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Collagenase Production by Nematode-Trapping Fungi†

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A number of species of nematode-trapping fungi, which capture and digest nematodes having keratin and collagen in their cuticles, were tested for the ability to produce extracellular collagenase and keratinase. Collagenase, which is active on ichthyocol, earthworm collagen, and procollagen from chicken embryo fibroblasts, was found in the growth medium of all tested species; keratinase was not found. The enzyme from *Arthrobotrys amerospora* was concentrated by precipitation with $(NH_4)_2SO_4$ and further purified by adsorption on collagen at 0°C. The collagenase was active over a pH range of 2.5 to 10.0. It was not inactivated by dialysis against ethylenediaminetetraacetic acid for 48 h or by the sulfhydryl group inhibitors *N*-ethylmaleimide and *p*-chloromercuribenzoate. The production of collagenase may aid the fungus to penetrate the cuticle of its prey.

Nematode-trapping fungi are an unusual group of predatory microcarnivores (19). Although they can live saprophytically, they can also capture living nematodes which they kill and digest. The mechanism by which nematode-trapping fungi penetrate the surface of their prey has not been determined, but it is generally assumed to involve enzymatic action (4, 16).

Most studies of the chemical composition of the nematode surface, or cuticle, have been performed with ascarids because they are individually large and can be collected in quantity from the intestines of domestic animals which they parasitize. The ascarid cuticle is a three-layered, fibrous structure which contains collagen and keratin of types unique to the *Nematoda* (3). Collagens are among the most complex of proteins and are but slowly degraded in natural soils and waters (23). In *Ascaris* cuticular collagen, the basic units are triple-stranded helices extensively cross-linked with disulfide bonds (5) to form a large, rigid fiber with a molecular weight of about 900,000 (1). Unlike most other collagens, that in *Ascaris* cuticles has little hydroxyproline (23). Although there is little known about the collagen of nematodes other than *Ascaris*, it was reported that *Panagrellus* cuticles have a high ratio of proline to hydroxyproline and collagen chains ranging from 89,000 to 105,000 in molecular weight (1).

Collagenolytic enzymes have been isolated from vertebrate (8, 9) and invertebrate (18) animal tissues and from bacteria (20, 21), but reports of production of collagenase by fungi are relatively rare (10). Although many proteases can hydrolyze denatured collagen (gelatin), few act on the triple helix configuration of unmodified collagen (22).

The following studies were performed to determine whether nematode-trapping fungi produce extracellular collagenases or keratinases that might facilitate penetration by the fungi of the cuticle of nematodes which they have captured and killed.

MATERIALS AND METHODS

Preliminary screening of fungi for enzyme production. The following eight species of nematode-trapping hyphomycetes were tested: *Arthrobotrys conoides, Arthrobotrys amerospora, Arthrobotrys musiformis, Arthrobotrys dactyloides, Arthrobotrys flagrans, Arthrobotrys candida, Monacrosporium rutgeriensis*, and *Monacrosporium ellipsosporum*. Cultures were grown on cornmeal extract agar slants and maintained by transfer every 3 to 4 months. Large quantities of mycelium were produced in a liquid medium which contained 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of NH_4SO_4, 10.0 g of proteose-peptone no. 3, and 1.0 g of yeast extract in 1,000 ml of Sorensen phosphate buffer (pH 6.5). Cultures were incubated for 6 days with agitation at 28°C and then filtered by using Whatman no. 2 paper to separate the mycelium from the broth.

Samples of liquid medium in which the fungi had grown were tested against Azocoll (Calbiochem), a substrate for nonspecific proteases which releases a red dye into solution when degraded (13). A 25-mg amount of Azocoll was added to 5 ml of medium and incubated at 37°C for up to 6 h; the reaction mixture was filtered, and absorbance of the filtrate was measured at 520 nm. Control tests were performed by using boiled culture filtrates. Tests for the presence of keratinases in the liquid medium were performed in a similar manner by using 0.01 g of Keratin-Azure (Calbiochem) per 5 ml of medium sample and incubating

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at 37°C for up to 6 h. Keratin-Azure releases a blue dye upon hydrolysis. Absorbance was read at 595 nm.

Since Azocoll is a denatured collagen gel that can be hydrolyzed by nonspecific proteases, it was necessary to test the fungal growth medium further for its activity against undenatured collagen. This was done with procollagen from 17-day-old chicken embryo fibroblasts containing ^{14}C-labeled amino acids (17). Collagen, at levels of 10, 50, 100, or 200 μg, was incubated with 2 ml of fungal medium for 2 h at 37°C by the method of Peterkofsky and Diegelmann (17). Control tests were performed on labeled collagen in buffer and on labeled collagen in uninoculated growth medium. After incubation, the undenatured collagen was precipitated with trichloroacetic acid and the radioactivity of both the pellet and the supernatant was measured. The presence of radioactivity in the supernatant was viewed as evidence that collagenase was present and had acted to release soluble amino acids or peptides.

