays. Four methods of detection of collagenolytic activity were used: (*a*) release of radioactive degradation products from reconstituted ^{14}C-glycine–labeled collagen fibrils (5), (*b*) viscometry at 25°C, (*c*) acrylamide gel electrophoresis (7), and (*d*) electron microscopy of segment long spacing aggregates of collagen (4).

The reconstituted ^{14}C-labeled collagen fibrils were prepared as follows. 2 mg of collagen were dissolved per ml of distilled water by gentle stirring overnight at 5°C. After the solution was dialyzed for 12 hr against 0.2 M sodium chloride in 0.05 M Tris buffer, pH 7.6, 5°C, it was centrifuged at 65,000 *g* for 2 hr. 200-μl aliquots of the supernatant (containing 1150 dpm) were pipetted into 3-ml test tubes and allowed to gel at 37°C overnight. To the opalescent gel, which consisted of organized collagen fibrils, was added 0.25 ml of 0.001 M calcium chloride in 0.05 M Tris buffer, pH 7.6, and 0.5 ml of the sample to be assayed. The mixture was incubated for 18 hr at 37°C and then filtered through a 0.9 μ pore size, 13 mm diameter Versapor [2] filter in a Swinney adapter. The filter retained insoluble collagen fibrils. Solutions containing intact collagen molecules and peptide reaction products passed through the filter and were counted. A 0.5 ml aliquot of the filtered solution was added to 20 ml of Bray's solution (22) and counted in a liquid scintillation spectrometer. When serum was included in the incubation mixtures the sample precipitated in Bray's solution. This was circumvented by treatment of the sample with 0.5 ml of 1.0 M sodium hydroxide at 60°C for 2 hr before addition of the counting mixture. Counts were determined to within a 2% error, and after correction for quenching by the channels ratio method, they were adjusted to 100% efficiency (23). All experiments included assays of buffer blanks. Concomitant trypsin [3] controls (25 μg) indicated the possible extent of nonspecific proteolytic breakdown of the collagen gel.

Kinetic experiments which measured the decrease in viscosity of collagen solutions with cleavage of the molecule were performed at 25°C in Ostwald viscometers (flow times 70–95 sec for 1.5 ml of water at 25°C). The 6 ml viscometry solution included 1 ml of enzyme, and contained final concentrations as follows: collagen 0.67 mg/ml, calcium chloride 0.015 M, sodium chloride 0.35 M, and Tris buffer 0.05 M, pH 8.5. The influence of pH was studied by using appropriate Tris-HCl or Tris-maleate buffers. All experiments were done in duplicate and included buffer blanks. Results were computed as the per cent reduction in specific viscosity with time from the initial measurement. Parallel optical rotation measurements were made on several viscosity experiments by following duplicates of the reaction mixture in a spectropolarimeter (model 80, O. C. Rudolph & Sons, Inc., Caldwell, N. J.) with an oscillating polarizer at 313 mμ at 25°C.

Both viscometry and the radiofibril assay were used to study potential modifiers of collagenase activity. Reduced glutathione,[4] L-cysteine,[5] and disodium EDTA were dissolved in the experimental buffers to make a final concentration of 0.01 M at pH 8.0 in the incubation mixtures. Similarly, in other reaction mixtures various dilutions of normal pooled human serum were added in volumes equal to that of the enzyme preparation.

At the completion of all viscometry experiments the products were studied by acrylamide gel electrophoresis. Reaction mixtures were precipitated by increasing the sodium chloride concentration to 20% (wt/vol). The pellet formed by centrifugation at 65,000 *g* for 15 min was dissolved in 8 M urea titrated to pH 5.3 with HCl. After dialysis against the pH 5.3 urea, the solution was subjected to electrophoresis according to the method of Sakai and Gross (7). Comparable results were obtained when, in other experiments, salt precipitation was omitted.

[1] Whatman DE 52, H. Reeve Angel and Co., Inc., Clifton, N. J.

[2] Gelman Instrument Co., Ann Arbor, Mich.

[3] Trypsin, 2 × crystallized, Worthington Biochemical Corp., Freehold, N. J.

[4] Sigma Chemical Co., St. Louis, Mo.

