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A Collagenase in Extracts of the Invertebrate *Bipalium kewense*

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The localization, isolation and partial characterization of a collagenolytic enzyme from the land planarian *Bipalium kewense* is described. This enzyme can be obtained by direct extraction of the organism, and can be separated from non-collagenous proteolytic activity by $(NH_4)_2SO_4$ precipitation and Sephadex-gel chromatography. Its mode of attack on collagen and sensitivity to a variety of inhibitors indicate that this enzyme differs from vertebrate collagenases and a previously described invertebrate collagenase.

The land planarian *Bipalium kewense* has as its natural prey the earthworm *Lumbricus*. It attacks by immobilizing the earthworm by secretion of an unknown toxin, and then appears to digest it by an extracellular secretion of lytic enzymes. Little is known about the nature or mechanism of action of these secretions.

In a continuing search for collagenolytic enzymes from invertebrate species, we surveyed *Bipalium kewense* for the presence of collagenase, in view of the known presence of collagen in the cuticle of its prey (Singleton, 1957; Watson, 1958; Maser & Rice, 1962; Josse & Harrington, 1964). A single instance of an invertebrate collagenase has been previously reported. Eisen & Jeffrey (1969) have demonstrated the presence of collagenolytic activity in extracts of the digestive organ (hepatopancreas) of the marine crustacean *Uca pugilator*. Of additional interest is that the mode of attack of this collagenase appears to be somewhat different from that of vertebrate and prokaryotic collagenases, suggesting a possible phylogenetic variation in the character of this class of enzymes.

In the present paper we describe the localization, isolation and partial characterization of a collagenolytic enzyme from *Bipalium kewense*. This enzyme can be obtained easily by direct extraction of the organism. Its physical and enzymic characteristics are different not only from vertebrate and prokaryotic collagenases but also from the invertebrate crustacean collagenase.

Materials and Methods

Preparation of extracts

Animals (*Bipalium kewense*) were obtained from Bio-Tech Co., Houston, Texas, U.S.A., and kept at room temperature (22–24°C) in a moist environment until ready for use. Extracts were prepared by quick-freezing the animals on a glass plate with solid CO_2, and slicing them into transverse sections with a razor blade while frozen. This procedure was necessary because the animals undergo rapid autodigestion if traumatized while alive. In addition, initial experiments showed that collagenolytic activity was located only in the centre section of the body of the worm (see below). For mass isolation the organisms were cut into three sections, of which the middle section was used for collagenase extraction. The sections were homogenized in the cold in 0.01 M-Tris–HCl buffer, pH 8.0, containing 0.005 M-$CaCl_2$ (Tris–$CaCl_2$ buffer), in a glass–Teflon homogenizer. The homogenate was then centrifuged at 15000*g* for 15 min. Essentially all of the collagenolytic and proteolytic activity of the homogenates was found in the supernatant after centrifugation, but only 30% of the protein content. Fractionation of the supernatants by $(NH_4)_2SO_4$ precipitation and Sephadex G-150 chromatography was carried out in the cold.

Enzymic activities

Proteolytic activity was measured with Azocoll (powdered azo-dye-coupled hide powder) (Calbiochem, Los Angeles, Calif., U.S.A.) as substrate. This substrate was chosen because of its sensitivity to a wide variety of proteolytic enzymes, and the ease and reproducibility of the assay for its hydrolysis. Incubation was in a volume of 1 ml containing 2 mg of Azocoll and Tris–$CaCl_2$ buffer, pH 8.0, at 35°C. After incubation for 30–120 min, the reaction mixtures were placed on ice, undigested Azocoll was removed by centrifugation, and absorbance in the supernatant measured at 540 nm in a Gilford spectrophotometer. Proteolytic activity against gelatin (1 mg/ml) was assayed in Tris–$CaCl_2$ buffer at 35°C by determining radioactivity released from [^{14}C]-glycine-labelled heat-denatured guinea-pig skin collagen as prepared by the method of Gross (1958).

