Enzymes of *Entamoeba hisolytica* I. Gelatinase.* (24642)

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Relatively little is known regarding the enzymatic makeup of *Entamoeba histolytica*. Amylase(1-3), phosphomonoesterase(4), glutaminase(5), maltase(2), esterase(6), succinic dehydrogenase(7), and proteolytic enzymes (8-10) have been reported in cultural forms of the amoeba. Since it is necessary to determine enzyme constitution of an organism to elucidate its metabolic pattern, it was considered important to make a systematic study of enzymes in *E. histolytica*. It is realized that enzymes present in amoebae *in vivo* may not be produced in culture. A case in point would be hyaluronidase, which is absent in amoebae maintained in cultures over a long time, yet has been detected in hamster liver abscesses (11). Until an axenic culture of *E. histolytica* becomes available, many studies dealing with enzymes, metabolism, and growth requirements will be beclouded by the activity of associate bacteria, minimal though it may be. However, with adequately controlled experiments, vital information can be obtained which may directly or indirectly lead to cultivation of amoebae in pure culture freed of all other living systems. In the study of enzymes, fairly large amounts of cell material are required; the bacteria-inhibited, growth-factor fortified culture method recently developed by Nakamura(12) seemed appropriate for obtaining large numbers of amoebae, which can be further purified by repeated washing and centrifugation. The reevaluation of enzyme gelatinase appeared valuable since information regarding the role of protein in amoebic nutrition is so poorly understood and also because information on proteolytic enzymes of *E. histolytica* may lead to a new approach regarding the pathogenic component of this protozoan parasite(13). Since most earlier work on presence of gelatin-hydrolyzing enzymes was performed with tubes containing high concentration of gelatin, some doubt exists as to whether liquefaction of gelatin was due to amoebic or bacterial associate activity. Employing a sensitive microtest for gelatinase developed by Thirst(14) and an agar-gelatin plate test developed by Smith(15), 6 strains of *E. histolytica* were studied for their ability to hydrolyze gelatin. Numerous control experiments and variations of the tests were included to show unequivocally that enzyme activity was due to amoebae.

Materials and methods. Six strains of *E. histolytica* (HUS-105, HUS-100, Washington, Griffith, ROK, and Conrad) were used. The HUS-100 and HUS-105 strains, both large races, were obtained from Dr. Chia-tung Pan; the other strains, all small races, were obtained from Dr. Russell M. McQuay, Jr. The amoebae were routinely carried on coagulated egg slants overlaid with Ringer-serum solution. Several methods were employed for detection of gelatinase. The microtest consisted of a thin film of gelatin as substrate on a glass slide; slides were prepared by dipping clean, dry microscope slides into melted aqueous gelatin solutions containing phenol, draining, and drying. Suspensions of amoebae were placed on the slides, which were incubated at 20°C for 12-24 hours in Petri dishes containing strips of moistened filter paper, to minimize evaporation. Hydrolysis of gelatin on these slides was indicated by appearance of clear areas on the slide, when stained with dilute carbolfuchsin, a protein stain. The presence of phenol prevented bacterial contamination without affecting gelatinase activity. The second method consisted of preparing agar places containing 0.4% gelatin upon which was placed the material to be assayed. Large drops of amoebae in suspension were placed on the surface of the gelatin-agar plates. The

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plates were incubated at 37°C for 12-24 hours, then flooded with approximately 10 ml of a solution of mercuric chloride in hydrochloric acid, a gelatin precipitant. A white precipitate indicated presence of gelatin and absence of precipitate (clear areas on plates) indicated gelatin hydrolysis. Amoeba materials assayed were prepared in several ways: (a) amoebae grown in absence of multiplying bacteria, with the aid of growth factors, adenosinetriphosphate and ribose-5-phosphate, and large concentrations of antibiotics, were pooled and washed with Ringer solution 5-7 times and finally made up into standard suspension containing 200,000 to 250,000 amoebae/ml; (b) pooled amoebae from stock cultures grown in association with a mixed bacterial flora were washed 6 times; portions of supernate from third and sixth washings and the sediment from sixth washing were assayed; (c) *E. histolytica* grown with *Escherichia coli* (a monobacterial associate) was used; *E. coli* does not hydrolyze gelatin. Control experiments consisted of: (a) placing drops of Ringer solution on slides and plates; (b) use of *Proteus vulgaris* and trypsin to detect gelatin hydrolysis; (c) inoculation of associated flora from amoeba cultures into conventional gelatin tubes to detect liquefaction of gelatin by the mixed bacterial flora; (d) destruction of amoebic gelatinase by autoclaving and inhibiting the enzyme with 20% copper sulfate and (e) alteration of enzymatic activity by alternate freezing and thawing of amoeba suspensions. Finally, to determine optimum pH for amoeba gelatinase by studying the enzyme at different pH values using phosphate buffer solutions.

Results. All 6 strains of *E. histolytica* hydrolyzed gelatin. The mixed bacterial flora were also capable of hydrolyzing gelatin. However, the aboebae were washed 5-7 times and the supernatant from final washing, which could conceivably contain a few bacterial cells, did not hydrolyze gelatin; therefore, it is evident that the activity was due to amoebae alone and not due to residual bacterial enzyme. After third washing, the supernate was without gelatinase activity. The amoebae also hydrolyzed gelatin when grown in a monoassociated culture with *Escherichia coli,* a species known not to hydrolyze gelatin. Amoebic gelatinase activity was inhibited by heat, copper sulfate, and freezing and thawing. The washed amoeba suspensions were diluted with saline to obtain a ½, ¼, ⅛, 1/16, and 1/32 dilution and tested for gelatinase activity on gelatin-agar plates. The degree of hydrolysis decreased with increased dilutions so that a 1/16 dilution showed only a faint zone of hydrolysis, and the 1/32 dilution showed no hydrolysis. Gelatinase activity of amoebae was not affected within a pH zone of 6.5 to 8; there was a decrease in activity at pH of 5. The pH values below 5 and above 8 could not be studied due to gelatin hydrolysis caused by acidity and alkalinity.

Summary. Six strains of *E. histolytica* were shown to hydrolyze gelatin. The gelatinase was inactivated by heat, copper salts, and freezing. The effects of concentration of enzyme and pH were studied.

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