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The Zymogen of Tadpole Collagenase*

Elvin Harper,†,‡ Kurt J. Bloch, and Jerome Gross

ABSTRACT: A precursor of tadpole collagenase, enzymatically inactive and incapable of binding to collagen, has been isolated from extracts of tadpole tail-fin tissue by fractionation techniques used to isolate the active enzyme from the media of tail-fin cultures. Both enzyme and zymogen have been purified by ammonium sulfate precipitation and agarose chromatography and appear as single bands of protein on polyacrylamide disc gel electrophoresis. The molecular weight of enzyme and zymogen, as estimated by sodium dodecyl sulfate–polyacrylamide electrophoresis in 5 and 10% gels, was 104,000 and 115,000, respectively; that of zymogen in 5 and 10% gels was 106,000 and 120,000, respectively. Activation of the zymogen was accomplished by incubation with collagenase-free tail-fin culture medium, but not with trypsin or chymotrypsin. The unidentified activator in the medium is heat labile and nondialyzable. The degradation products of soluble native guinea pig skin collagen produced by activated zymogen are similar to those produced by the purified tadpole collagenase obtained from tissue culture medium. The γG fraction of rabbit anti-collagenase antiserum blocked the activity of the activated zymogen as well as that of the purified tadpole collagenase.

The animal collagenases have generally required tissue culture methods for both detection and isolation. Collagenolytic activity was first detected in the culture media of living tadpole tissue incubated on a collagen substrate in physiologic salt solutions (Gross and Lapiere, 1962). Freezing and thawing of the explant or the application of low concentrations of puromycin (Eisen and Gross, 1965), blocked the appearance of the enzyme. In addition, collagenolytic activity could not be demonstrated in tissue extracts. These observations led to the tentative conclusion that the enzyme was synthesized *de novo* in the cultured tissues, or that a zymogen requiring protein synthesis for activation (Lapiere and Gross, 1963) was the source.

By employing the methods devised for detection, isolation, and characterization of the tadpole collagenase, it has been possible to obtain collagenolytic enzymes with very similar properties from a variety of animal tissues, including inflamed gingiva (Fullmer and Gibson, 1966), human rheumatoid synovia (Evanson *et al.*, 1968), resorbing rat uterus (Jeffrey and Gross, 1967, 1970), human skin (Eisen *et al.*, 1968), skin wounds (Grillo and Gross, 1967; Donoff *et al.*, 1971), regenerating newt limb (Grillo *et al.*, 1968; Dresden and Gross, 1970), middle-ear cholesteatoma (Abramson, 1969), bone (Shimizu *et al.*, 1969; Fullmer and Lazarus, 1969), and corneal ulcerations (Brown *et al.*, 1969; Slansky *et al.*, 1969). These enzymes all have neutral pH optima, are inactive at low pH, and cleave native collagen molecules below the denaturation temperature in a limited and characteristic manner (Gross and Nagai, 1965; Sakai and Gross, 1967; Eisen *et al.*, 1968; Jeffery and Gross, 1970). The ongoing studies of tadpole collagenases continue to serve as a prototype for other collagenases in normal and diseased tissues.

Continuing investigations on the control mechanisms for the elaboration of the tadpole collagenase led to a search for an inactive enzyme precursor using an antibody prepared against highly purified tadpole collagenase (Harper *et al.*, 1970). The present study reports the detection, partial characterization, and activation of the zymogen found in extracts of tadpole tail fin.

Materials and Methods

Preparation and Purification of Collagenase from Tail-Fin Culture Medium. Crude tadpole collagenase in lyophilized form was prepared as described previously (Nagai *et al.*, 1966). Briefly, bullfrog tadpoles in late legless stages were sterilized for 24 hr with antibiotics added to the aquarium water. Strips of tail fin were placed on Whatman No. 1

