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HUMAN SKIN COLLAGENASE. ISOLATION AND MECHANISM OF ATTACK ON THE COLLAGEN MOLECULE*

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SUMMARY

1. A collagenolytic enzyme has been isolated from the culture medium of tissue cultures of normal human skin but not from tissue extracts. The enzyme is capable of reducing the specific viscosity of native collagen at 28° and neutral pH in addition to lowering the denaturation temperature by approximately 5° without altering its helical content.

2. Enzymatic attack on the collagen molecule results in the appearance of new, faster moving bands on disc electrophoresis. Electron micrographs of segment long spacings prepared from the enzyme–collagen reaction mixtures reveal the presence of an "A" end fragment (TC^A) 75% the length of the collagen molecule and a "B" fragment (TC^B) representing the remaining one-quarter.

3. Human skin collagenase also acts on collagen in the native fibrillar form at 37° with a pH optimum of 7 to 8. No enzyme activity is present below pH 5.0; above pH 8.0 activity falls off markedly. The enzyme is inhibited by low concentrations of EDTA, cysteine and human serum but not by soybean trypsin inhibitor.

INTRODUCTION

Observations on the metabolic turnover of collagen[1,2] confirm the fact that although the bulk of body collagen may be relatively inert, forms of collagen do exist that normally are rapidly degraded. A system for collagen degradation undoubtedly exists in collagen-containing tissues, such as normal skin, which may be mediated by specific enzymatic action. Indeed, collagenolytic activity has recently been detected[3,4] and quantitatively measured in both normal and diseased human skin[5] by means

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of a sensitive tissue culture radioassay *in vitro*. Although collagenases have been detected in a variety of other tissues[6,7,9,10] they have been isolated, partially purified and characterized, only from tadpole tail fin[11,12], rat uterus[13] and rheumatoid synovial tissue[14]. Since none of these enzymes has, so far, been found in tissue extracts, their preparation requires the use of tissue culture techniques.

The present study deals with the isolation of a collagenase from the media of short term cultures of normal human skin and the mechanism by which it specifically degrades the collagen molecule.

METHODS

Tissue culture of normal human skin

Specimens of normal skin were obtained by surgical excision unter sterile operating room conditions. Immediately after excision the skin was placed in a sterile vial containing Dulbecco's modified Eagle's medium (Grand Island Biological Co.) with 100 mg/l of penicillin and streptomycin, and gassed with 95% O_2–5% CO_2. Complete processing of the tissue was accomplished within 30 to 45 min after excision, during which time the specimens were maintained at room temperature. Tissue, trimmed of subcutaneous fat and lower dermis was cut into pieces, approx. 4–6 mm in size, placed in 3.0 ml Dulbecco's modified Eagle's medium and cultured in disposable plastic flasks (Falcon Plastics) at 37° in an atmosphere of 95% O_2–5% CO_2. Culture medium was routinely changed at 24-h intervals and assayed for collagenolytic activity. Examination of enzymatically active culture media for bacterial growth both aerobically and anaerobically on numerous occasions revealed no evidence of contamination.

Media having collagenolytic activity was pooled, centrifuged at 50 000 × *g* for 30 min at 4° and dialyzed in the cold for 24 h against several changes of distilled water. The enzyme solution was lyophilized and stored at −20°. For use, the lyophilized enzyme powder was redissolved in 0.05 M Tris–HCl (pH 7.5), containing 0.005 M $CaCl_2$ to a final concentration of approx. 1.0 mg protein per ml.