Undigested gelatin was precipitated with trichloroacetic acid (5%)–sodium tungstate (0.25%) (Miller & Udenfriend, 1970). Proteolytic activity against casein (2mg/ml) and bovine serum albumin (2mg/ml) was assayed in Tris–$CaCl_2$ buffer by measuring acid-soluble u.v. absorbance at 280nm after the reaction was terminated by the addition of trichloroacetic acid (5%). Since *Bipalium* collagenase did not hydrolyse these substrates appreciably incubation was for 6–24h.

Collagenolytic activity was assayed by using both native reconstituted collagen fibres and soluble collagen. Activity on collagen fibres (100–200μg of collagen) was measured by solubilization of radioactivity from [^{14}C]glycine-labelled guinea-pig skin collagen (7500c.p.m./mg of collagen) at 35°C for 6–18h by the method of Nagai *et al.* (1966). Collagen gel blanks exposed to 0.01% trypsin solutions were included in all experiments. Activity on acid-extracted soluble collagen was measured viscosimetrically by using Ostwald Semi-Micro viscometers (Cannon Instrument Co., State College, Pa., U.S.A.), as well as by the method used for gelatin (described above). Activity against benzyloxycarbonyl-Gly-Pro-Gly-Gly-Pro-Ala (Mann Research Laboratories, New York, N.Y., U.S.A.) was measured spectrophotometrically by the method of Grassmann & Nordwig (1960).

Protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as a standard.

Results

Localization of enzymic activity

To determine the localization of enzyme activity, frozen worms were dissected into ten equal sections (A–J) (Fig. 1). Each section was homogenized in 1.0ml of Tris–$CaCl_2$ buffer and centrifuged. The supernatants were then analysed for protein content, proteolytic (Azocollytic) activity, and collagenolytic activity (Nagai *et al.*, 1966). The results are shown in Fig. 1 and indicate that collagenolytic activity was localized primarily in sections C, D and E. This area corresponds to the pharynx of this organism.

In contrast, proteolytic activity with an Azocoll substrate as well as the protein content of the extracts was distributed more uniformly along the sections, probably reflecting activity from the intestine of the organisms, which runs the length of the body.

Partial purification of collagenolytic activity in homogenates from sections C, D and E was then undertaken. This activity was partially separated from proteolytic activity by $(NH_4)_2SO_4$ fractionation (Fig. 2). Most of the collagenolytic activity was precipitated at 70% saturation. The material that precipitated between 30 and 60% saturation was further purified by chromatography on Sephadex G-150. Fig. 3 shows that collagenolytic activity appeared as a single, fairly broad peak before the bulk of the proteolytic activity. Fractions containing collagenase activity were combined, concentrated by $(NH_4)_2SO_4$ precipitation and rechromatographed on Sephadex G-150. The resultant elution profile yielded a single

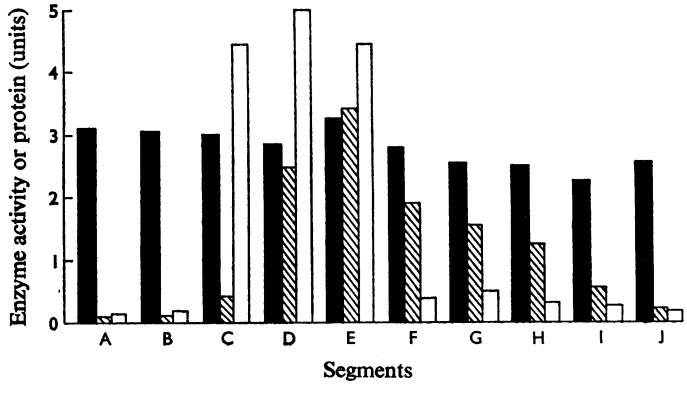


Fig. 1. *Localization of proteolytic and collagenolytic activities*

Frozen worms were dissected into ten equal sections. Each section was homogenized in 1ml of 0.01M-Tris–HCl (pH8.0)–0.005M-$CaCl_2$ buffer. The homogenate was centrifuged at 15000*g* for 10min, and portions (50–100μl) of the supernatant were assayed for protein content (■), proteolytic activity on Azocoll (■), and collagenolytic activity on reconstituted collagen fibres (□). One unit = 1.0mg of protein/ml of extract; hydrolysis of 10mg of Azocoll/h per ml of extract at 35°C; hydrolysis of 1mg of collagen/h per ml of extract at 35°C.

peak of collagenolytic activity and a coincident peak of proteolytic activity. These columns were pre-calibrated with aldolase, ovalbumin, chymotrypsinogen and ribonuclease by using a standard molecular-weight calibration kit (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.). On the basis of the calibration curve obtained, the *Bipalium* collagenase had an apparent molecular weight of 52000. When the Sephadex-purified collagenase is analysed by polyacrylamide-gel electrophoresis in a 7.5% gel at pH 8.7, a single band is obtained after staining with Amido Black.

