SHORT COMMUNICATIONS

Degradation of Cysts of *Hartmannella culbertsoni* by Culture Filtrates of *Alternaria* sp.

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Intestinal or hepatic amoebiasis caused by *Entamoeba histolytica* is estimated to affect about 10% of the world population (W.H.O. Expert Committee on Amoebiasis, 1969). Free living amoebae such as *Acanthamoeba* and *Naeglaria* are now implicated in fatal meningo-encephalitis in man (Culbertson, Ensminger & Overton, 1966; 1968). Although a number of drugs are available which are effective against the vegetative or trophic form of pathogenic amoebae, the discovery of a cysticidal agent remains a challenge. With the urgency of the need for cysticidal agents in the eradication of amoebiasis, the W.H.O. Expert Committee on Amoebiasis (1969) has stressed the importance and relevance of basic studies directed towards the understanding of the nature of the amoebic cyst wall and the process of encystation and excystation.

Employing the free-living amoeba *Hartmannella culbertsoni*, previous studies from this laboratory have shown that trophozoites can be transformed axenically into viable cysts by exposure to taurine and several other biogenic amines (Raizada & Krishna Murti, 1971*a*; Raizada, Verma & Krishna Murti, 1972). These amines exert their triggering action on encystation through the intermediary agency of cyclic 3',5'-adenosine monophosphate (Krishna Murti, 1971; Raizada & Krishna Murti, 1972*a*; Raizada *et al.* 1972). These cysts differ from trophozoites in having cellulose and mucopolysaccharides as their major cell components (Raizada & Krishna Murti, 1971*b*; 1972*b*). Availability of enzymes degrading the cyst wall should lead to improved understanding of the localization of these components and the chemical linkages between them.

An enrichment culture technique has been employed, using cysts of *Hartmannella culbertsoni*, to isolate organisms able to degrade the cysts. The present report summarizes the experiments on the isolation, characterization and cystolytic activity of *Alternaria* sp. capable of lysing the cysts of *H. culbertsoni*.

Cysts of *Hartmannella culbertsoni* (Singh & Das, 1970) were prepared by methods described earlier (Raizada & Krishna Murti, 1972*b*). Portions of 50 mg wet wt of cysts were suspended in 20 ml 50 mM-phosphate buffer (pH 5.0 for fungus and pH 7.0 for bacteria), autoclaved at 15 lb/in² for 20 min and cooled; to this was added 0.2 ml of a salt solution containing (g/l): MgSO₄·7H₂O, 20; FeSO₄·7H₂O, 5; CaCl₂, 2.0; MnCl₂, 0.2; sodium molybdate, 0.1. The flasks were inoculated with soil samples and incubated with shaking at 30 °C. Samples were removed and examined periodically for disappearance of cysts. A fungus grew in one flask, which when inoculated into fresh medium containing cysts resulted in the complete disappearance of cysts in 3 to 4 days. This fungus was isolated by repeated streaking on potato-dextrose agar medium (Difco, Detroit, Michigan, U.S.A.) and was stored as spores preserved in sterile sand.

Fig. 1. Release of protease, cellulase and chitinase in culture filtrate of *Alternaria* sp. grown on cysts of *H. culbertsoni*. ■, Protease (according to Jönsson & Martin, 1965): 1.5 ml of reaction mixture, consisting of 0.5 ml of 1% (w/v) casein in 200 mM-acetate buffer, pH 5.0, and 1.0 ml of culture filtrate, were incubated at 37 °C for 60 min and deproteinized by 10% trichloroacetic acid, and tyrosine was estimated. Activity is expressed as mg tyrosine liberated in 60 min by 100 ml of culture filtrate. ●, Cellulase (according to Pettersson & Porath, 1966): 2.0 ml of reaction mixture consisting of 1.0 ml of 1% (w/v) carboxymethyl cellulose in 50 mM-acetate buffer, pH 5.0, and 1.0 ml of culture filtrate, were incubated at 37 °C for 60 min and deproteinized by barium-zinc precipitation, and reducing substances were estimated according to the method of Park & Johnson (1949). Activity is expressed as μg glucose liberated in 60 min by 100 ml of culture filtrate. ▲, Chitinase (according to Jeuniaux, 1966): 2.0 ml of reaction mixture consisting of 0.5 ml of 0.5% (w/v) colloidal chitin and 0.5 ml of 200 mM-acetate-buffer, pH 5.0, were incubated at 37 °C for 60 min and deproteinized by barium-zinc precipitation, and reducing substances were estimated. Activity is expressed as μg N-acetyl glucosamine liberated in 60 min by 100 ml of culture filtrate.

The fungus grew well and sporulated on potato-dextrose agar medium. Colonies were circular, greenish to greenish black, and on ageing turned black. The mycelium was richly branched with sterile, creeping, septate hyphae, 3 to 5 μm in diameter. Conidiophores were straight to slightly curved, septate and 15 to 20 μm in length. The apical region of the conidiophore was bluntly rounded and slightly swollen. Each conidiophore bore a chain of 3 to 5 conidia at its apex and a geniculate by lateral growth of its apical cell. These extensions gave rise to further chains of conidia. Conidia were greenish black 20 to 40 μm long and 8 to 12 μm in diameter; ellipsoidal, ovoid, obclavate or obpyriform; multicellular, muriform, with 2 to 7 transverse septa and 1 to 3 longitudinal and slightly oblique septa. The conidium wall was distinctly constricted at major transverse septa. The majority of conidia had tapering beaks 5 to 18 μm long.

We place the fungus in the genus *Alternaria* Nees ex Fries (Gilman, 1950; Funder, 1953; Simmons, 1967).

The fungus grew on the cysts of *Hartmannella culbertsoni* and utilized them as the sole source of carbon and nitrogen. On the tentative assumption that the major polymers forming the cyst wall are proteins, mucopolysaccharides and cellulose (Neff & Neff, 1969; Raizada & Krishna Murti, 1972b), it was considered likely that the fungus exerted its cystolytic action by secreting proteases, mucopolysaccharidases and cellulase into the culture filtrate while growing on the cysts. The activities of these three enzymes were therefore assayed in culture filtrates. The fungus was inoculated into a medium containing 2.5 g wet wt of cysts in 100 ml of medium and incubated with shaking at 30 °C. At intervals the activity of the three enzymes was assayed in samples of culture filtrates (Fig. 1).

Protease activity appeared early, reached a maximum on the second day and then declined. Cellulase also appeared early, reached a peak value on the fifth day and then declined. In contrast, the chitinase activity appeared only on the sixth day and remained at the same level on the seventh day. Culture filtrates from the second day (maximum protease activity), fifth day (maximum cellulase activity) and seventh day (maximum chitinase activity) were collected and seitz filtered. Individually these fractions did not cause any change in the morphology of *H. culbertsoni* but when added together, they reduced the cysts to unrecognizable debris within two to three days.

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