Assay for collagenase

This has been described in detail by LAPIERE AND GROSS[15] and NAGAI, LAPIERE AND GROSS[12], and depends on the release of soluble [^{14}C]glycine-containing peptides from native, reconstituted, guinea-pig skin collagen fibrils. The assay was slightly modified to accomodate smaller volumes of radioactive collagen and enzyme solution. A typical reaction mixture contained 50 µl of 0.2% ^{14}C-labeled collagen (specific activity of 23 000 counts/min per mg) that had been allowed to gel for 14 h at 37° in a 1.0-ml plastic centrifuge tube; 50 µl of 0.05 M Tris–HCl (pH 7.5), containing 0.005 M $CaCl_2$ and 50 µl of enzyme solution. After incubation the reaction was inhibited with EDTA at a final concentration of 0.01 M and the tubes centrifuged at 50 000 × *g* at room temperature. For liquid scintillation counting, aliquots of the reaction mixture were suspended in BRAY's[16] solution containing 4.0% Cab-O-Sil[17]. Use of a thixotrophic gel eliminated difficulties encountered due to the precipitation of protein and EDTA when samples were added to BRAY's solution. When necessary, quenching corrections were made by the addition of an internal standard.

Non-collagenolytic protease activity, using casein as a substrate was determined at neutral pH by the method of KUNITZ[22]. Protein was determined by the method of LOWRY *et al.*[23].

Preparation of collagen substrate

Acid-extracted calf skin collagen or neutral-salt extracted, ^{14}C-labeled guinea-pig collagen was prepared according to the methods of GROSS AND KIRK[18,19]. For use purified lyophilized collagen was dissolved at a concentration of 0.2% in cold phosphate buffer (pH 7.6), $\Gamma/2 = 0.4$, by shaking 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 100 000 × *g* for 1 h to remove any undissolved material.

Viscosity measurements and disc electrophoresis

Measurements of specific viscosity as a function of time were made in Ostwald viscometers with water flow times at 28° ranging from 22 to 70 sec. The usual reaction mixture contained 2.5 ml of 0.2% collagen, 1.0 ml 0.05 M Tris–HCl buffer (pH 7.6), in 0.4 M NaCl, 1.5 ml enzyme resuspended in 0.05 M Tris–HCl (pH 7.5), *plus* $5.0 \cdot 10^{-3}$ M $CaCl_2$. The final enzyme concentration ranged between 0.3 and 0.5 mg/ml. A 1.0-ml aliquot of the reaction mixture was 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.

Samples were taken at varying intervals during the reaction, made 0.01 M with respect to EDTA and examined, after denaturation at 40°, by disc electrophoresis on polyacrylamide gels according to the method of NAGAI, GROSS AND PIEZ[20]. Occasionally samples were brought to 30% saturation at 0° with respect to ammonium sulfate. After 1 h the precipitates were collected by centrifugation, dissolved in 0.1 M acetic acid and dialyzed for 24 h in the cold against several changes of 0.05 M acetic acid. It has been shown[21] that most of the collagen in such reaction mixtures can be recovered by this method of ammonium sulfate fractionation. The procedure enhances the resolution of the electrophoretic pattern. Optical rotation was followed simultaneously with viscosity measurements in a Cary 60 automatic spectropolarimeter at 230 mμ. The temperature was maintained constant at 28°. Thermal denaturation characteristics were followed in the spectropolarimeter at pH 7.5 and a temperature increment of 1° per 20 min. For both control and enzyme reaction mixtures that had been dialyzed to pH 4.8 in 0.15 M potassium acetate buffer, the denaturation temperature was measured in a Rudolph Photoelectric spectropolarimeter equipped with an oscillating polarizer, at 365 mμ.

Preparation of segment long spacing for electron microscopy

Samples of the reaction mixture, containing approx. 1.0 mg of collagen per ml, were dialyzed in the cold against several changes of 0.05 M acetic acid for 48 h.

Segment long spacing* crystallites[24] were formed by adding 0.2 ml of a 1% aqueous solution of the free acid of ATP to 0.5 ml of the reaction mixture at 4°. After standing 30 to 60 min in the cold, drops of the cloudy suspension were placed on collodion-coated standard 400-mesh grids. The grids were drained, positively stained with 1% phosphotungstic acid at pH 3.5 and examined in an RCA EMU 3G electron microscope. Photographs were taken at magnifications of 26 000 to 50 000 times.

* Segment long spacing crystallites are arrays of collagen molecules induced by adenosine triphosphoric acid in which the rod shaped molecules are lined up in parallel and in close register, giving rise to a highly ordered asymmetrically banded crystallite as seen in the electron microscope.

