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Pseudocollagenase: A protease from *Clostridium histolyticum*

The current definition of true collagenase activity requires that the enzyme degrade native collagen as well as the heat-denatured parent gelatin. However, numerous reports on the specificity of *Clostridium histolyticum* collagenase[1-4] enabled WÜNSCH[5] to synthesize an N-protected synthetic hexapeptide (Z-Gly–Pro–Gly–Gly–Pro–Ala) which GRASSMAN AND NORDWIG[6] first utilized as a specific collagenolytic substrate. The purpose of this communication is to report a proteolytic enzyme from *C. histolyticum* which rapidly degrades WÜNSCH's synthetic substrate as well as gelatin but is inactive against native collagen.

The crude material is commercially available as Worthington Biochemicals' Collagenase preparation CLS which is initially chromatographed (2 g in 100 ml 0.10 M sodium phosphate, pH 6.7) at room temperature on hydroxylapatite (Bio-Gel HTP) with a column having 2.5 cm × 12 cm dimensions at 60 ml/h. Following the initial peak, which is discarded, is a "step" buffer change to 0.20 M sodium phosphate, pH 6.7, when absorbance of the effluent falls below 0.1. The resultant peak is concentrated with Sephadex G-25 and chromatographed on a 3.5 cm × 125 cm column of Sephadex G-75 in 0.05 M sodium phosphate, pH 6.7, at 50 ml/h. Two distinct, well-separated peaks emerge. The first elutes in the column void volume and contains

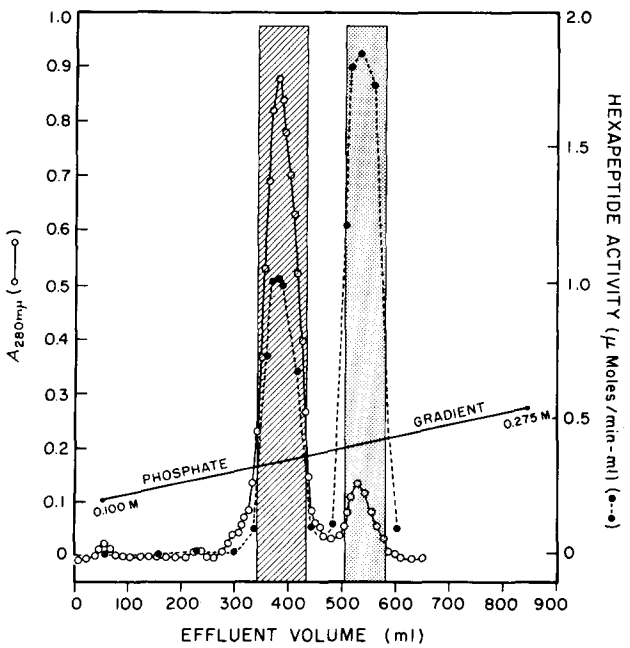


Fig. 1. Elution profile of Peak 1 (Sephadex G-75 chromatography) rechromatographed on hydroxylapatite. Shaded areas indicate the pooled fractions. Peak 1 is the collagenase fraction; Peak 2 is the pseudocollagenase fraction. Enzymatic activity against Z-Gly–Pro–Gly–Gly–Pro–Ala is indicated by ●---● on the right ordinate.

TABLE I

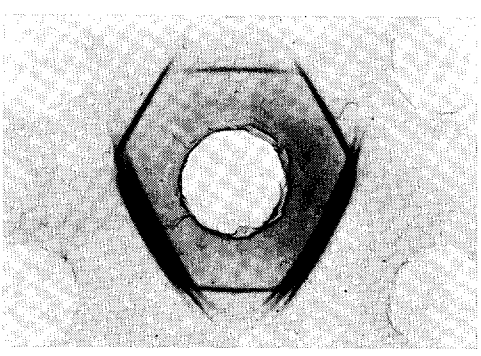
SUMMARY OF TYPICAL PURIFICATION DATA FOR PSEUDOCOLLAGENASE

Step	*Protein (mg)*	*Units of activity**	*Specific activity*	*Activity recovery (%)*
Crude	2000	780	0.39	100
Hydroxylapatite No. 1	281	365	1.3	47
Sephadex G-75	120	351	2.9	45
Hydroxylapatite No. 2	12	280	23	36
Hydroxylapatite No. 3	5.4	211	39	28

* Units of activity are expressed as μmoles Z-Gly–Pro–Gly–Gly–Pro–Ala hydrolyzed per min at 25° (100 mM sodium citrate, pH 6.3, containing 10 mM calcium acetate).

collagenases and pseudocollagenase. The second peak consists of clostridiopeptidase B (ref. 7). The collagenase peak is pumped directly on a second column of hydroxylapatite equilibrated at 0.05 M sodium phosphate using similar dimensions as previously described. Following column loading, a linear gradient from 0.100 M sodium phosphate to 0.275 M sodium phosphate in a total volume of 800 ml is utilized as column influent. Two peaks are resolved in this manner (Fig. 1). The first containing the major portion of the applied protein is the collagenase peak. The second peak containing the major fraction of the hexapeptide activity is the pseudocollagenase peak. Rechromatography of the material obtained from either peak results in elution in its proper chromatographic profile. Table I illustrates purification data for pseudocollagenase using the synthetic hexapeptide (Mann Research Laboratories, New York) as enzyme substrate.

