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PROPERTIES OF A COLLAGENOLYTIC ENZYME FROM *BIPALIUM KEWENSE*

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A collagenolytic enzyme from the land planarian *Bipalium kewense* has been purified by preparative isoelectric focusing. The enzyme has a molecular weight of 47 000 ± 2 000 and appears to be dimeric. It has an isoelectric point of 4.6 ± 0.1 and a high content of acidic amino acids. The amino acid composition of the *Bipalium* collagenase is similar to that of human skin fibroblast collagenases but clearly different from previously reported collagenolytic proteases from other invertebrates, *Uca pugilator* and *Hypoderma lineatum*. In its action on guinea-pig collagen, the enzyme produces distinct products, at low incubation temperatures, different from those produced by vertebrate and other invertebrate collagenolytic enzymes. These products have glycine as their N-terminal amino acids. As determined by viscosity measurements, the *Bipalium* collagenase is more active on invertebrate, earthworm, collagen than it is on the vertebrate, Type I guinea-pig skin, collagen. The *Bipalium* collagenase differs from both bacterial and vertebrate collagenases as well as from invertebrate, collagenolytic serine proteases.

Introduction

The isolation and initial characterization of a collagenolytic enzyme from the flatworm, *Bipalium kewense*, has been previously reported [1]. This enzyme is likely to have a digestive function, since its natural prey is the earthworm, *Lumbricus terrestris*.

This collagenase is of interest because its physical and enzymatic characteristics differ not only from vertebrate and procaryotic collagenases but also from other invertebrate collagenases. While the *Bipalium* collagenase is a metalloprotease [1] and is not inhibited by serine protease inhibitors, the other reported invertebrate collagenolytic enzymes, from the fiddler crab, *Uca pugilator* [2], and the warble fly, *Hypoderma lineatum* [3–5], are serine proteases. On the other hand, the *Bipalium* collagenase does not appear to produce the characteristic 75–25% cleavage of collagen carried out by the vertebrate collagenases [1].

Bipalium collagenase can digest native, soluble and reconstituted fibrillar collagen and also denatured collagen, gelatin [1]. In contrast to the other invertebrate collagenases, it does not appreciably digest non-collagenous proteins such as casein or bovine serum albumin, nor does it hydrolyze synthetic peptides used to assay bacterial (*Clostridium histolyticum*) collagenase activity [1].

This report described the further purification and additional properties of the *Bipalium* collagenase.

Materials and Methods

Enzyme purification. Flatworms (*B. kewense*) were collected from local garden nurseries. Previous experiments [1] had shown that the collagenolytic activity was located in the midsection (pharyngeal region) of the worm. Therefore, this section was dissected from the animal, quick-frozen on dry ice and stored at −20°C prior to use.

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Abbreviation: SDS, sodium dodecyl sulphate.

Extracts were prepared by homogenization in a glass-Teflon homogenizer in cold 0.01 M Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$ (buffer A) plus 0.2% Triton X-100; the homogenate was then centrifuged at 15 000 × *g* for 15 min.

The supernatants were concentrated by ammonium sulfate precipitation at 90% of saturation or alternatively by ultrafiltration with an Amicon PM-10 membrane (Amicon Corp., Lexington, MA) to approx. 1.5–1.8 ml, and dialysed overnight against 1.0% glycine/NaOH, pH 8.0, plus 5 mM $CaCl_2$. Preparative isolectric focusing was carried out on a 50-ml bed of Ultrodex (LKB, Bromma, Sweden) using ampholines in the pH range from pH 4 to 6 for 15 h at 8 W. Following electrofocusing the gel bed was divided into 30 fractions using an LKB template and each fraction was eluted with 0.2 M NaCl in buffer A. Small samples of each fraction were also diluted with distilled water overnight in order to determine the pH of each fraction.

Amino acid analyses. Amino acid analyses were performed on a Durrum D-502 Amino Acid Analyzer (Dionex Corp., Sunnyvale, CA) equipped with the DOS-2 operating system using ninhydrin detection. Prior to analysis samples were hydrolyzed in vacuo with 6 M HCl at 110°C for 20 h, then dried over NaOH.

Collagenase and protease assays. Collagenase activity was measured using the soluble collagen assay system of Terato et al. [6]. The collagen used in these assays was obtained by salt (0.5 M NaCl/0.005 M Tris-HCl, pH 7.6) extraction from guinea-pig skin which had been labeled in vivo by intraperitoneal injection of [^{14}C]glycine.

