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# Extracellular Collagenase from *Trichophyton schoenleinii*

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The dermatophyte *Trichophyton schoenleinii* elaborates an extracellular collagenase which is specific for native collagen and gelatin and is separable from an enzyme which is active against Azocoll. The fungal enzyme differs from the bacterial collagenases in its acid *p*H and small molecular weight. In addition, the fungal enzyme is irreversibly inhibited by ethylenediaminetetraacetic acid, whereas the bacterial enzymes can be restored by divalent cations, such as $Mg^{++}$ and $Ca^{++}$.

In a recent review of microbial collagenases, Mandl (3) concluded that only the enzymes of *Clostridium histolyticum* and *Clostridium perfringens* were valid collagenases. The criterion for a specific collagenase is an increase in flow rate of solubilized undenatured collagen (referred to as decrease in specific viscosity) accompanied by a release of hydroxyproline-containing units. An extracellular enzyme produced by a streptomycete meets this criterion (6). The organism *Streptomyces madurae* is an etiological agent of maduromycosis in man, and collagenolytic activity was also noted for the dermatophyte *Trichophyton schoenleinii* (6), the first reported for a fungus. In 1966, Schoellman and Fisher (9) described a collagenic enzyme for a strain of *Pseudomonas aeruginosa* which had been isolated from an ocular infection. The present study is concerned with the isolation and characterization of collagenase from *T. schoenleinii*.

## MATERIALS AND METHODS

*Crude collagenase. T. schoenleinii* was grown in 12-liter lots, with Trypticase Soy Broth at 30 C for 5 days, in a New Brunswick Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.), with agitation at 300 cycles/min and aeration at 4 liters of air per min. The culture fluid was collected by filtration, brought to 40% saturation with solid ammonium sulfate, and allowed to stand at 4 C overnight. The precipitate was separated by centrifugation, dialyzed overnight against distilled water in the cold, and lyophilized.

*Substrate.* Highly purified acid-soluble mammalian collagen was prepared according to the method presented in a previous publication (6). This material was resistant to trypsin and pepsin at *p*H 7.5 and appeared homogeneous when examined in an electron microscope (the characteristic banding and periodicity of reconstituted fibers were seen). The assay of collagenase was based on the release of ninhydrin-positive material from particulate dried material. In the viscosity measurements, dried collagen was dissolved in 3% acetic acid, dialyzed first against phosphate buffer at *p*H 7.5 (0.45 ionic strength) and then against 0.85% sodium chloride (0.45 ionic strength).

*Assay for hydrolysis of Azocoll.* A 20-mg amount of Azocoll (Worthington Biochemical Corp., Freehold, N.J.) was suspended in 4.9 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer, and 0.1 ml of enzyme preparation (1 mg/ml) in the Tris chloride buffer was added. The reaction mixture was incubated in a shaking water bath at 37 C. After specified intervals, the unhydrolyzed Azocoll was removed by filtration and the red solution was read at 540 mμ in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The initial linear rate of dye release from Azocoll was expressed in Q units (3).

*Assay for hydrolysis of collagen.* The change in the flow rate of solubilized collagen (specific viscosity) with time, in the presence of collagenase, was determined by the modified Seifter method (10) with the use of low-shear Ostward viscometers. The reaction mixture contained 1.4 ml of dissolved collagen (1 mg/ml) and 0.1 ml of enzyme preparation (0.05 to 0.1 mg/ml). Increase in flow rate was observed at intervals during incubation at 30 C. Activity was expressed as increase in flow rate/0.1 mg of enzyme protein per unit time.

The release of soluble hydroxyproline-containing units was also followed in the above reaction mixture. After precipitation by the addition of an equal volume of 10% trichloroacetic acid, free and bound hydroxyproline was determined according to the method of Prockop and Udenfriend (5).

