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CHARACTERIZATION OF A COLLAGENASE FROM RAT SKIN

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SUMMARY

A collagenase from normal rat skin has been isolated and purified extensively. The mechanism of action of the enzyme on native collagen closely resembles that of the collagenase from post-partum rat uterus. The rat skin collagenase cleaves native collagen in solution at a number of specific sites, producing fragments 75, 67 and 62% the length of the collagen molecule from the NH_2-terminus. In addition, the enzyme lowers the denaturation temperature of collagen by approx. 6° without appreciably altering its helical content. At 37° and neutral pH rat skin collagenase, like the rat uterine enzyme, extensively degrades native collagen fibrils to smaller peptides with greater than 60% of the reaction products being dialyzable.

In some respects the rat skin collagenase closely resembles other vertebrate collagenases. The enzyme is maximally active at neutral pH and inactive at pH 5. Calcium is required for activity; EDTA and cysteine are inhibitory. The inhibition of rat skin collagenase by these compounds is only partially restored by the addition of excess calcium, suggesting the possibility of a second metal requirement.

The results indicate that the collagenase obtained from rat skin is very similar to the rat uterine enzyme but differs from the collagenases obtained from the skin of two other species, human and tadpole.

INTRODUCTION

The recent demonstration that human skin, amphibian[1], and synovial[2] collagenases are present *in vivo*, suggests that collagenase can be present at tissue levels which are of physiologic significance in the degradative phase of collagen metabolism. This indicates that a wide variety of animal species depend on a collagenolytic system for the control of collagen remodeling.

Specific neutral collagenases, capable of degrading native collagen in an almost identical manner, have been obtained from the medium of cultures of a variety of human as well as amphibian tissues[3]. Human collagenases from a number of tissues[4–9],

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as well as the enzyme from tadpole tailfin[10], all cleave the native collagen molecule at a point one-quarter the distance from the C-terminus. The collagenase obtained from the culture medium of resorbing post-partum rat uterus[11], however, not only cleaves the native collagen molecule at this three-quarter–one-quarter point (TC^{A}_{75}), which is the only point of attack by human and tadpole collagenases, but is also capable of catalyzing further cleavages in the native molecule, producing pieces which are 67% and 62% from the "A" (N-terminal) end of the molecule.

Of further interest is the fact that a collagenase from another murine source, the mouse tibia, cleaves the collagen molecule not like the rat uterus enzyme but in a fashion very similar to that of human and tadpole collagenases[12]. In addition, an enzyme obtained from another amphibian source, the regenerating newt limb[13], has been shown to produce cleavages very much like those catalyzed by the rat uterine collagenase and unlike that produced by tadpole skin collagenase. The only other collagenase so far identified that is capable of producing several cleavages in the collagen molecule is that obtained from the crustacean hepatopancreas[14].

The similarities and differences observed in the properties of collagenases derived from a number of tissues from various species present an interesting problem in comparative enzymology. It seemed important, then, to determine whether skin from an animal source, such as the rat, contains a collagenase and whether its sites of action and characteristics resemble those of the collagenase of another rat tissue, the uterus, or those of the enzyme from the skin of another species, the human.

The present paper reports on the properties of rat skin collagenase purified from the culture medium of newborn rat skin and characterizes its mechanism of attack on the collagen molecule.

METHODS

Preparation of enzyme from culture medium

Skin from newborn Sprague–Dawley rats was excised from the animals, under sterile conditions, within 24–36 h after delivery and washed several times in Hank's balanced salt solution containing 200 units of penicillin and 200 μg streptomycin per ml. The panniculus was removed by scraping the under surface of the skin with a sharp scalpel blade. The skin was then cut into pieces about 2 mm^2 in size, and approx. 250 mg of tissue 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 O_2–CO_2 (95:5, v/v). Culture medium was changed daily for eight days and each day's medium centrifuged, augmented with one-twentieth volume of 1 M Tris–HCl (pH 7.5) and stored frozen until the termination of the experiment. Media were pooled, dialyzed at 4° against several changes of distilled water, lyophilized and stored at —20°.

Crude rat skin collagenase powder was dissolved in 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ to a concentration of approx. 30 mg protein per ml. A saturated solution of $(NH_4)_2SO_4$, adjusted to pH 7.5 with concentrated NaOH at 0°, was added to a final saturation of 60%. The precipitate was collected by centrifugation at 18 000 rev./min for 20 min and dissolved in 6–8 ml of 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$.