[5] L-cysteine hydrochloride, Nutritional Biochemicals Corp., Cleveland, Ohio.

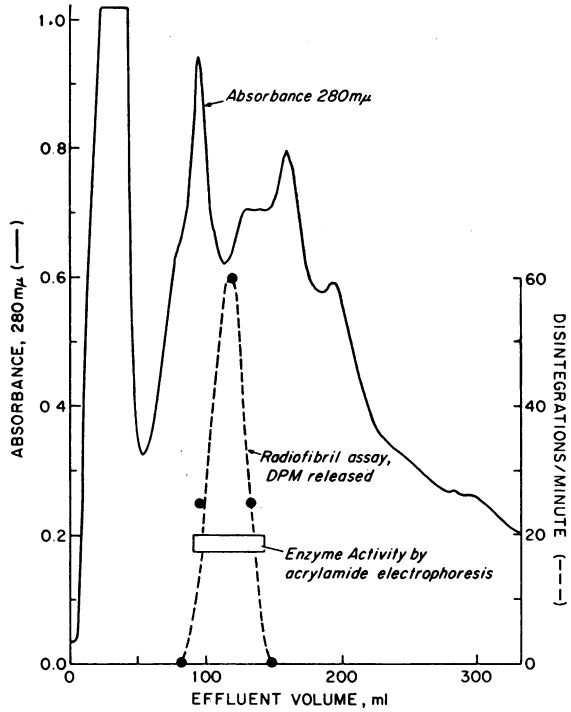

FIGURE 1 Purification of collagenolytic activity from 8×10^8 granulocytes on a DEAE column at 1°C. The eluting buffer was 0.002 M calcium chloride in 0.01 M Tris, pH 8.5, on which was superimposed a linear salt gradient. Shown are optical density at 280 mμ and collagenolytic activity as measured by acrylamide gel electrophoresis and reconstituted radioactive collagen fibril assays.

To study the morphology of the collagen molecules after granulocyte collagenase cleavage, segment long spacing collagen (SLS) was prepared from standard viscometry mixtures according to the method of Gross and Nagai (4). Electron micrographs were taken using a Siemens Elmiskop I electron microscope with double condenser illumination and a 50 μ aperture at 40,000–80,000 magnification.

RESULTS

The granulocyte collagenase was extracted from a heterogeneous population of white cells without regard to lymphocyte contamination, because these cells have been shown not to contain collagenase (2).

An extract of 10^{10} white cells (80% granulocytes) yielded 350 mg of protein. 17 mg of this crude extract was able to solubilize 28% of a radioactive collagen fibril gel and had caseinolytic activity equivalent to 50 μg of trypsin. Chromatography of this material on a diethylaminoethyl cellulose column is presented in Fig. 1. Collagenase activity, as measured by both radiofibril and acrylamide gel electrophoresis assays, was confined to 60 ml eluted over a sodium chloride concentration range of 0.045–0.075 M. The fraction with maximum collagenase activity was free of caseinolytic activity and had a protein content of 0.6 mg/ml. The degree of purification could not be determined since the crude preparation was not stable until other leukocytic proteases were removed by DEAE chromatography.

In experiments with collagen in solution, a 65% decrease in specific viscosity was effected by the partially purified granulocyte collagenase when the reaction went to completion. No change in optical rotation could be detected when viscosity and polarimetry were observed in parallel experiments (Fig. 2). This indicated that the over-all helical structure of the products had been maintained.

The activity of the partially purified enzyme as a function of pH was also studied by viscometry (Fig. 3). Maximal activity was found close to pH

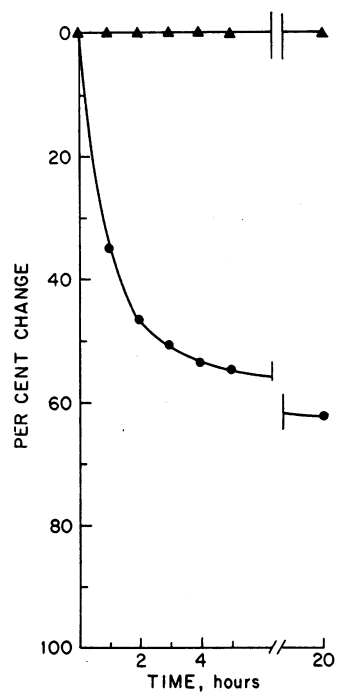

FIGURE 2 Parallel determinations of per cent change in optical rotation (▲—▲) and per cent decrease in specific viscosity (○—○) during incubation of collagen with the partially purified granulocyte collagenase with time (pH 8.5).

