Collagenase From the Culture Medium of Rabbit Colon Wall With Special Reference to the Latent Type and Substrate Specificity

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Latent and active forms of collagenase were detected in culture media of the normal rabbit colon. During culture, the collagenase appeared to be produced by surviving and growing mucosas on the degenerated or necrotic colon wall. Type III collagen was most readily degraded by the collagenase, followed by type I and II collagens. The collagenase did not attack type IV or V collagens. The latent collagenase was activated by trypsin and chaotropic agents such as 3 M NaSCN or NaI, and autoactivated gradually during storage. Activated latent collagenase showed the properties of metalloproteinase as in the active collagenase. The apparent molecular weights, determined by calibrated Sephadex G-75, were 39,000 and 31,000 for the latent and active enzymes, respectively. After 12 h of tissue culture, the latent collagenase appeared in the culture media 10–20 h earlier than the active collagenase. The collagenase in the culture media of the early period was mainly the latent form, while the media of the late period contained a large amount of the active form.

Animal collagenase (EC 3.4.24.7) and its latent form, which is activated by trypsin or other means, have been found in different tissues of various kinds of animals (1–12). By the tissue explant method, we detected collagenolytic activity in the cancerous mucosa of the human stomach and also in the mucosa of patients with chronic gastritis (13). Using a similar tissue explant method, Hawley et al. (14) reported a significant increase in collagenolytic activity in the postoperative intestinal tract of rabbits. We detected collagenolytic activity in the culture medium of intestinal tracts of normal rabbits and found that the activity from the lower colon was higher than that from other portions of the intestinal tract. However, the collagenase activity was not detected in a direct extract from the fresh intestinal tissue (15). In the present study, we mainly used the normal rabbit colon. The rabbit colon collagenase was thought to be derived from the mucosa of cultured colon wall.

Physiologic and pathological significance of the colon collagenase is not clear. The enzyme is possibly related to collagen metabolism in the colon wall, especially the mucosa. Particularly in the pathological conditions of the mucosa, such as injury or inflammation, it may act on the epitheliomesenchymal junction and serve to accelerate the exfoliation and subsequent regeneration of epithelial cells and renewal of the epithelial layer. In such states, it may work in the suppression of fibrosis in the mucosa.

In the culture medium of normal rabbit colons, we further found latent collagenase, a part of which was activated by chaotropic agents such as 3 M NaSCN or NaI; the remainder was not activated by these agents but was activated by trypsin.

Our paper reports on the release of the active and latent collagenase from the rabbit colon tissues and on some properties of the enzymes, including the substrate specificity by collagen types.

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Materials and Methods

Tissue Culture: Preparation and Partial Purification of Collagenase

The rabbit colon was cultured as described (15).

(a) The colon was removed from mature male rabbits after 3-day starvation, emptied, and fully washed with sterilized Tyrode solution. Thin strips of tissues, 1.0 g wet wt in each dish, were spread on a filter paper disk floating in 10 ml of Tyrode solution (pH 7.6) containing antibiotics in a 90-mm Petri dish. These were then incubated at 37°C in a moist atmosphere including 5% CO_2. The culture media were harvested and replenished at 24-h intervals, and each 24-h medium was centrifuged at 10,000 g for 20 min at 4°C. One molar Tris-HCl buffer (pH 7.6) was added to the supernatant to a final concentration of 0.05 M; the resultant solution was used as the crude enzyme solution.

(b) To examine the beginning and subsequent time-course of release of the latent collagenase from the cultured tissue into the medium, the media of some dishes were harvested after 4, 8, 12, 16, 20, and 30 h, and each medium was subjected to the collagenase assay.

(c) The crude collagenase solution was concentrated by ultrafiltration through a Diaflo PM-10 membrane under a pressure of 4 kg/cm^2 N_2 or by precipitation between 20% and 70% saturation with $(NH_4)_2SO_4$. Then the concentrated enzyme solutions were applied to a column of Sephadex gel equilibrated with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.2 M NaCl and 5 mM $CaCl_2$ (Tris-NaCl-$CaCl_2$) and eluted with the same buffer in a cold room. The fractions containing the enzyme were concentrated through a Diaflo membrane and used as a partially purified collagenase.

Collagenase Assay Using Isotope-Labeled Collagen

Gelatinous reconstituted fibrils of [U-^{14}C]glycine-labeled soluble collagen from the guinea pig dermis was used as the substrate for the collagenase assay (16).

The enzyme activity was represented as units per milliliter of the enzyme solution; 1 U means the degradation of 1 μg of reconstituted collagen fibrils per minute at 37°C.

Generally, all assays were carried out in duplicate.

Activation of Latent Collagenase

Trypsin. The solution containing latent collagenase was allowed to react with trypsin (NBC Co., 2 × Cryst.) for 15–30 min at pH 7.6 and room temperature (2). There was no difference in resulting collagenase activity by trypsin activation for this range of time. Eleven to 30 μg/ml of trypsin was used for the crude culture medium and 6–11 μg/ml for the partially purified one. Because protein concentration of the latter was reduced (~1/10 to 1/30), a smaller amount of trypsin was used. After activation, soybean trypsin inhibitor (STI, Sigma Chemical Co., St. Louis, Mo.) was put into the mixture at a final concentration of 40–120 μg/ml. Ten minutes later, the collagenase activity was determined. Trypsin and soybean trypsin inhibitor within the range of used concentrations had no effect on the collagen substrate.

Chaotropic agents (3 M NaSCN or NaI). The solution containing latent collagenase was dialyzed against 3 M NaSCN- or NaI-containing buffer (Tris-NaCl-$CaCl_2$) for 16 h at 4°C and subsequently these chaotropic agents were removed by dialysis against Tris-NaCl-$CaCl_2$ buffer (17). The solution obtained was subjected to the collagenase assay.