Viscosimetric assay of collagenolytic activity against salt-extracted guinea-pig collagen and earthworm cuticle collagen substrates was determined using Ostwald Semi-Micro viscometers (Cannon Instrument Co., State College, PA). Earthworm cuticle collagen (EWCC) was extracted from *L. terrestris* (Carolina Biological Supply; Burlington, SC) cuticle by the method of Josse and Harrington [7] using 0.5 M NaCl.

The bacterial collagenase used for the studies shown in Fig. 6 was obtained from Worthington (Freehold, NJ) and had been further purified by the method of Peterkofsky and Diegelmann [8].

General protease activity was measured using Azocoll (Sigma, St. Louis, MO), an azo-dye coupled hide powder, as previously described [1].

Collagen gel electrophoresis. Polyacrylamide gel electrophoresis of the collagen reaction products of the action of *Bipalium* collagenase was carried out in 7.5% acrylamide gels by the method of Nagai et al. [9] and stained with Amido Black.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using pore-gradient slab SDS-polyacrylamide gel electrophoresis modelled after the method of Laemmli [10]. The running gels were cast with a linear gradient of acrylamide (7.5 to 25%; 1 : 37.5 crosslinker : acrylamide ratio) and a parallel gradient of urea (0 to 4.0 M). The gel buffer and running buffer were as used by Laemmli except that β-alanine was substituted for glycine.

Prior to loading, the samples and standards were boiled 2–3 min in loading buffer. Protein bands were detected by staining with Coomassie Brilliant Blue R-250 at 37°C for 30–60 min. Molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were included in all experiments.

Determination of N-terminal amino acids. Activity of the *Bipalium* collagenase on guinea-pig skin collagen was assessed following incubation of 2 mg salt-soluble collagen with 5 μg purified collagenase at 24°C in 0.01 M Tris-HCl/5 mM $CaCl_2$. The reaction was terminated with 6 M HCl when the viscosity of the collagen solution has decreased to 30–40% of the original viscosity. The samples were lyophilized and the amino terminal amino acids of the reaction products were determined by reaction with dansyl chloride by the method of Hartley [11]. After hydrolysis in 6 M HCl for 18 h at 105°C, DNS-amino acids were separated by chromatography on polyamide sheets (Schleicher and Schuell; Keene, NH) using Hartley's three-solvent system. The identity of the new amino termini was confirmed by use of the appropriate DNS-amino acid standards.

Results

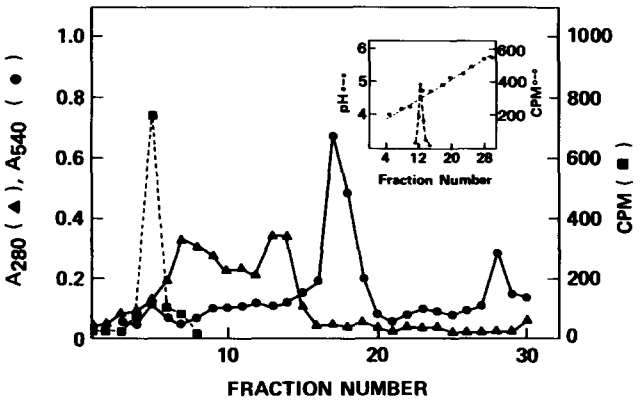
Purification of the Bipalium collagenase

Initial examination of the *Bipalium* enzyme was undertaken by preparative isoelectric focusing of crude extracts in polyacrylamide gels over the pH range 3.5–9.5. Following electrophoresis, 0.25-cm slices of the gel were incubated overnight in 20 mM

Tris-HCl, pH 7.6, plus 5 mM $CaCl_2$ to elute proteins and assayed for protein content, collagenolytic activity and general proteolytic activity. The results shown in Fig. 1 demonstrate a single peak of collagenolytic activity, at a pH of approx. 4.6–4.8, which was not coincident with general proteolytic activity. A similar result was seen when crude extracts were subjected to acrylamide gel electrophoresis at pH 8.3 and the resultant gel fractionated and assayed for collagenase and proteolytic activity (data not shown).