Chromatography

All chromatographic procedures were carried out at 4°. Gel filtration of the $(NH_4)_2SO_4$ enzyme preparation was performed using reverse flow on a column (2.5 cm × 100 cm) of Sephadex G-150 (Pharmacia) equilibrated with 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$. Fractions having collagenase activity were pooled, dialyzed against several changes of distilled water at 4° and lyophilized. Ion exchange chromatography was carried out on microgranular DEAE-cellulose (DE-32, Whatman) equilibrated in 0.005 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ (starting buffer). The lyophilized enzyme powder obtained from gel filtration was dissolved in the starting buffer, dialyzed against the same buffer for 12 h and pumped onto a DE-32 column (1.2 cm × 25 cm) at a rate of 25 ml/h. The enzyme was eluted using a linear gradient established from the starting buffer to 0.4 M NaCl. Fractions containing collagenase activity were pooled, dialyzed against distilled water and lyophilized. The concentrated enzyme powder was dissolved in 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ immediately prior to use for affinity chromatography.

Preparation of collagen–Sepharose for affinity chromatography

Collagen was conjugated to Sepharose 4B (Pharmacia) as described by BAUER *et al.*[2,15] by a method similar to that developed by CUATRECASAS[16] for other ligands. Briefly, 25 ml of cyanogen bromide activated Sepharose 4B was suspended in 50 ml of cold 0.2 M $NaHCO_3$ (pH 9) and 100 mg of the purified collagen in 25 ml of 0.4 M NaCl was added immediately. The mixture was stirred gently in the cold for 18 h, washed with cold water and then equilibrated with cold 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$.

The enzyme preparation obtained from DEAE-cellulose was applied to a column (1.2 cm × 4 cm) of collagen–Sepharose and developed with 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ followed by 1.0 M NaCl in the same buffer.

Assay procedures

Collagenase activity was determined either viscometrically or by release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils[17]. Viscosity was measured in Ostwald viscometers with flow times for water ranging from 25–32 sec at 25°. Guinea pig skin collagen was purified by the method of GROSS[18]. Reconstituted native collagen fibrils were prepared from solutions of [^{14}C]glycine-labeled guinea pig skin collagen (specific activity 30 000 counts/min per mg). A typical reaction mixture contained 50 μl of 0.4% ^{14}C-labeled collagen fibrils that had been allowed to gel for 24 h at 37° in 1.0-ml glass (Kimax) centrifuge tubes; 50 μl of 0.05 M Tris–HCl (pH 7.5) containing $5 \cdot 10^{-3}$ M $CaCl_2$ and 50 μl of enzyme solution containing approx. 25–100 μg of protein. All results were compared to collagen controls in which trypsin was added to a final concentration of 0.01%. In every case the trypsin blank represented less than 10% of the total counts indicating that no appreciable amount of denatured collagen was present in any of the preparations used.

Non-collagenase protease activity, using casein as a substrate was determined at neutral pH by the method of KUNITZ[19]. Protein was determined by the method of LOWRY *et al.*[20].

Optical rotation and electron microscopy

Optical rotation of collagen in reaction mixtures as a function of incubation time was monitored in a Cary 60 spectropolarimeter at 240 nm and 25° simultaneously with viscosity measurements. Thermal denaturation characteristics were followed in the spectropolarimeter for enzyme reaction mixtures at pH 7.5 and after dialysis to pH 4.8 in 0.15 M potassium acetate buffer with a temperature increment of 1° per 20 min. Controls were run at pH 4.8 only.

Segment-long-spacing crystallites of intact collagen and enzymatic products of collagen were prepared for electron microscopy according to GROSS AND NAGAI[10], stained with uranyl acetate, and examined in a Phillips EM 300 electron microscope.

Disc gel electrophoresis

Thermally denatured collagen in reaction mixtures was subjected to electrophoresis in polyacrylamide gels according to NAGAI *et al.*[21]. The enzyme was inactivated either by adding EDTA (10^{-2} M) or by adding sufficient 0.1 M HCl to the reaction mixtures to reduce the pH to approximately 2 (0.03 M) before denaturation at 40°.

For monitoring enzyme purification disc gel electrophoresis was carried out according to the method of DAVIS[22], using a 7.5% concentration of acrylamide.

Removal and readdition of cations

To determine the effects of calcium and other cations on rat skin collagenase, the enzyme purified by affinity chromatography was dialyzed overnight in the cold against 0.05 M Tris–HCl (pH 7.5). The enzyme was then passed through a column 0.5 cm × 5 cm of Chelex (Bio-Rad), which had been adjusted to pH 7.5 with 6 M HCl and washed with 0.05 M Tris–HCl (pH 7.5). All cations were added as their chloride salts after adjusting their pH to 7.5.

RESULTS

Measurements of collagenase activity in the culture media from explants of rat skin assayed daily indicate a delay of approximately 36 h before enzyme activity can be demonstrated. Collagenase activity reaches a peak at 48–72 h and can be detected in the medium for at least 8 days after cultures have been initiated. A significant level of non-collagenase neutral protease activity is present in the culture medium which correlates closely with the collagenase activity. These findings are similar to those described for the production of human skin collagenase in tissue culture except that non-collagenolytic neutral protease activity from human skin is always present at very low levels[1,4].

Enzyme purification

Even though a relatively good yield of rat skin collagenase activity can be obtained by passing crude enzyme preparations directly through collagen–Sepharose, a large number of contaminating proteins also adhere to the matrix. It was, therefore, necessary to perform a variety of purification procedures prior to the use of affinity chromatography.