Enzyme purification. *A. amerospora* was used to study enzyme production because it grew rapidly and had high collagenase activity. Crude enzyme was recovered from the medium 6 days after inoculation. The broth was filtered to remove the fungal mycelium, 400 g of $(NH_4)_2SO_4$ was added per liter of filtrate (20), and the preparation was left undisturbed for 24 h at 4°C. The brown precipitate was concentrated by centrifugation, dissolved in 50 ml of distilled water, and dialyzed for 24 h against cold distilled water. The solution was then lyophilized and yielded approximately 0.2 g of crude enzyme preparation per liter of original medium.

The crude enzyme was partially purified by adsorption on ichthyocol (7). Equal volumes of a 1% ichthyocol solution and a 2% enzyme solution in distilled water were mixed at 0°C for 10 h and then centrifuged. The precipitate was washed three times with cold distilled water and then suspended in 10 ml of distilled water. Digestion of the collagen by the enzymes was evident after 1.5 h when the initially turbid preparation became clear. The solution was then dialyzed for 18 h against distilled water to remove amino acids and peptides and lyophilized.

Preparation of substrates. Ichthyocol was used as the test substrate. It is similar in some ways to *Ascaris* cuticle collagen (23) and can be easily prepared from cod swim bladders by the method of Gallop and Seifter (6). The preparation was lyophilized and stored at 4°C until used.

Methylated ichthyocol was prepared from cod swim bladder collagen by treatment with 37% aqueous formaldehyde and $NaBH_4$ (12, 14). Reductive methylation of the free amino groups made them unreactive to trinitrobenzenesulfonic acid and gave a much more sensitive test of collagenase action, since only the new terminal amino groups resulting from enzyme hydrolysis were measured.

Earthworm collagen was purified from the animal's cuticle (11). Freshly collected cuticular tissue was ground, stirred in 0.5 M NaCl for 48 h, and then centrifuged. A 25-ml amount of saturated $(NH_4)_2SO_4$ (pH 8) was added per 100 ml of the supernatant. The preparation was centrifuged, and the pellet was suspended in a small volume of 0.5 M NaCl and stored at 4°C.

Characterization of crude and partially purified enzymes. Collagenase activity was assayed by using methylated ichthyocol as the substrate at a concentration of 4 mg/ml of water. Typically, 1.0 mg (by weight) of crude of partially purified enzyme was added to 3.0 ml of ichthyocol suspension and incubated for 20 min at 37°C. A 2-ml amount of trinitrobenzenesulfonic acid and 2 ml of 4.0% $NaHCO_3$ were added to 2 ml of the sample, and the mixture was incubated for 90 min in the dark (12). Trinitrobenzenesulfonic acid combines with enzyme-generated α-amino groups to yield yellow picramide derivates of the amino acids. Finally, 2 ml of 10% sodium dodecyl sulfate and 1.0 ml of 1 N HCl were added, and absorbance was measured at 340 nm. Enzyme activity was determined by reference to a standard curve prepared by using alanine. Boiled enzyme was used as a control. When earthworm collagen or nonmethylated ichthyocol was used as substrate, the undigested protein was removed by precipitation with trichloroacetic acid and sodium deoxycholate before addition of the trinitrobenzenesulfonic acid and $NaHCO_3$ (2).

The presence of nonspecific proteinases in the collagenase preparations was determined by using casein as a substrate (7, 15). Casein was denatured by boiling in 0.05 M tris(hydroxymethyl)aminomethane (Tris) (pH 10). A 4-ml amount of the 1% casein solution (pH 7) was incubated with 1.0 mg of enzyme or 1.0 ml of fungal medium for 2 h at 37°C. The reaction was stopped, and casein was precipitated by adding 2 ml of 24% trichloroacetic acid. After centrifugation, the absorbance of the solution was measured at 280 nm.

RESULTS AND DISCUSSION

Culture filtrates from all eight fungal species grown on proteose-peptone were able to degrade Azocoll, but samples from the faster-growing cultures were the more active. Culture filtrates from only two fungi, *A. amerospora* and *A. flagrans*, degraded Keratin-Azure at all. However, the reaction was minimal, and, consequently, no further tests of keratinase activity were performed. *A. amerospora* was the most active producer of collagenase and was used in subsequent studies to purify and characterize the enzyme.

