Determination of Fungal Elastase

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The elastin-agar plate is useful for determining elastolytic activity. Determination of fungal elastase, however, requires partial purification of culture extracts and the orcein elastin or gravimetric method.

The first report of the occurrence of elastase among species of fungi was made from this laboratory in 1967 (1) and 1968 (2). For survey purposes, the organisms were maintained on Trypticase soy agar (Difco) at 25°C and transferred on a Czapek-Dox agar (Difco) plate containing 1% elastin. Elastase activity was defined as a zone of clearing 3 mm around a growing colony within 5 days. Since that time, we and others have encountered difficulties and inconsistencies even with our standard strains in regard to elastase activity by this method. This has prompted an investigation into the factors that influence elastolytic activity, elastase production, and more accurate methods of enzyme assay.

Growth of many fungi on media containing animal protein induces the formation of several proteolytic enzymes, some of which also attack elastin. For this reason stock cultures have been maintained on Czapek-Dox agar, Trypticase soy agar, or potato dextrose-agar. Several fungi, such as some dermatophyte strains, digest elastin incorporated in Czapek-Dox agar. Strains of the same species often show no clearing of elastin agar initially, but clearing occurs eventually. Other strains do not show clearing even after 6 weeks of incubation. Organisms showing these several reaction patterns were grown in Czapek-Dox broth (supplemented with 0.1% yeast extract) and tested for the production of extracellular elastase. Previous work has shown a uniformity of the elastase isolated from several fungal species as to inhibitors and pH optimum (2). Advantage was taken of the selective inhibition of proteolytic and elastolytic activity found for pancreatic elastase, depending on the pH of the reaction mixture (3).

Spores (10⁶) of Allescheria boydii were inoculated in 100 ml of Czapek-Dox (supplemented with 0.1% yeast extract) in a 250-ml flask and grown for 3 weeks with shaking in a water bath at 25°C. At the end of this time, the mycelial mat was separated from the culture broth by filtration through Whatman no. 3 filter paper.

Culture filtrate was brought to 30% saturation with solid ammonium sulfate. After being allowed to stand overnight at 4°C, the precipitate was centrifuged and contained no elastase activity when estimated by the elastin-orcein method (2).

The supernatant fluid was brought in sequence to 70 and 100% (fraction AFIV) saturation with ammonium sulfate and similarly processed. The sediment from the 70% saturation was redissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5. Acetone was added (at 4°C) to a concentration of 44%, and the sediment was removed (AF I). The supernatant fluid was brought to 64% acetone concentration, again at 4°C, the sediment was removed (AF II), and the supernatant fluid was dialyzed at 4°C and lyophilized (AF III). When tested for elastase activity, all sediments were redissolved in 0.05 M Tris buffer (pH 8.0). The concentration was 1 mg/ml. A 0.5-ml amount of enzyme solution was placed in a 25-ml Erlenmeyer flask with 1 ml of the same buffer and 20 mg of elastin (alkali prepared, Worthington Biochemical Corp.). The reaction mixture was incubated for 20 hr at 25°C and stopped by the addition of 2 ml of 0.5 M phosphate buffer (pH 6.0). The undissolved elastin was filtered, washed, dried, and weighed (Table 1).

The enzyme in fraction AF II was further purified on a diethylaminoethyl cellulose column dissolved in 4 ml of 0.05 M Tris buffer (pH 8.5). It was eluted by an 0.05 M Tris buffer sequence (pH 8.5, 8.0, 7.5, 7.0, and 7.0) with 0.05 M NaCl followed by 0.1, 0.15, 0.2, 0.4, and 0.4 M NaCl. Fractions (4 ml) were collected and tested for elastase activity which was concentrated in the 0.05 M Tris buffer (pH 8.0) eluate. Similar preparations from the (+) mating type of Nannizzia fulva (six isolates), the (a) mating type of Arthroderma benhamii (three isolates), Allescheria boydii (four isolates), Trichophyton schoenleinii (three isolates), Trichophyton verrucosum (three isolates), and Ajellomyces dermatitidis 784 contained elastase activity but did not digest casein. Two of the A. benhamii A isolates and one of the N. fulva showed some clearing on elas-
Table 1. Digestion of elastin by crude fungal extracts of Allescheria boydii

| Fraction | Elastin dissolved (mg) |
|----------|------------------------|
| AF I (44% acetone fraction sediment) | 3 |
| AF II (64% acetone fraction sediment) | 10 |
| AF III (64% acetone fraction supernatant fluid) | 2.1 |
| AF IV (70-100% ammonium sulfate fraction) | 0.0 |

Elastin plates not exceeding more than 2 mm beyond the colony edge at 14 days. The above cultures and the *A. dermatitidis* had activity against casein at pH 7.0. Several other dermatophytes *Epidermophyton floccosum*, *Microsporum canis*, *Microsporum van breuseghemii* x 322, and *Arthroderma quadrifidum*, as well as some soil fungi such as *Aspergillus terreus*, *A. sydowii*, *Fusarium oxysporum*, and *Penicillium lilacinum*, were also grown and their culture filtrates were processed similarly. They were tested for elastase activity by the gravimetric method and were found to contain no activity.

However, several of the above showed clearing of agar plates containing particulate elastin.

An incorporation of 0.1% elastin in the broth quantitatively increased the yield of both proteolytic activity as measured by casein digestion and elastase in fungi that produce elastase and proteolytic activity in fungi that do not elaborate elastase. Whereas 100 units per mg of N elastase (3) were obtained in broth without added elastin, the increase was 10-fold in most strains and 100-fold with *N. fulva* (+). As has been noted before, the amount of elastase extractable with 0.85 M NaCl (2) is much greater in agar plates than in broth cultures.

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