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filter paper floating in sterile amphibian Ringer solution in 105-mm plastic Petri dishes and incubated at 37°. The culture medium was removed and replaced with fresh fluid daily. The fluid obtained from as many as 20 dishes was combined, dialyzed against 0.01 M Tris–0.001 M calcium chloride buffer (pH 7.4), and then lyophilized. Purification of the enzyme from the crude powder was accomplished as described previously (Harper, 1970; Harper and Gross, 1970), with certain modifications. In general, 500 mg of lyophilized third-day culture medium was dissolved in 70 ml of 0.05 M Tris–0.005 M $CaCl_2$ buffer (pH 7.4) (Tris-$CaCl_2$) and centrifuged at 27,000*g* at 0° for 20 min. The supernatant was brought to 30% saturation by the gradual addition of solid $(NH_4)_2SO_4$ (special enzyme grade, Mann Research Laboratories, New York) in an ice bath. The resulting precipitate was dissolved in a small volume of Tris-$CaCl_2$ and dialyzed against 0.01 M Tris–0.001 M $CaCl_2$ buffer (pH 7.4). After dialysis, 5 ml of solution containing approximately 4 mg of protein/ml was applied to an 8% Agarose column (1.5 × 70 cm) and 1.5-ml fractions were eluted every 6 min with the latter buffer made 0.2 M with respect to NaCl.

Preparation of Tadpole Tail-Fin Extract. The fin tissue from five tadpole tails was minced and homogenized in 10 ml of Tris–$CaCl_2$ using a glass homogenizer maintained at ice-bath temperature. The homogenate was centrifuged at 18,000*g* for 15 min at 2°. The supernatant was purified by $(NH_4)_2SO_4$ fractionation and Agarose column chromatography following the protocol outlined above. A peak of protein, devoid of collagenase activity, was eluted in the same region of the chromatogram as the active enzyme from the culture medium. Protein fractions constituting the ascending portion of this peak were fractionated by gel slab electrophoresis (Raymond, 1962; Harper and Gross, 1970); at least five bands of protein were resolved by this method. Of these bands, one corresponded in electrophoretic mobility to that of the enzyme. Protein constituting this band will be referred to as "peak 1 extract" and the corresponding active enzyme from the culture medium as "peak 1 enzyme." This latter designation is useful since a second collagenase fraction of lower molecular weight has been reported in fractions obtained by Agarose chromatography (Harper and Gross, 1970).

Preparation of "Activator" from Culture Medium. A suspension of insoluble purified guinea pig skin collagen fibrils in Tris–$CaCl_2$ buffer was added to crude culture medium in an ice bath in order to remove active collagenase by adsorption to solid substrate. The ratio of suspended collagen to protein in solution was 1:1 on a weight basis. The mixture was gently stirred for 1 hr, followed by centrifugation at 18,000*g* for 20 min at 2°, and separation of the clear supernatant.

Preparation of Antisera to Collagenase. Peak 1 enzyme (1 mg) was dissolved in 1 ml of saline and combined with 1 ml of complete Freund's adjuvant; the resulting emulsion was injected into the four footpads of rabbits. Antisera were obtained 4–6 weeks after the start of immunization. Anti-egg albumin antisera were similarly prepared; anti-sheep erythrocyte antisera were obtained from rabbits initially injected with 1.0 ml of 50% sheep erythrocytes emulsified with 1.0 ml of complete Freund's adjuvant.

Rabbit antiserum was fractionated by the method of Campbell *et al.* (1964). The fractions representing the initial peak of protein eluted from a DEAE-cellulose column were pooled and tested by gel diffusion and immunoelectrophoresis using goat antisera to rabbit serum proteins or rabbit γG-globulin. The pooled eluate was found to contain only γG-globulin, no other serum proteins were detected.

Hemolysin titration was performed as outlined in Kabat and Mayer (1961).

Measurement of Enzyme Activity. Collagenolytic activity was assayed by the decrease in viscosity of undenatured guinea pig skin collagen in solution at pH 7.4 at 27° using an Ostwald capillary viscometer (flow time for water about 90 sec at 20°). The release of [^{14}C]glycine-containing collagen degradation products from a reaction mixture containing enzyme and reconstituted acetic acid extracted guinea pig skin collagen fibrils (Nagai *et al.*, 1966) was also used as follows. Gels were prepared in plastic Microfuge tubes (Beckman-Spinco Instrument Co., Wakefield, Mass.) containing 200 μg of labeled collagen in 2 μmoles of NaCl in Tris–$CaCl_2$ buffer; the total radioactivity per tube varied from 1200 to 1800 cpm. The tubes were incubated for 16 hr at 37° in a water bath and the gels that formed were disrupted by use of a vibrator and reincubated for 1 hr at 37°. Solutions to be assayed were added to these suspensions, mixed by vibration, and incubated at 37° for varying periods of time, usually for 4 hr. The reaction was terminated by centrifugation in a Beckman-Spinco 152 microfuge for 5 min at 15,000 rpm. Aliquots of the supernatant were suspended in Brays' (1960) solution for determination of radioactivity in an automated liquid scintillation counter.