Based upon this result, purification was undertaken by preparative isoelectric focusing using narrow range ampholines (pH 4–6). After electrofocusing, fractions were assayed for collagenolytic activity and yielded a single sharp peak at pH 4.6, as shown in Fig. 1 (inset).

When this material was examined by analytical iso-



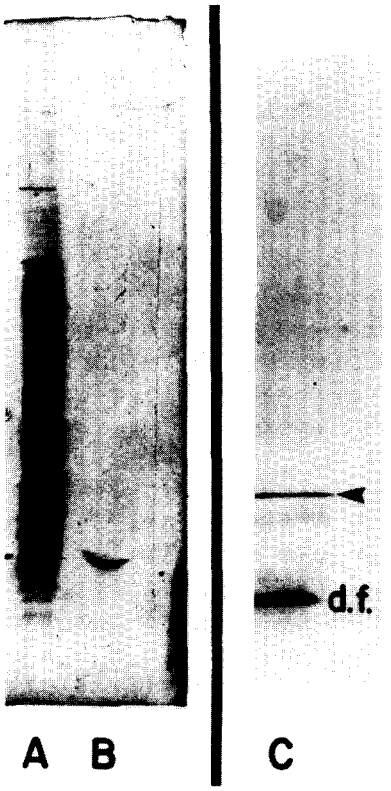


Fig. 1. Isoelectric focusing of the *Bipalium* collagenase. An aliquot of *Bipalium* extract (80 μg protein), concentrated by ultrafiltration, was applied to a pH 3.5–9.5-wide range ampholine PAG plate (LKB, Bromma, Sweden) and electrophoresed for 2 h. Gel segments (2.5 mm) were eluted overnight in 20 mM Tris-HCl, pH 7.6/5 mM $CaCl_2$ and aliquots were assayed for protein content (▲——▲), general proteolytic activity (●——●) and collagenolytic activity (■- - - - -■). Inset. Preparative isoelectric focusing. *Bipalium* extract (240 mg protein), partially purified by ammonium sulfate precipitation at 90% saturation, was applied to a 100-ml Ultrodex bed containing pH 4–6 ampholines prefocused by overnight electrophoresis. The sample was electrophoresed for 2 h and samples eluted and assayed for collagenolytic activity (○- - - - -○). The pH (●- - - - -●) of each sample was determined by elution with distilled water.

Fig. 2. Analytical isoelectric focusing. Aliquots of crude *Bipalium* extract (A) and purified *Bipalium* collagenase (B) were subjected to isoelectric focusing for 2 h at 5°C. Following electrophoresis the gels were stained with Coomassie Brilliant Blue R-250. The isoelectric point of the purified enzyme is pH 4.6. In part C an aliquot of the purified *Bipalium* collagenase (12 μg) was subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The *Bipalium* sample shows a single major band at 24 500 ± 1 000 (indicated by the arrow) as well as a faint band not reproducibly present, plus a strong band near the dye front (d.f.) due to ampholines remaining from isoelectric focusing.

electric focusing, the enzyme appeared to be essentially homogeneous, as shown in Fig. 2.

The major disadvantage of this rapid technique is the low yield of active enzyme. Overall yields varying from 6 to 15% of the original enzyme activity were obtained in four different experiments. The specific activity of the enzyme preparations was increased by 35- to 50-fold in these four experiments. Typically 250–500 μg enzyme was recovered from these experiments, with specific activity at 37°C of 0.4–0.45 mg collagen digested/min per mg enzyme protein. Since most collagenolytic enzymes are metalloproteases, it was hoped that the addition of $ZnCl_2$ or $CaCl_2$ might be useful in increasing the recovery of enzyme activity. However, the immediate addition of $ZnCl_2$ or $CaCl_2$ up to 1 mM to the fractions following elution failed to increase the amount of activity recovered.

Physical-chemical properties

The isoelectric point of the *Bipalium* collagenase was determined from the results of preparative isoelectric focusing (Fig. 1) as well as from analytical isoelectric focusing (Fig. 2) and was shown to be pH = 4.6 ± 0.1.

Molecular weight determinations of the enzyme were made by gel filtration on Ultrogel AcA54. The molecular weight of the native enzyme by this technique was 47 000 ± 2 000, which compares with a previous estimate of 52 000 obtained by Sephadex G-150 gel filtration [1].

