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PREPARATION AND PROPERTIES OF COLLAGENASES FROM EPITHELIUM AND MESENCHYME OF HEALING MAMMALIAN WOUNDS

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SUMMARY

Collagenolytic activity has been isolated from the medium during culture of proliferating epithelium and new mesenchymal tissue obtained from healing open cutaneous wounds in the rabbit. Specific collagenase activity, distinct from other proteolytic enzymes, has been demonstrated by both radioactive and viscometric assays. Neutral protease activity appeared in conjunction with collagenase activity in the tissue culture medium. Differences in the time of appearance of collagenolytic activities in the component tissues may be attributed to endogenous digestion of collagen present in mesenchyme-containing tissues. Collagenolytic enzymes from the epithelium and granulation tissue do not appear to be identical as evidenced by their different responses to inhibitors and their different molecular weights, although both enzymes cleave the native collagen molecule at a point three quarters of its length from the amino terminal end. The functional significance of these collagenolytic enzymes remains unclear. Potential roles exist during cell migration and collagen resorption in the processes of epithelization, contraction and wound remodeling.

INTRODUCTION

The amount of collagen in a unit area of a cutaneous open wound in a mammal may show net resorption at certain times during healing[1-3] and falls rapidly following wound closure (H. C. GRILLO, unpublished results). Mechanisms for removal of collagen are required for remodeling of connective tissue to occur and, also, to permit the re-arrangement of both mesenchymal and epithelial cell populations which occur during repair. Since native collagen is not degraded by usual proteases, specific collagenases are probably necessary to permit controlled degradation and resorption of collagen in the healing wound.

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True collagenolytic enzymes in tissues were first demonstrated by GROSS AND LAPIÈRE[4] who showed the ability of such an enzyme in the culture medium of living explants of tadpole tissue to degrade a specific substrate of undenatured collagen under physiologic conditions. No enzyme was found in tissue extracts, breis or cultures of dead cells or those treated with inhibitors of protein synthesis such as puromycin. They concluded that the enzyme was produced *de novo* and not stored for release. Characterization of that enzyme isolated from the culture medium indicated a neutral or slightly alkaline pH optimum, inhibition by EDTA, cysteine and serum and a highly specific mode of attack on the collagen molecule. Subsequently, similar collagenases have been detected in a variety of other tissues[5–8] and have been isolated, partially purified and characterized from cultures of tadpole tail fin[9], rat uterus[10], rheumatoid synovial tissue[11], normal human skin[12], mouse bone[13] and human gingiva[14]. The use of tissue culture technique is generally required for enzyme preparation; human polymorphonuclear leukocyte collagenase[6] and crustacean hepatopancreatic collagenase[15] are exceptional in that they have been obtained by direct extraction.

GRILLO AND GROSS[16] demonstrated high levels of collagenolytic activity in cultures of epithelial and mesenchymal tissue from the margins of open cuteneous wounds in guinea pigs and, also, from proliferating marginal epithelium alone. Granulation tissue from the center of the open wound was much less active. GRILLO *et al.*[17] found similar collagenases in human wounds, with differing intensities of activity.

MATERIALS AND METHODS

Experimental wounds

Five pound rabbits were anesthetized with intravenous sodium pentobarbital, shaved around the thorax and abdomen, depilated with Nair (Carter Wallace, Inc., New York) and washed thoroughly with water. 3-cm^2 wounds were made down to the panniculus muscularis on each side, using a flexible polyethylene template which was then fixed to the edge of the wound with interrupted dermalon sutures to prevent contraction during healing and thus permit epithelial spread. The wound was covered with vaseline gauze and sponge padding, wrapped with gauze bandage and a light plaster cast was applied around the entire area for protection.

Culture of wound tissue

On the 12th day after wounding the animals were killed with an overdose of intravenous pentobarbital and the overlying scab was removed. The healing central granulation tissue and the proliferating epithelium, which was clearly visible at the wound edge, were cut into 2-mm strips while still *in situ*; the strips were elevated, sharply dissected free from underlying panniculus and placed in mammalian Tyrode's solution containing 2000 units/ml, of penicillin and streptomycin. Pre-existing dermis lay behind the epithelial edge and was not included in the sample. The initial "epithelial" sample included underlying, new-formed mesenchyme. The tissues were washed in two changes of Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, New York) with added antibiotics. Wound edge from which epithelium was to be separated from mesenchyme was incubated immediately

in medium containing 0.05% elastase (Worthington Biochemical Corp., Freehold, N.J.) at 37° for 30 min, following the method of KLEIN AND FITZGERALD[18]. Epithelial strips were separated from mesenchyme under a dissecting microscope. Central granulation tissue was harvested only from the center of the wound and was visibly and histologically free of epithelium. Tissue was cut into pieces approx. 2–3 mm in size, placed in 3.0 ml of Dulbecco's modification of Eagle's medium with added antibiotics and cultured in disposable plastic flasks (Falcon Plastics, Los Angeles, Calif.) at 37° in an atmosphere of O_2–CO_2 (90:10, by vol.). Culture medium was harvested at 24-h intervals and replaced with fresh fluid. Cultures were examined for bacterial growth both aerobically and anaerobically. Unseparated samples of wound edge, containing both proliferating epithelium and new mesenchymal tissue ("granulation tissue") are called "whole edge" samples. Epithelial, mesenchymal and whole edge samples similarly prepared were used for microslide culture as well.