Collagenase Assay Using Non-Isotope-Labeled Collagen

This assay procedure was used only for examination of the substrate specificity against the collagenase by collagen types.

Preparation of collagens. Type I and III collagens were extracted from rat skin with 0.45 M NaCl, followed by repeated differential salt precipitation with NaCl and dissolution, using modifications of the methods of Timpl et al. (18) and Fujii and Kühn (19). Type II collagen was isolated from bovine fetal articular cartilage after pepsin digestion, using a modification of the method of Miller (20). Type IV collagen was prepared from pepsin extracts of rat kidney cortex according to the method of Kresina and Miller (21). Type V collagen was prepared from pepsin extracts of human placentas as described by Sage et al. (22).

Viscometry. For viscometric assay the collagens were dissolved in 5 mM acetic acid at 4°C at a concentration of 2 mg/ml, followed by centrifugation at 80,000 g for 60 min. Two milliliters of the collagen solution and 3 ml of buffer [0.1 M Tris-HCl(pH 7.6), 0.3 M NaCl, 50 mM $CaCl_2$, 0.6 M glucose] were mixed in a test tube, and 2 ml of the collagenase solution (~9 U) was added to the mixture. Six milliliters of the reaction mixture was immediately placed into an Ostwald capillary viscometer (a flow time for water of ~253 s at 25°C) and kept at 21 ± 0.5°C in a water bath. The viscosity of the collagen was measured at appropriate intervals, and results were calculated as percentage of original specific viscosity. Original specific viscosity of each reaction was based on a control experiment without enzyme.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and densitometry. After incubations with collagenase at 21°C, the reactions were stopped by heating at 80°C for 10 min. The reaction mixtures were then subjected to SDS-polyacrylamide gel electrophoresis by the method of Hayashi and Nagai (23) using 7.5% polyacrylamide gels and Tris-glycine buffer system. The gels were stained with 0.25% Coomassie blue in methanol-acetic acid-water (5:1:5 by vol), destained with 7.5% acetic acid and 5% methanol, and scanned at 570 nm with a densitometer (model-PAN II, Jookoo Sangyo Co. Ltd, Japan). The protein content was calculated on the integrated value of the area of densitometric chart showing the protein band patterns of the gels; the value was linearly related to the protein content according to the method of Hayashi et al. (24). The undegraded α-chain (%) was calculated using the following formula: undegraded α-chain (%) = (area of α-chain)/(area of α-chain + 4/3 area of α^A-chain) × 100.

Miscellaneous

Protein concentration was determined (25) using bovine serum albumin as a standard. All reagents used were of analytic grade. Some pieces of the cultured tissues were fixed in 10% formalin for light microscopy.

Results

Collagenase Activity in the Culture Media of the Rabbit Digestive Tracts

Collagenase activity appeared in the culture media of rabbit intestinal tracts. It showed a time-dependent increase, reached a peak in the culture of day 3 or 4, and decreased thereafter. Culture media from the lower colon showed the greatest amount of collagenase activity, followed by the cecum, upper colon, and the small intestine (Figure 1). After the mucous membrane was physically separated from the colon wall, the mucosa and the colon wall without it were cultured separately. The former produced collagenase, but the latter did not. No collagenase activity was detectable in the culture media of the intestinal contents, normal stomach wall, liver, kidney, and spleen, under our experimental conditions. Bacterial contamination markedly lowered the collagenase activity in the colon culture media.

Disk electrophoresis of collagen incubated with the collagenase from intestinal tracts showed the presence of α^A and β^A bands, which are typical reaction products of collagen degradation by the animal collagenase. The collagenase was inhibited by rabbit and human serum, L-cysteine, and chelating reagents. Its activity was dependent on Ca^{2+} and the pH optimum was 7.5–8.5. The molecular weight

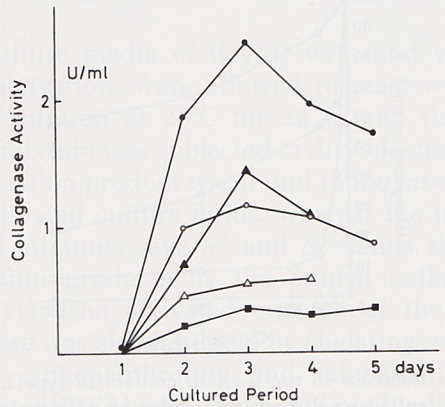

Figure 1. Comparison of manifest collagenase activity in culture media of rabbit intestinal tracts. Duplicate samples of each culture medium (refreshed every day; *closed circles*, lower part of colon; *open circles*, upper part of colon; *closed triangles*, middle part of cecum; *open triangles*, apex of cecum; *closed squares*, small intestine) were assayed for collagenase activity.

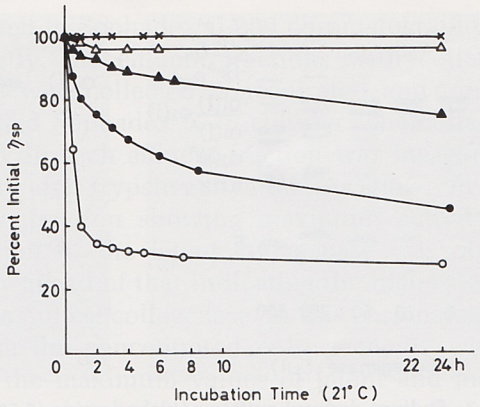

Figure 2. Decrease in specific viscosity of collagen solutions on incubation with the rabbit colon collagenase. Type I *(closed circles)* and type III *(open circles)* collagens from salt-soluble fractions of rat skin, type II *(closed triangles)* collagen from pepsin extracts of bovine fetal articular cartilage, type IV *(open triangles)* collagen from pepsin extracts of rat kidney cortex, and type V (×) collagen from pepsin extracts of human placenta.

of the collagenase was calibrated as being between 35,000 and 45,000 by Sephadex G-200 gel filtration.