Examination of the subunit structure of this collagenase was carried out by SDS pore-gradient gel electrophoresis [10]. The results of such experiments are shown in Fig. 2C. A single major band is obtained which has a molecular weight of approx. 24 500 ± 1 000 as determined by the use of appropriate standards. The heavy fast-running band near the bottom of the gels represents ampholines remaining from the preparative isoelectric focusing. The minor band at a molecular weight of approx. 21 000 ± 1 000 was not reproducibly present. When the 24 500 dalton band was removed from the gel, the protein extracted with 60% formic acid and subjected to acid hydrolysis with 6 M HCl, its amino acid composition was identical to that of the purified enzyme.

The amino acid composition of the collagenase was determined on purified enzyme isolated by isoelectric focusing, and is shown in Table I. These results represent triplicate analyses of three different collagenase preparations. The *Bipalium* enzyme is clearly rich in acidic amino acids as well as high in cysteine. The latter may be involved in disulfide bond formation since the enzyme is strongly inhibited by cysteine, 2-mercaptoethanol and glutathione but not by *N*-ethylmaleimide [1]. The amino acid composition of the *Bipalium* enzyme clearly differs from the invertebrate, *U. pugilator* [12] and *H. lineatum* [3], collagenases.

TABLE I

THE AMINO ACID COMPOSITION OF THE *BIPALIUM* COLLAGENASE COMPARED WITH THOSE OF HUMAN SKIN FIBROBLAST AND INVERTEBRATE COLLAGENASE

Results are presented as mol percent of total residues recovered. The analysis represents the average of triplicates on three different samples.

	Collagenase			
	Bipalium	Human [a]	*Uca* [b]	*H. lineatum* [c]
Asp	16.6	12.2	12.3	12.4
Thr	5.2	5.7	9.7	5.5
Ser	5.9	6.0	6.2	8.3
Glu	9.0	9.9	5.8	9.2
Peo	4.5	6.4	5.3	4.1
Gly	8.5	8.5	11.5	9.6
Ala	6.6	6.6	8.4	5.0
Cys	8.4	1.2	2.7	2.8
Val	4.3	5.1	9.3	8.7
Met	1.4	1.5	1.3	1.4
Ileu	2.7	4.4	7.1	8.3
Leu	5.2	6.2	5.8	6.4
Tyr	1.3	4.3	3.1	5.0
Phe	2.5	6.9	3.5	3.7
His	2.5	3.4	1.8	2.3
Lys	6.3	4.9	0.4	1.8
Arg	9.0	5.1	1.8	2.8
Trp	n.d.	1.9	1.8	2.8

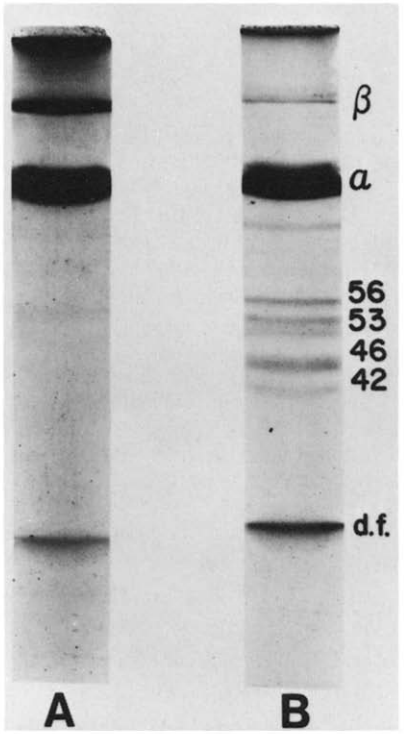
[a] Ref. 15.
[b] Ref. 12.
[c] Ref. 3.
n.d., not determined.

Specificity of Bipalium collagenase on collagens

We have previously shown that when the *Bipalium* collagenase is incubated at 24°C with guinea-pig skin