When unstained gels are sliced and eluted in Tris–$CaCl_2$ buffer, a single peak of collagenolytic activity against reconstituted collagen fibrils, coincident with this band, is observed.

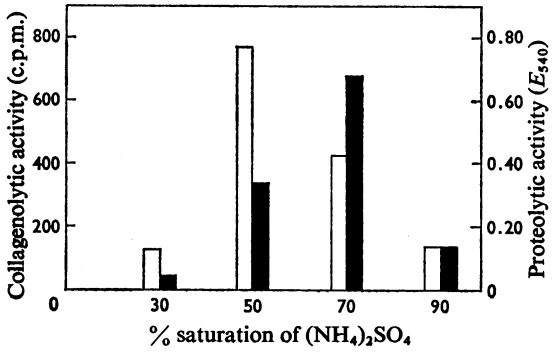


Fig. 2. *$(NH_4)_2SO_4$ fractionation of Bipalium extracts*

Sections, C, D and E from five frozen worms were combined and prepared as described in the Materials and Methods section. Solid $(NH_4)_2SO_4$ was added to the supernatant and consecutive fractions were collected at 30, 50, 70 and 90% saturation. The precipitated materials were dissolved in Tris–$CaCl_2$ buffer and assayed for proteolytic activity on Azocoll (■) and collagenolytic activity (□).

Partial characterization of enzyme

Most collagenolytic enzymes exhibit little activity against non-collagenous proteins. Further, remaining non-specific proteolytic activity can be separated from collagenolytic activity by chromatographic techniques or specific inhibitors (Harper & Gross, 1970; Peterkofsky & Diegelmann, 1971). The relative activity of the Sephadex-purified *Bipalium* enzyme against collagen and non-collagen protein substrates is shown in Table 1. This enzyme was essentially inactive against serum albumin and casein, although it was highly active against both native and denatured collagen. Activity against reconstituted collagen fibrils is approx. 5–10% of the activity against soluble collagen. The *Bipalium* enzyme is not active against the synthetic peptide benzyloxycarbonyl-Gly-Pro-Gly-Gly-Pro-Ala, a substrate originally used to assay *Clostridium histolyticum* collagenase. The invertebrate

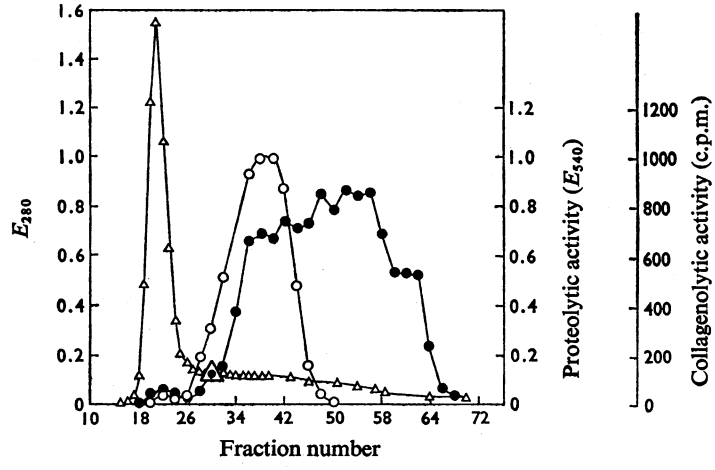


Fig. 3. *Sephadex G-150 chromatography of Bipalium extracts*

Extracts of *Bipalium* were fractionated with $(NH_4)_2SO_4$ between 30 and 60% saturation. The 60%-saturated-$(NH_4)_2SO_4$ precipitate was applied to a column (45cm×2.5cm) of Sephadex G-150 in the cold and eluted with Tris–$CaCl_2$ at a flow rate of 25ml/h. Fractions (3ml) were collected and assayed for protein content (E_{280}, △), proteolytic activity on Azocoll (●), and collagenolytic activity (○).