As shown in Fig. 1 a single peak of collagenase activity is obtained following gel filtration on Sephadex G-150 of the $(NH_4)_2SO_4$ fraction. Chromatography of the area

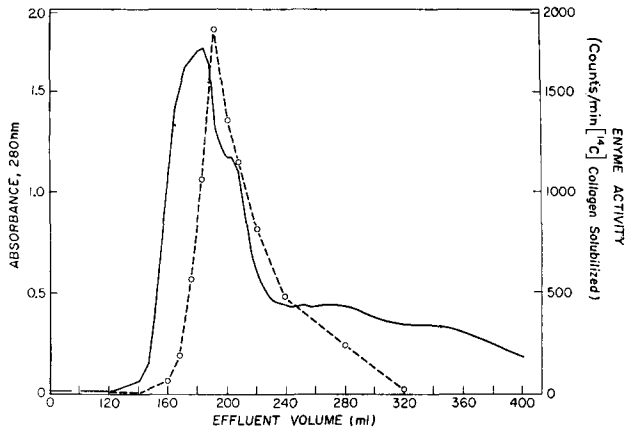


Fig. 1. Gel filtration of an $(NH_4)_2SO_4$ preparation of rat skin collagenase containing approx. 120 mg of enzyme protein on Sephadex G-150 using reverse flow. A column (2.5 cm × 100 cm) was equilibrated with 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ at 4°. Effluent fractions of 3.6 ml were collected at a rate of approximately 15 ml/h. Collagenase activity was measured by incubating 50 μl of the eluant fractoins for 2 h in a shaken water bath at 37° with [^{14}C]collagen fibrils. ●—●, absorbance 280 nm; ○---○, enzyme activity.

of collagenase activity on DEAE-cellulose (Fig. 2) results in an 11-fold purification of the rat skin collagenase but only a slight decrease in caseinolytic activity. Rechromatography of the area of enzyme activity on DEAE-cellulose gives a single well defined peak of enzyme activity, coincident with the single peak of ultraviolet-absorbing material but does not result in a further increase in specific activity. Throughout these stages of purification non-collagenase neutral protease activity coincides with the collagenase peak.

Fig. 3 demonstrates a typical affinity chromatogram obtained by passing the

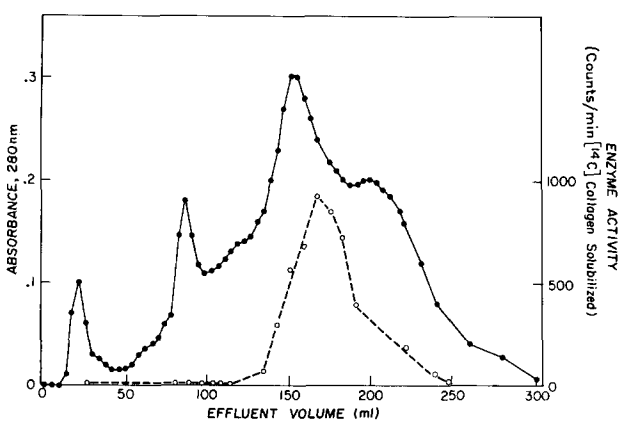


Fig. 2. DEAE cellulose chromatography of rat skin collagenase at 4°. Approx. 65 mg of enzyme protein in 0.005 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ was pumped onto a column (1.2 cm × 25 cm) at a rate of 25 ml/h and effluent fractions of 4 ml were collected. The enzyme was eluted using a linear gradient established from the starting buffer to 0.4 M NaCl. Collagenase activity was measured by incubating 50 μl of the eluant fractions as described in Fig. 1. ●—●, absorbance at 280 nm; ○---○, enzyme activity.

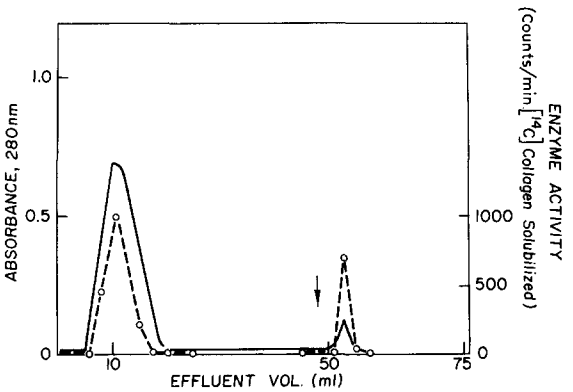


Fig. 3. Affinity chromatography of rat skin collagenase on collagen–Sepharose at 0°. A sample of 16 mg of enzyme protein obtained following DEAE chromatography was applied to a column (1.2 cm × 4 cm) and effluent fractions of 2.5 ml were collected at a rate of 25 ml/h. Elution was accomplished by the addition of 1 M NaCl to the eluant buffer (arrow). Collagenase activity was measured by incubating 25 μl of the eluant fractions with [^{14}C]collagen fibrils for 2 h in a shaken water bath at 37°. ●—●, absorbance at 280 nm; ○---○, collagenase activity.