collagen, it initially converts cross-linked collagen β-chains to monomeric α-chains, followed by degradation of the collagen to dialyzable peptides [1]. At the temperature (24°C) used for those experiments, we were unable to detect discrete degradation products. However, more careful analysis using purified collagenase incubated with guinea-pig skin collagen at lower incubation temperatures (15°C) revealed the presence of distinct cleavage fragments by polyacrylamide gel electrophoresis (Fig. 3). These fragments were determined to consist of approx. 78, 56, 53, 46 and 42% of the original size of the collagen α-chains. The 78% fragment was not reproducibly present. Electron microscopy of segment-long-spacing crystallites [13] shown in Fig. 4 reveals the 42% fragment (which contains the C-terminus of the collagen molecule). We have not been able to detect the remaining fragments to date. The amino terminal amino acids of these products were analyzed by reaction with dansyl chloride and chromatography on polyamide sheets. The results shown in Fig. 5 demonstrate that the reaction products from *Bipalium* collagenase digestion contained primarily N-terminal glycine; although an apparent increase in dansyl lysine and arginine (large oval spot at the bottom of Fig. 5a) is seen, this is probably due to increased accessibility of these amino acids during digestion of the collagen by collagenase, and subsequent thermal denaturation of the fragments [1].

During feeding, *Bipalium* is able to digest the colla-



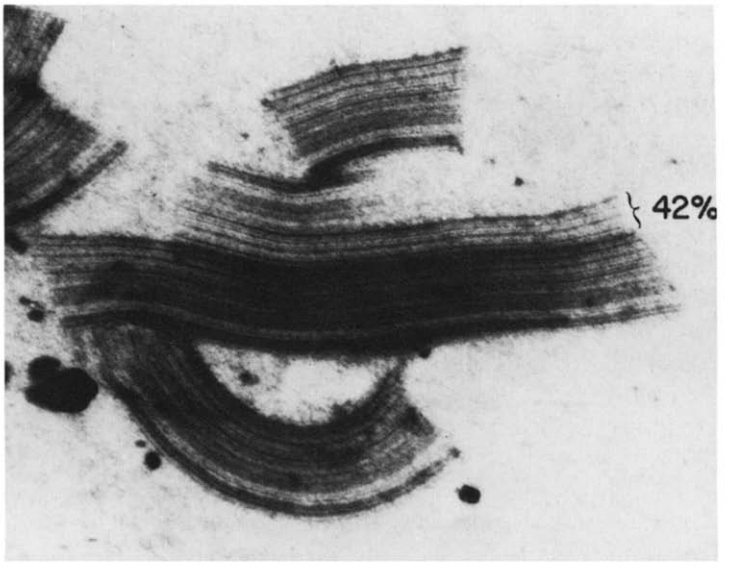


Fig. 3. Polyacrylamide gel electrophoresis of collagen degradation products. Purified *Bipalium* collagenase (6 μg) was incubated with salt-extracted guinea pig skin collagen (1 mg) at 15°C for 4 h. Samples were removed and electrophoresed by the method of Nagai et al. [9] and stained with Amido Black. Control collagen is shown in A, *Bipalium* collagenase-treated collagen in B. A conversion of β- to α-chains is seen as well as the appearance of new bands at approx. 78, 56, 53, 46 and 42% of the original α-chains. The 78% band was not reproducibly present.

Fig. 4. Segment-long-spacing crystallites. An aliquot of the material used in Fig. 3B was used to prepare segment-long-spacing crystallites [13]. The fragment representing 42% of the original collagen molecule, including the carboxyl terminal, is indicated by a bracket. Magnification ×42 500.