Preference for Degrading Type III Collagen Over Type I and Type II Collagens

According to changes in the specific vicosity of collagen solution by the rabbit colon collagenase, type III collagen proved to be most readily degraded by the enzyme, followed by type I and II collagens; the collagenase did not degrade type IV or V collagens under the conditions used (Figure 2). A similar tendency was shown in the SDS-polyacrylamide gel electrophoresis of the reaction mixtures of these types of collagens treated with the collagenase; the α^A-peptides appeared with a smaller amount of the enzyme active on type III than on type I and II collagens (Figure 3). No reaction products were shown in the mixtures of type IV or V collagens. The degradation rates of these collagens were not changed by proteinase inhibitors, such as 1 mM *N*-ethylmaleimide (NEM) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Figure 4).

To further confirm these results, the percentage of remaining α-chain content in the treated collagen solution was calculated from the densitometric analysis of the SDS-polyacrylamide gel electrophoresis of the reaction mixtures. Diminution of α1(III) chain from type III collagen occurred much more effectively than that of α1(I) chain from type I collagen (Figure 5). Decrease in the specific viscosity of these three types of collagens by the rabbit colon collagenase (Figure 2) corresponds well with decrease in the α-chain content of the collagens (Figure 5).

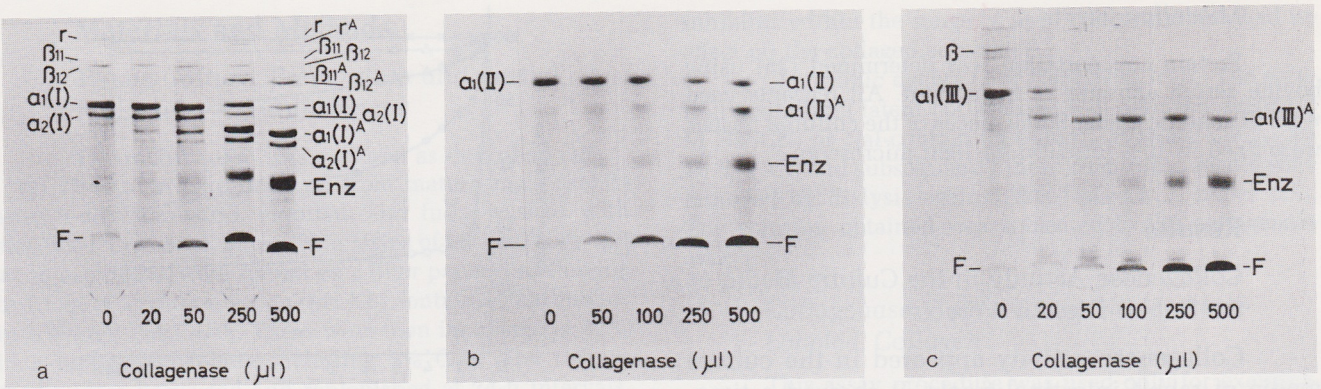

Figure 3. Sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis of type I, II, and III collagens after incubation with the rabbit colon collagenase of various volumes at 21°C for 24 h. The collagenase in various volumes was put into the reaction mixture and the final volume of the mixtures was made up to 7 ml with the enzyme buffer solution. Part *a*: type I collagen, *b*: type II collagen, *c*: type III collagen.

Histology of the Incubated Rabbit Colon Wall

The mucosa of the normal rabbit colon consisted of the orderly surface epithelium and the lamina propria in which many intestinal glands were regularly distributed (Figure 6*a*), with a small number of spindle-shaped fibroblastic cells and migratory cells (Figure 6*a*,*b*). During incubation, a large part of the colon wall rapidly fell into degeneration and necrosis. In the cultured colon tissues showing collagenase activity, however, surviving and growing mucosas were always present in places on a large part of the degenerated or necrotic colon wall (Figures 7*a*, 8*a*). The surviving mucosa consisted of living epithelial cells and a thin layer of lamina propria containing a small number of mesenchymal cells and migratory cells, many of which had pycnotic nuclei (Figures 7*b*,8*b*). The height of the surface epithelium in the surviving mucosa appeared to be slightly lower than that of the normal one. Histology was essentially similar in the surviving mucosa of the incubated colon wall during several days, although glandular structures appeared to be much decreased in the late period of culture. Growth of these mucosa seemed to slow after ~5 days.

Collagenase in a Latent Form

In the culture medium we found latent collagenase that could be activated by trypsin or chaotropic agents such as 3 M NaSCN and NaI. Some

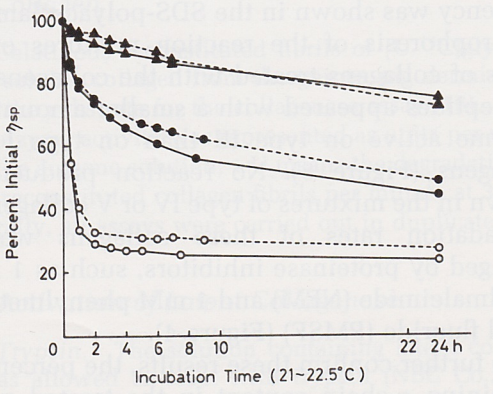

Figure 4. Decrease in specific viscosity of collagen solutions on incubation with the rabbit colon collagenase treated with proteinase inhibitors. The collagenase solution containing 1 mM phenylmethyl sulfonyl fluoride and 1 mM *N*-ethylmaleimide was allowed to stand overnight at 4°C, and the collagen solutions with the previously described enzyme solution were assayed in viscosity *(dotted lines)*. *Solid lines* are from control experiments without proteinase inhibitors. Type I *(closed circles)*, type II *(closed triangles)*, and type III *(open circles)* collagens.