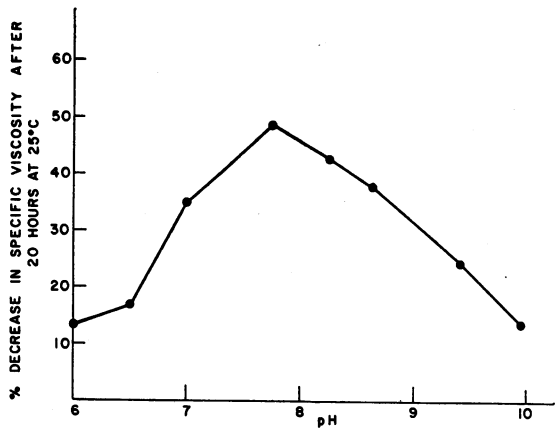

FIGURE 3 Activity of the partially purified granulocyte collagenase as a function of pH. Activity is measured as the per cent decrease in specific viscosity after 20 hr at 25°C.

7.8. Little activity could be detected either below pH 6.5 or above pH 9.5.

The acrylamide gel electrophoresis patterns of the denatured products of incubation of collagen with both the purified and crude enzyme preparations are shown in Fig. 4. The control pattern consists of monomers (α), dimers (β), and higher molecular weight species. Incubation of collagen with the partially purified granulocyte collagenase results in discrete products of lower molecular weight which are similar to those seen with other animal collagenases (6, 7, 10). The products are denoted by superscripts A and B which are the N-terminal 3/4 and C-terminal 1/4 of the molecule respectively (6). The double bands for each species reflect the heterogeneous chain structure of the collagen molecule (6). The complex pattern produced after incubation with the crude extract is also presented for comparison. The numerous bands indicate extensive hydrolysis of the entire molecule.

Fig. 5 is an electron micrograph of segment long spacing collagen (SLS) aggregates. On the right is SLS formed from collagen which had been incubated with heat-inactivated purified granulocyte collagenase and shows the usual length (2700–2800 A) and periodicity. On the left is SLS collagen formed after incubation with active granulocyte collagenase. The carboxy terminal one-quarter of the molecule has been cleaved off. The shortened molecules (2150 A) were all of the same length and no SLS between 2150 and 2750 A was seen. The electron micrographic site of cleavage

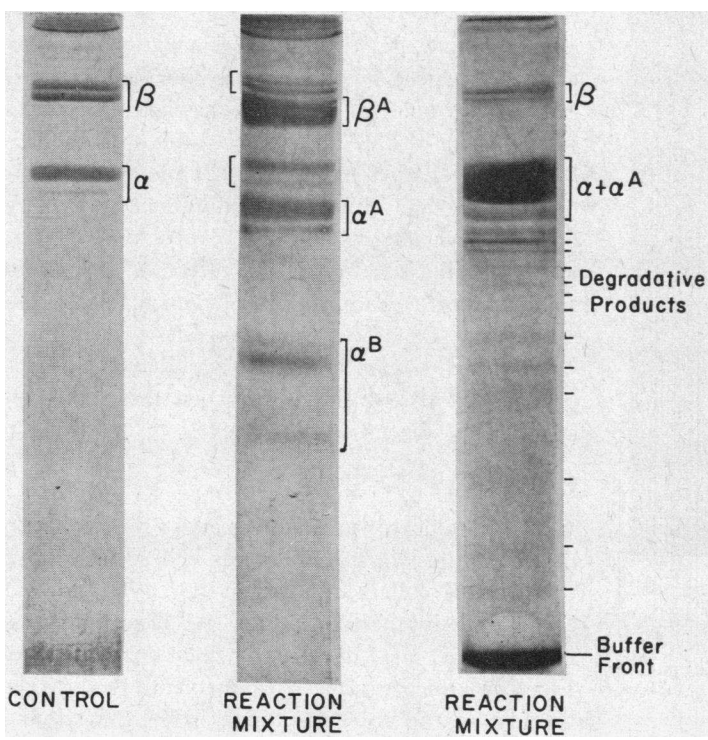

FIGURE 4 Acrylamide gel electrophoresis patterns of products from incubations of collagen with heat inactivated granulocyte collagenase (left), partially purified granulocyte collagenase (middle), and unpurified granulocyte collagenase (right). α, monomeric chain; β, dimeric chain; superscript A, N-terminal 3/4 cleavage product; superscript B, C-terminal 1/4 cleavage product.

