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Biochemical and Histochemical Localization of Invertase in *Neurospora crassa* During Conidial Germination and Hyphal Growth

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The intracellular localization of *Neurospora* invertase, an enzyme partially secreted and partially retained by *Neurospora* at the cell periphery, was investigated. A cell wall fraction was isolated, to which 24% of the cell-bound invertase was firmly attached. A sensitive osmiophilic stain for invertase was developed and used in conjunction with the technique of indirect immunofluorescence to follow the pattern of invertase localization during the development of *Neurospora* from the germination of conidia to the mature hypha. These studies revealed that: (i) conidial invertase was uniformly distributed along the cell periphery; (ii) growing hyphal tips of germinating conidia showed pronounced invertase activity as the rest of the conidial cell wall lost its peripheral activity; (iii) hyphae in early log-phase growth had strong enzyme activity associated with the cell wall, and in late log phase the activity became associated with the plasma membrane and points where new hyphal branches were being formed; and (iv) hyphae in early stationary phase had strong fluorescence at incipient branching points, in "dots" close to the plasma membrane, and in the cytoplasm.

A large number of enzymes which function in the catabolic processes of microorganisms are located at the cell periphery in the intramural space between the cell wall and the plasma membrane, or alternatively are mural enzymes located in or on the cell wall. The biochemical experiments of Metzenberg (13) on *Neurospora* invertase and those of Lampen et al. (10) on yeast invertase have indicated that most of the cell-bound enzyme is localized in the space between the cell membrane and the cell wall, the intramural space. In contrast, the localization of the α-amylase of *Aspergillus aryzae* by immunofluorescence (17) strongly suggests that its location is mural. This has been reviewed in a previous paper (18).

These enzymes of the cell periphery, of which the invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.32) of *Neurospora* is a typical example, are freely accessible to the external milieu, and often are released in amounts which vary depending on a multiplicity of factors during growth of the microorganisms in liquid media. The finding that molecular sieving by the cell wall of *Neurospora* occurred during the secretion of invertase isozymes (18), and the report by Manochoa and Colvin of the discovery of discrete pores in the cell wall of *Neurospora* (9), suggested that the secretion of the intramural invertase during log-phase growth occurred through these pores. Thus, further investigations of the localization of invertase inside the cell during different stages of growth were undertaken to illuminate the mechanism by which intramural enzymes are distributed within the cell in response to variations in the growth conditions and age of the mycelium.

Our studies and those reported by others during the course of this work (7, 15) have indicated that, in addition to the invertase which is intramural, a significant fraction of this enzyme is mural in localization. The investigation has proceeded from this point to define the intracellular localization of this enzyme more exactly than would be possible by biochemical techniques. This localization has been independently substantiated by a sensitive histochemical method which made possible the detection of this enzyme with a 10-fold increase in sensitivity over that of the method previously used (2), and by fluorescent staining by use of the indirect immunofluorescence method (16) with an antiserum specifically directed against invertase (15).

MATERIALS AND METHODS

Mycelial culture. Conidia of the wild-type *N. crassa* containing the Emerson genetic background were collected from an agar plate culture maintained in Fries

basal growth medium (1) with 1.4% sucrose as a carbon source. The conidia were suspended in 10 ml of distilled water. The suspension was filtered through a pad of glass wool and centrifuged at 650 × *g* for 5 min. About 0.5 ml of packed conidia was inoculated into 100 ml of Fries basal growth medium supplemented with 0.15 M galactose and 0.04 M sodium succinate buffer at *p*H 5.2. The culture was grown at 30 C in a Metabolyte Gyrotory Water Bath Shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at a speed of 200 rev/min for 12 to 24 hr. Mycelia in logarithmic growth were harvested by filtration through a membrane filter (type HA, 0.45 μm; Millipore Corp., Bedford, Mass.) and washed two times with phosphate-buffered saline (0.9% sodium chloride buffered with 0.01 M sodium phosphate buffer at *p*H 7.1, abbreviated as PBS). *P*-Nitroblue Tetrazolium from Calbiochem is used as the conventional dye component for comparison.