RESULTS

Collagenolytic activity in the culture media reached its peak after 24–48 h of incubation. At this time, non-collagenolytic neutral protease activity was barely detectable even in the concentrated enzyme solutions. Further details on the kinetics of appearance and disappearance of the enzyme in tissue culture will be presented in detail elsewhere. Attempts to extract the enzyme from homogenates of whole skin prior to culture have been unsuccessful.

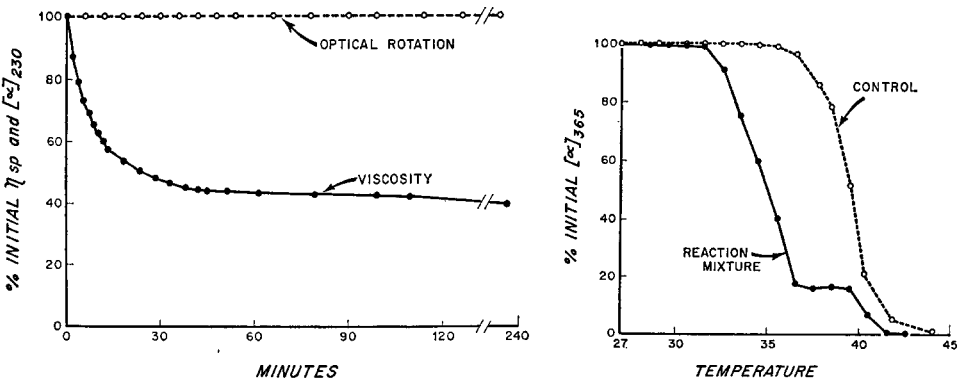


Fig. 1. Effect of human skin collagenase on viscosity and optical rotation at 28°. Reaction mixtures contained 0.1% purified acid-extracted calf skin collagen in 0.05 M Tris–HCl, 0.2 M NaCl, 0.0025 M $CaCl_2$ (pH 7.5), starting viscosity, $\eta_{sp} = 3.81$. Control viscosity remained unchanged over the experimental time period. The optical rotation was monitored simultaneously in an aliquot of the reaction mixture.

Fig. 2. Thermal denaturation curves of enzyme–collagen reaction mixture and control collagen. Both enzyme and control reaction mixtures, containing 0.1% acid-extracted calf skin collagen, were dialyzed to pH 4.8 in acetate buffer. Temperature increments are 1° per 20 min.

Human skin collagenase acts on collagen in solution and is capable of reducing the specific viscosity at pH 7.5 and 28° to approx. 50 to 60% of the control values after 2 h (Fig. 1). Fresh enzyme added after 2 h had no further effect. Over a 4-h period there was no accompanying change in optical rotation (Fig. 1).

The denaturation temperature midpoint (Tm) of the altered collagen in a 4-h reaction mixture at pH 4.8 was approx. 5° lower than that for native collagen (Fig. 2). This was also true of the Tm at pH 7.5. Although not determined in this study the Tm value for native calf skin collagen at neutral pH is approximately 39° (refs. 18, 25). In the experiment described in Fig. 2 a plateau appeared after 80% denaturation of the reaction mixture followed by an abrupt fall which closely paralleled the same region of the control curve.

Human skin collagenase degrades collagen in its fibrillar form (Fig. 3); breakdown was linear with enzyme concentration and with time of incubation when determined by the release of soluble radioactive peptides from reconstituted fibers.