Polyacrylamide Disc Gel Electrophoresis. Protein fractions from tissue extracts and culture media were examined by the method of Davis (1964) and Ornstein (1964). For this procedure, riboflavin was substituted for ammonium persulfate (Brewer, 1967; Fantes and Furminger, 1967). Disc gel electrophoresis of collagen reaction products were performed as described by Nagai *et al.* (1964). The split gel electrophoresis procedure of Clarke (1964) as modified by Schwabe (1969) was used for comparison of mobilities of peak I enzyme and peak I extract.

Molecular Weight Determinations. The method of Dunker and Rueckert (1969), based on the electrophoretic separation on polyacrylamide gel of urea-denatured protein treated with sodium dodecyl sulfate, was employed for estimation of molecular weight of peak I enzyme and peak I extract. Molecular sieve gel filtration using Sephadex G-200 according to the method of Andrews (1965) was also used for this purpose. In both procedures, calibration was accomplished with a series of proteins ranging in molecular weight from 12,000 to 160,000.

Preparation of Segment Long Spacing for Electron Microscopy. Samples of the mixture resulting from the reaction of activated peak I extract and soluble collagen substrate were dialyzed overnight against 0.1 M acetic acid at 4°. An equal volume of 0.4% adenosine triphosphoric acid was added to 0.5 ml of the above reaction mixture and drops of the resulting suspension were deposited on carbon-coated grids, drained, and positively stained with uranyl acetate. A Voelco 100B electron microscope was used.

Results

Column Chromatography of Tadpole Collagenase. A representative chromatogram of the redissolved 30% saturated $(NH_4)_2SO_4$ precipitate, containing the peak I enzyme is shown in Figure 1A. Collagenolytic activity was estimated by measuring the release of [^{14}C]glycine-containing peptide from guinea pig skin collagen. Protein fractions constituting the ascending portion of peak I were combined and examined

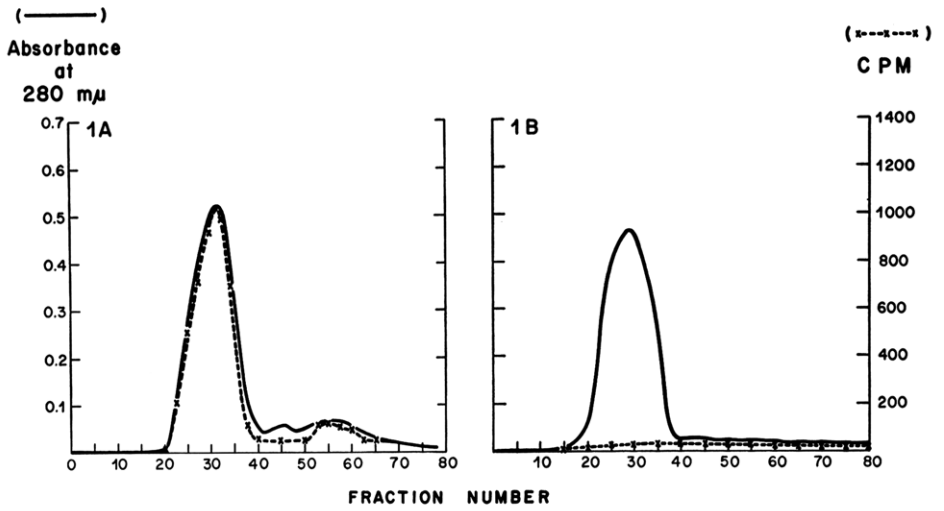

FIGURE 1: (A) Third-day culture medium was brought to 30% saturation with $(NH_4)_2SO_4$, the redissolved precipitate applied to an 8% Agarose column, and eluted with Tris–$CaCl_2$ buffer made 0.2 M with respect to NaCl. Solid line represents protein content of the eluted fractions; broken line represents collagenolytic activity as determined by [^{14}C]glycine-containing peptide release from collagen. (B) Fresh tadpole tail-fin extract was treated with $(NH_4)_2SO_4$ followed by chromatography as above.

by polyacrylamide disc gel electrophoresis. A single band of protein was seen; this protein is referred to as peak I enzyme.