appears identical with that of the tadpole collagenase (4).

Collagenase activity was inhibited by boiling the enzyme for 5 min, addition of sodium EDTA to a final concentration of 0.01 M, omission of calcium, or addition of reduced glutathione or cysteine to a final concentration of 0.01 M (Fig. 6). Because serum has been shown to inhibit collagenase activity derived from other tissues (8) its effect on the granulocyte enzyme was studied. A clear difference was observed: the addition of serum, or serum diluted 1 : 10 with buffer, to the partially purified enzyme did not inhibit viscosity fall or

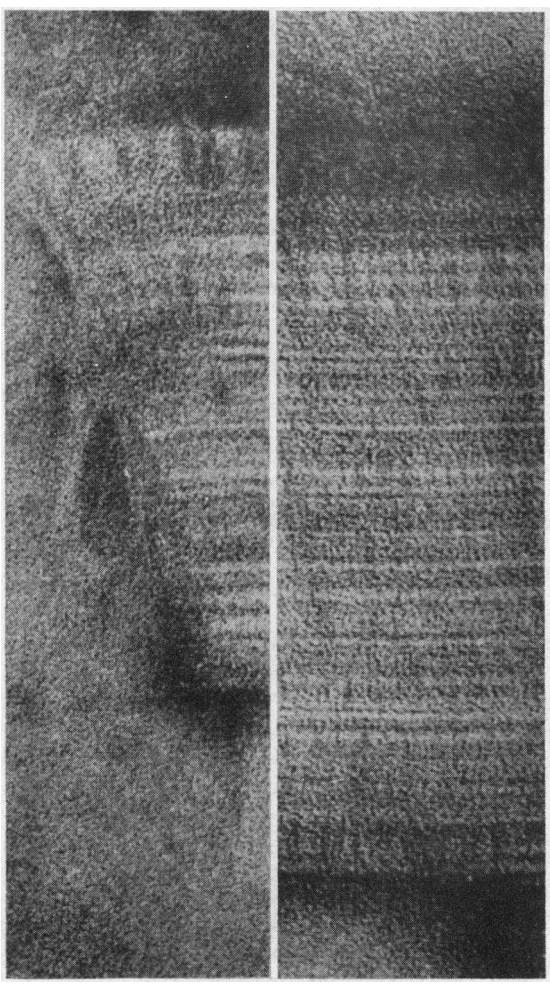

FIGURE 5 Comparison of SLS aggregates from collagen incubated with active partially purified granulocyte collagenase (left) and heat inactivated granulocyte collagenase (right). The SLS formed after incubation with active enzyme is missing the carboxy terminal 1/4 of the molecule.

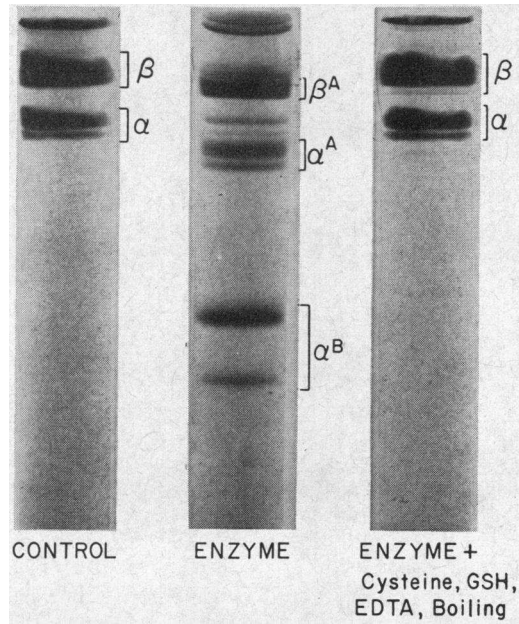

FIGURE 6 The effect of cysteine 0.01 M, reduced glutathione (GSH) 0.01 M, EDTA 0.01 M, and boiling on purified granulocyte collagenase activity as determined by acrylamide gel electrophoresis (pH 8.5, 25°C, 20 hr).