The collagenase isolated from human skin culture media is also capable of degrading intact tissue collagen. A 0.02% enzyme solution incubated with approx. 2–3 mg of whole fresh human skin dermis at 37° for 14 h resulted in the release of

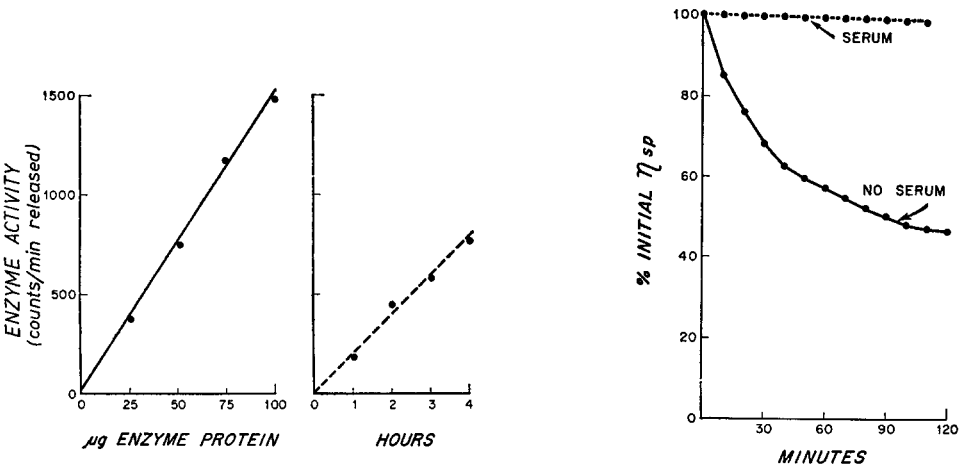


Fig. 3. Degradation of collagen by skin collagenase as a function of time and concentration. Measured by release of radioactivity from [^{14}C]glycine-labeled collagen gels. Concentration dependence determined after a 2-h incubation at 37°. (See text for experimental details.)

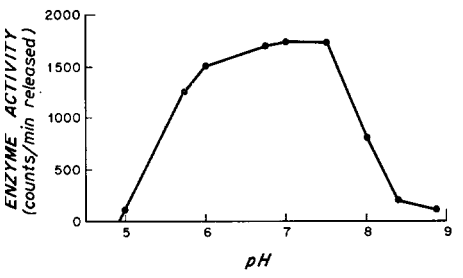
Fig. 4. Effect of normal human serum diluted 1:1000 on the viscosity reducing activity of human skin collagenase. The reaction mixture is the same as for Fig. 1 but contains approx. 8.0 μg of whole serum protein and 50.0 μg enzyme protein.

10–12 μg of hydroxyproline (about 1/4 of the total) into the medium as compared with no liberation of hydroxyproline in the absence of enzyme.

Enzymatic activity is completely inhibited by EDTA at a concentration of $5 \cdot 10^{-3}$ M. This is also the case for the other isolated collagenases. Histidine, which is also capable of chelating certain heavy metals[25], appears to have no inhibitory effect at 10^{-3} M. Soybean trypsin inhibitor at 10^{-5} M does not inhibit skin collagenase. Cysteine, which blocks the action of the tadpole enzyme[7], will inhibit human skin collagenase at 10^{-2} but not at 10^{-3} M. It is of interest that dialyzed, whole normal human serum is capable of inhibiting human skin collagenase activity almost completely in dilutions up to 1:1000 (Fig. 4). This is also true for sera from a variety of species which are capable of inhibiting human skin as well as tadpole collagenase (EISEN, BLOCH, SAKAI AND GROSS, unpublished observations).

The pH optimum of the unpurified enzyme is between pH 7 and 8 (Fig. 5). Whether the sharp decline in activity above pH 8 is significant in terms of differentiating the skin enzyme from other collagenases will require further studies with a purified preparation.

Acrylamide gel electrophoresis of the denatured reaction products from mixtures incubated at 28° are shown in Fig. 6. In the 0 time mixture only the α and β bands[11] are clearly seen with the γ and higher molecular weight aggregates remaining at the top of the running gel. At a time when the specific viscosity of the reaction mixture had decreased by 15–20%, one new band is seen below the original β band and a single new band is present below the original α. In addition, two faster moving components are always present just above the solvent front. With increased time of incubation both the original α and β components of collagen disappear completely and are replaced by the new components. The electrophoretic band pattern closely resembles that of collagen degraded by tadpole collagenase[11].