enzymatically active peak obtained from DEAE-cellulose chromatography through collagen-Sepharose. As previously shown with other vertebrate collagenases[15] only partial adsorption of collagenase activity occurs. The fraction that does bind can be eluted as a sharp peak of collagenase activity with a buffered solution of 1.0 M NaCl. This material is completely free of caseinolytic activity even after assay for 14 h at 37°. The yields and purification of a typical enzyme preparation are summarized in Table I. The rat skin collagenase purified in this fashion degrades native reconstituted collagen fibrils at pH 7.5 and 37°, in a linear fashion both as a function of time and enzyme concentration.

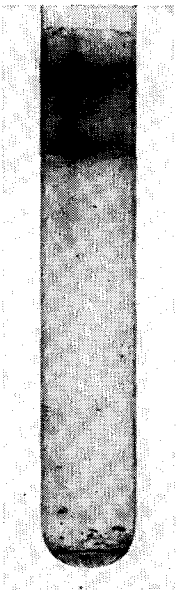
The enzymatically active eluant fraction from collagen–Sepharose, however, still shows several protein bands on polyacrylamide gel electrophoresis (Fig. 4). It has not been possible to purify the enzyme further by rechromatography on collagen–

TABLE I

PURIFICATION OF RAT SKIN COLLAGENASE

Purification step	*Total protein (mg)*	*Specific activity* (units/mg protein)*	*Total activity (units)*	*Recovery (%)*	*Caseinolysis*	
					*Specific activity** (μg trypsin/mg protein)*	*Total activity (units)*
Crude enzyme powder	467	22	10 274	100	0.9	420
$(NH_4)_2SO_4$ ppt.	120	58	6 960	68	2.6	312
Sephadex G-150	64	70	4 480	44	3.1	198
DEAE-cellulose	16	245	3 920	38	1.9	30
Collagen–sepharose	0.43	2017	867	8	0	0

* Specific activity refers to the μg collagen solubilized per min per mg protein.
** Caseinolytic activity was determined after a 1-h incubation at 37°. Specific activity expressed in equivalent μg of trypsin.



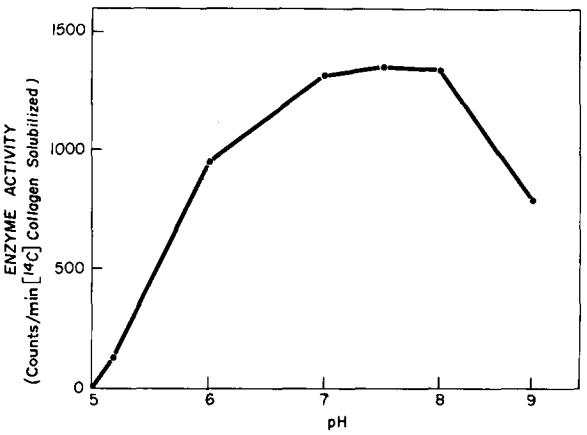


Fig. 4. Polyacrylamide gel electrophoresis of rat skin collagenase purified on collagen–Sepharose. Sample contained approx. 80 μg of protein. 7.5% acrylamide was used at 5 mA per tube.

Fig. 5. Dependence of rat skin collagenase activity on pH measured by the release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils as described under METHODS. Buffers used to obtain the appropriate pH values in the range of 5 to 9 were Tris–maleate and boric acid-KCl at a final concentration of 0.1 M. The final pH at each point was determined using appropriate blanks in the absence of enzyme protein or collagen.

Sepharose, as is the case for certain other vertebrate collagenases[2,15]. Repeated attempts to isolate collagenase activity by extracting the protein containing areas of polyacrylamide gels were also unsuccessful.

The pH optimum of the purified enzyme is between 7.4 and 7.8 (Fig. 5) with almost complete loss of activity below pH 6.0.

TABLE II

EFFECT OF DIVALENT CATIONS ON THE ACTIVITY OF CHELEX TREATED RAT SKIN COLLAGENASE

Reaction mixtures contained 50 μl of 0.2% [^{14}C]glycine-labeled collagen as a substrate gel (4468 counts/min per gel) and 75 μg of purified enzyme protein in a total volume of 400 μl. Incubation was carried out for 3 h at 37° in a shaken water bath. Results are expressed as counts/min above a 0.01% trypsin blank which represents approx. 9% of the total counts in the substrate gel. Prior to Chelex treatment 75 μg enzyme protein liberated 2807 counts/min in 3 h at 37°.

