NOTES

LOCALIZATION OF CELL-BOUND α-AMYLASE IN *ASPERGILLUS ORYZAE* DEMONSTRATED BY FLUORESCENT-ANTIBODY TECHNIQUE

KENZO TONOMURA AND OSAMU TANABE

Fermentation Research Institute, Chiba-city, Japan

Received for publication 14 June 1963

The activity of α-amylase (α-1,4-glucan 4-glucanohydrolase) in *Aspergillus oryzae* is generally found in the mycelium as well as in the culture medium. Formation of α-amylase can be induced by the addition of isomaltose, maltose, and starch [Tonomura et al., Agr. Biol. Chem. (Tokyo) **25**:1, 1961]. For the liberation of the enzyme from mycelium, pH, ionic strength, and the addition of an acidic substance such as phosphopeptides prepared from peptone were found to be significant [Tonomura et al., Agr. Biol. Chem. (Tokyo) **26**:434, 1962]. However, very little is known about endocellular location of the enzyme. It was recently found that S^{35}-labeled α-amylase was bound to *A. oryzae* mycelium at an acidic pH, and that bound α-amylase was liberated in solutions of basic pH [Tonomura et al., Agr. Biol. Chem. (Tokyo) **27**:128, 1963]. Further study revealed that the analogous binding occurred on the isolated cell walls, and that the α-amylase bound to mycelium was neutralized by antiserum against α-amylase when the mycelium was incubated with the antiserum. These results suggested a possibility that α-amylase might be located on the cell surfaces. An attempt was made, by use of a fluorescent-antibody technique, to observe the localization of α-amylase on the mycelium and on the cell walls.

The mold was grown in the defined medium (glucose, 30 g; $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and 1 liter of distilled water) on a shaker for 40 hr. The mycelium, suspended in 0.1 M phosphate buffer (pH 7.9), was disrupted twice, each time for 30 min by a sonic disintegrator (10 kc). The cell-wall fraction was obtained by centrifugation at 5,000 × *g* for 10 min and washed with (in the following order) distilled water, 5% trichloroacetic acid, distilled water, 1 N NaOH, distilled water, ethanol, and ether at room temperature. The cell-wall fraction did not contain α-amylase, but fixed α-amylase when incubated in 0.01 M acetate buffer (pH 5) containing the crystallized α-amylase. The antiserum was prepared by injection of rabbits with crystallized α-amylase. A titer of 1:64 was obtained after 11 injections given at intervals of 2 or 3 days; the amount of antigen injected was gradually increased from 5 to 50 mg (dry weight). Staining was performed indirectly (Beutner, Bacteriol. Rev. **25**:49, 1961) with sheep antirabbit globulin fraction conjugated with fluorescein isothiocyanate which was prepared by Sylvana Chemical Co. (Millburn, N.J.). To eliminate a nonspecific reaction, the antiserum (1:2) and the globulin fraction (1:4) were absorbed twice with about one-fifth the weight of the mycelium or the cell-wall preparation which was without α-amylase. Mycelium not containing α-amylase was obtained when grown in a medium containing glycerol as the sole carbon source. Smears of mycelium and cell walls were made on glass slides, and fixed for 20 min in 99% methanol. The fixed smears were stained by overlapping with antiserum for 15 min, and washing thoroughly with buffered saline (pH 6.8); they were finally stained with the labeled globulin fraction (1:4) for 15 min, and washed. Observations were made under a fluorescence microscope equipped with a high-pressure mercury lamp (Permaray Co., Japan) and filters.