Microcultures for collagenase detection

Salt extracted guinea pig collagen dissolved at neutral pH was used to prepare gels for the culture substrate, as previously described[16]. The rigid opalescent gels were formed from 125 μl of a 0.1% solution of collagen in cold mammalian Tyrode's medium containing added amino acids, by warming at 37° for 3-h in microslide chambers made of Lucite. Pieces of tissue were placed on the collagen gels and incubated at 37° in a moist atmosphere containing O_2–CO_2 (90:10, by vol.) and were observed daily for lytic activity and bacterial growth. Visible lysis was recorded on a scale of 1+ to 4+. 1+ indicated definite lysis and 4+ total lysis of the collagen gel. These cultures served as a check for collagenolytic activity. The assay is highly specific for collagenase[4,16].

Preparation of enzyme powder

Medium harvested daily from the cultures with minimal disturbance of tissue was centrifuged at 50 000 × *g* at 4° for 30 min and dialyzed in the cold for 24-h against distilled water with added 0.05 M Tris–HCl containing 0.005 M $CaCl_2$ at pH 7.5. The enzyme solution was lyophilized and stored at −20°. For use, the crude enzyme powder was redissolved in the same buffer. Fresh medium was added to the tissue after each harvesting.

Purification of epithelial and granulation tissue enzymes

Crude lyophilized enzyme powder (200–400 mg) was dissolved in 15 ml of 0.05 M Tris–HCl buffer (pH 7.6) *plus* 0.005 M $CaCl_2$ at 5°. The solution was centrifuged in the cold at 10 000 × *g* for 10 min and the residue discarded. To the supernatant solution in an ice bath solid ammonium sulfate was added to 20% saturation and the preparation was allowed to stand for 30 min. Any precipitate was removed by centrifugation. More ammonium sulfate was added to 50% saturation and the suspension was allowed to stand 1 h or overnight. The precipitate was removed after centrifugation at 10 000 × *g* for 10 min. The supernatant was saved for assay. The precipitate was dissolved in 1–2 ml of the original buffer, dialyzed against 0.01 M Tris–HCl (pH 7.6)–0.2 M NaCl–0.005 M $CaCl_2$ and centrifuged to remove any undissolved residue.

Gel filtration of the partially purified enzymes was accomplished on a column

(0.9 cm × 60 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.). 0.01 M Tris buffer–0.2 M NaCl–0.005 M $CaCl_2$ was used for elution. The effluent was monitored at 280 nm. Collagenolytic and proteolytic activites were assayed in alternate fractions.

Measurement of degradation of collagen in explants

Preliminary experiments had demonstrated that collagenolytic activity from granulation tissue and whole edge was released into the culture medium later than the epithelial enzyme[17]. A possible explanation of this finding might be that collagenase from granulation tissue was bound to the collagen of the tissue and had to digest it first before being released into the medium. As an index of possible cleavage of endogenous collagen, changes in hydroxyproline soluble in cold trichloroacetic acid and insoluble hydroxyproline (hot trichloroacetic acid soluble) were measured. Granulation tissue was harvested and divided into flasks containing 2.0 ml of Dulbecco's modified medium. Wet weight was obtained by difference. Flasks were incubated at 37° in an atmosphere of O_2–CO_2 (90:10, by vol.). On the day of culture and at daily intervals for five days the total tissue from each flask was homogenized in its own culture medium. The preparations were centrifuged and the supernatant brought to double its volume with ice cold 5% trichloroacetic acid. The ground tissue pellet was extracted twice with cold trichloroacetic acid and the extract combined with the trichloroacetic acid soluble material from the culture medium. This solution, containing free and small peptide hydroxyproline, serves as a measure of collagenolytic activity in the tissue itself, since this is the only source of collagen. Insoluble residue from each flask was then extracted twice with 5% trichloroacetic acid at 90° for 30 min[19]. Both the cold and hot trichloroacetic acid soluble solutions were taken to dryness, suspended in 6 M HCl, and hydrolyzed at 110° for 24 h and the hydrolysates were assayed for hydroxyproline by the method of BERGMAN AND LOXLEY[20], with standards run for each assay.

