BBA 65734

EXTRACELLULAR COLLAGENASE PRODUCED BY *STREPTOMYCES MADURAE*

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(Received January 2nd, 1968)

SUMMARY

1 A collagenase from *Streptomyces madurae*, specific for native collagen and its gelatin, is described. The enzyme is similar to that elaborated by *Clostridium histolyticum*

2 The pH optimum is around 7 5 and activity is irreversibly inhibited by cysteine and urea Although EDTA inhibits activity it is reversed by added calcium. Sulfhydryl-inhibiting agents have no detectable effect on activity.

3 The molecular weight of the enzyme (approx. 35 000) is smaller than the values reported for other bacterial collagenases.

INTRODUCTION

Collagenolytic activity was reported for *Streptomyces madurae* in 1964 (ref 1) and represented the second bacterial enzyme fulfilling the criteria of a specific collagenase as defined by MANDL[2]. The other bacterial enzyme, derived from *Clostridium histolyticum* and *C perfringens*, has been purified and characterized[3,4] A third bacterial collagenase has recently been reported by SCHOELLMANN AND FISHER[5] in strains of *Pseudomonas aeruginosa*. A collagenase of fungal origin was first reported for the dermatophyte *Trichophyton schoenleinii*[1]. The present communication describes the isolation and properties of collagenase from *S madurae*

EXPERIMENTAL

Substrate preparation. Newly weaned guinea pigs were first depilated with Nair and then sacrificed The trunk and back-skin was removed, scraped to remove excess flesh and fatty tissue, frozen in solid CO_2, and ground, using solid CO_2, to a fine powder in a Disposal tissue grinder After the solid CO_2 evaporated, the powder was dissolved in 3% acetic acid and allowed to stand overnight at 4°. The following procedures were carried out at 4° The material was centrifuged at 12 000 rev /min for 30 min and filtered The sediment after centrifugation was again shaken overnight with 3% acetic acid Following centrifugation and filtration, both filtrates were com-

bined To the filtrate was added 10% NaCl and after standing for 6 h in the cold, the solution was centrifuged at 12 000 rev /min for 30 min. The sediment was redissolved in 3% acetic acid after shaking overnight in a water bath A second salt precipitation was carried out and the sediment dissolved a third time in 3% acetic acid. After shaking overnight, the filtrate was centrifuged, the supernate dialyzed against 0.05% acetic acid for 24 h and lyophilized. The material was resistant to trypsin and pepsin at physiological pH and appeared homogeneous in electron micrographs, showing the characteristic banding and periodicity of reconstituted collagen fibers For viscosity studies, dried collagen was redissolved in 3% acetic acid, dialyzed against phosphate buffer (pH 7 5) ($I = 0$ 45), then 0.45 ionic strength NaCl. The particulate dried material was used for the ninhydrin–collagenase assay

Assay methods

(1) Hydrolysis of collagen The change in the flow rate of solubilized collagen (specific viscosity) with time in the presence of collagenase was determined by a method modified from SEIFTER *et al.*[4] Low shear Ostwald viscosimeters were used The reaction mixture contained 1 4 ml of dissolved collagen (1 mg/ml) and 0 1 ml of enzyme preparation (0 05–0 1 mg/ml) The increase in flow rate was determined at various periods at 30° Activity was expressed as the increase in flow rate (specific viscosity) per 0 1 mg enzyme protein. The ratio (η/η°) represents the time of flow of the reaction mixture at the end of 1 h relative to distilled water

The release of soluble hydroxyproline-containing units was determined for the reaction mixture following precipitation by 10% trichloroacetic acid in a ratio of 1 1 Free and bound hydroxyproline-containing units were determined by the method of PROCKOP AND UDENFRIEND[6] The release of ninhydrin-positive groups from particulate collagen was determined by a modification of the MANDL, KELLER AND MANAHAN[7] method 10 mg of particulate collagen were suspended in 5 ml phosphate buffer (pH 7 4, 0 067 M) containing 0 45% NaCl and 0 1 ml enzyme solution (1 mg/ml) The mixture was incubated on a Dubnoff shaking water bath at 37° for 18 h or for specified intervals The undissolved collagen was removed by filtration To 1 ml of filtrate was added 0 5 ml cyanide acetate buffer and 0 5 ml ninhydrin solution[8]. The reaction mixture was boiled 15 min followed by rapid cooling and addition of 5 ml of diluent (isopropyl alcohol–water in a ratio of 1:1) After cooling, the developed color was read in a Beckman DU spectrophotometer at 570 mμ Leucine was used as the standard and activity was expressed as μmoles of leucine equivalent released per unit time.