Two kinds of negative controls were employed. First, the mycelium and the cell walls without α-amylase were stained by the same method as above. Second, the mycelium and the cell walls which contained α-amylase were stained without the antiserum only by the globulin fraction, or by the antiserum which was completely absorbed with the antigen α-amylase before the staining. These controls showed no fluorescence. The cell-wall preparation showed no autofluorescence, but a few hyphae showed a dark-red or a whitish-yellow autofluorescence. However, these could be

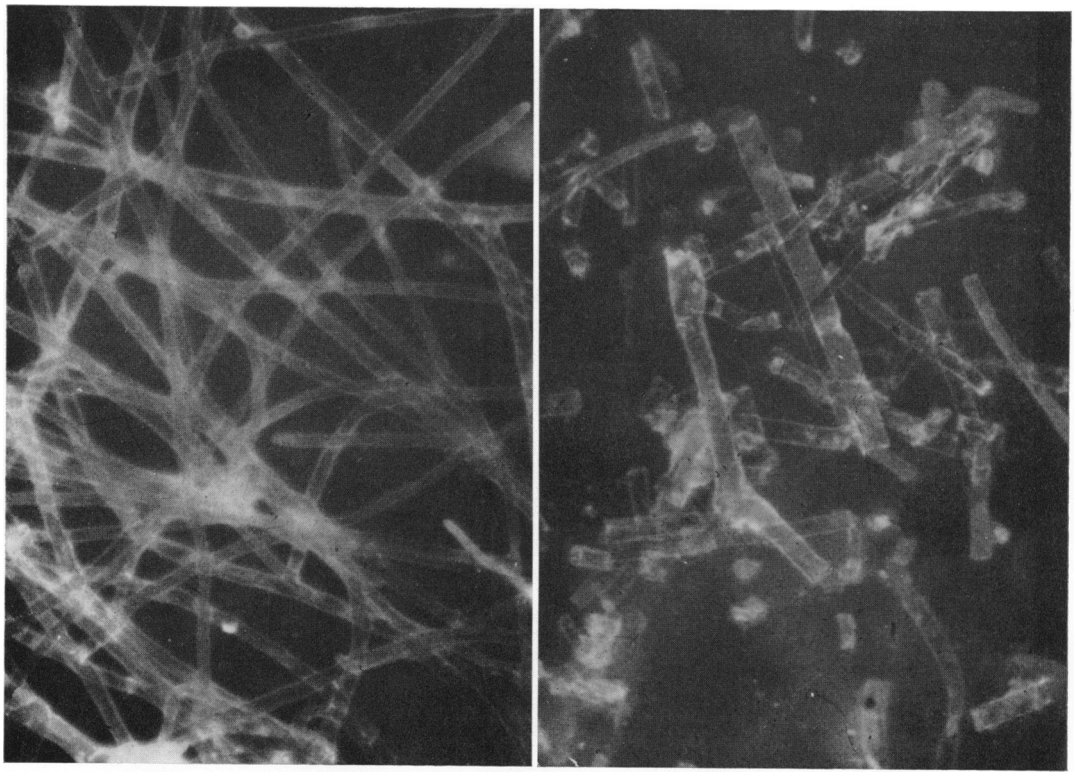

FIG. 1. *Mycelium (left) and cell walls (right) of Aspergillus oryzae stained with the antiserum against α-amylase by an indirect fluorescent-antibody technique.* × *400.*

distinguished from the color of fluorescein isothiocyanate. Figure 1 shows the mycelium and the cell walls stained by the method described. The staining reaction of these preparations could be observed as far as a 1:16 dilution of the antiserum. These results suggest that α-amylase may be located on the cell surfaces where the antibody molecules were accessible, although there is no information concerning the site where the exocellular enzyme is formed.

The authors are grateful to T. Arai and S. Kuroda, Chiba University, for their kind guidance.

GROUPING OF BETA-HEMOLYTIC STREPTOCOCCI ON CELLULOSE ACETATE MEMBRANES

M. GOLDIN AND ARLEEN GLENN

Division of Bacteriology, Department of Pathology, Mount Sinai Hospital and Research Foundation, Chicago, Illinois

Received for publication 1 August 1963

Serological grouping of beta-hemolytic streptococci by the classical capillary precipitin method is not entirely satisfactory because of zone- and cross-reactions and other factors. Lancaster (Am. J. Clin. Pathol. **34**:131, 1960) described an agar diffusion microtechnique. Bergner-Rabinowitz (Bull. Res. Council Israel, Sect. E **9**:117, 1961) modified this procedure for use on cellulose acetate membranes. Feinberg (Nature **195**:985, 1962) reported a "microspot" test for antigen-antibody reactions on cellulose acetate membranes, claiming simplicity and