The release of ninhydrin-positive groups from particulate collagen was determined. A 10-mg amount of particulate collagen was suspended in 5 ml of 0.067 M phosphate buffer (*p*H 7.4) containing 0.45% NaCl, and 0.1 ml of enzyme solution (1 mg/ml concentration) was added. The mixture was incubated in a Dubnoff shaking water bath at 37 C for a specified

period of time, after which undissolved collagen was removed by filtration. To 1.0 ml of the filtrate, 0.5 ml of cyanide-acetate buffer and 0.5 ml of ninhydrin solution were added (7). This reaction mixture was boiled for 15 min, and then 5 ml of isopropyl alcohol in water (1:1) was immediately added. After shaking, the mixture was allowed to cool and was read in a Beckman DU spectrophotometer at 570 mμ. Leucine was used as the ninhydrin standard and activity was expressed as micromoles of leucine equivalent released per unit time.

*Assay for hydrolysis of casein.* A 4.0-ml amount of 0.6% casein solution in 0.1 M Tris buffer (*p*H 7.8) was incubated with 0.1 ml of enzyme solution (0.5 mg/ml of protein) for various periods at 37 C (11). The reaction was stopped by adding 2 ml of 25% trichloroacetic acid, the material was filtered, and the optical density (OD) of the filtrate was read at 278 mμ. Crystalline bovine albumin was used as the standard, and activity was expressed as milligrams of protein released per milligram of enzyme per minute.

*Gel filtration.* Filtrations were carried out in jacketed columns, 2.5 × 100 cm, by use of G-50, G-75, G-100, or G-200 Sephadex gels. The columns were filled with swollen gels and equilibrated with 1.5 M Tris chloride buffer (*p*H 7.5) containing 4 mM $CaCl_2$. Crude enzyme (300 mg), from the 40% ammonium sulfate fraction in 4 ml of Tris chloride-$CaCl_2$ buffer, was placed on the column. Flow rates of 15 ml/hr were used, and 5-ml samples were collected. Each sample was read spectrophometrically at 276 mμ for protein and was also tested for Azocoll and collagenase activity.

Further purification was achieved by use of diethylaminoethyl (DEAE)-Sephadex A-50 columns (2 × 10 cm). Equilibration and elution procedures were first conducted with 0.05 M Tris chloride buffer (*p*H 7.5) and an NaCl-gradient (Buchler Varigrad; Buchler Instruments, Inc., Fort Lee, N.J.) ranging from 0 to 2.5 M. Subsequently, 5 mM Tris chloride (*p*H 7.5), with molarity gradients of 0.005, 0.05, and 0.005 M Tris chloride-0.1 M NaCl, and 0.005 M Tris chloride-0.5 M NaCl, was used. All buffers also contained 5 mM $CaCl_2$.

*Molecular weight.* Estimates of molecular weight were made by use of calibrated gel filtration, according to the Andrews method (1). Diffusion constants were measured in a Neurath cell contained in glycinate buffer (*p*H 10) at 27 C (11), and sedimentation constants were obtained by use of a Spinco model E analytical ultracentrifuge (glycinate buffer, *p*H 10, $\Gamma/2 = 0.1$). Densities were determined with an Ostwald pycnometer (*p*H 10, 25 C, glycinate buffer).

*Inhibitors and pH optimum.* The effects of inhibitors and the determination of the optimal *p*H were assessed by incubating the enzyme in the presence of the inhibiting agent or in the buffers followed by a standard activity test. In a second series, the inhibiting agents or *p*H buffers were included in the reaction mixture. Tris chloride and phosphate buffers were used in the standard tests. Enzyme activity was the same in both buffers.

## RESULTS

Most of the proteolytic activity of *T. schoenleinii* culture filtrate for Azocoll, casein, and collagen was found in the 0 to 30% ammonium sulfate fraction (Table 1).