Preparation of assay substrate

Native, neutral salt-soluble guinea pig skin collagen was purified by the technique described GROSS AND KIRK[21] and GROSS[22]. Labeled collagen was prepared by injection of [^{14}C]glycine (1 μC/g body weight.) One half of the dose was given 6 h prior and one half 3 h prior to flaying. Specific activity of the labeled collagen was 6000 counts/min per mg. For use, purified lyophilized collagen was dissolved at a concentration of 0.4% in cold phosphate buffer (pH 7.6, $I = 0.4$) by agitating overnight on a wrist shaker at 4°. The solutions were then dialyzed against large volumes of 0.4 M NaCl in the cold for 24 h, followed by centrifugation at 50 000 × g for 1 h to remove any undissolved material. The solutions were diluted with cold mammalian Tyrode's solution or 0.4 M NaCl before use.

Collagenase assay on culture media

This assay, described in detail by NAGAI *et al.*[9] depends on the release of soluble [^{14}C]glycine-containing peptides from native, reconstituted, guinea pig skin collagen fibrils. A typical reaction mixture contained 50 μl of radioactive collagen (specific activity 1100 counts/min) that had been allowed to gel for 14 h at 37° in a 1.0-ml plastic centrifuge tube, 50–200 μl of 0.05 M Tris–HCl (pH 7.5) containing 0.005 M

$CaCl_2$, and 50–100 μl of crude culture medium. After incubation for 3 h the reaction was stopped with 50 μl of 0.3 M EDTA at pH 7.5, and the tubes were centrifuged at 18 000 rev./min for 15 min. For liquid scintillation counting, aliquots of the reaction mixture were suspended in the solution of BRAY[23] containing 4.0% Cab-O-Sil. Cab-O-Sil was later found to be unnecessary in these experiments. The activity was expressed as counts/min released in the reaction mixture *minus* the counts/min in the supernatant of control gels. Control gels contained 0.1% trypsin (Worthington Biochemical Corp., Freehold, N.J.) which served to measure nonspecific proteolytic breakdown of collagen substrate.

Non-collagenolytic protease activity, using casein as a substrate, was determined at neutral pH by the method of KUNITZ[25]. The reaction mixture consisted of 1 ml of 0.6% casein in 0.05 M Tris–HCl buffer containing 0.0001 M $CaCl_2$ at pH 7.0 and 200 μl of enzyme solution. After incubation for 1 h at 37°, 2 ml of 0.4 M trichloroacetic acid was added and 30 min later the tubes were centrifuged and the optical density determined at 280 nm.

Protein was estimated by the method of KALCKAR[26] from the optical density at 280 nm and 260 nm.

pH dependence

The activity of enzyme solutions as a function of pH was tested in the range pH 5.0–9.0 using 0.1 M Tris–HCl and 0.1 M Tris–maleate buffers adjusted to the desired pH. Blanks were run at each pH value as controls.

Viscosity, optical rotation, disc electrophoresis and thermal denaturation

Measurements of specific viscosity as a function of time were made in Ostwald viscometers with water flow times at 28° of about 60 sec. The usual reaction mixture contained 2.5 ml of 0.2% collagen, 1.0 ml of 0.05 M Tris–HCl in 0.4 M NaCl (pH 7.5), 1.5 ml of crude enzyme resuspended in 0.05 M Tris–HCl *plus* 0.005 M $CaCl_2$. Final concentrations of enzyme preparations ranged from 0.3 to 0.5 ml/ml. Aliquots of the initial reaction mixtures were transferred to a viscometer and the remainder incubated simultaneously in the viscometer bath at 28°. Controls, with or without heat inactivated enzyme, were run simultaneously. Optical rotation changes were followed simultaneously in a Cary 60 automatic spectropolarimeter at 230 nm at 28°.

Samples were taken for disc electrophoresis at varying intervals during the reaction, 0.3 M EDTA was added and they were denatured for 5 min at 40°. Samples were examined by disc electrophoresis on polyacrylamide gels according to the method of NAGAI *et al.*[28].

Thermal denaturation characteristics of reaction products were observed in a Perkin–Elmer spectropolarimeter equipped with an oscillating polarizer at 313 nm. Samples for thermal denaturation study were brought to 30% saturation at 0° with respect to ammonium sulfate. After 1 h the precipitates were collected by centrifugation, dissolved in 0.15 M acetic acid. Failure to remove the contaminating enzyme from the reaction mixtures resulted in poor denaturation data. The procedure also enhanced electrophoretic resolution. Temperature increments were 1° per 20 min.

Segment long spacing preparations for electron microscopy

Samples of the reaction mixture were dialyzed at 4° against several changes

of 0.5 M acetic acid during 48 h and then against 0.4% salt free ATP at pH 2.8 after the method of KUHN AND ZIMMERMAN[28]. Drops of suspension were placed on collodion coated standard 400 mesh grids, followed by positive staining with 1% uranyl acetate in water, and were examined in an RCA EMU 3 electron microscope.