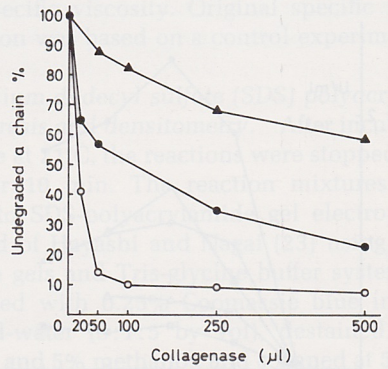

Figure 5. Decrease in the α-chain content of type I, II, and III collagen solutions on incubation with the rabbit colon collagenase of various volumes. Incubation was performed at 21°C for 24 h. These data were derived from quantitative densitometric scans of the gels of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reaction mixtures. Type I *(closed circles)*, type II *(closed triangles)*, and type III *(open circles)* collagens. Results are the mean of five experiments.

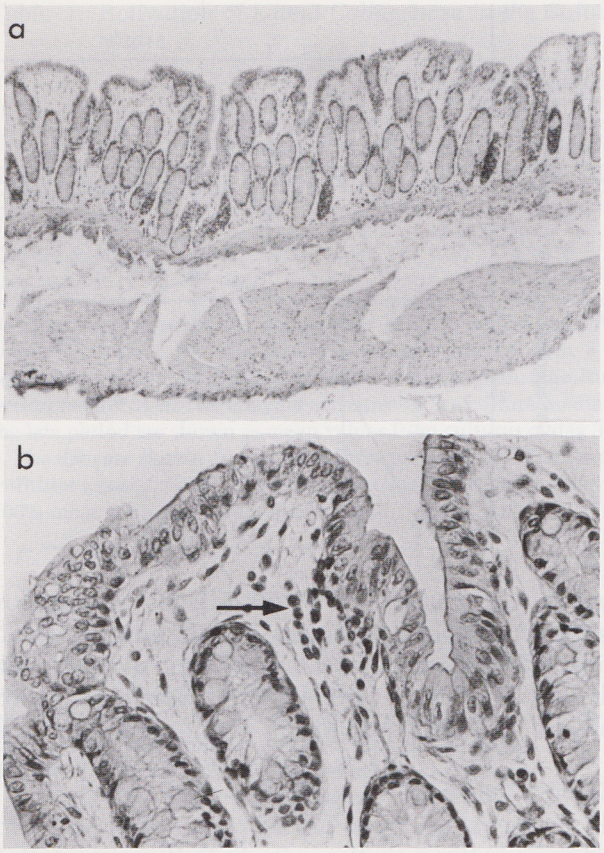

Figure 6. Normal rabbit colon. *a*. The mucosa is covered by the orderly surface epithelium and many intestinal glands are regularly distributed in the lamina propria. (H&E, ×50.) *b*. The lamina propria contains some spindle-shaped fibroblastic cells and a small number of migratory cells (*arrow*). (H&E, ×250.)

properties of the latent collagenase were examined using the 1-day culture medium, in which the collagenase was largely the latent form (Figures 9,10, Table 1).

The culture media of day 1, with and without trypsin activation, were allowed to react with the collagen solution at 20°C for 22 h, and then the reaction mixture was subjected to disk electrophoresis; no reaction product was found in the mixture by the unactivated culture fluids, whereas the trypsin-activated mixtures gave α^A and β^A bands showing the reaction products by the animal collagenase (Figure 11). The activated enzyme of the latent collagenase was also inhibited by chelating reagents, such as *o*-phenanthroline and ethylenediaminetetraacetate (EDTA), and serum, but not by diisopropyl fluorphosphate (DFP), PMSF, and *p*-chloromercuribenzoic acid (PCMB) (Table 2). These properties were the same as those of the manifest collagenase (15). Rather, PCMB activated the latent collagenase to some extent.

The culture media of day 1 were concentrated and subjected to Sephadex G-200 chromatography. Subsequently, the effluent fractions with collagenase activity were collected, concentrated, and applied to calibrated Sephadex G-75 column. The collagenase activity in each effluent fraction was measured before and after trypsin activation. As shown in Figure 12, the fraction showing maximum activity after activation of the latent collagenase was obtained slightly ahead of that indicating the highest activity of the manifest collagenase. In this chromatography, because the concentrated culture media were applied, the maximum values of latent and manifest collagenase were higher to some extent than those from the crude culture media. Apparent molecular weights of the latent and manifest collagenase were ~39,000 and 31,000, respectively. The molecular weight of the latent collagenase was calculated as 5,000–10,000 larger than that of the active one from the results obtained on Sephadex G-75, G-100, and G-200. In the chromatography, collagenase activities