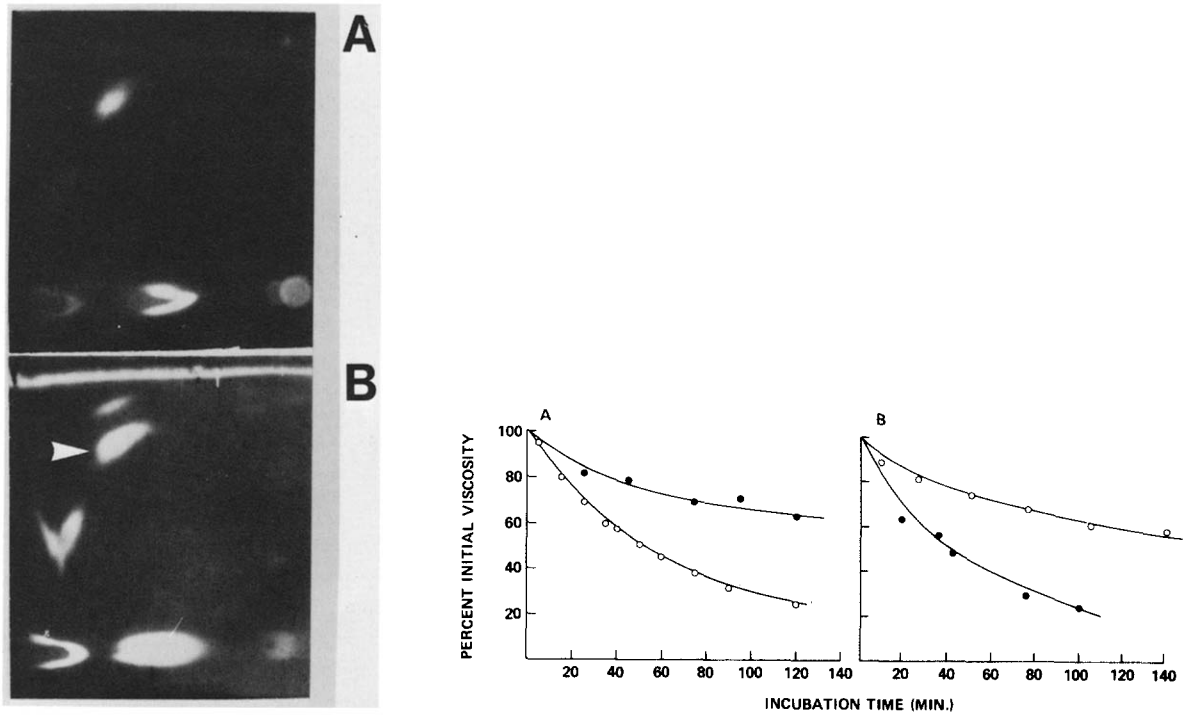


Fig. 5. Determination of N-terminal amino acids. The N-terminal amino acids of *Bipalium* collagenase-treated collagen were determined as described in Materials and Methods. The dansylated mixture was applied in the lower right hand corner. Equivalent amounts of undigested native (A) and collagenase-treated (B) collagens were used. The large spot indicated by the arrow in B corresponds to dansyl glycine, as does a much smaller spot in A. The other spots seen in B are dansyl hydroxide, dansylated lysine and arginine.

Fig. 6. Effect of *Bipalium* and *C. histolyticum* collagenases on collagen viscosity. *Bipalium* collagenase purified as described previously [1] and *C. histolyticum* collagenase purified as decribed by Peterkofsky and Diegelmann [8] were incubated with salt-extracted guinea-pig skin and earthworm cuticle collagens at 18°C. In A, 9.35 μg *C. histolyticum* collagenase was used with equimolar amounts (0.45 mg and 0.9 mg, respectively) of guinea-pig skin (o——o) and earthworm cuticle (•——•) collagens. In B, 17.5 μg *Bipalium* collagenase was incubated with the same collagen concentrations.

genous cuticle of its earthworm, *L. terrestris*, prey. It was, therefore, of interest to compare the action of the *Bipalium* collagenase on both earthworm and vertebrate (guinea-pig skin) collagens. In addition, the activity of bacterial (*C. histolyticum*) collagenase was also tested on the same substrates. In Fig. 6A is shown the relative activity of bacterial collagenase versus equimolar mixtures of guinea-pig skin and earthworm cuticle collagen, while in Fig. 6B is shown the relative activity of the *Bipalium* collagenase against the same concentrations of the two collagens.

Discussion

The land planarium, *B. kewense*, produces a collagenolytic enzyme which may function in the digestion of its natural prey, the earthworm *L. terrestris*. Previous work established that this enzyme was active only against collagenous substrates and that it resembled most vertebrate collagenases as a metalloprotease [1].

In these studies we report the purification of the *Bipalium* collagenase and examine some of its prop-

erties. Previous estimates of its molecular weight [1] as 52 000 by gel filtration on Sephadex G-150 were confirmed by estimates of 47 000 using Ultrogel AcA54. SDS-acrylamide gel electrophoresis revealed a single band with molecular weight of approx. 25 000, suggesting that the collagenase is dimeric and is made up of subunits with similar or identical molecular weights (Fig. 2C). In this respect the *Bipalium* enzyme differs from the *U. pugilator* [2] and *H. lineatum* [3] collagenases, whose molecular weights have been reported to be approx. 25 000. Although the vertebrate collagenases differ more widely in molecular weight, from 33 000 to approx. 55 000 for various human collagenases [14,15], they have been shown to be single polypeptide chains, as determined by SDS-polyacrylamide gel electrophoresis. In contrast, the bacterial enzymes from *Achromobacter iophagus* [4] and *Vibrio* B-30 [16] are reported to be dimeric proteins with molecular weights of approx. 70 000 and 105 000, respectively.