Inhibition studies

Inhibitors were prepared by addition to the 0.05 M Tris–HCl–0.005 M $CaCl_2$ buffer at the desired concentration and adjustment of the final solution to pH 7.6. Cysteine or EDTA in varying concentrations and enzyme were preincubated for 30 min at 37° and then added to the remainder of the radioactive assay mixture. Preliminary experiments demonstrating inhibition of collagenolytic activity by serum[16] led to a search for the serum protein responsible for inhibition. Serum protein fractions were studied in an attempt to localize the inhibitory fraction. Rabbit normal serum, γ free serum (Hyland Laboratories, Los Angeles, Calif.) and albumin, α- and β-globulin fractions were used (Mann Research Laboratories, New York, N.Y.). α-1 deficient serum was kindly supplied by Dr. Richard Talamo, Massachusetts General Hospital. Appropriate blanks were run in each case to eliminate nonspecific substrate solubilization as artifact. The radioassay used has been described previously.

RESULTS

Activity in microassay cultures of wound tissues

Explants of the component tissue of the open healing wound cultured on collagen gels were observed through a 5-day period of lytic activity. Dissociated epithelium produced lysis on reconstituted collagen gels in 40% of cases, central granulation tissue showed activity in 60% and whole edge tissue gave activity in 90%. These findings are in contrast with the uniformly high activity of guinea pig epithelium and considerably lower central granulation activity[16].

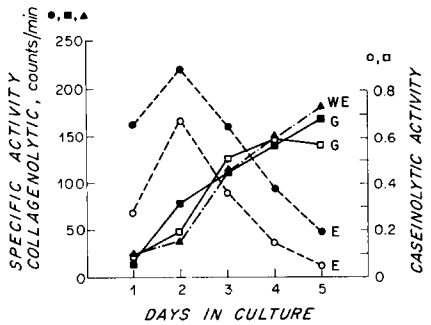
Tissue culture

Collagenolytic activity of the components of the open healing wound are shown in Fig. 1. The lifetime of these cultures is not known since they were terminated on the 5th day routinely. Activity appeared earlier in epithelial cultures than in cultures of granulation tissue or whole edge. The kinetics of enzyme production were identical in four different preparations obtained from the culture medium of tissue with 8–10 rabbits in each preparation. Neutral proteolytic activity paralleled collagenolytic activity (Fig. 1). This is in contrast to the early release of proteolytic activity in cultures of human skin and tadpole tailfin.

Attempts to extract collagenolytic activity from freshly sampled tissues were unsuccessful. Cultures which were infected with microorganisms produced less collagenolytic activity and were discarded.

Degradation of collagen in explants

Fig. 2 shows the ratio of insoluble to soluble hydroxyproline of granulation tissue of the cultures expressed as μg of hydroxyproline per mg of granulation tissue.



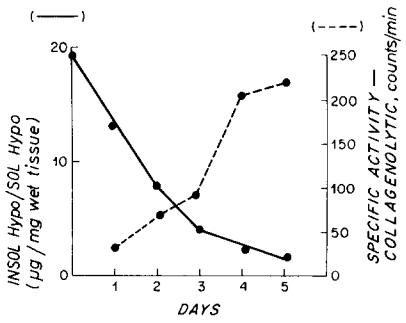


Fig. 1. Enzyme production in culture with time. E, epithelium; G, granulation; WE, whole edge. Caseinolytic activity coincides with collagenolytic activity production. Complete lysis of 50 μl of the collagen gel used gave 1100 counts/min.

Fig. 2. Enzyme production with time and decreasing insoluble hydroxyproline in cultures of granulation tissue. Activity in culture fluid increases as endogenous tissue collagen is digested by enzyme which is produced.

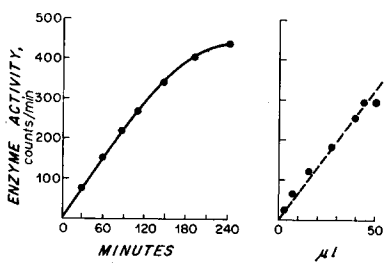
The soluble hydroxyproline increased daily while the insoluble values decreased. The rate of change was paralleled by an increase in measurable enzyme activity.

Kinetics of enzyme activity

Collagenase activity was dependent on time of incubation and on enzyme concentration when determined by the release of soluble radioactive peptides from reconstituted collagen fibrils (Fig. 3). The data shown is for granulation tissue collagenase, but epithelial and whole edge enzymes gave similar results.

pH dependency

The pH optima for collagenolytic activity of the unpurified preparations is near neutrality (Fig. 4). The data suggest that the epithelial enzyme is somewhat more active at alkaline values than the granulation tissue enzyme.



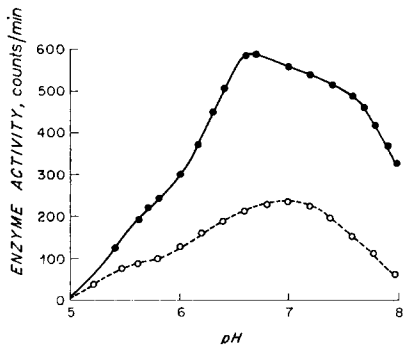


Fig. 3. Degradation of collagen by granulation tissue collagenase as a function of time and concentration. Measured by release of radioactivity from [^{14}C]glycine-labeled collagen gels. Concentration dependence determined after incubation at 37° for 3 h.