Metal	*Concn. (M)*	*[^{14}C]Collagen solubilized (counts/min)*
None		131
$CaCl_2$	10^{-3}	2651
$ZnCl_2$	10^{-3}	136
$CuCl_2$	10^{-3}	124
$MnCl_2$	10^{-3}	159
$MgCl_2$	10^{-3}	162

Effects of divalent cations

As shown in Table II, rat skin collagenase requires calcium for activity. Unlike rat uterine collagenase[11] the rat skin enzyme does not lose activity after extensixe dialysis against calcium free buffer. A single passage of the dialyzed collagenase through a column of Chelex ion exchange resin, however, results in a loss of approximately 95% of the original enzyme activity. Other divalent cations (Zn^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}) are unable to replace calcium, and are ineffective in restoring collagenase activity.

Effects of inhibitors

Enzymatic activity is completely inhibited by 10^{-3} M EDTA. The addition of excess calcium ($5 \cdot 10^{-2}$ M) to the EDTA inhibited rat skin collagenase restores only about 30% of the original enzyme activity. Cysteine totally inhibits this skin collagenase at 10^{-1} M, approximately 45% at 10^{-2} M but is not inhibitory at 10^{-3} M. This is similar to the action of cysteine on human skin collagenase[4]. Preliminary observations suggest that calcium only partially restores activity to the cysteine inhibited rat skin enzyme. Tadpole collagenase appears more sensitive to this amino acid, being irreversibly inhibited by 10^{-3} M cysteine[17].

Viscosity and optical rotation

The purified rat skin collagenase acts on collagen in solution (pH 7.5) and is capable of reducing the initial specific viscosity at 25°, a temperature well below the denaturation temperature of collagen, by approx. 25% after 5 h with no significant change in optical rotation (Fig. 6). After 16 h at 25° and a loss of 50% of the specific viscosity, optical rotation measurements of the collagen substrate indicated a loss of slightly less than 7% of the helical structure. The enzyme continues to act on collagen so that by the end of 24 h of incubation approximately 75% of the initial specific

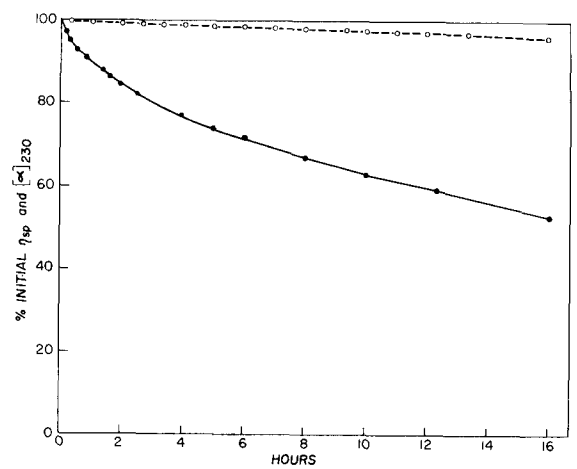


Fig. 6. Effect of rat skin collagenase on viscosity and optical rotation at 25° and pH 7.5. Reaction mixtures contained 0.05% collagen in 0.05 M Tris–HCl, 0.2 M NaCl and 0.005 M $CaCl_2$. Starting viscosity, η_{sp} = 2.1. Control viscosity containing heat inactivated enzyme remained unchanged over the experimental time period. The optical rotation was monitored simultaneously in an aliquot of the reaction mixture. Enzyme protein concentration was 75 μg/ml. ●—●, viscosity; ○---○, optical rotation.

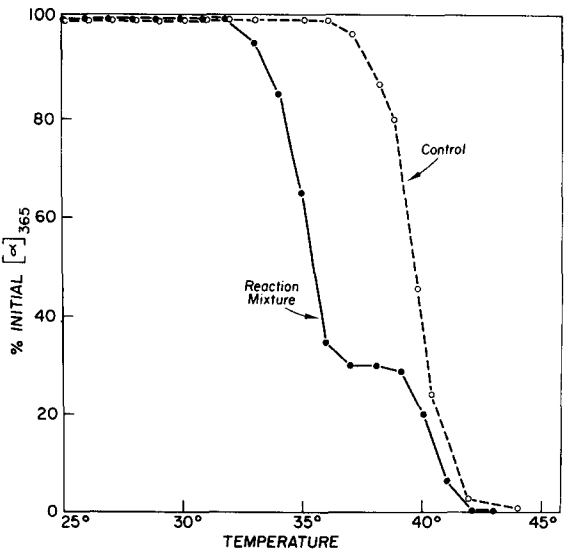


Fig. 7. Thermal denaturation curves of enzyme–collagen reaction mixture and control collagen. The reaction mixture used had lost 61% of the initial η_{sp} at 25° and was subsequently dialyzed to pH 4.8 in acetate buffer. Temperature increments are 1° per 20 min.

viscosity was lost. Exhaustive dialysis of a reaction mixture incubated at 25° until 60% of the initial specific viscosity was lost, yielded only approx. 5% dialyzable peptides.

At 28° the rate of enzyme activity was much greater than at 25°, with a loss of more than 70% of the initial specific viscosity after 5 h. Measurements of optical rotation at this time period indicate a loss of approximately 9% of the helical content and yielded 12% dialyzable peptides. At 37° greater than 60% of the reaction products are dialyzable.