(2) Hydrolysis of Azocoll 20 mg of Azocoll (Worthington) were suspended in 4 9 ml 0 05 M Tris–HCl buffer and 0 1 ml of enzyme preparation (1 mg/ml) in Tris–HCl buffer was added The reaction mixture was incubated at 37° on a shaking water bath for 18 h or for specified intervals Unhydrolyzed Azocoll was removed by filtration and the red color of the solution was read at 540 mμ in a Beckman DU spectrophotometer The initial rate of dye release from Azocoll was expressed in Q units[9]

(3) Hydrolysis of casein 4 ml of 0 6% casein solution in Tris–HCl buffer (0 1 M, pH 7 8) were incubated with 0.1 ml of enzyme solution at 37° for various intervals The reaction was stopped by adding 2 ml of 25% trichloroacetic acid. The material was filtered and the filtrate read at 278 mμ, a modification of the method published by YOSHIDA AND NODA[3]. Crystalline bovine albumin was used as the standard, activity was expressed as mg protein released per mg enzyme protein per min.

Enzyme preparation

Streptomyces madurae was grown as microcolonies in a New Brunswick Microferm in trypticase soy broth in 12-l lots (at 30°, agitation at 300 rev /min, aeration at 4 l per min, 5 days) The organisms were separated by filtration and the filtrate brought to 40% saturation with solid ammonium sulfate The resulting precipitate was centrifuged and the crude enzyme preparation was dialyzed against distilled water and lyophilized. The enzyme was further purified using Sephadex G-100 and G-150 columns The columns (2 5 cm × 100 cm) were equilibrated with pH 7 5 Tris–HCl buffer (1 5 M) containing 4 mM $CaCl_2$ 300 mg of crude enzyme were usually dissolved in 4 ml of the same buffer and placed on the column. An elution rate of 15 ml per h was used and samples of 5 ml were collected. Each tube was read in a spectrophotometer at 278 mμ for protein concentration and then tested for enzymatic activity against Azocoll and collagen. Samples showing maximal activity were placed on a DEAE-Sephadex A-50 column (2 cm × 10 cm). Equilibration and elution procedures were first conducted with pH 7.5 Tris–HCl (0 05 M) buffer using an NaCl concentration-gradient ranging from 0 to 2 5 M In later experiments, elution was achieved with 5 mM Tris–HCl (pH 7.5) buffer followed by molarity gradients of 0.05 M Tris–HCl, 0 005 M Tris–HCl with 0 1 M NaCl, and 0 005 M Tris–HCl with 0 5 M NaCl All the above buffers contained 4 mM $CaCl_2$ Gradients were achieved using a Buchler Varigrad. Only peaks that showed one protein band corresponding to collagenase activity, as determined by disc and starch gel electrophoresis, were considered as the isolated enzyme

Molecular weight estimations were determined by the ANDREWS[10] method of calibrated gel filtration, by measuring diffusion constants in a Neurath cell at 27° (glycinate buffer, pH 10) (ref. 3) and by noting sedimentation constants in a Spinco Model E analytical ultracentrifuge (glycinate buffer, pH 10, $I = 0.1$) Densities were determined with an Ostwald pycnometer (pH 10, 25°, glycinate buffer)

The effect of inhibitors and the determination of the optimum pH were done by incubating the enzyme in the presence of the inhibitory agent or in the buffers and using the standard activity test for hydrolysis of collagen or by inclusion of the agent or pH buffer in the reaction mixture. Tris–HCl and phosphate buffers were used in these studies. There was no detectable difference in activity for these buffers.

RESULTS

Preliminary purification using ammonium sulfate precipitation indicated that most of the collagenase activity was found in the 50% saturated fraction In the stepwise increments of ammonium sulfate, most of the enzyme was precipitated in the 30% fraction (Table I) The various fractions were tested for increase in flow rate of solubilized collagen and gelatin, release of α-amino groups from particulate collagen, release of dye from Azocoll and digestion of casein. The half-time for increase in flow rate for collagen using the 30% fraction was found to be 60 min and for gelatin, 10 min

Further purification was achieved using Sephadex column chromatography On a Sephadex G-100 column the collagenase fraction still contained enzymatic activity for Azocoll and by disc electrophoresis gave 4 detectable protein bands. There was no activity against casein and no pigments were present in this preparation Using Sephadex G-150, the enzyme specific for collagen was separated from the material active against Azocoll and gave one detectable protein band by disc and gel electrophoresis

TABLE I

RECOVERY OF COLLAGENASE ACTIVITY FROM $(NH_4)_2SO_4$ FRACTIONATION OF CULTURE FILTRATE

Percent saturation with $(NH_4)_2SO_4$	*Increase in flow rate (specific viscosity)* (η/η°)	*Hydrolysis of collagen* (μmoles/5 mg protein)*	*Hydrolysis of Azocoll (Q units)*
0–30	2 63	2360	1377 6
30–40	8 47	350	62 7
40–50	12 97	310	4 48
50–60	13 41	240	—
60–100	13 13	—	—
Viscosity control	14 2		
Whole culture filtrate		617 0	208 32