Fig. 4. Dependence of activity from epithelial (○— — —○) and granulation tissue (●—●) preparations on pH. Reaction mixtures were made to a final concentration of 0.01 M with respect to the buffers Tris–HCl and Tris–maleate.

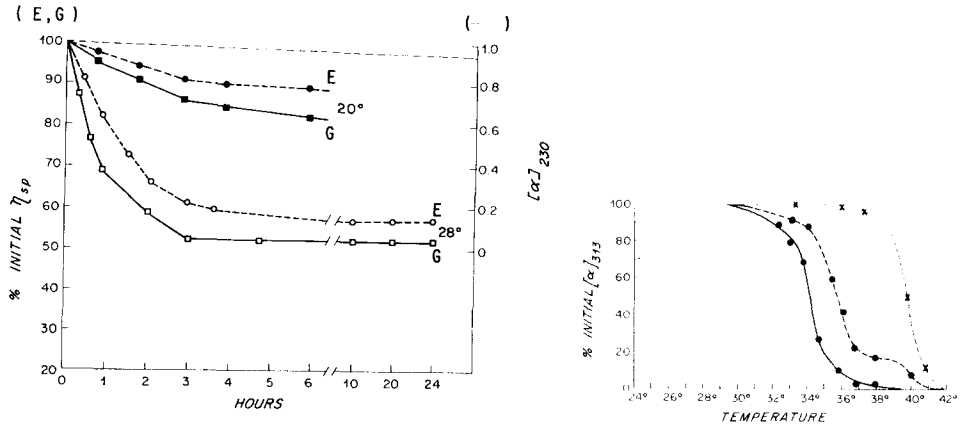


Fig. 5. Effect of epithelial (E) and granulation tissue (G) collagenase on viscosity at 20° and 28° and on optical rotation at 28°. Reaction mixture contained 0.1% salt extracted guinea pig collagen in 0.05 M Tris–HCl, 0.2 M NaCl, 0–0025 M $CaCl_2$ (pH 7.5), starting viscosity $\eta_{sp} = 3.74$. Control viscosity remained unchanged over the experimental time period. The optical rotation was monitored simultaneously in an aliquot of the reaction mixture.

Fig. 6. Thermal denaturation curves of enzyme–collagen reaction mixture and control collagen. ●—●, granulation; ●— —●, epithelial; ×— —×, control. Temperature increments are 1°/20 min.

Viscosity reduction and thermal denaturation

Epithelial and granulation tissue collagenases act on collagen in solution and are capable of reducing the specific viscosity at pH 7.5 and 28° to approx. 50% of the control values after 3 h. No significant change in optical rotation occurred during viscosity reduction. At 20° viscosity reduction was less marked (Fig. 5). The denaturation midpoint (T_m) of the altered collagen in a 3-h reaction mixture with granulation tissue collagenase at pH 4.8 was approx. 5° lower than that for native collagen (Fig. 6). Epithelial enzyme reduced the T_m 4° and the curve showed a plateau with 80% denaturation suggesting incomplete digestion. Although not determined in this study the T_m value for native calf skin collagen at neutral pH is approx. 39° which agrees with our control value for the T_m.

Disc electrophoresis of reaction products

Acrylamide gel electrophoresis of the denatured reaction products from mixtures incubated at 28° are shown in Fig. 7. In the control only the α and β bands are clearly seen with the γ and higher aggregates remaining at the top of the separating gel. Reaction products of epithelial enzyme and collagen resulted in one new band below the original β band and a single new band below the original α. In addition, two faster moving components are always present just above the solvent front. With increasing time of incubation, both the original α and β components of collagen disappear completely and are replaced by the new components. Granulation enzyme resulted in more than one new α and β band. Semipurified granulation enzyme resulted in the same gel pattern of reaction products, suggesting differences between the products of epithelial and granulation tissue collagenases.

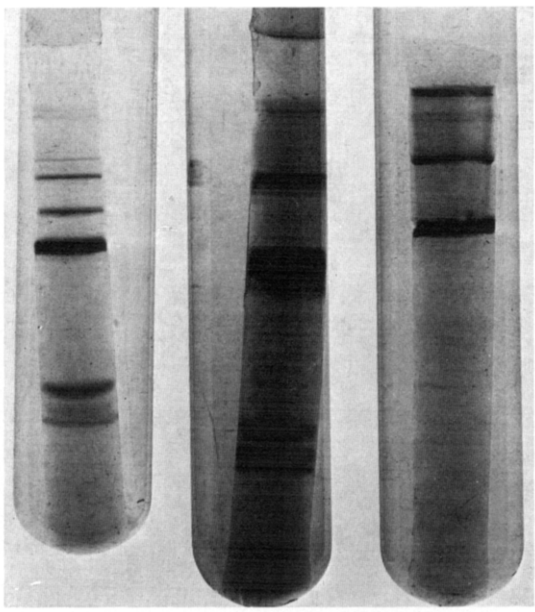


Fig. 7. Disc electrophoretic patterns of thermally denatured reaction mixture of enzyme and guinea pig skin collagen after incubation for 3 h. Left to right: epithelial enzyme, granulation tissue enzyme, control.