The denaturation temperature midpoint (T_m) of altered collagen in an enzyme–collagen reaction mixture at 25° after a loss in specific viscosity of 50% was approximately 6° lower than native collagen (Fig. 7). This was true both at pH 4.8 as well as at pH 7.5. Although not determined in this study the T_m value for native collagen at neutral pH is approximately 39° (ref. 23). Since the degradation of collagen by rat skin collagenase in the reaction mixtures at 25° was not complete, the melting curve was diphasic with a plateau appearing after 70% denaturation (Fig. 7) followed by an abrupt fall closely paralleling the same region of the control curve. This portion of the curve probably represents the remaining intact collagen having a T_m of 40.3°.

Acrylamide gel electrophoresis

Acrylamide gel electrophoretic patterns of thermally denatured reaction products from mixtures incubated at 25° are shown in Fig. 8. At viscosity losses up to 20%, one new band is seen below the original β band and another beneath the original α component. Two faster moving bands are present just above the buffer front. The electrophoretic pattern at this time closely resembles that of collagen degraded by tadpole[10], human skin[4], and synovial collagenases[5]. With increased time of incubation

the electrophoretic pattern becomes more complicated with a progressive decrease of the original β and α components and the appearance of four faster migrating bands beneath each of the original collagen components. At viscosity losses of 60% or greater a larger number of fast-moving bands are present beneath the original β chains (Fig. 8). At equivalent viscosity losses the reaction products at 25° are identical with those

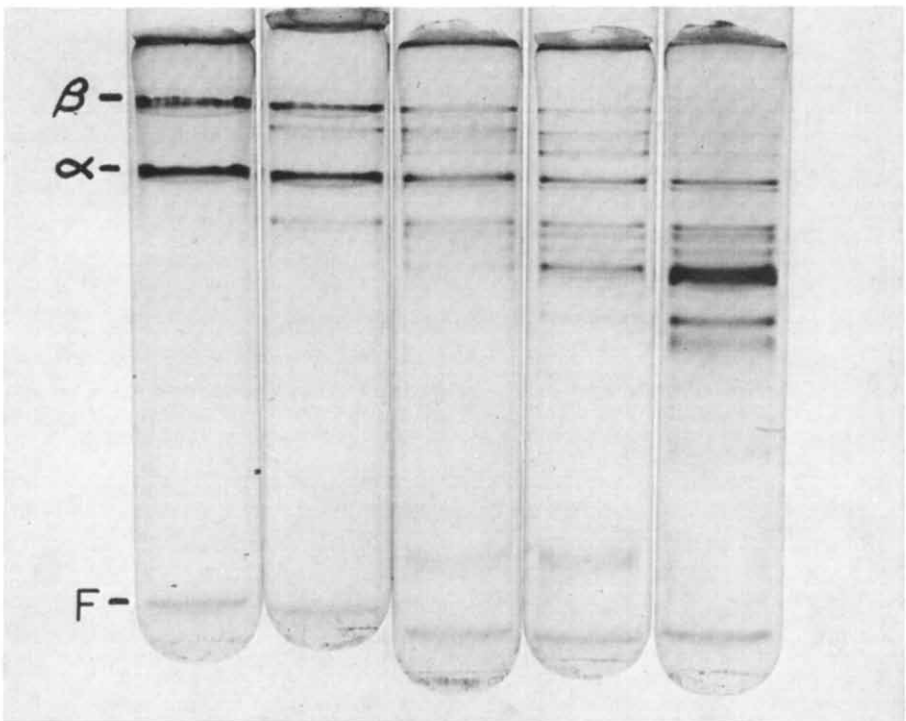


Fig. 8. Acrylamide gel electrophoresis patterns of thermally denatured enzyme–guinea pig skin collagen reaction mixtures at 25°. Left to right: zero time reaction mixture and after 20, 40, 60 and 75% reduction in specific viscosity. α refers to the single polypeptide chain, β to the cross-linked dimers of the α chains and F to the buffer front.

obtained at 28°. These electrophoretic patterns are almost identical to those obtained during the degradation of collagen by rat uterus collagenase[11].