Chromatographic Analysis of Extract. A peak of protein, also from a 30% saturated $NH_4(SO_2)_4$ precipitate, but devoid of collagenase activity, was eluted in the same region of the chromatogram as peak I enzyme (Figure 1B). Protein fractions constituting the ascending portion of this peak were fractionated by gel slab electrophoresis (Raymond, 1962; Harper and Gross, 1970); at least five bands of protein were resolved by this method. Of these bands, one corresponding in electrophoretic mobility to the peak I enzyme, is referred to as "peak I extract." Peak I enzyme and peak I extract were compared by split gel electrophoresis as shown in Figure 2; a single band of protein with similar electrophoretic mobility was seen in each half of the gel. A large amount of peak I extract fraction was deliberately applied in order to reveal any minor components; none was seen.

Reaction of Purified Enzyme with the γG Fraction of Rabbit Anti-collagenase Antiserum. Peak I purified enzyme was sub-

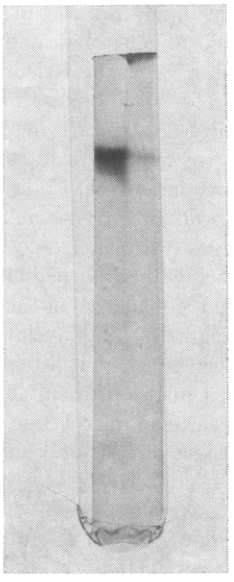

FIGURE 2: Polyacrylamide split gel electrophoresis of peak I extract (left) and peak I enzyme (right); 58 μg of extract and 22 μg of enzyme protein were applied to the gel.

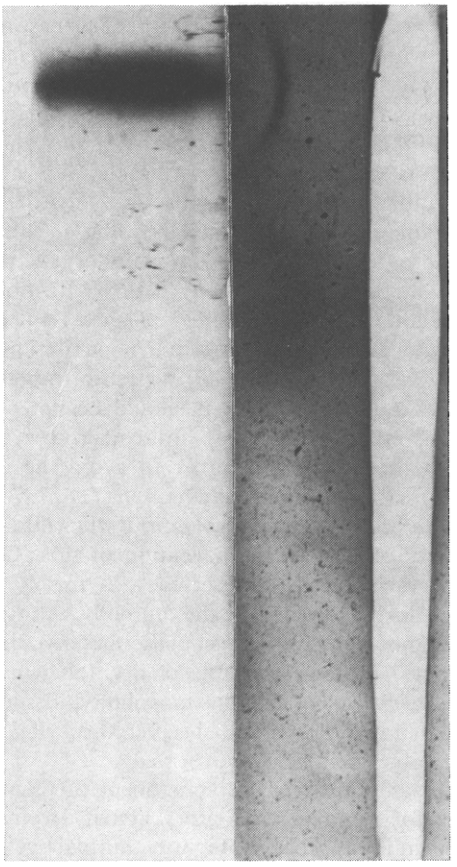

FIGURE 3: Polyacrylamide disc gel electrophoresis in 7% gel of peak I enzyme (left); 65 μg of enzyme protein was applied to the gel. Following electrophoresis the gel cylinder was embedded in agar and reacted with the γG fraction of anti-collagenase antiserum (20 mg of protein/ml in trough, right). A single line of precipitation is seen in the gel adjacent to the enzyme band.

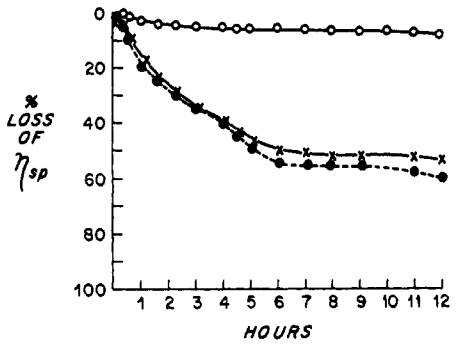

FIGURE 4: Viscometric assay of collagenolytic activity of peak I enzyme alone (●), and peak I enzyme preincubated with the γG fraction of anti-collagenase (○) or anti-egg albumin antiserum (x). The reaction mixture consisted of 0.1 ml of peak I enzyme solution containing 92 μg of enzyme protein, 0.3 ml of collagen solution containing 1200 μg of collagen in 0.4 M NaCl, and 0.6 ml of Tris-$CaCl_2$ buffer. For the inhibition studies, enzyme solution (0.1 ml) and the γG fractions (0.1 ml containing 2.0 mg of protein) were mixed and held at 27° for 30 min prior to the addition to the collagen solution. Collagenolytic activity was inhibited by preincubation of peak I enzyme with antibody.