Immunodiffusion and disc electrophoresis of the pseudocollagenase preparation indicates a high level of purity. With the immunodiffusion technique[8], a single band



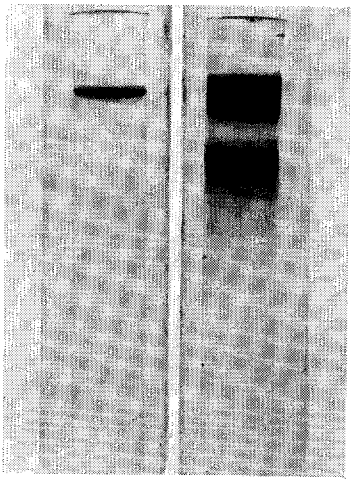


Fig. 2. Immunodiffusion of crude enzyme (No. 3 and 5), purified collagenase (No. 2 and 6), and pseudocollagenase (No. 1 and 4). No. 1 is the top well and No. 4 the bottom well.

Fig. 3. Disc electrophoresis of pseudocollagenase and crude enzyme at pH 9. Purified enzyme is on the left. Direction of migration is top (cathode) to bottom (anode).

can be demonstrated in the purified preparation with lines of non-identity against true collagenases from the same starting material (Fig. 2). Disc electrophoresis[9] shows essentially a single band as illustrated in Fig. 3.

Pseudocollagenase, in contradistinction to collagenase, has little if any proteolytic potential against native ichythyocol collagen. The enzyme possesses negligible activity against ichythyocol collagen in the standard collagenase viscometric assay[10]. Table II further demonstrates the lack of activity against native collagen. However, the activity against the parent gelatin is similar to that against the synthetic hexapeptide, Z-Gly–Pro–Gly–Gly–Pro–Ala, as well as the proteolytic potential of colla-

TABLE II

COMPARISON OF EFFECT OF PARTIALLY PURIFIED COLLAGENASE AND PSEUDOCOLLAGENASE ON THE SYNTHETIC HEXAPEPTIDE (Z-Gly–Pro–Gly–Gly–Pro–Ala) AND NATIVE ICHYTHYOCOL COLLAGEN AT 25° *versus* PROTEOLYTIC ACTIVITY ON ICHYTHYOCOL GELATIN AT 37°*

Enzyme	*25°*		*37°*
	Hexapeptide	*Ichythyocol*	*Ichythyocol*
Collagenase	1.2	3.6	13.5
Pseudocollagenase	13.7	0	14.2

* Reported as μmoles Gly–Pro–Ala ninhydrin equivalents released per min per mg enzyme, initial rates.

genase against ichythyocol gelatin. If collagenase is allowed to hydrolyze the synthetic hexapeptide to completion, subsequent addition of pseudocollagenase results in no further degradation of the substrate. In addition the latter shows no activity against Gly–Pro–Ala or Leu–Gly–Gly. Lyophilization completely inactivates the enzyme, whereas collagenase tolerates the procedure well. Pseudocollagenase is relatively stable in phosphate buffer, pH 6.7, but loses 75% of its activity after 24-h dialysis against water.

MANDL AND ZAFFUTO[11] suggested the existence of this protease by a differential immunological method, and a possible specific gelatinase was described in a brief communication by OGLE AND LOGAN[12]. The apparent specificity for randomly oriented collagenase substrates (*i.e.*, gelatin and the synthetic hexapeptide) but the inability to digest native collagen has stimulated adoption of the term, pseudocollagenase.

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A low molecular weight arginase in the earthworm

Mammalian liver arginase (L-arginine ureohydrolase, EC 3.5.3.1) has a molecular weight of about 140 000 (refs. 1, 2) whereas the molecular weights of the arginases present in chicken (*Gallus domesticus*) and lizard (*Ctenosaura pectinata*) liver and *Neurospora crassa* are around 280 000 (refs. 3, 4). In this report, we show that the arginase in the earthworm (*Lumbricus terrestris*) gut is unique in being a much smaller protein than any of the previously studied arginases. The metabolic role and certain properties of this enzyme have been described[5]. The molecular weight of earthworm arginase is estimated to be around 27 000. Because it is in the size range of several protein subunits[6], the earthworm enzyme could conceivably represent the natural occurrence of a "monomer unit" of arginase.

The molecular weight of earthworm gut arginase, present in unfractionated tissue extracts, was estimated from data obtained by density gradient centrifugation and gel filtration[7,8]. For gel filtration studies, gut tissue was homogenized in an equal volume 0.1% (w/v) cetyltrimethylammonium bromide with a Potter–Elvehjem tissue grinder. The homogenate was then diluted with a solution containing 50 mM Tris chloride (pH 7.5), 100 mM KCl and 5 mM $MnCl_2$ to give a final tissue concentration of 20% (w/v). This Tris–KCl–$MnCl_2$ solution was also used as the eluting buffer for the Sephadex columns. The diluted homogenate was centrifuged at 27 000 $\times$ *g* for 20 min at 0°. A portion (3–4 ml) of the supernatant fluid was placed on the column after sucrose (5 mg/ml) had been added to increase its density. For density gradient centrifugation, the gut tissue was homogenized in a solution containing 50 mM Tris chloride (pH 7.5) and 5 mM $MnCl_2$ to give a 20% tissue homogenate. This Tris–$MnCl_2$ solution was also used for preparing the sucrose gradients. The homogenate was centrifuged as above and a portion (0.2 ml) of the supernatant fluid was layered on the gradient. The same procedures were followed with rat liver and snail hepatopancreas, except that 10% tissue homogenates were prepared.

Columns of Sephadex G-100 (Pharmacia, Uppsala) were prepared and calibrated as described by ANDREWS[9]. The size of the column was 2.5 cm $\times$ 50 cm and the temperature was maintained at 0° with a water jacket. Elution was with the Tris–KCl–$MnCl_2$. solution and 3 ml fractions were collected. Gel filtration data were calculated as the parameters (K_D ref. 10) and K_{av} (ref. 11). Plots of either $K_D^{\frac{1}{3}}$ *vs.* Stokes radius or