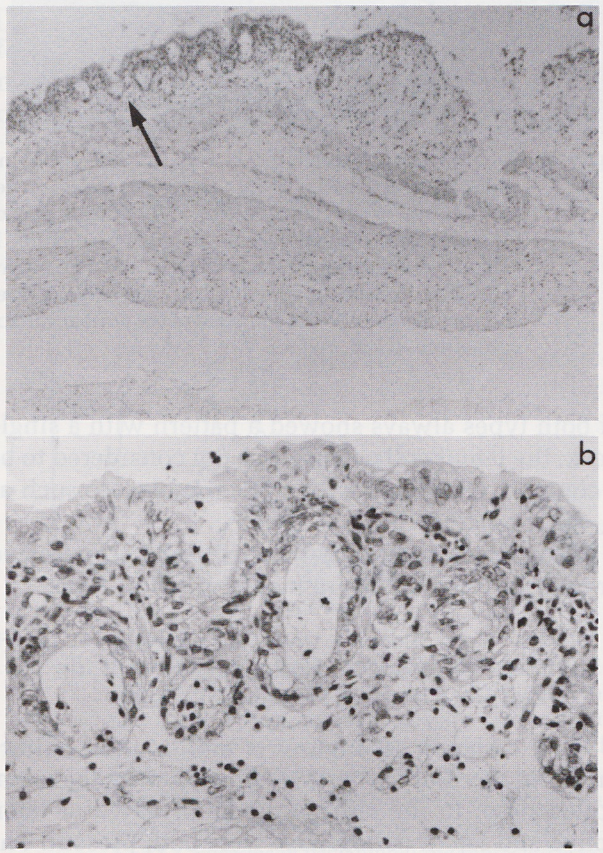

Figure 7. Rabbit colon cultured for 24 h. *a*. A part of the mucosa appears to survive *(arrow)* and grow to some extent on a large part of the degenerated colon wall. (H&E, ×50.) *b*. The surviving mucosa consists of the living surface epithelium, a small number of glandular structures, and a thin layer of lamina propria containing a small number of mesenchymal cells and migratory cells, many of which have pycnotic nuclei. (H&E, ×250.)

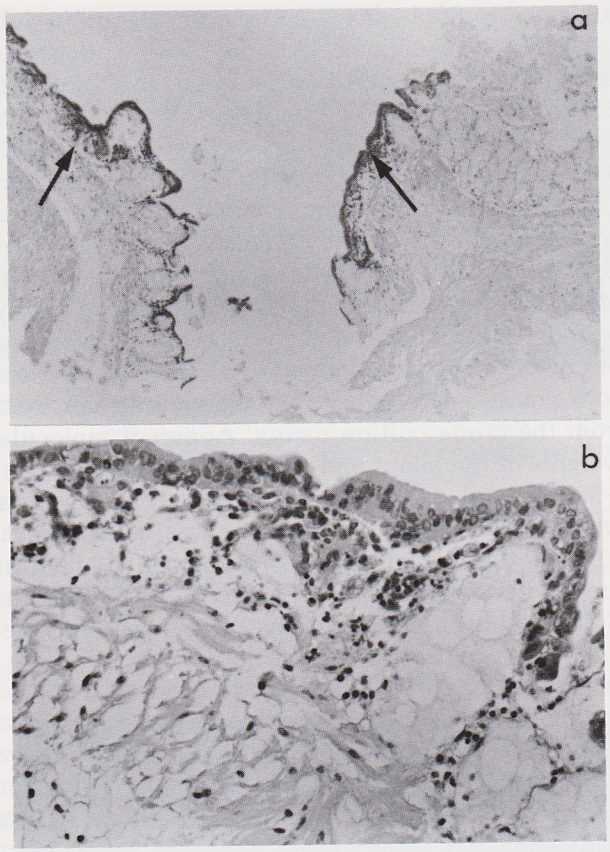

Figure 8. Rabbit colon cultured for 3 days. *a.* The surviving mucosa (*arrow*) partly covers the degenerated or necrotic tissue of the colon. (H&E, ×50.) *b.* This part of the surviving mucosa has no glandular structure. It contains some mesenchymal cells and a few migratory cells. The cytoplasm of epithelial cells looks denser and their height is lower than that of the normal surface epithelium. (H&E, ×250.)

of both types always showed a pattern with a single peak; the latent collagenase was not considered to be a complex combined with a macromolecule such as α_2-macroglobulin.

No collagenase activity was detected in nontreated or trypsin-treated culture media of rabbit feces.

Release of Latent Collagenase From the Cultured Tissues of Rabbit Colon

As shown in Figures 9 and 10, the culture media of day 1 or earlier contained mainly the latent collagenase. In the culture media of day 2 or later, active and latent collagenase were both present. The collagenase activity of the trypsin-treated culture media also showed a time-dependent increase, reached a peak in the culture of day 3 or 4, and decreased thereafter. The trypsin treatment of the culture media up to day 3 elevated its collagenase activity with a statistically significant difference ($p < 0.05$) (Figure 9). Figure 10 shows a time-course

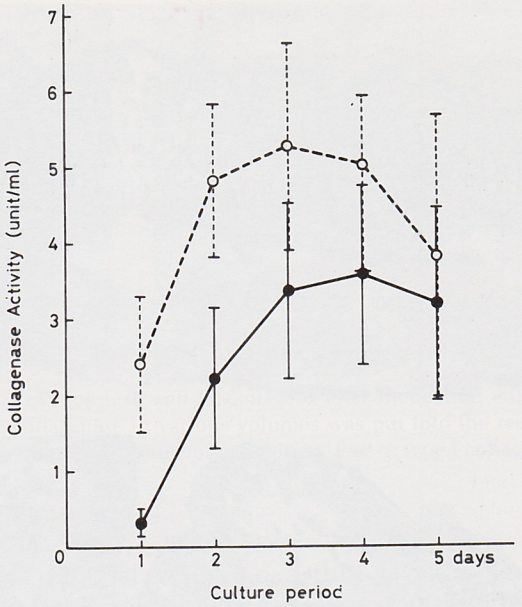

Figure 9. Time-course of collagenase activity in culture media of rabbit colon explants during 5 days. Duplicate samples of each 24-h culture medium (refreshed every day) were assayed for collagenase activity directly (*closed circles*) and after trypsin treatment (*open circles*). Each point is the mean of activities from six experiments; the *vertical bars* correspond to 2 SD.

of collagenase activity in the culture media of the early period. The latent collagenase began to be released after a time lag of 12 h from the beginning of the culture, while the active one was detected in very small amounts even after 30 h; the latent