Table 1. *Effect of Bipalium collagenase on various substrates*

The assay methods are described in the Materials and Methods section. Each reaction mixture contained 2mg of substrate and 15μg of *Bipalium* collagenase (from Sephadex chromatography) in a volume of 1.0ml. The value in parentheses for native soluble collagen represents measurement by decrease in viscosity ($\eta_{sp.}$). All assays were done at 35°C.

Substrate	Substrate hydrolysed (mg/h)
Azocoll	1.0–1.2
Bovine serum albumin	No detectable hydrolysis
Casein	0.006
Collagen (native, soluble)	0.4–0.6 (2.5)
Gelatin	0.5–0.7

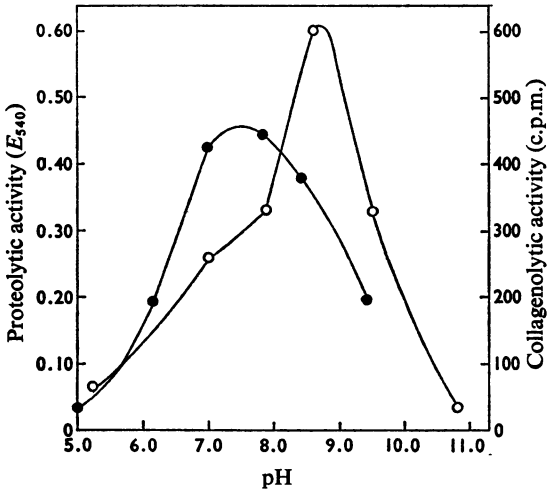


Fig. 4. *pH-dependency of proteolytic and collagenolytic activities*

Extracts (containing 50–100μg of protein) of *Bipalium* were assayed for proteolytic activity (●) on Azocoll (sections F, G, H) and for collagenolytic activity (○) (sections C, D, E) at various pH values. Buffers were prepared from 0.05M-Tris–HCl and 0.05M-Tris–maleate and contained 5mM-$CaCl_2$. At each pH value incubations without extract were done to measure the extent of substrate solubilization.

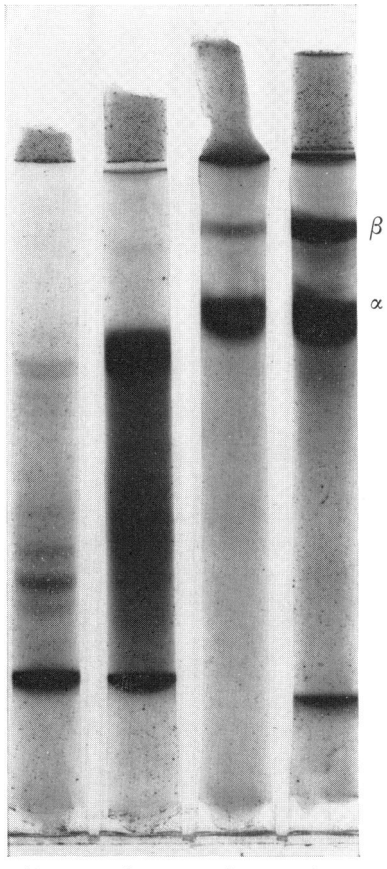
crustacean enzyme, however, retains tryptic and/or chymotryptic activity even after purification (Eisen & Jeffrey, 1969; A. Z. Eisen, personal communication). It should be noted that the apparent activity against Azocoll in *Bipalium* extracts can be attributed to the hydrolysis of gelatin in this substrate, since Azocoll is prepared by heat extraction of powdered bovine skin. The lytic activity against Azocoll, gelatin and casein is inhibited by EDTA and cysteine, as is collagenolytic activity (see below).