Inhibition studies

Results of EDTA and cysteine on the collagenolytic activities of epithelial and granulation tissue enzymes are shown in Table I. The data suggest non-identity of these collagenases. Appropriate blanks eliminated the factor of non-specific solubilization. Granulation tissue activity was inhibited by cysteine while epithelial

TABLE I

EFFECT ON COLLAGENASES FROM EPITHELIUM AND GRANULATION TISSUE OF CYSTEINE AND EDTA

Data are averages of five experiments. Activity is counts/min above 0.1% trypsin blank of 97 counts/min. Total counts/min of 50 μl collagen was 1024.

	Activity released (counts/min)	
	Epithelial collagenase	*Granulation tissue collagenase*
Enzyme only	180	376
Enzyme and cysteine		
0.001 M	203	330
0.01 M	246	225
0.1 M	310	173
Enzyme only	176	332
Enzyme and EDTA		
0.001 M	133	224
0.01 M	36	180
0.1 M	25	67

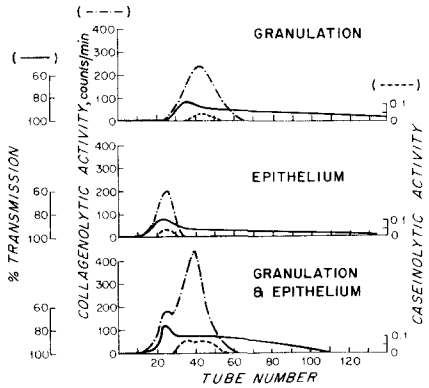


Fig. 8. Molecular sieve of activities of enzymes from tissue components on Sephadex G-200. Proteolytic activity is recorded as the optical density of the supernate at 280 nm in assay using casein as the substrate. % Transmission is at 280 nm. Collagenase activity was determined by radioassay. Flow rate, 12 ml/h.

activity was enhanced. Preliminary experiments suggest that EDTA inhibition is not reversed by calcium in contrast with other collagenases studied.

Sera from human and rabbit sources inhibit both epithelial and granulation tissue activity. Rabbit γ-globulin free serum is also inhibitory for both activities, while serum albumin produces no inhibition. Studies with α- and β-globulin showed no inhibition while α- 1 deficient serum produced inhibition of granulation tissue activity.

Purification and gel filtration

Ammonium sulfate fractionation of epithelial and granulation tissue enzymes resulted in an expected increase in collagenolytic specific activity and decrease in proteolytic specific activity. Crude enzyme activity measured as counts/min per mg per h was increased 140% by ammonium sulfate fractionation. Sephadex filtration further increased purification by 1400%. Ammonium sulfate fractionation reduced caseinolytic activity by 50% and Sephadex filtration reduced this to 20% of the original activity present in the crude enzyme powder. Although overnight salting

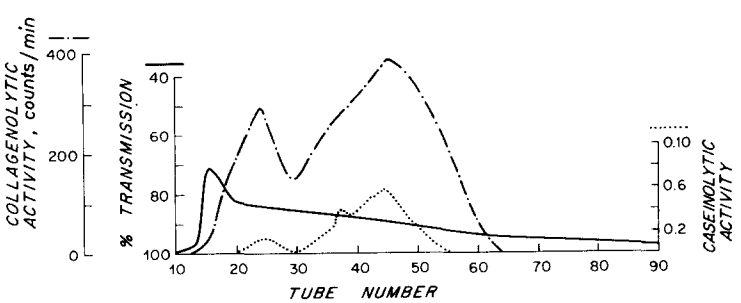


Fig. 9. Separation of epithelial–granulation enzyme mixture on Sephadex G-200. Activity of first peak was enhanced by cysteine. Activity of second peak was inhibited by cysteine.