Incubation of culture filtrates with Azocoll at 37°C and at a series of pH values (pH 3 to 10) gave peak Azocoll degradation at pH 7.0. Dialysis of medium against 10^{-2} M ethylenediaminetetraacetic acid (EDTA) for 48 h did not reduce its ability to degrade Azocoll. Likewise, the sulfhydryl group inhibitors, *N*-ethylmaleimide and *p*-chloromercuribenzoic acid did not inhibit Azocoll degradation.

When *A. amerospora* culture filtrate was incubated with various concentrations of ^{14}C-labeled procollagen from 17-day-old chicken em-

bryo fibroblasts, the ratio of counts per minute of the supernatant to that of the pellet (undigested collagen) showed the collagen to be almost completely degraded in all cases.

Crude enzyme preparations precipitated from *A. amerospora* culture filtrate also degraded Azocoll. They were brown and contained nonspecific proteases in greater concentration than the original growth medium. They also were active on native ichthyocol, methylated ichthyocol, and earthworm cuticle collagen. Micro-Kjeldahl analysis of the crude enzyme gave an average nitrogen content corresponding to 34 mg of protein per 50 mg of sample.

When the enzyme was purified further by adsorption on ichthyocol, it was still brown, but the pigment was removed completely by treatment with diethylaminoethyl Sephadex (pH 6.7 or 8.5). Some nonspecific protease activity remained, but it was reduced from that present in the crude preparations. Although it is possible that *A. amerospora* collagenase degrades casein, the partially purified enzyme preparation was much less active than the crude preparation. This suggests that the fungal collagenase was contaminated with nonspecific proteases, which is in agreement with what others have observed in studies of collagenase from different sources (7, 15).

Crude enzyme was slightly more active against native ichthyocol than was the partially purified enzyme which contained less protease. When 1.0 mg of crude enzyme was incubated with ichthyocol for 20 min, the absorbance of the supernatant solution at 340 nm was 1.55. This was comparable to the absorption of a 0.35 mM solution of picramide-alanine. Partially purified enzyme (1.0 mg) gave a final reading of 1.19 or 0.2 mM alanine equivalent on the amino acid standard curve. Incubation of ichthyocol with *A. amerospora* collagenase for various lengths of time resulted in increased degradation with increasing time (Fig. 1).

A. amerospora collagenase activity was not reduced by dialysis against EDTA. This suggests that cations are not needed for activation of the enzyme. However, there is need to confirm this preliminary observation before concluding that the fungal enzyme differs from bacterial or tadpole collagenases, whose activation by calcium and zinc (7, 15) is destroyed by dialysis against EDTA. However, it does not rule out activation by more tightly bound metal ions. Incubation of the enzyme with the sulfhydryl group inhibitors *N*-ethylmaleimide and *p*-chloromercuribenzoate did not significantly reduce its ability to degrade ichthyocol.

When the crude enzyme was dissolved in 2.5 mM HCl (pH 2.6) for 10 min, its ability to degrade Azocoll at pH 7.0 was not significantly altered. However, lowering the pH from 2.6 to 2.4 caused a sharp, irreversible decrease in activity. By comparison, purified tadpole collagenase was irreversibly inactivated at pH 3.5 (15). The fungal enzyme was not inactivated by pH changes over the range of 2.6 to 10.0 but was irreversibly inactivated at pH 11.0.

Placing the enzyme solution in a water bath at a series of increasing temperatures for 10 min resulted in a diminishing capacity to degrade ichthyocol at 37°C (Fig. 2). The decrease in activity followed a sigmoid curve with the sharpest drop being between 60 and 70°C. The fungal enzyme is more heat stable than purified tadpole

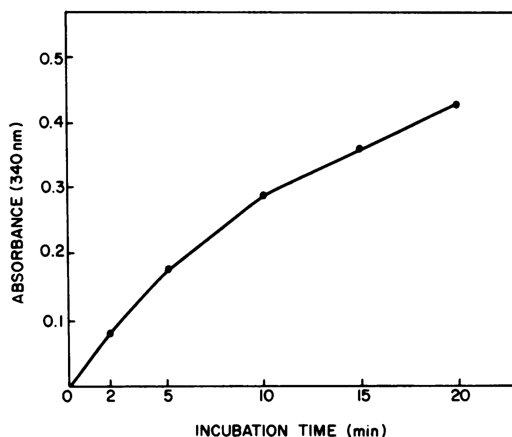

FIG. 1. *Hydrolysis of ichthyocol by A. amerospora collagenase. The amount of collagen hydrolyzed was measured spectrophotometrically as yellow picramide derivatives of enzyme-generated α-amino groups.*

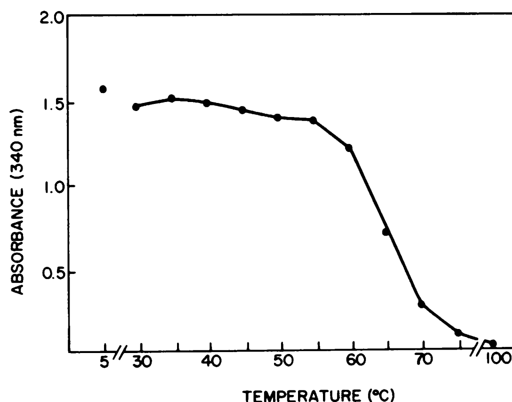

FIG. 2. *Temperature inactivation of A. amerospora collagenase. The effect of 10 min of incubation at various temperatures on the ability of the collagenase to hydrolyze ichthyocol at 37°C is as shown.*

collagenase, which was reported to be inactivated by heating at between 50 and 60°C for 10 min (15).