Collagenolytic and proteolytic activity in crude *Bipalium* homogenates were differentiated by pH optimum (Fig. 4). Collagenolytic activity was maximal around pH8.5–9.0, as demonstrated for many other collagenases (Seifter & Harper, 1971), whereas proteolytic activity on Azocoll was maximal at pH7.0–7.5.

The effects of various inhibitors on *Bipalium* collagenase are shown in Table 2. The *Bipalium* enzyme was inhibited by the chelating agent EDTA, as well as by cysteine, 2-mercaptoethanol and GSH. On the other hand, phenylmethylsulphonyl fluoride, an inhibitor of serine proteases, which inhibited the crustacean enzyme (Eisen & Jeffrey, 1969), did not affect appreciably the collagenolytic activity of the *Bipalium* enzyme.

Mode of enzyme attack

Incubation of Sephadex-purified *Bipalium* collagenase with soluble guinea-pig skin collagen, at temperatures between 15° and 25°C, caused a rapid and essentially complete loss in viscosity of collagen solutions (Fig. 5). In contrast, in similar experiments with vertebrate collagenases viscosity was decreased to a stable value at 40–60% of the original specific viscosity (Nagai *et al.*, 1966; Seifter & Harper, 1971). Examination of these reaction mixtures at various times during reaction by polyacrylamide-gel electrophoresis (Nagai *et al.*, 1964) showed that initially dimeric collagen (β chains) was rapidly converted into monomeric α chains (Plate 1). Further degradation occurred by conversion of the α chains into small peptides. Dialysis for 24h in the cold of portions of these reaction mixtures (with [^{14}C]glycine-labelled guinea-pig skin collagen) revealed that essentially all of the collagen remained non-diffusible during the early stages (0–60% viscosity loss) of the reaction. Continued incubation rendered increasing proportions of the collagen diffusible so that after 8–12h of incubation at 24°C, when the reaction mixtures retained less than 10–15% of the original viscosity, less than 20% of the collagen was found in the dialysis residue. Intermediate degradation forms were detect-



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of collagen–collagenase mixtures

Samples (50 μl for *a*, 25 μl for *b*, *c*, *d*) of the reaction mixtures used to obtain the results in Fig. 5 were removed at (*a*) 100% of initial viscosity, (*b*) 74% of initial viscosity, (*c*) 51% of initial viscosity, and (*d*) 12% of original viscosity. Further reaction was stopped by the addition of HCl (final concn. 0.1 M). The samples were then subjected to polyacrylamide-gel electrophoresis by the method of Nagai *et al.* (1964) and stained with Amido Black. α and β show the positions of the α and β chains respectively.

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Table 2. *Effect of various inhibitors on Bipalium collagenase*

Collagenase assays were carried out by the [^{14}C]glycine micro-gel technique of Nagai *et al.* (1966) for 18h at 35°C in a volume of 0.25ml, containing 25μl of heat-gelled radioactive guinea-pig skin collagen (4mg/ml), 175μl of Tris–$CaCl_2$ buffer, pH8.0, and 25μl of Sephadex-purified *Bipalium* collagenase (15μg of protein). Full lysis of the collagen gels occurred in the control tubes. Inhibitors in a volume of 25μl were added to the final concentrations shown above.

Inhibitor	Inhibition (%)
EDTA (disodium salt; 0.01M)	97
Soya-bean trypsin inhibitor (100μg/ml)	0
Phenylmethylsulphonyl fluoride (100μg/ml)	<10
Pooled human serum (1:10 dilution)	95
Cysteine	
10mM	92
1mM	72
GSH	
10mM	93
1mM	57
2-Mercaptoethanol	
10mM	94
1mM	64
N-Ethylmaleimide	
10mM	26
1mM	0