The fraction active for casein was eluted with the void volume of the G-50 Sephadex, followed by the fraction active for Azocoll. There was a complete separation of these proteases from the fraction specific for collagen (Fig. 1). The tubes showing peak activity for collagenase were further purified and were used for viscosity, reaction rate, and inhibition studies. Further purification was achieved on the DEAE A-50 Sephadex column. The peak corresponding to the collagenase activity was a single unit, as determined by disc electrophoresis. This preparation was used for further characterization studies.

The optimal *p*H for the decrease of viscosity and the release of ninhydrin-positive material

TABLE 1. *Proteolytic activity in ammonium sulfate fractions of culture filtrate*

| Percntage of saturation $(NH_4)_2SO_4$ | Hydrolysis of collagen (μmoles/hr per mg of protein) | Hydrolysis of Azocoll (Q units) | Hydrolysis of casein (mg/mg of protein) |
|---|---|---|---|
| 0–30 | 3.7 | 43.7 | 1.95 |
| 30–40 | 0.7 | 34 | 0.02 |
| 40–80 | — | 13 | 0.02 |

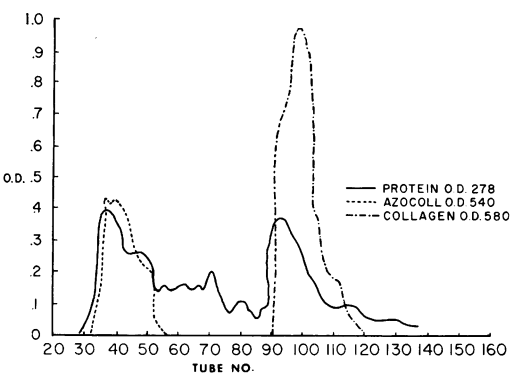


FIG. 1. *Gel filtration of collagenase with Sephadex G-50. A 2 × 100 cm jacketed column was loaded with 300 mg of crude enzyme in 4 ml of buffer. The elution buffer was 1.5 M Tris chloride (pH 7.5) containing 4 mM $CaCl_2$. A flow rate of 15 ml/hr was used, and each 5 ml was collected. Optical density (OD) of each sample was read at 276 mμ. Each sample was tested for hydrolysis of Azocoll (OD was read at 540 mμ) and for hydrolysis of collagen (OD was read at 580 mμ).*

was 6.5 (Table 2). Preincubation of the enzyme in a milieu with an unfavorable *p*H did not irreversibly alter the activity.

The effect of preincubating the enzyme in the presence of the inhibitors is summarized in Table 3.

Crude filtrate containing both Azocoll and collagenase activity was heated for 1 hr at 25, 37, 50, 80, and 100 C. Each preparation was then tested for activity against Azocoll and collagen. Azocoll activity diminished at 50 C and was not detected at 80 C. Collagenase activity, however, was only slightly altered at 80 C (Table 4).

A value for *Ve* on Sephadex G-100 of 143 ml gave an estimated molecular weight of 19,900 ± 200 (1) for the collagenase. The partial specific volume ($\bar{v}$) was found to be 0.689 ± 0.007. Substituting this figure in Schachman's formula (8), the value for the molecular weight of 20,100 ± 300 was obtained.

TABLE 2. *Effect of pH on viscosity reduction and α-amino release by collagenase*

| Assays | *p*H | | | |
|---|---|---|---|---|
| | 5.5 | 6.5 | 7.5 | 8.5 |
| Increase in flow rate[a] ($\eta/\eta^\circ - \eta/\eta$ 1 hr).... | 0.33 | 2.4 | 2.21 | 1.22 |
| Hydrolysis of collagen (μmoles/min per mg)..... | 0.50 | 3.0 | 1.80 | 0.78 |

[a] Boiled enzyme control, 0.02 (*p*H 7.5).