TABLE I: Inhibition of Tadpole Collagenase by the γG Fraction of Rabbit Anti-collagenase Antiserum.

	Dilution of γG Fractions	Cpm Released	Inhibn (%)
Collagenase		1450[a]	
Collagenase + γG (anti-enzyme)	1:1[b]	0	100
	1:10	530	64
	1:100	1270	12
	1:1000	1440	0
	1:10,000	1460	0
Collagenase + γG (anti-egg albumin)	1:1	1520	0

[a] The reaction mixture consisted of 0.1 ml of peak I enzyme solution containing 85 μg of protein, 0.05 ml of collagen solution containing 100 μg of [^{14}C]glycine-labeled guinea pig skin collagen (1800 cpm) in 0.4 M NaCl, and 0.25 ml of Tris-$CaCl_2$. For the inhibition studies, 0.1 ml of peak I enzyme plus 0.1 ml of γG-globulin fraction were incubated at 37° for 15 min prior to being added to the labeled collagen fibrils. [b] Initial concentration of the γG fraction was 20 mg of protein/ml.

ected to electrophoresis in polyacrylamide gel. The gel cylinder was then embedded in agar and reacted with the γG fraction of rabbit anti-collagenase antiserum. A single precipitin line formed in the agar gel in the region adjacent to the band of enzyme protein localized in the polyacrylamide gel (Figure 3).

The ability of the γG fraction of anti-collagenase antiserum to interfere with the action of the enzyme was demonstrated by its ability to block the enzymatic cleavage of native guinea pig skin collagen in solution, thus preventing a fall in viscosity (Figure 4). Preincubation of enzyme with the γG fraction of rabbit anti-egg albumin antiserum did not inhibit the enzyme. Antibody to collagenase also inhibited the degradation by enzyme of ^{14}C-labeled collagen fibrils. Table I illustrates the progressive decrease in inhibitory activity as a function of dilution of the antibody. The γG fraction of anti-collagenase antiserum also inhibited collagen gel degradation by live explants of tadpole tail-fin tissue; no inhibition occurred in control gels containing the γG fraction of rabbit anti-egg albumin antiserum (Figure 5). It should be noted that the γG fraction, rather than whole anti-collagenase antiserum was used in these studies in order to avoid the inhibitory effects of normal serum α globulins (Eisen *et al.*, 1970).

Evidence for a Collagenase Zymogen. In the course of these studies it was observed that incubation of the γG fraction of rabbit anti-collagenase antiserum with freshly extracted tadpole tail-fin tissue removed the antibody. Although such extracts did not contain collagenolytic activity, they might have contained proteases capable of degrading mammalian antibodies *in vitro*. To test this possibility, tissue extracts were incubated with rabbit anti-sheep erythrocyte antiserum; no loss of antibody activity was observed.

On the assumption that the component of tissue extracts responsible for removing inhibitory activity from an anti-collagenase antibody fraction might be an inactive precursor of collagenase, fresh tadpole tail-fin extracts were subjected to the same procedure followed for the isolation of active enzyme, plus certain additional steps outlined in Methods. The fraction isolated by this method, peak I extract (Figure 1B) was compared to peak I enzyme by diffusion in agar gel *vs.* the γG fraction of anti-collagenase antiserum. A reaction of identity was observed (Figure 6). Incubation of peak I enzyme with insoluble collagen at 0° resulted in binding of the enzyme to the substrate and its removal from solution; the supernatant did not yield a precipitin line with antibody. In contrast treatment of peak I extract with collagen fibrils at 0° did not remove the antibody. Based on these experiments it was tentatively concluded that peak I extract might constitute a collagenase precursor which does not bind to a collagen substrate.

Activation of Peak I Extract. Peak I extract was incubated with trypsin and chymotrypsin (three-times crystallized Worthington Biochemical Corp.) at a ratio of 1:100 of enzyme to substrate (Hartley, 1964; Charles *et al.*, 1963) for 10, 20, 30, and 60 min at 0, 24, and 37° prior to assay for collagenolytic activity. No collagenolytic activity was obtained.