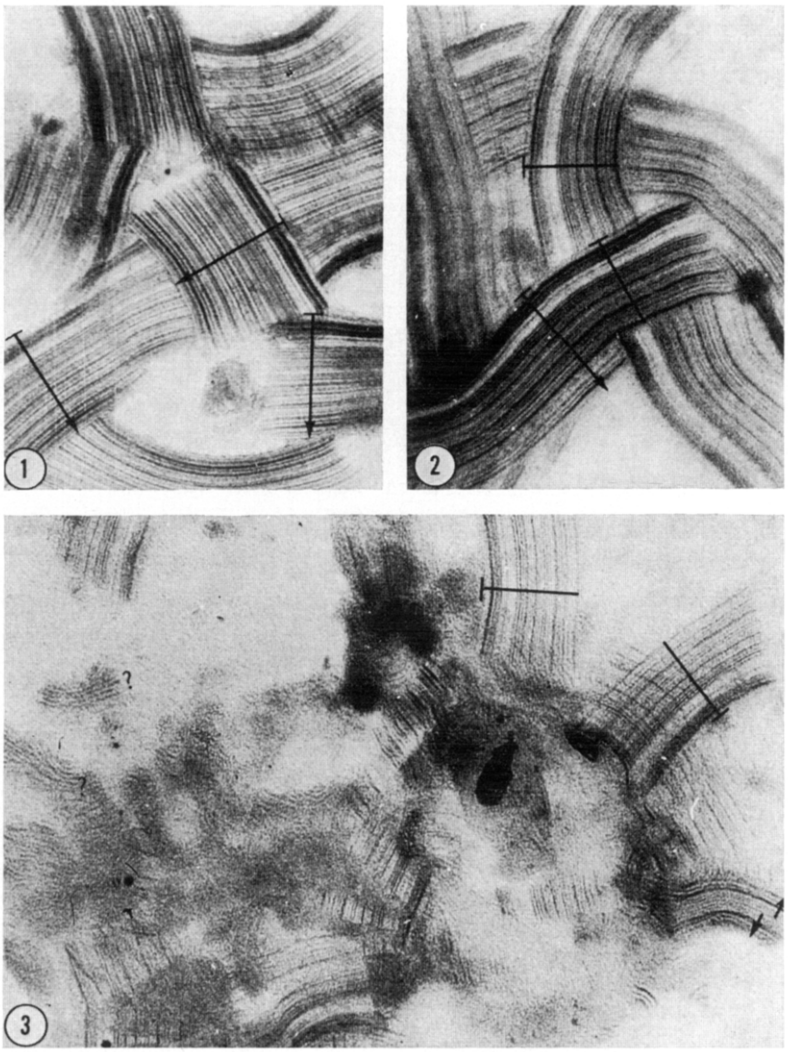


Fig. 10. Segment long spacing electron micrographs. (1) Normal collagen. (2) Epithelial enzyme reaction products. (3) Granulation tissue enzyme reaction products. Magnification × 60 000. The arrows indicate the length of the tropocollagen molecules, arranged in segment long spacing register. TC_A and TC_B fragments are indicated by appropriate broken segments of arrows. The question marks point to unidentified fragments.

out resulted in more complete recovery of collagenolytic activity in the precipitate, an approx. 1000% increase, caseinolytic activity was 2–3 times that obtained when the precipitate was collected after 1 h.

Results of gel filtration of semipurified component collagenolytic activities are shown in Fig. 8. Epithelial enzyme was eluted before granulation tissue enzyme. Fig. 9 shows the resolution of a mixture of semi-purified epithelial and granulation tissue collagenases. Activity of Peak I was enhanced by cysteine, while Peak II activity was inhibited by similar treatment. This data again suggest non-identity,

based on a semipurified preparation. Separation of crude whole edge enzyme on Sephadex G-200 and Bio-Gel, 1.5 m., 200–400 mesh, (Cal. Biochem. Corp., Los Angeles, Calif.) also suggests two peaks of collagenolytic activity.

Segment long spacing preparations

Segment long spacing preparations of the epithelial reaction products showed TC_A fragments 3/4 of the length of the collagen molecule and TC_B fragments 1/4 of the length of the collagen molecule. Hybrids of these two were also observed. Fig. 10 shows representative examples of these preparations. Segment long spacing preparations of the granulation enzyme reaction products showed TC_A, TC_B and hybrids. Small fragments not seen in segment long spacing preparations from the epithelial enzyme were observed in preparations from granulation tissue collagenase (Fig. 10-3.)

DISCUSSION

Short term cultures of the components of healing open rabbit wounds in enriched synthetic medium result in the diffusion into the culture medium of an enzyme capable of degrading collagen under physiological conditions. The assay systems employed in the present study are specific for collagenase[4,16]. Only 7–10% of the total counts present in the collagen substrate were released as a result of 0.1% trypsin action. Heat inactivated enzyme and trypsin controls produce minimal reduction in viscosity of a reconstituted collagen substrate. The negligible change in optical rotation during viscosity reduction is evidence of the limited nature of the digestion of the collagen molecule by the collagenase enzymes at 28°.

Measurements of collagen degradation in explanted mesenchymal tissue suggests that apparent late release of granulation tissue activity is related to endogenous splitting of tissue collagen in culture, since there was a daily increase in soluble hydroxyproline with a decrease in insoluble hydroxyproline values. The rate of change was in accord with enzyme production. In culture, enzyme may be bound to endogenous collagen and detection of collagenase may not be possible until the endogenous collagen is digested, thus releasing the enzyme into the culture medium.