TABLE 3. *Effect of inhibitors on decrease in viscosity of collagen by collagenase*

| Assays | Inhibitors[a] | | | | | |
|---|---|---|---|---|---|---|
| | Cysteine | Urea | CMB | EDTA | EDTA ($Ca^{++}$) | IA |
| Increase in flow rate[b] ($\eta/\eta^\circ - \eta/\eta$ 1 hr)..... | 0.61 | 0.31 | 2.48 | 0.44 | 0.40 | 2.39 |
| Hydrolysis of collagen (μmoles/min per mg)..... | 0 | 0 | 4.2 | 0 | 0 | 4.0 |

[a] Cysteine, 0.05 M; urea, 7 M; *p*-chloromercuribenzoate (CMB), $10^{-2}$ M; ethylenediaminetetraacetic acid sodium salt (EDTA), $10^{-4}$ M; $Ca^{++}$ as $CaCl_2$, 4 mM; iodoacetate (IA), $10^{-2}$ M.
[b] Boiled enzyme control, 0.11 (*p*H 7.5).

TABLE 4. *Effect of heat on Azocoll and collagenase activity of crude enzyme preparation*[a]

| Assays | Temperature | | | | |
|---|---|---|---|---|---|
| | 25 C | 37 C | 50 C | 80 C | 100 C |
| Azocoll (OD 520)..... | 0.250 | 0.237 | 0.200 | 0 | 0 |
| Ninhydrin (OD 570).. | 0.225 | 0.275 | 0.290 | 0.190 | 0 |

[a] Solution of protein (1 mg/ml concentration) incubated at temperature for 1 hr, followed by assay at 37 C for remaining activity in usual manner.

## DISCUSSION

Three enzymes of microbial origin are now known to fulfill the criteria for specific collagenase. All these enzymes are from bacterial species: *C. histolyticum* (3), *C. perfringens* (3), *P. aeruginosa* (10), and *S. madurae* (6). The extracellular enzyme for *T. schoenleinii* is the first reported collagenase of fungal origin. Recent work has demonstrated a role for this enzyme in the pathogenicity of *S. madurae* (J. W. Rippon and G. Peck, *in press*).

The fungal enzyme is significantly different in several respects from the bacterial enzymes. The *p*H optimum of the collagenase of *C. histolyticum* and *S. madurae* is 7.4 in phosphate buffer (4), whereas the fungal enzyme has optimal activity at *p*H 6.5 in either phosphate or Tris chloride buffer. Incubation at either high or low *p*H does not irreversibly alter activity in the fungal collagenase, whereas incubation at low *p*H does irreversibly alter activity in clostridial collagenase.

In general, the tested inhibitors had the same effect on all the collagenases. All collagenases are irreversibly inhibited by cysteine and the hydrogen bond-breaking agent, urea. Since these are not SH enzymes, cysteine is probably acting as a metal sequestering agent. One difference was obtained for ethylenediaminetetraacetate, a chelating agent which inhibits all enzymes, but only irreversibly inhibits the fungal enzyme. Activity can be restored in the bacterial enzymes by divalent cations, such as $Ca^{++}$ or $Mg^{++}$, but this cannot be accomplished in the fungal enzyme. The —SH inhibitors, iodoacetate and *p*-chloromercuribenzoate, had no effect on any of the enzymes.

One difference between the Azocoll- and collagen-active enzymes was demonstrated by the effect of heat on their activity. Activity against

Azocoll is lost at temperatures lower than those which inhibit activity against collagen.

Harper et al. (2), Mandl (4), and Yoshida and Noda (11) have recently shown that *C. histolyticum* elaborates two collagenases. Yoshida and Noda (11) separated and characterized both enzymes. Collagenase II attacked collagen at a greater rate than collagenase I and was almost without activity for Azocoll. The fungal enzyme does not appear to include more than one molecular species and has no Azocoll activity. Yoshida and Noda reported molecular weights of 95,000 and 79,000 for collagenases I and II, respectively. The molecular weight of enzyme from *T. schoenleinii*, approximately 20,000, is quite small by comparison.

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