Successful activiation was achieved using culture medium in which living tadpole tail-fin tissue had been maintained for several days. Third-day culture medium was pretreated with collagen in an ice bath in order to remove the active collagenase present in such media. Following such treatment, the medium was devoid of enzyme activity as detected by the release of fragments from labeled collagen fibrils. Incubation of peak I extract with this medium at pH 7.4 for 15 min at 37° produced collagenolytic activity detectable by viscometric assay (Figure 7) and by labeled fibril assay, as well as by the appearance of typical reaction products, TC^A and TC^B, precipitated in the form of segment long spacing, and visualized in the electron microscope (Figure 8). A further examination of the effect of time and temperature of incubation of activator with peak I extract is outlined in Table II. There was a progressive increase in collagenolytic activity generated from 162 μg of protein at both 27 and 37°. A peak value was attained after incubation at 37° for 60 min, this value corresponded to the collagenolytic activity of 180 μg of peak I enzyme under identical conditions. Incubation for 90 and 120 min did not increase the collagenolytic activity

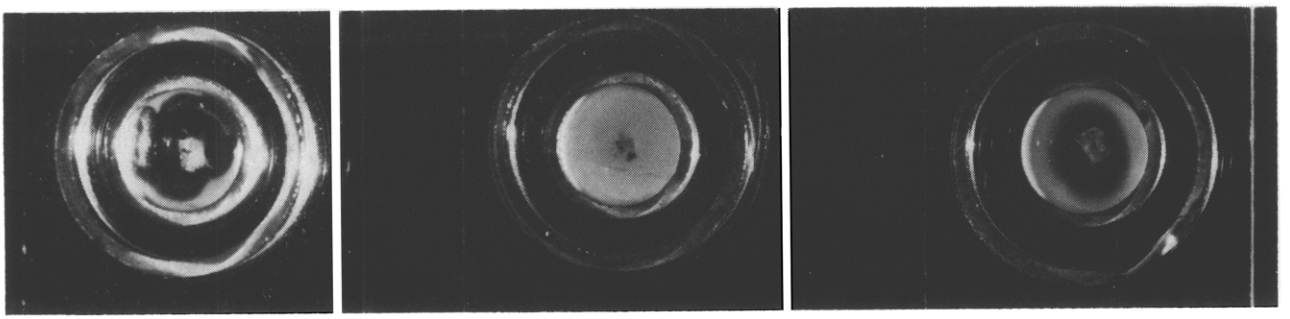

FIGURE 5: Incubation of tadpole tail-fin tissue explants on collagen gel (left), and collagen gel containing 10 mg/ml of the γG-globulin fraction of anti-collagenase (center) and anti-egg albumin antiserum (right). No lysis of the gel occurred in the center well.

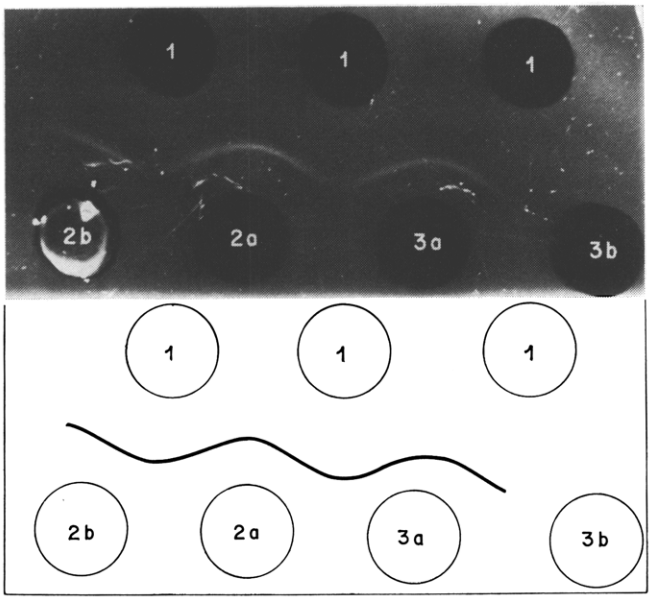

FIGURE 6: Agar gel diffusion reaction between the γG fraction of rabbit anti-collagenase antiserum (20 mg of protein/ml in wells labeled 1) and peak I enzyme (920 μg/ml) before (3a), and after (3b), exposure to collagen fibrils; as well as peak I extract (800 μg/ml) before (2a) and after (2b) exposure to collagen fibrils. A reaction of identity is shown between antibody and peak I extract; only the peak I enzyme was removed by adsorption to collagen.