Collagenolytic activity cannot in most cases be obtained by extraction of tissue homogenates but requires living tissue for its production. Freeze-thawing the tissue completely prevents the appearance of wound[16], skin[12] and tadpole[9]collagenases, suggesting that there is little or no storage of active enzyme in the cells. Noncollagenolytic neutral protease activity of the culture medium as determined by its activity on denatured casein was found in conjunction with the collagenolytic activity. This finding contrasts with that in human skin cultures[12] where neutral protease production occurred before release of collagenolytic activity and became nearly undetectable at the time of maximal collagenolytic activity. The occurrence of neutral proteolytic activity in conjunction with collagenolytic activity at physiologic temperature could have significance for further digestion of products of collagenase action. At 37° more than 50% of the reaction products of wound collagenase activity are dialyzable[16].

Rabbit epithelial and granulation tissue collagenase attack the collagen molecule in a specific and limited manner at temperatures below the denaturation temperature. Products of the epithelial reaction are similar to those produced by

tadpole[8], rheumatoid snyovia[11], mouse bone[13], human skin collagenase[12] and human gingiva[14]. These enzymes cleave the collagen molecule 1/4 from the B end producing TC_A pieces 75% the original length and TC_B pieces 25% the original length. Postpartum rat uterine collagenase[10] digests the substrate to a greater extent than other demonstrated collagenases. TC_A fragments produced initially are further digested from the cut end, producing pieces 67% and 62% the normal length. Granulation tissue collagenase appears to digest collagen fibrils to a greater extent than epithelial collagenase. Although the presence of a second enzyme in the granulation tissue cannot be discounted, the fact that crude and semi-purified enzyme give the same reaction product pattern on polyacrylamide disc electrophoresis suggests that this is due to the collagenase and not to other proteolytic activity present. Segment long spacing observations have not yet shown additional TC_A zone pieces in granulation enzyme reaction products, but do show large numbers of fragments smaller than TC_B.

In common with other collagenases, except for that from human granulocytes[6], the activity of component rabbit tissues is inhibited by serum. Serum inhibition is of particular interest as a possible physiologic control mechanism. Our studies with albumin, α, β, γ deficient, and α-1 deficient serum have not yet pinpointed the source of the serum inhibition. The α-1 deficient serum which was inhibitory in this study contained 10% normal α-1 globulin levels. This might be sufficient for inhibition. α-1 globulin is of interest because it has been shown to possess antitryptic[29], antielastase[30] and anticollagenase activity[31]. The α and β fractions used in this study were prepared by Cohn fractionation, which might have destroyed the activity of these serum proteins against trypsin and, possible, collagenase. Albumin and γ-globulin may be eliminated as sources of serum inhibition. Another common feature shared by collagenase is inhibition by EDTA, presumably on the basis of chelation. This effect is reversed by calcium in all other collagenases studied except the uterine enzyme[10]. Similarly, the activities of the enzymes from the open wound are not restored by addition of calcium after preincubation and inhibition by EDTA.

Epithelial and granulation tissue collagenases produce similar reductions in denaturation temperature without altering the helical structure of the collagen fragments. It has been postulated[8,9] that the lowered denaturation temperature has physiological significance, since the fragments are unstable and will lose their helical structure spontaneously at 37°, thus becoming susceptible to attack by other tissue proteases or the collagenase itself.

Epithelial and granulation tissue collagenases appear to be different on chemical and physical grounds. In contrast to all other demonstrated collagenases, only collagenase from the rat uterus[10] is not inhibited by cysteine. Granulation tissue enzyme is inhibited by cysteine but epithelial enzyme shows enhanced activity in the presence of cysteine. Chemical evidence of non-identity is supported by the gel filtration data which demonstrated two peaks of collagenolytic activity. The finding of two enzymes may have physiological significance. Collagenase from granulation tissue appears to have greater activity against reconstituted collagen than does the epithelial enzyme. This may be important, since the granulation tissue enzyme generally functions in an area of greater collagen concentration.

It is unlikely that collagenase activity of the granulation tissue is due in any significant measure to white blood cells in this tissue. Relatively few granulocytes

are found in 12-day wounds. Although extracts of granulocytes are active in collagenolysis, extracts of 12-day granulation tissue show no such activity and frozen-thawed tissue has no lytic activity.

The increase in activity of epithelial collagenase during a period of rapid epithelial migration might facilitate cellular detachment from the connective tissue base. KAHL AND PEARSON[32] noted clean dermal-epidermal separation of guinea pig skin after intracutaneous injection of Clostridial collagenase; the basement membrane rather than dermal collagen was the affected structure. The epidermal cellular pattern did not become disorganized. We have found that Clostridial collagenase readily separates the epithelium from the mesenchyme of "whole edge" samples from wounds in both guinea pigs and rabbits (H. C. GRILLO, unpublished results). ROSS AND ODLAND[33] pointed out that as the epidermis advances into an incised skin wound the cells remain attached by desmosomes but move as a sheet. WINTER[34] observed that the epidermal cells migrated at a relatively deep level in a dry skin wound having to remove dermal collagen—presumably by enzymatic activity. The appearance of collagenase activity in subcutaneous mesenchymal tissue only during a period of active repair of the wound suggests a role in collagen remodeling. Differences in biochemical behavior of epithelial and mesenchymal collagenases might reflect different functions.

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