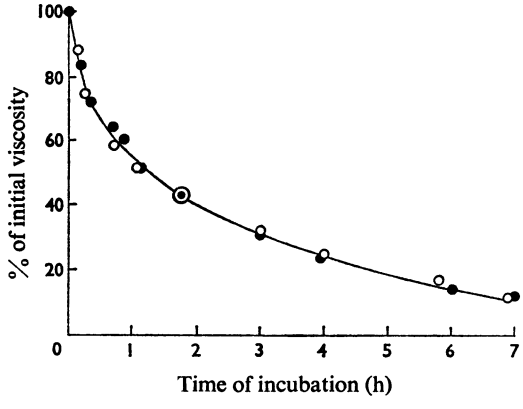

Fig. 5. *Effect of Bipalium collagenase on the viscosity of soluble collagen*

Mixtures containing 0.1% salt-extracted guinea-pig skin collagen, 15μg of Sephadex-purified *Bipalium* collagenase, 0.25M-NaCl, 0.01M-Tris–HCl buffer, pH8.0, and 0.5mM-$CaCl_2$ in a total volume of 1ml were incubated in Ostwald viscometers at 24°C (●). The viscosity is plotted as % of initial viscosity ($\eta_{sp.}$). ○, represents a duplicate mixture containing, in addition, 100μg of phenylmethylsulphonyl fluoride/ml.

ed by polyacrylamide-gel electrophoresis, but not in amounts that permitted determination of the precise sites of proteolytic cleavage by electron microscopy.

It is noteworthy that *Bipalium* collagenase did not bind to collagen covalently linked to Sepharose, under the same conditions used by Bauer *et al.* (1971) to purify mammalian collagenases by affinity chromatography. Bacterial (*Clostridium histolyticum*) collagenase was bound to Sepharose–collagen under these conditions (M. H. Dresden, unpublished work).

Discussion

The present paper describes the localization, isolation and partial characterization of an extractable collagenolytic enzyme from the invertebrate *Bipalium kewense*. It appears to be a true collagenase in that it degrades collagen under physiological conditions and shows low activity toward non-collagenous proteins.

Its physical and enzymic parameters do not entirely resemble any of the prokaryotic, invertebrate or vertebrate collagenases previously reported, although it has some characteristics of all of these. The molecular weight, 52000, is greater than that of the vertebrate enzymes, which are 25000 and 40000–45000 (Harris *et al.*, 1969; Fullmer *et al.*, 1972), and less than that of the major species of bacterial (*Clostridium histolyticum*) collagenase (A), 105000 (Seifter & Harper, 1971). The molecular weight may be similar to that of collagenase B from *Clostridium histolyticum*, which has been assigned molecular weights of 57400 (Seifter & Harper, 1971) and 79000 (Yoshida & Noda, 1965).

Bipalium collagenase is inhibited by both EDTA and cysteine, as are bacterial and vertebrate enzymes,

whereas the crustacean enzyme is not. Like the bacterial and vertebrate enzymes, *Bipalium* collagenase is not inhibited appreciably by trypsin and chymotrypsin inhibitors, which do inhibit the crustacean collagenase. Inhibition by cysteine, 2-mercaptoethanol and GSH but not by *N*-ethylmaleimide suggests that (1) a labile disulphide bridge is critical to enzyme activity, or (2) the inhibition is due to chelation of metal ions, as has been suggested by Seifter & Harper (1971) for bacterial collagenases. Degradation of dimeric collagen β chains to monomeric α chains, which is probably due to cleavage of the extrahelical peptide region in collagen containing a cross-link between two α chains, is also characteristic of the crustacean and bacterial enzymes. This activity appears to be an intrinsic part of the *Bipalium* enzyme, since it is not inhibited by phenylmethylsulphonyl fluoride.

The mode of attack on collagen resembles that of the bacterial and crustacean collagenases. Although optical-rotation measurements have not yet been carried out, dialysis experiments and the decrease in stainable material on polyacrylamide-gels (Plate 1*d*) during prolonged incubation suggest that this enzyme degrades the collagen triple-helix extensively under conditions in which the triple-helix is not thermally denatured. The vertebrate enzymes require denaturation of the triple-helix for complete degradation of collagen. In contrast to the results with crustacean enzyme, we were not able to detect appreciable amounts of intermediate degradation fragments even by incubation at 15°C, although very careful analyses such as those done for bacterial collagenase by Stark & Kühn (1968) might be profitable.

The collagen substrate used in these experiments was guinea-pig skin collagen. The natural substrate of *Bipalium* enzyme is earthworm cuticle collagen, which has been shown to be different, both in molecular weight and amino acid composition (Maser & Rice, 1962; Josse & Harrington, 1964), from most mammalian collagens.

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