Electron microscopy

Examination of ATP-precipitated segment-long-spacing aggregates from collagen enzymatically degraded by rat skin collagenase revealed segments of different lengths. In early reaction mixtures normal length segment-long-spacing crystallites (Fig. 9A) and segments three-quarters the molecular length from the "A" end (TC^{A}_{75}, Fig. 9B) are readily observed. In reaction mixtures in which the initial specific viscosity had decreased to 40 to 60% of the controls two additional fragments, one 67% (TC^{A}_{67}; Fig. 9C) and the other 62% (TC^{A}_{62}; Fig. 9D) also from the "A" end of the molecule have so far been identified. One-quarter length fragments (TC^{B}_{25}), most frequently seen as stacks of segments, were poorly visualized. Very small segments,

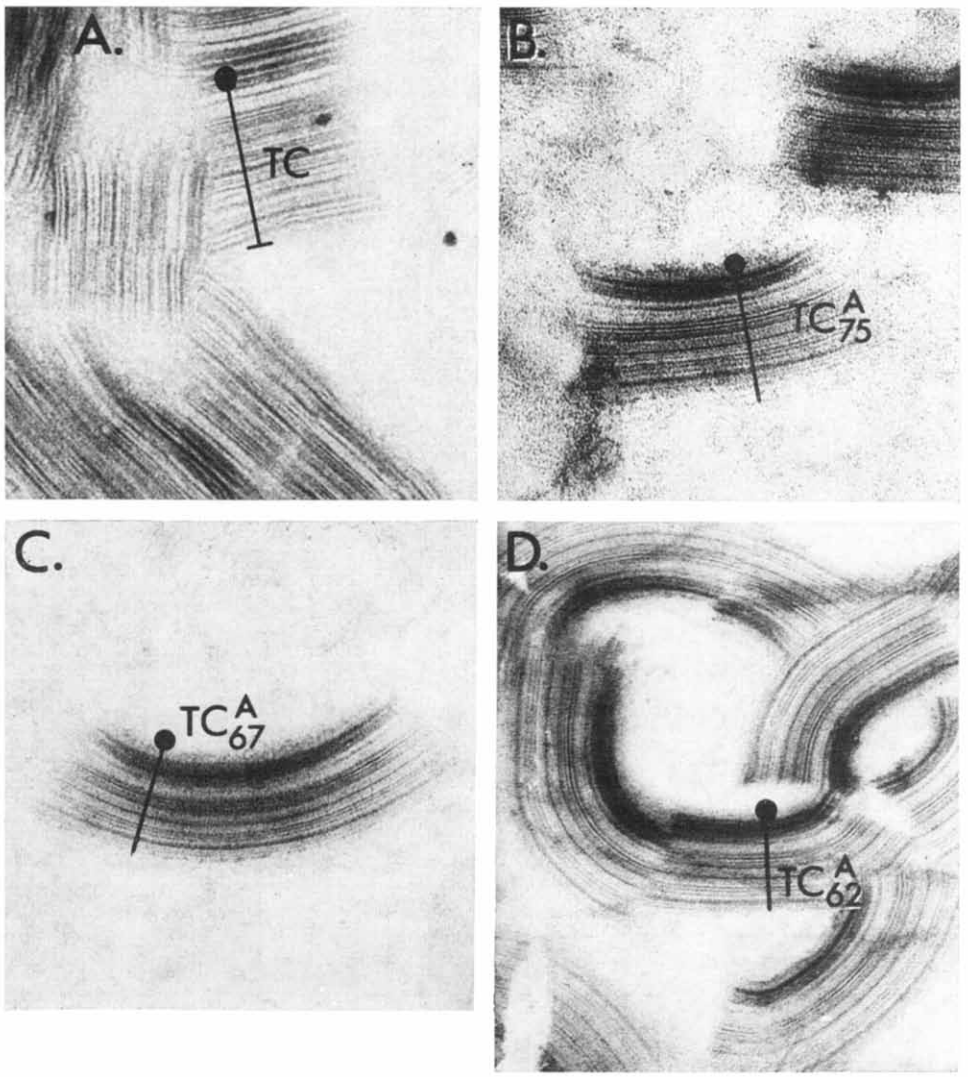


Fig. 9. Electron micrographs of segment long spacing crystallites of rat skin collagenase-modified collagen. A, collagen segments of normal length (TC). B, depicts a three-quarter length (TC^{A}_{75}) segment. C, illustrates a segment long spacing segment that is 67% normal length from the A end (TC^{A}_{67}). D, shows a modified segment approx. 62% normal length (TC^{A}_{62}). Magnification 81 500 ×.

representing the fragments removed from TC^{A}_{75} to produce TC^{A}_{67} and TC^{A}_{62}, were not observed.

DISCUSSION

This study is a continuation of our efforts to isolate and characterize specific neutral collagenases from both human and animal tissues to determine whether

enzymatic differences exist between species and if within a given species collagenases obtained from different organs degrade the collagen molecule in a similar fashion. The collagenase obtained from rat skin clearly differs from human[4] and tadpole skin[10] collagenases in its mechanism of attack on the collagen molecule. Rat skin collagenase, at temperatures below those which will denature the substrate, cleaves the native collagen molecule at the three-quarter–one-quarter point producing TC^{A}_{75}, which is characteristic of human and tadpole collagenases[4,10]. However, unlike these enzymes, it is also capable of catalyzing further cleavages at points 67% (TC^{A}_{67}) and 62% (TC^{A}_{62}) from the "A" (NH_2-terminal)end of the collagen molecule. The characteristics of the catalytic activity of rat skin collagenase appear, then, to be identical to that of rat uterine collagenase[11].