The isoelectric point of the *Bipalium* collagenase, 4.6 (Figs. 1 and 2) is considerably below that reported by Stricklin et al. [15] for the human collagenase, 6.7, but is consistent with the differences in amino acid composition of the two enzymes. Examination of the data in Table I demonstrates that the *Bipalium* enzyme is more abundant in acidic amino acids than the human skin fibroblast collagenase [15] and even more so than the collagenase from the fiddler crab, *U. pugilator* [2,12]. A major difference in cysteine content is seen between the human and *Bipalium* enzyme, as well as differences in aspartate, phenylalanine and tyrosine contents. On the whole, however, the amino acid content of the *Bipalium* enzyme resembles much more that of the human enzyme rather than that of the invertabrate, *U. pugilator*, collagenase.

The *Bipalium* collagenase also differs from the invertebrate enzyme isolated by Lecroisey et al. [3] from the insect, *H. lineatum*. While this latter enzyme also has an acidic p*I*, 4.10 and 4.15, and a high content of aspartic and glutamic acid, it differs from the *Bipalium* enzyme in amino acid composition and molecular weight (approx. 25 000). Keil and his coworkers [3,4] have suggested that the insect and crab enzymes may be related to the trypsin family of serine proteinases, and have recently demonstrated sequence homology between the *Hypoderma* collagenase and proteases of the trypsin family [5].

The specificity of the *Bipalium* collagenase appears to be similar to that of clostridial collagenase [17] in that the enzyme cleaves X-Gly bonds releasing N-terminal glycine (Fig. 5). Other bacterial collagenases, such as *A. iophagus* collagenase, also liberate N-terminal glycine from rat skin collagen [4,18] and *Vibrio* B-30 collagenase liberates primarily glycine from the bacterial peptide Z-Gly-Pro-Gly-Gly-Pro-Ala [16]. This is in contrast to the action of vertebrate collagenases which hydrolyze Gly-Leu(Ile) bonds [19].

Incubation of the *Bipalium* collagenase under controlled conditions produces distinct cleavage products, as shown in Fig. 3. The distinct nature of these products (rather than a smear of multiple degradation products) suggests that the collagenase is acting endoproteolytically. While the 75–25% cleavage seen with vertebrate collagenases is not observed with the *Bipalium* enzyme, we have been able to visualize the smallest (42%) of these degradation products by examination of segment-long-spacing crystallites (Fig. 4). The site of cleavage appears to be between bands 35 and 36 in the nomenclature of Bruns and Gross [13]. This site may be similar to a minor cleavage site for *C. histolyticum* [18]. As pointed out previously [1], incubation at higher temperatures and/or for prolonged periods of time led to digestion of guinea-pig skin collagen to dialyzable fragments.

Because *B. kewense* encounters earthworm collagen in vivo, it was of interest to test the relative activity of the collagenase on both guinea-pig skin and earthworm cuticular collagens. The data shown in Fig. 6B indicate that *Lumbricus* collagen is relatively more sensitive to the action of *Bipalium* collagenase than is guinea-pig skin collagen. This contrasts with our results (Fig. 6A) and those of Goldstein and Adams [20] and Kimura and Tanzer [21] who reported that invertebrate collagens from *Lumbricus* and *Nereis virens* were more resistant to digestion with clostridial collagenase than calf skin collagen. Our observations suggest that the susceptibility of earthworm cuticle collagen to *Bipalium* collagenase may reflect adaptation of the enzyme to its physiological substrate, although the biochemical basis for this observation is not known. Attempts to analyse the degradation products of earthworm cuticle collagen using the *Bipalium* enzyme, on 3% acryl-

amide gels in SDS, showed three main bands able to penetrate the gel while undigested collagen does not penetrate (unpublished observations).

We have shown that the *Bipalium* collagenase clearly has a number of properties in common with both bacterial and vertebrate collagenase, while differing from both groups of enzymes. This enzyme is very different from the other invertebrate collagenases described to date. Further studies on this unique collagenase may be useful in elucidating the structures of collagen and the mode of action of the collagenases.

Acknowledgements

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