These results show that nematode-trapping fungi do produce, even on nonspecific media, a collagenase capable of degrading substrates similar to *Ascaris* collagen and, presumably, of degrading the collagen of their natural prey. It is not possible to determine from these studies if the fungal collagenase is constitutive or inducible. However, the presence of nematodes, the prey upon which the fungus can feed, is not essential for collagenase production.

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LITERATURE CITED

1. **Adams, E.** 1978. Invertebrate collagens. Science **202**:591–598.
2. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**:241–250.
3. **Bird, A. F.** 1971. The structure of nematodes. Academic Press, Inc., New York.
4. **Dowsett, J. A., and J. Reid.** 1979. Observations on the trapping of nematodes by *Dactylaria scaphoides* using optical, transmission and scanning-electron-microscopic techniques. Mycologia **71**:379–389.
5. **Evans, H. J., C. E. Sullivan, and K. A. Piez.** 1976. The resolution of *Ascaris* cuticle collagen into three chain types. Biochemistry **15**:1435–1439.
6. **Gallop, P. M., and S. Seifter.** 1963. Preparation and properties of soluble collagens. Methods Enzymol. **6**: 635–641.
7. **Gallop, P. M., S. Seifter, and E. Meilman.** 1957. Studies on collagen. I. The partial purification, assay, and mode of activation of bacterial collagenase. J. Biol. Chem. **227**:891–906.
8. **Gross, J., and C. M. Lapiere.** 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc. Natl. Acad. Sci. U.S.A. **48**:1014.
9. **Gross, J., and Y. Nagai.** 1965. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc. Natl. Acad. Sci. U.S.A. **54**:1197.
10. **Hurion, N., H. Fromentin, and B. Keil.** 1979. Specificity of the collagenolytic enzyme from the fungus *Entromophthroa coranata*: comparison with the bacterial collagenase from *Achromobacter iophagus*. Arch. Biochem. Biophys. **192**:438–445.
11. **Josse, J., and W. F. Harrington.** 1964. Role of pyrrolidine residues in the structure and stabilization of collagen. J. Mol. Biol. **9**:269–287.
12. **Lin, Y., G. E. Means, and R. E. Feeney.** 1969. The action of proteolytic enzymes on *N,N*-dimethyl proteins. J. Biol. Chem. **244**:789–793.
13. **Mandl, L., J. D. MacLennan, and E. L. Howes.** 1953. Isolation and characterization of proteinase and collagenase from *Cl. histolyticum*. J. Clin. Invest. **32**:1323–1329.
14. **Means, G. E., and R. E. Feeney.** 1968. Reductive alkylation of amino groups in proteins. Biochemistry **7**:2192–2201.
15. **Nagai, Y., C. M. Lapiere, and J. Gross.** 1966. Tadpole collagenase. Preparation and purification. Biochemistry **5**:3123–3130.
16. **Nordbring-Hertz, B., and M. Stahlhammar-Carlemalm.** 1978. Capture of nematodes by *Arthrobotrys oligospora*, an electron microscope study. Can. J. Bot. **56**:1297–1307.
17. **Peterkofsky, B., and R. Diegelmann.** 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry **10**:988–993.
18. **Phillips, J., and M. H. Dresden.** 1973. A collagenase in extracts of the invertebrate *Bipalium kewense*. Biochem. J. **133**:329.
19. **Pramer, D.** 1964. Nematode-trapping fungi. Science **144**: 382–388.
20. **Seifter, S., and P. M. Gallop.** 1962. Collagenase from *Clostridium histolyticum*. Methods Enzymol. **5**:659–665.
21. **Seifter, S., P. M. Gallop, L. Klein, and E. Meilman.** 1959. Studies on collagen. II. Properties of purified collagenase and its inhibition. J. Biol. Chem. **234**:285–293.
22. **Seifter, S., and E. Harper.** 1970. Collagenase. Methods Enzymol. **19**:613–635.
23. **Weiss, J.** 1976. Enzymatic degradation of collagen. Int. Rev. Connect. Tissue Res. **7**:101.