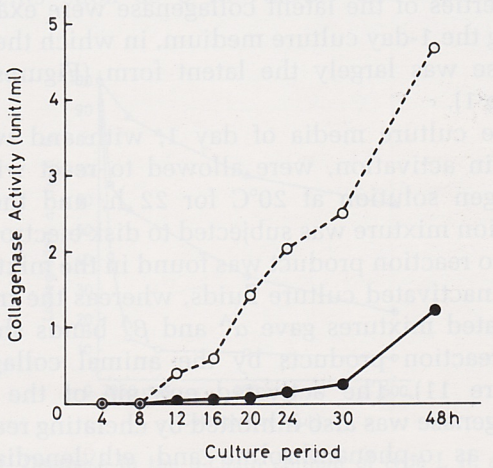

Figure 10. Time-course of collagenase activity in culture media of rabbit colon explants during 48 h from the beginning of culture. Rabbit colon explants were continuously cultured, and the media were harvested at each time point on the horizontal line. Duplicate samples of each culture medium were assayed for collagenase activity directly (*closed circles*) and after trypsin treatment (*open circles*).

Table 1. Activation of Latent Collagenase in the Culture Media

	Active form	Latent form + active form	
	Nontreated	Salt-treated	Trypsin-treated
Culture medium Early period	0.35 ± 0.21[a] (15)[b]	1.36 ± 0.39 (56)	2.41 ± 0.78 (100)
Culture medium Late period	2.81 ± 0.11 (66)	3.24 ± 0.30 (76)	4.26 ± 0.76 (100)

Samples were assayed for collagenase activity before and after activation with 3 M NaSCN. Some samples treated with 3 M NaSCN underwent additional activation with trypsin. The early period means 24 h. The enzyme activities in the culture media of the late period are shown as mean values from cultures of day 3 to 5. The data are shown as a mean of three experiments. [a] Units per milliliter (means ± SD). [b] Percent in the total collagenase activity; 100% means the activity after additional trypsin treatment.

Table 2. Effect of Inhibitors on the Trypsin-Treated Latent Collagenase in the Culture Medium of Rabbit Colon Explants

Inhibitor	Concentration	Activity (% *of control*)
DFP[a]	10^{-3} M	100
PMSF[b]	3×10^{-3} M	100
PCMB[c]	10^{-3} M	100
Dithiothreitol	10^{-2} M	15
Dithiothreitol	10^{-3} M	43
Mercaptoethanol	2×10^{-2} M	55
Cysteine	10^{-2} M	26
o-Phenanthroline	1.24×10^{-4} M	41
EDTA[d]	10^{-2} M	5
Human serum	1:15	7

Partially purified latent collagenase was activated by trypsin and then its aliquots were preincubated with inhibitors at room temperature for 20 min before collagenase assay. The preincubation mixture consisted of 0.4 ml of enzyme solution and 0.1 ml of each compound solution. [a] DFP, diisopropyl fluorophosphate. [b] PMSF, phenylmethyl sulfonyl fluoride. [c] PCMB, p-chloromercuribenzoic acid. [d] EDTA, ethylene diaminetetraacetate.

collagenase usually appeared 10–20 h earlier than the active one. No collagenase, latent or active, was detected in extracts of fresh colon tissues. The latent collagenase was spontaneously converted into the active form when the culture medium was allowed to stand for several months at 4°C.

Chaotropic agents such as 3 M NaSCN or NaI could activate a part of the latent collagenase. The collagenase activity in the culture medium was first enhanced to some extent by such agents and further elevated by the subsequent treatment with trypsin (Table 1). The collagenase in the culture medium of the early period (≤24 h) consisted predominantly of the latent form (>80%), while that of the late period (≥3 days) contained a large amount of the active form (>60%) (Table 1, Figure 9,10). In the culture media from day 1 to 5, it appeared that ~30%–50% of the latent collagenase could be activated by chao-

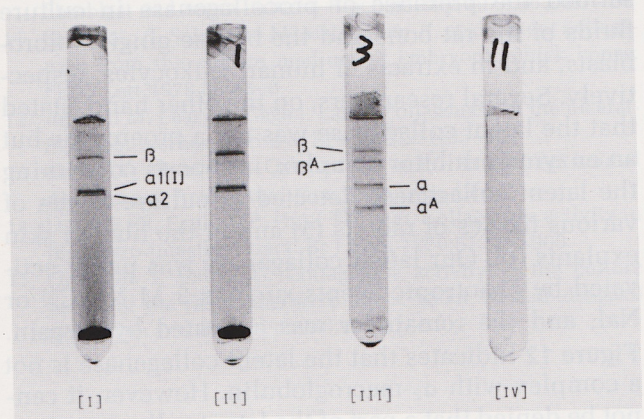

Figure 11. Disk gel electrophoresis of reaction products from enzymatic hydrolysis of acid-soluble collagen from the guinea pig in solution. Incubation of collagen and crude enzyme was made at pH 7.6 and 20°C for 22 h. Polyacrylamide gel electrophoresis was performed with modification of the method of Nagai et al. (26). The electrophoresis was carried out using a current of 5 mA/tube for 1.5 h at room temperature. (I) collagen control; (II) collagen + culture medium of day 1; (III) collagen + trypsin-activated culture medium of day 1; (IV) culture medium of day 1.