* Collagen concentration 2 mg/ml

TABLE II

PURIFICATION OF COLLAGENASE USING SEPHADEX COLUMN CHROMATOGRAPHY

Assays	*Hydrolysis of collagen* (μmoles/mg protein)*	*Hydrolysis of casein (mg/mg protein)*	*Hydrolysis of casein with cysteine,* 10^{-2} *M (mg/mg protein)*	*Hydrolysis of Azocoll (Q units)*
Whole culture filtrate	198 5	3 1	2 41	353 9
Sephadex G-100 fraction	483 3	—	—	72 3
Sephadex G-150 fraction	15 500	—	—	—

* Collagen concentration 1 mg/ml

(Table II). This material eluted as a single peak from a DEAE Sephadex A-50 column This preparation was active against collagen and gelatin The rate of gelatin hydrolysis was approx. 10 times greater than for collagen. This preparation was also active against Z-glycyl-prolyl-glycyl-glycyl-prolyl-alanine and 4 phenyl-azo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Mann Research Laboratories, New York, N.Y) It was not active against casein, casein with cysteine, elastin, hemoglobin or Azocoll.

TABLE III

EFFECT OF pH ON COLLAGENASE ACTIVITY

pH	*Increase in flow rate (specific viscosity)* (η/η°)	*Hydrolysis of collagen* (μmoles/mg per h)*
5 5	2 39	4 53
6 5	1 98	8 62
7 5	1 71	20 12
8 5	1 27	22 14
Control	3 11	—

* Collagen 1 mg/ml Tris–HCl buffer with 1 5 M $CaCl_2$, 30°.

TABLE IV

EFFECT OF INHIBITORS ON COLLAGENASE ACTIVITY

Collagen 2 mg/ml Tris–HCl buffer with 1.5 M $CaCl_2$, 30°

*Inhibitor**	*Cysteine*	*Urea*	*p-Chloromercuribenzoate*	*Iodoacetate*	*EDTA*	*EDTA Ca^{2+}*	*Control*
Specific viscosity (η/η^0)	5 4	5 0	1 0	1 3	4·3	1 6	1 0

* Cysteine 0 05 M, Urea 7 M, *p*-chloromercuribenzoate 10^{-2} M, EDTA (sodium salt) 10^{-4} M, Ca^{2+} as $CaCl_2$ 4 mM, iodoacetate 10^{-2} M

The pH optimum was found to be a broad range from 7 5 to 8 5 (Table III). The activity curve could not be carried out at more acid or alkaline pH values as these denature the substrate Incubation of enzyme for 10 min in buffer more acid than pH 4 5 caused irreversible inactivation Above this pH, however, no effect was noted.

In the inhibition studies, preincubation of the enzyme in cysteine or urea irreversibly inactivated the enzyme. The inhibition shown by ethylenediamine–tetraacetic acid (sodium salt) was reversed by addition of calcium ions Neither iodoacetate nor *p*-chloromercuribenzoate (in glycyl-glycine buffer) had any effect on activity The observations are summarized in Table IV

With a value for V_e of 124 ml on Sephadex G-100 column the estimated molecular weight by the method of ANDREWS[10] was 35 000 ± 100. The $s_{20,w}$ was 3 285, the D_{20} (in centrifuge) was $8\ 1 \cdot 10^{-7}$ cm^2 sec^{-1}, and a partial specific volume ($\bar{v}$) 0 731 $cm^3 \cdot g^{-1}$ From these values a molecular weight of 36 100 ± 200 was calculated.

DISCUSSION

The collagenase from *S madurae* is similar to those isolated from other bacterial species The alkaline pH optimum, inhibition by cysteine and urea and lack of inhibition by *p*-chloromercuribenzoate and iodoacetate have been reported for the clostridial enzyme[2] The inhibition by EDTA can be reversed by replacement of calcium The effect of cysteine is considered a metal sequestering effect since this does not appear to be sulfhhydryl-containing enzyme

Several workers have recently established that 2 collagenases are elaborated by *C. histolyticum*[3,7,11] YOSHIDA AND NODA[3] were able to separate these enzymes and to demonstrate that they had different attack rates and sites on collagen One of the enzymes (collagenase II) was almost without activity for Azocoll and had a greater attack rate on collagen than the other. The streptomycete collagenase was easily separated from all Azocoll activity and appears to be a single unit with a molecular weight smaller than that for collagenases I and II of YOSHIDA AND NODA The recently reported fungal collagenase[2] from *T. schoenleinii* is quite different from the bacterial enzymes. The enzyme from *T schoenleinii* has a pH optimum of 6 5, is irreversibly inhibited by EDTA and has a molecular weight of about 20 000 (ref. 12).

The collagenase from *S madurae* has been shown to have a significant role in the pathogenicity of the organism[13] In experimental infections, mycetoma-like lesions and skin loss were produced by inoculation of the wild type organism Mutants unable

to elaborate the enzyme are also avirulent. Revertants with collagenase activity regained virulence

ACKNOWLEDGMENTS

This research was supported in part by U S Public Health Service grant No. AI-06444, Medical Training grant No TI-AM-5261 and Career Award (RCDA-Rippon) K03-A107926. The technical assistance of Mrs D AADERSON, Mrs M A. KRONKE and Mrs M KIDRON is gratefully appreciated

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