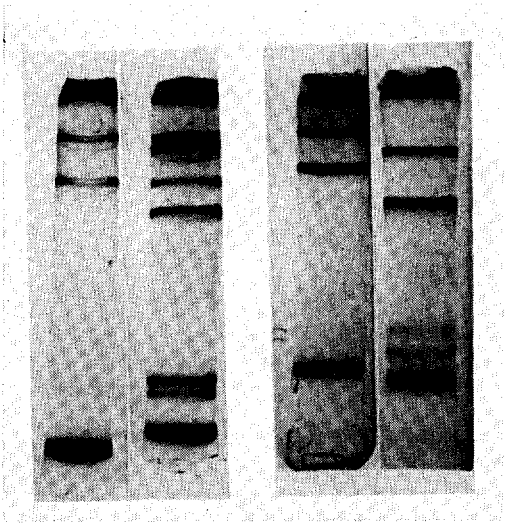


Fig. 5. Dependence of collagenolytic activity on pH. Reaction mixtures contained 150 μg ^{14}C-labeled glycine reconstituted collagen fibrils, 0.005 M $CaCl_2$, 0.2 M NaCl, and were made to a final concentration of 0.1 M with respect to the following buffers. 2(*N*-Morpholino) ethanesulfonic acid (pH 5 to 6.5), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (pH 7 to 7.5), and *N*,*N*-bis(2-hydroxyethyl) glycine (pH 8 to 9)(ref. 30).

Fig. 6. Disc electrophoretic patterns of thermally denatured enzyme–calf skin collagen reaction mixture. Two experiments. Left to right: 0 time reaction mixture, and after 20% reduction in specific viscosity; 0 time, and after 55% reduction in specific viscosity.

Extensive dialysis of reaction mixtures in which human skin collagenase was incubated for 24 h, both with collagen in solution, at 28° and with collagen in the native fibrillar form, at 37°, yield no dialyzable material. This suggests that the skin enzyme is not capable of degrading the native collagen molecule or fragments with intact helical structures to low molecular weight peptides.

In electron microscope studies of segment long spacings produced by the action of tadpole collagenase GROSS AND NAGAI[11] demonstrated that the native collagen molecule is severed at the b_2^2 locus of HODGE AND SCHMITT[27] into two segments, one being three-quarters the molecular length from the "A" end and the other one-quarter the length from the "B" end. This occurred without disrupting the helical conformation of either fragment. Segment long spacings prepared from collagen enzymatically attacked by human skin collagenase also revealed the presence of crystallites 75% the length of the collagen molecule, representing the fragment termed TC^A by GROSS AND NAGAI[11]. In some preparations TC^A segments frequently aggregated with their "A" ends in apposition. That human skin collagenase also severs the collagen molecule at the b_2^2 band is clearly demonstrated in Fig. 7. Over large areas of the many grids examined this was the only "A"end fragment found. Short segments representing the remaining 25% of the length of the collagen molecule from the "B"end, called TC^B (ref. 11) have, also been identified. The TC^B segments were most frequently seen as stacks of segments in which the terminal or "B" ends were abutting.