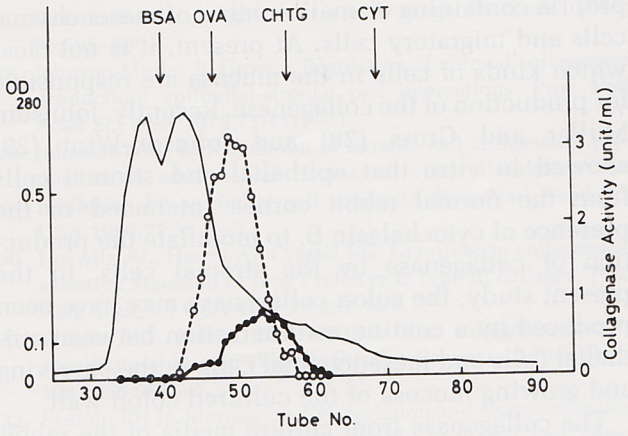

Figure 12. Sephadex G-75 column chromatography of rabbit colon collagenase after Sephadex G-200 gel filtration. Effluent fractions with collagenase activity on Sephadex G-200 column chromatography were collected, concentrated by Diaflo PM-10 membrane, applied to Sephadex G-75 column (2.6 × 100 cm) equilibrated with Tris-NaCl-$CaCl_2$ buffer, and eluted with the same kind of buffer (4.4-ml fractions; flow rate 18.8 ml/h; 3°–7°C). Portions of each eluted fraction were assayed for collagenase activity directly (*closed circles*) and after trypsin treatment (*open circles*). The *solid line* represents absorption at 280 nm. Under the same condition, the standard proteins were gel filtrated and the molecular weight of collagenase was determined by the method of Andrews (27). BSA, bovine serum albumin, OVA, ovalbumin; CHTG, α-chymotrypsinogen; CYT, cytochrome c.

tropic agents, and ~50%–70% could not be activated by such agents (Table 1).

Discussion

In the present study, collagenase activity was detected in the culture medium of the rabbit colon, but not in a direct extract from the fresh colon tissue.

By tissue culture, the mucosa physically separated from the colon wall also produced collagenase, while the colon wall without the mucosa did not show the activity. The collagenase appeared in the culture medium after a certain time lag from the beginning of tissue culture. The culture medium in an initial stage (<12 h) of tissue incubation had no collagenase activity, although the cultured tissue rapidly underwent marked degeneration. After 12 h of tissue culture, the latent collagenase was first found in the culture medium, followed by an appearance of the active collagenase 10–20 h later. In the cultured tissues exhibiting collagenase activity from 1 to 5 days, surviving and growing mucosas were always present in places on a large part of the degenerated or necrotic colon wall. This suggests that the collagenase may be produced by such remaining and living mucosas. The surviving mucosa was composed of a living epithelial layer with a few glandular structures and a thin layer of lamina propria containing a small number of mesenchymal cells and migratory cells. At present, it is not clear which kinds of cells in the mucosa are responsible for production of the collagenase. Recently, Johnson-Muller and Gross (28) and Johnson-Wint (29) showed in vitro that epithelial and stromal cells from the normal rabbit cornea interacted in the presence of cytochalasin B, to modulate the production of collagenase by the stromal cells. In the present study, the colon collagenase may have been produced by a continuous interaction between epithelial cells and mesenchymal cells in the surviving and growing mucosa of the cultured colon wall.

The collagenase from culture media of the rabbit colon degraded type I, II, and III collagens to a different degree, but did not affect type IV or V collagens. Horwitz et al. (30) reported that the collagenase from acute-phase inflammatory cells (human polymorphonuclear leukocytes) degraded human type I collagen preferentially (15:1) to human type III collagen, while late-stage inflammatory cells (rabbit pulmonary alveolar macrophages) digested type I and III collagens at an equal rate. In contrast, our collagenase degraded type III collagen more effectively than type I collagen. Type II collagen was more resistant to the collagenase than type I and III collagens. The degradation rates of these collagens were not changed by proteinase inhibitors, such as 1 mM PMSF and 1 mM NEM; this means that such collagen degradation is undoubtedly caused by the metalloproteinase, such as collagenase. According to Welgus et al. (31), human skin fibroblast collagenase degraded, at different rates, type I, II, and III collagens from human and several species of animals (calf, guinea pig, and rat), respectively, and it was more specific for human type III collagen than human type I collagen from the placenta. They proposed that the substrate specificity of animal collagenase was dependent upon at least two factors: collagen types and species of substrate origin. In the present study, the rabbit colon collagenase proved to degrade type I and III collagens from rat skin particularly well, especially type III. Because plenty of type III collagen is contained in the intestine (32), it seems reasonable that the rabbit colon collagenase degrades type III collagen preferentially to other types of collagen examined.

The latent collagenase in the culture medium of the rabbit colon was not detected in a direct extract from the fresh colon tissue either. The collagenase in the culture medium of the early period (≤24 h) was mostly the latent form (>80%), whereas that of the late period (≥3 days) contained a large amount of the active form (>60%). A drop of ~5,000–10,000 in the molecular weight of the latent collagenase occurred after its activation by trypsin. This result is in agreement with that obtained by activation of the latent collagenase derived from other sources (1,5–12). There is a considerable controversy concerning the nature of latent collagenase. Vaes (2), Birkedal-Hansen et al. (3), and Kurze and Wojtecka (4) described the presence of procollagenase in culture fluids of the rat bone and the bovine gingival fibroblasts, and in extracts of human leukocytes, respectively. Several researchers, on the other hand, stated that the latent collagenase was not a proenzyme but an enzyme-inhibitor complex, in reports concerning the latent collagenase detected in culture media of various tissues of rabbits (5) and of the human skin explants (8). Our latent collagenase was partly activated by chaotropic agents such as 3 M NaSCN or NaI, and the remainder was activated by trypsin. Figure 12 indicates that the latent collagenase is not a complex with α_2-macroglobulin. However, it cannot be denied that a part of the latent collagenase is a complex with a low molecular weight inhibitor. After several months, the latent collagenase stored at 4°C proved to be activated spontaneously. There may be a possibility that the latent collagenase in the form of proenzyme was gradually activated by some immanent activator during storage.