effect the acrylamide gel electrophoresis pattern (Fig. 7). Furthermore, the addition of serum to the crude enzyme preparation prevented production of multiple digestion products and resulted in an acrylamide gel electrophoresis pattern similar to that produced by the partially purified enzyme. A crude granulocyte preparation which effected a viscosity fall of 25% in 4 hr was able to solubilize 28% of a collagen gel while a sample of the purified granulocyte enzyme which reduced viscosity 55% in 4 hr solubilized only 17% of the collagen fibrils (Table I). When the effect of serum on solubilization of radioactive collagen fibrils by the crude and partially purified enzyme preparations was studied, partial inhibition was found. The apparent lack of correlation between these two assays will be discussed below.

DISCUSSION

The granulocyte enzyme cleaves the collagen molecule into two specific pieces. These distinctive products of collagenase action (β^A, α^A, and α^B) are shown in the acrylamide gel electrophoresis patterns of the denatured reaction mixtures. Electron micrographs demonstrate the larger product of the cleavage which is the N-terminal

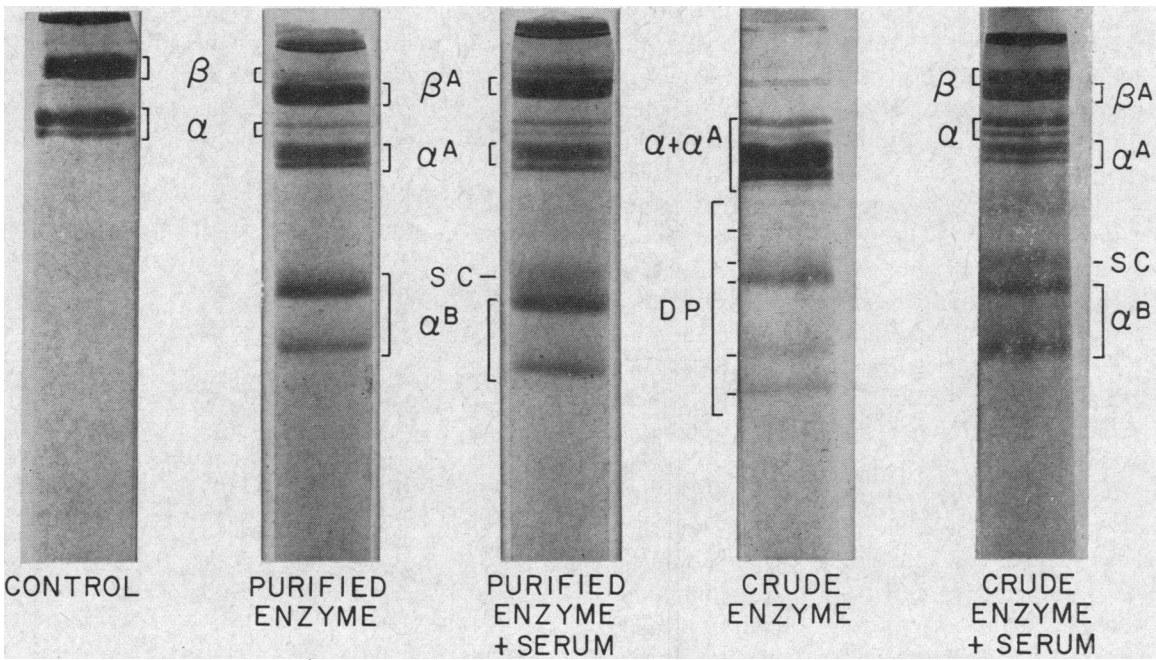

FIGURE 7 Acrylamide gel electrophoresis patterns of the products from incubations of collagen with partially purified and crude granulocyte collagenase with and without human serum (pH 8.5, 20 hr). α, monomeric chain; β, dimeric chain; superscript A, N-terminal 3/4 cleavage product; superscript B, C-terminal 1/4 cleavage product; *DP*, nonspecific degradation products; *SC*, serum components.