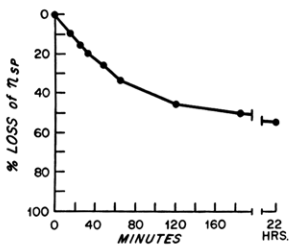

FIGURE 7: Viscometric assay of collagenolytic activity generated by treatment of zymogen with "activator." Zymogen (0.1 ml) containing 124 μg of protein and activator (0.1 ml) containing 1200 μg of protein were mixed and incubated for 30 min at 27°. Thereafter, 0.1 ml of this solution, 0.3 ml of collagen solution containing 1200 μg of collagen in 0.4 M NaCl, and 0.6 ml of Tris–$CaCl_2$ buffer were combined and assayed at the intervals shown.

generated at 37° The characteristics of the activator have not been determined; it is known that activity persists upon dialysis of the absorbed culture medium at 4° for 24 hr, but is destroyed by heating at 55° for 10 min.

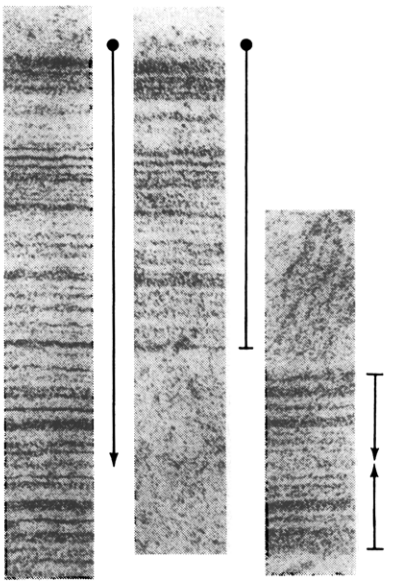

FIGURE 8: Electron micrographs of segment long spacings of collagen fragments obtained by the action of activated collagenase zymogen: (left) native tropocollagen, polymeric form (TC); (center) TC^A segment; (right) TC^B segment (dimeric form).

Incubation of the activated peak I extract with the γG fraction of rabbit anti-collagenase antiserum, under conditions in Table I, completely inhibited collagenolytic activity; the γG fraction of anti-egg albumin antiserum had no effect (Table III). On the basis of this evidence, it is appropriate to refer to peak I extract as a collagenase zymogen.

The molecular weight of enzyme, as estimated by sodium dodecyl sulfate–polyacrylamide electrophoresis in 5 and 10% gels, was 104,000 and 115,000, respectively; that of zymogen in 5 and 10% gels was 106,000 and 120,000, respectively. A molecular weight of 100,000 for enzyme was obtained using molecular sieve chromatography.

The slight difference in molecular weight of enzyme and zymogen was reflected in the sodium dodecyl sulfate gels in which both substances were tested separately and as mixtures; the latter revealed a double band as seen in Figure 9.

Discussion

Although Lapiere and Gross (1963) had originally considered the possibility of a collagenase zymogen which might require protein synthesis for activation, they preferred the alternative possibility namely, that the enzyme was synthesized *de novo* by living cells as needed. The latter view accounted

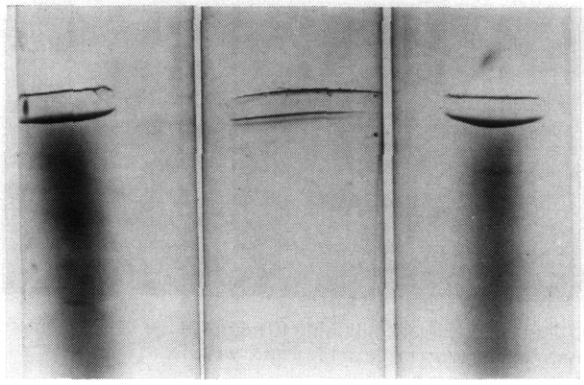

FIGURE 9: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of collagenase, 0.5 μg (left); collagenase, 0.2 μg plus zymogen, 0.2 μg (center); and zymogen, 0.5 μg (right).

for the apparent absence of enzyme from tissue extracts, as well as from the culture medium of dead or puromycin-treated tissues. The evidence presented here strongly supports the other hypothesis, namely that a zymogen is present in the tissue which can be extracted and activated by a factor found in the culture medium from living tissues.

The activating factor was a thermolabile, nondialyzable component present in the culture medium of living tissues; it has not yet been further characterized. Trypsin and chymotrypsin did not substitute for this component in the activation process. Activation was demonstrated by showing the ability of the newly formed enzyme to degrade native collagen in both viscometric and radioactive fibril assays, and by demonstrating typical reaction products, TC^A and TC^B, by electron microscopy. Furthermore, the activated zymogen was inhibited by antibody to enzyme. The evidence for a zymogen, is further strengthened by the observation that the molecular weights of both the active and inactive forms are almost identical, and that in purified form they each appear as a single component on sodium dodecyl sulfate gel electrophoresis.