The physiologic and pathological significance of the rabbit colon collagenase is not clear. The enzyme is possibly related to collagen metabolism in the

colon wall, especially the mucosa. The collagenase activity of the normal colon is probably low, but in pathological conditions such as injury or inflammation, the enzyme may become high in activity and act on the epitheliomesenchymal junction, accelerating the exfoliation and subsequent regeneration of the epithelial cells and renewal of the epithelial layer. In such conditions the enzyme may play some role in the suppression of fibrosis in the mucosa.

References

1. Cawston TE, Mercer E, Tyler JA. The activation of latent pig synovial collagenase. Biochim Biophys Acta 1981;657:73–83.
2. Vaes G. The release of collagenase as an inactive proenzyme by bone explants in culture. Biochem J 1972;126:275–89.
3. Birkedal-Hansen H, Cobb CM, Taylor RE, et al. Synthesis and release of procollagenase by cultured fibroblasts. J Biol Chem 1976;251:3162–8.
4. Kruze D, Wojtecka E. Activation of leucocyte collagenase proenzyme by rheumatoid synovial fluid. Biochim Biophys Acta 1972;285:436–46.
5. Sellers A, Cartwright E, Murphy G, et al. Evidence that latent collagenase are enzyme-inhibitor complexes. Biochem J 1977;163:303–7.
6. Woessner JF. A latent form of collagenase in the involuting rat uterus and its activation by a serine protease. Biochem J 1977;161:535–42.
7. Horwitz AL, Kelman JA, Crystal RG. Activation of alveolar macrophage collagenase by a neutral protease secreted by the same cell. Nature 1976;264:772–4.
8. Shinkai H, Nagai Y. A latent collagenase from embryonic human skin explants. J Biochem 1977;81:1261–8.
9. Wize J. A latent collagenase from rheumatoid synovial fluid: purification and partial characterization. Biochim Biophys Acta 1980;615:199–207.
10. Stricklin GP, Bauer EA, Jeffrey JJ, et al. Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ culture. Biochemistry 1977;16:1607–15.
11. Sakamoto S, Sakamoto M, Matsumoto A, et al. Latent collagenase from the culture medium of embryonic chick bones. FEBS Lett 1978;88:53–8.
12. Berman M, Leary R, Gage J. Latent collagenase in the ulcerating rabbit cornea. Exp Eye Res 1977;25:435–45.
13. Usuku G, Honya M, Oyamada I, et al. Studies on animal collagenase. Acta Pathol Japn 1974;24:1–19.
14. Hawley PR, Faulk WP, Hunt TK, et al. Collagenase activity in the gastro-intestinal tract. Br J Surg 1970;57:896–900.
15. Oyamada I, Usuku G. Collagenase activity in the culture media of the rabbit digestive tracts. Igaku To Seibutsugaku (Medicine and Biology) 1976;92:331–6.
16. Nagai Y, Hori H. Entrapment of collagen in a polyacrylamide matrix and its application in the purification of animal collagenases. Biochim Biophys Acta 1972;263:564–73.
17. Abe S, Nagai Y. Interaction between tadpole collagenase and human α_2-macroglobulin. Biochim Biophys Acta 1972; 278:125–32.
18. Timpl R, Glanville RW, Nowack H, et al. Isolation, chemical and electron microscopical characterization of neutral-salt-soluble type III collagen and procollagen from fetal bovine skin. Hoppe-Seyler's Z Physiol Chem 1975;356:1783–92.
19. Fujii T, Kühn K. Isolation and characterization of pepsin-treated type III collagen from calf skin. Hoppe-Seyler's Z Physiol Chem 1975;356:1793–801.
20. Miller EJ. Isolation and characterization of a collagen from chick cartilage containing three identical α chains. Biochemistry 1971;10:1652–9.
21. Kresina TF, Miller EJ. Isolation and characterization of basement membrane from human placental tissue. Evidence for the presence of two genetically distinct collagen chains. Biochemistry 1979;18:3089–97.
22. Sage H, Woodbury RG, Bornstein P. Structural studies on human type IV collagen. J Biol Chem 1979;254:9893–900.
23. Hayashi T, Nagai Y. Separation of the α chains of type I and III collagens by SDS-polyacrylamide gel electrophoresis. J Biochem 1979;86:453–9.
24. Hayashi T, Nakamura T, Hori H, et al. Degradation of rates of type I, II, and III collagens by tadpole collagenase. J Biochem 1980;87:809–15.
25. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265–75.
26. Nagai Y, Gross J, Piez KA. Disc electrophoresis of collagen components. Ann NY Acad Sci 1964;121:494–500.
27. Andrews P. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem J 1965; 96:595–606.
28. Johnson-Muller B, Gross J. Regulation of corneal collagenase production: epithelial-stromal cell interactions. Proc Natl Acad Sci USA 1978;75:4417–21.
29. Johnson-Wint B. Regulation of stromal cell collagenase production in adult rabbit cornea: in vitro stimulation and inhibition by epithelial cell products. Proc Natl Acad Sci USA 1980;77:5331–5.
30. Horwitz AL, Hance AJ, Crystal RG. Granulocyte collagenase: selective digestion of type I relative to type III collagen. Proc Natl Acad Sci USA 1977;74:897–901.
31. Welgus HG, Jeffrey JJ, Eisen AZ. The collagen substrate specificity of human skin fibroblast collagenase. J Biol Chem 1981;256:9511–5.
32. Epstein EH Jr, Munderloh NH. Isolation and characterization of CNBr peptides of human $[\alpha1(III)]_3$ collagen and tissue distribution of $[\alpha1(I)]_2\alpha2$ and $[\alpha1(III)]_3$ collagens. J Biol Chem 1975;250:9304–12.