3/4 of the molecule. Cleavage of the collagen molecule does not cause any major change in helical structure of the products since the initial negative optical rotation is maintained. The enzyme is active over a physiological pH range. The pH profile presented is semiquantitative in that no attempt has been made to establish reaction rates at saturating substrate concentration. The enzymatic activity can be blocked by EDTA and the free sulfhydryl compounds cysteine and reduced glutathione. These properties are shared with collagenases from other tissue sources (3–8, 11–13).

TABLE I

Effect of Normal Human Serum and Reduced Glutathione (GSH) on Crude and Partially Purified Granulocyte Collagenase Activity as Measured by the Radioactive Reconstituted Collagen Fibril Assay

	DPM	Collagen gel solubilized
		%
Crude granulocyte collagenase	300	28 ±2
Crude granulocyte collagenase + serum	125	11 ±1
Purified granulocyte collagenase	175	17 ±1.5
Purified granulocyte collagenase + serum	90	9 ±1
Purified granulocyte collagenase + GSH 0.01 M	35	4 ±1
Trypsin 50 μg	50	5 ±1

Results presented have been corrected for the buffer blank of 75 DPM. Each gel initially contained 0.4 mg collagen (1150 DPM). Values expressed are the mean of four determinations ±SD.

There are, however, two major differences between the granulocyte collagenase and other reported animal collagenolytic enzymes of this type. First, the enzyme is readily detected on extraction of the cells of origin and is apparently stored in the leukocyte granule (2). Collagenolytic activity from other sources requires tissue culture for detection (3, 8–14). Its presence in the granulocyte is distinctive since attempts to extract similar activity from comparable numbers of human lymphocytes (2) and rabbit alveolar macrophages [6] have failed.

Second, granulocyte collagenase is not inhibited by human serum. When specific cleavage of the collagen molecule is studied by viscometry and acrylamide gel electrophoresis, no inhibition of

[6] Lazarus, G. S., and J. Goggins. Unpublished data.

granulocyte enzyme action is found in the presence of serum. This is at variance with observations made with other collagenases since all these enzymes are inhibited by serum when studied by identical techniques (8, 24). In contrast, the addition of serum to either the crude or partially purified granulocyte enzyme inhibits dissolution of collagen fibrils when the radioactive fibril assay is employed. Because of the observations on collagen in solution, this finding cannot be ascribed to interference with the specific cleavage of the molecule into A and B pieces. Furthermore, the partially purified granulocyte collagenase is less effective in solubilizing collagen fibrils than comparable amounts of the crude granulocyte collagenase as measured by viscometry. These observations suggest that the specific cleavage of the molecule is inefficient in dissolving the fibril and secondary proteolytic activity which is inhibitable by serum, facilitates effective solubilization. This impression was strengthened by observations made with the crude granulocyte extract. When incubation mixtures using this extract were studied, not only was solubilization of collagen fibrils marked, but numerous products were seen on acrylamide gel electrophoresis. Upon addition of serum, however, only products specific for granulocyte collagenase were found: this condition was associated with a decrease in dissolution of radioactive collagen fibrils. Thus, these experiments demonstrate the presence of serum-inhibitable protease activity in the crude granulocyte preparation which increases the effectiveness of granulocyte collagenase in solubilizing collagen fibrils. Whether the serum-inhibitable, solubilizing activity of the partially purified granulocytic collagenase is a property of the enzyme itself or results from persistent contamination by other enzymes cannot yet be stated.

Sakai and Gross (7) have shown that the products of tadpole collagenase action are more susceptible to tryptic hydrolysis than the intact molecule. They correlated this observation with the lowered melting points of the specific products. That the specific cleavage products of the granulocytic enzyme can be further degraded by other proteases in the white cell is shown by the numerous bands found on acrylamide gel electrophoresis after action by the crude granulocyte preparation. These observations suggest that during granulocyte-mediated dissolution of collagen fibrils the primary effect of the collagenase is to cleave the collagen molecule into two fragments which are then more susceptible to hydrolysis by other proteases.

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