Eisen *et al.* (1971) have reported the presence of an immunoreactive collagenase in human skin extracts that had no dectable enzyme activity. Gel filtration of the skin extracts permitted the separation of collagenase in its active form from other proteins in the mixture; the latter included the serum antiproteases, α2M macroglobulin and α1 anti-trypsin. In the present study, no evidence was found for an enzyme–inhibitor complex in tadpole tissues. It is possible that the initial purification step employing differential precipitation with ammonium sulfate, removes both activator and protein inhibitors. By contrast, the procedures followed by Eisen *et al.* (1971) may not separate activator and zymogen; thus enzyme activated in the process of extraction may be available for inhibition by serum proteins also present in the extract.

It is of interest that the zymogen has neither catalytic nor substrate binding capacity and that activation uncovers both properties. Based on the difference in molecular size between zymogen and enzyme of approximately 10,000, it would appear that removal of a small portion of the zymogen molecule reveals both sites.

TABLE II: Effect of Temperature and Time on Zymogen Activation.

	Cpm Released	
Incubn (min)[a]	27°	37°
15	0	0
30	80	320
60	260	660

[a] The reaction mixture consisted of 0.1 ml of zymogen, containing 162 μg of protein and 0.1 ml of "activator," containing 1425 μg of protein; these reactants were mixed and incubated under the conditions indicated prior to addition to labeled collagen fibrils (total cpm/tube 1500). Control tubes containing 180 μg of peak I enzyme or 1425 μg of "activator" were treated similarly; under all conditions tested, the former released approximately 620 and 25 cpm of solubilized collagen, respectively.

TABLE III: Inhibition of Activated Zymogen by Antibody to Enzyme.

	Cpm Released[a]	
	Expt I	Expt II
Zymogen	0	0
Activator	0	0
Activated zymogen	404	380
Activated zymogen + γG (anti-enzyme)	0	0
Activated zymogen + γG (anti-egg albumin)	568	528

[a] The reaction mixture consisted of 148 μg of zymogen, 1400 μg of activator, 0.1 ml of γG (anti-enzyme) fraction containing 20 mg of protein/ml or 0.1 ml of γG (anti-egg albumin) fraction containing 20 mg of protein/ml, in a final volume of 0.2 ml. These components were mixed and incubated at 37° for 15 min prior to addition to labeled collagen fibrils (total cpm/tube 1500).

Acknowledgment

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Amino- and Carboxyl-Terminal Amino Acid Sequences of the *Peptostreptococcus elsdenii* and *Clostridium pasteurianum* Flavodoxins*

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ABSTRACT: The NH_2-terminal sequences of 2 flavodoxins were examined in the protein sequencer. The first 51 residues of the *Clostridium pasteurianum* and the first 41 residues of the *Peptostreptococcus elsdenii* flavodoxin were determined. The carboxyl-terminal sequences of the two flavodoxins were found to be Leu-Val-COOH and Lys-Ala-COOH for the *C. pasteurianum* and *P. elsdenii* proteins, respectively. The cysteine residue which is involved in binding the single FMN is not in the portion of the molecule which was sequenced. A number of constant residues were observed for the flavodoxins but the most interesting portion was a 12-residue section from residues 6 to 17 in the *P. elsdenii* protein and from residues 7 to 18 in the *C. pasteurianum* protein which probably has an important function. The flavodoxins have a relatively low molecular weight of 14,000 and are ideal proteins for studying the structure–function relationships and should serve to act as a model for other flavoproteins whose structures are as yet unknown. The fact that the X-ray structural studies of crystalline flavodoxin are in progress simply added further impetus for investigating the primary structure of flavodoxin. In addition, the capabilities of the sequencer are of general interest to biochemists at this time and the sequencer results obtained in our laboratory with different proteins are presented.

Flavodoxin, a FMN protein, was initially isolated in crystalline form from *Clostridium pasteurianum* (Knight *et al.*, 1966; Knight and Hardy, 1966). These investigators demonstrated that flavodoxin was produced by *C. pasteurianum* when the iron content in the growth medium was low (Knight *et al.*, 1966; Knight and Hardy, 1966, 1967). Furthermore,

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