The three-quarter–one-quarter cleavage is the most favorable site of attack for the enzyme from the standpoint of rate since it is the first cleavage to occur at lower temperatures but it is not unlikely that all three cleavages can occur simultaneously. The absence of non-collagenase proteolytic activity as measured against casein suggests that this collagenase is capable of catalyzing the additional cleavages in the collagen molecule observed at 37°. The role of the neutral protease which is associated with the collagenase in cultures of rat skin is as yet unknown. This enzyme could be of importance in the further degradation of the collagen molecule as well as the smaller segments removed from TC^{A}_{75} following attack on collagen by rat skin collagenase.

At 25° enzymatic attack results in a loss of 50–60% in the viscosity of collagen in solution at neutral pH and occurs with a minimal loss of helical content and no significant dialyzable material is produced. At 28°, and a loss of over 70% of the initial specific viscosity of the collagen solution, measurements of optical rotation indicate a modest loss of helical content (approx. 9%) and a concomitant increase in dialyzable peptides to approx. 12%. It is unlikely, however, that this accounts for further cleavages in the major helical fragments, since the acrylamide gel patterns at 25 and 28° are identical. It seems possible that the decrease in helical content and increase in dialyzability noted with increasing temperature may be the result of the instability of the small new fragments formed as TC^{A}_{75} is further cleaved, producing TC^{A}_{67} and TC^{A}_{62}. A similar modest decrease in helical content has been observed with the collagenase from regenerating newt limb[13] even at temperatures below 25°. Like uterine collagenase[11], at 37° the rat skin enzyme is capable of degrading the collagen substrate extensively to smaller polypeptides and in this respect differs from human skin collagenase[4].

Many of the characteristics of rat skin collagenase are similar to those of other human and animal collagenases so far identified (see review EISEN *et al.*[3]). These enzymes all have neutral pH optima and rapidly lose activity below pH 6 and above pH 8. They also produce a similar decrease in the denaturation temperature of collagen without appreciably altering the helical structure of the collagen fragments.

These collagenases require calcium and are completely inhibited by EDTA. Inhibition of tadpole collagenase[17] is reversible but in the case of the rat uterus enzyme[11] inhibition is irreversible after treatment with this chelating agent. However, with inhibited rat skin collagenase partial enzymatic activity can be restored by the addition of calcium but not by other divalent cations. In addition, dialysis of rat uterus collagenase apparently removes calcium with a total loss of activity[11] which is completely restored by addition of calcium. In contrast, rat skin collagenase retains

enzymatic activity even after prolonged dialysis. The rat skin enzyme, however, does appear to specifically require calcium, since only this cation is capable of restoring activity to enzyme inactivated by Chelex. Whether a second metal is required for enzymatic activity of rat skin collagenase, as has been postulated for the rat uterine enzyme[11], remains to be determined.

Cysteine which inhibits tadpole[17] and human skin[4] collagenases also inhibits rat skin collagenase but only at levels above 10^{-3} M. Rat uterine collagenase, like the rat skin enzyme, is not inhibited at 10^{-3} M cysteine[11] but at 10^{-2} M, this amino acid also inhibits uterine collagenase activity approx. 30% (unpublished observations) and it seems likely that enzymatic activity will be further inhibited by higher levels of cysteine. The nature of the inhibition of these enzymes by cysteine is not known, but SEIFTER *et al.*[24] have shown that cysteine chelates zinc in the collagenase from *Clostridium histolyticum*. Whether the same is true for vertebrate collagenases is, at present, a matter of conjecture.

Recently it has been suggested[25] that the epidermis and granulation tissue of healing cutaneous wounds from another murine species, the rabbit, each produces a specific collagenase. These enzymes differ in their mode of catalytic action and susceptibility to cysteine inhibition. The significance of these findings is presently unclear, since comparisons between the enzyme from wounds and normal rabbit skin have not been made.

In an attempt to further characterize collagenases recent immunologic studies[26] have shown that species specificity exists between human and animal collagenases. These findings have been extended[2] to show that collagenases from two human tissues, skin and rheumatoid synovium, which are known to have the same mechanism of action[5], are immunologically identical and that organ specificity for the collagenases from these tissues is unlikely. These observations indicate that it is not unreasonable to predict that within a given organism or species the collagenases produced by various organs will have very similar properties. This possibility is at least partially borne out by the demonstration that the collagenases from rat skin and rat uterus degrade the collagen molecule in an identical fashion, and have in common a number of other biochemical similarities. However, the intriguing studies by SHIMIZU *et al.*[12] demonstrate that mouse bone collagenase cleaves the collagen molecule in a manner similar to that of human[4] and amphibian[10] collagenases rather than like the rat skin and rat uterus enzymes[11]. Our observations on a collagenase from mouse skin (unpublished) indicates that its mechanism of action is almost identical to that of rat skin and uterus and not like that of the mouse bone enzyme. It may be, then, that organ specific collagenases will ultimately be identified in some species.

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