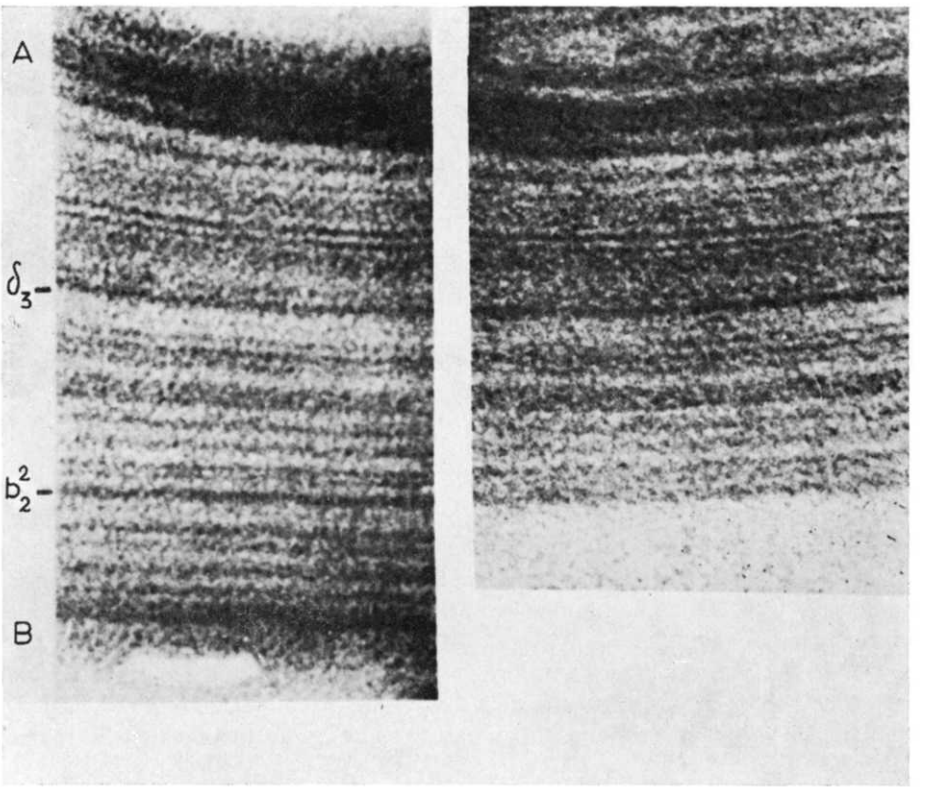


Fig. 7. Segment long spacing of normal collagen compared with that of a three-quarter length (TC^A) fragment. Magnification × 300 000.

DISCUSSION

Short term cultures of normal human skin grown in enriched synthetic medium result in the diffusion into the culture medium of an enzyme capable of degrading collagen under physiological conditions. The culture medium containing the enzyme appeared to be free of noncollagenolytic neutral proteases as determined by its activity on denatured casein.

As with the other animal collagenases recently isolated[12-14], the human skin enzyme cannot be obtained by extraction of tissue homogenates and requires living tissue for its production. Freeze-thawing the tissue completely blocks the appearance of both skin[5] and tadpole collagenases[6,8] suggesting that there is little or no storage of active enzyme in the tissues. These findings, in addition to the fact that both enzymes operate maximally at neutral pH, argue against their being lysosomal in origin[8]. Like the tadpole enzyme[8] human skin collagenase is produced by the epithelium (EISEN, unpublished observations) and not by the dermal mesenchyme. The human skin enzyme is capable of attacking collagen in whole dermis as indicated by the release of a significant amount of hydroxyproline when the enzyme is incubated with pieces of normal skin.

The specificity and limited nature of attack by human skin collagenase on the collagen molecule is similar to that produced not only by tadpole collagenase[11] but also by the enzyme recently isolated from rheumatoid synovium[14]. In common with bacterial collagenase[28] these enzymes are also inhibited by EDTA and cysteine[11,12] but their mode of attack on the collagen molecule is markedly different[12,28]. Electron microscopic investigations[29] of segment long spacing fragments resulting from the action of bacterial collagenase on dissolved collagen at low temperatures (10°) showed digestion of the molecules from both ends with the initial attack proceeding from the "A" end. In contrast with the skin enzyme, the collagenase from post-partum rat uterus[13] not only produces TC^A initially but continues to digest the cut end of TC^A producing pieces which are 67% and 62% the normal length. It differs from other collagenases in that it is not inhibited by cysteine[13]. In addition, while the human skin enzyme produces virtually no dialyzable peptides after 24 h digestion at 37°, the products of uterine enzyme attack are almost completely dialyzable under the same conditions[13].

Both the human skin and tadpole collagenases produce a similar fall in denaturation temperature without altering the helical structure of the collagen fragments. It has been postulated[11,12] 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.

The possible physiological significance of this specific collagenase in normal human skin is its relationship to the degradative phase of collagen metabolism. In addition its elevation in certain skin diseases[5] suggests its involvement in human pathology.

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