Preparation of cell wall. All steps were carried out at or below 4 C unless otherwise stated. The harvested mycelia were suspended in 20 ml of PBS in a metal tube and disrupted with a Sonifier (Branson Soni Power S 125) at maximal output for about 3 min. The temperature of the suspension was kept below 10 C by using an ethylene glycol-dry ice bath and turning on the Sonifier only intermittently. A sample of the disrupted suspension was taken for determination of total invertase activity. The rest of the suspension was overlaid on 20 ml of 30% sorbitol and centrifuged at 650 × *g* in a model PR-2 centrifuge (International Equipment Co., Needham Heights, Mass.) for 20 min. This sorbitol centrifugation made it possible to sediment the dense cell walls cleanly from the cytoplasmic content by use of a medium which, unlike sucrose, permits assay of invertase. The supernatant fluid consists of a PBS layer containing the liberated cytoplasmic content and a heavier sorbitol layer. These were removed separately by aspiration and saved for assay of invertase activity. The residue was resuspended in 30 ml of PBS with a magnetic stirrer and was centrifuged again at 100 × *g* for 15 min, after which the supernatant fluid and the orange rim of unbroken conidia on the surface of the pellet were removed by aspiration. The last washing procedure was repeated five times. The residue by then was a white fluffy mass consisting of at least 95% isolated cell walls; very little cell debris was observed with a phase-contrast microscope.

Glucosamine determination. A known amount of mycelium or cell wall fraction dried at 80 C for 24 hr was hydrolyzed in vacuo with 6 N HCl at 110 C for 18 hr. The hydrolysate was centrifuged to remove the insoluble residue. The supernatant fluid was carefully removed. The residue was washed with distilled water and centrifuged, and the supernatant fluids were combined and evaporated to dryness in vacuo. This residue was dissolved in buffer and assayed for glucosamine with a Beckman model 120 C amino acid analyzer, with authentic glucosamine as a standard.

Assay of invertase activity. Invertase activity was assayed by two successive incubations with reagents containing sucrose and glucose oxidase, according to the method of Metzenberg (12).

Histochemical enzymatic staining for invertase. The harvested and washed mycelia were embedded in Tissue-Tek (Ames Co., Division Miles Laboratories, Inc., Elkhart, Ind.) and kept at −20 C in an Ames Lab-Tek Cryostat. Frozen sections 2 μm thick were cut and incubated at 37 C. The incubating medium was prepared by combining 2.5 mg of 3,3′-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, Mo.) dissolved in 5 ml of potassium phosphate buffer (*p*H 7, 0.05 M), 1 ml of 2 M sucrose, 1 mg of horseradish peroxidase (type II from Sigma Chemical Co.) dissolved in 1 ml of phosphate buffer, and 0.05 ml of glucose oxidase at 114 Worthington units/ml (Worthington Biochemical Corp., Freehold, N.J.). To obtain maximal staining, the incubating medium over the section was changed three times over a period of 90 min. The sections were then drained and fixed in 10% Formalin-saline (0.9% sodium chloride in 10% Formalin) for 10 min, rinsed through glass-distilled water, left in 15% alcohol for 5 min, and mounted in immersion oil (n_D = 1.515, Carl Zeiss).

The color of the deposit was further intensified if necessary with 1% osmium tetroxide. In this case, after incubation, the sections were rinsed thoroughly in three changes of glass-distilled water for 10 min each and overlaid with the osmium tetroxide solution at room temperature for 10 min. The slide was then rinsed in distilled water, fixed, and mounted as before.

Two kinds of control sections were used. First, sucrose was replaced by water in the incubation medium, to check for any endogenous glucose present in the tissue section. Second, sucrose was replaced by glucose. The incubation mixture was allowed to develop its maximal color reaction prior to incubation with the tissue section to check for any preferential adsorption of the dye deposit to the tissue.

Indirect immunofluorescence staining for invertase. Sections (2 μm thick) from frozen fresh mycelia were cut and laid on slides that had been soaked in PBS overnight. They were fixed in 95% alcohol and stained with the "sandwich technique" according to Nairn (14). Purified serum from rabbits immunized against *N. crassa* invertase was kindly donated to us by H. D. Braymer. This was used as the middle layer that reacted with the invertase in situ in the mycelial sections. The antigen (invertase)-antibody (rabbit anti-invertase gamma globulin) complex thus formed made up the "sandwich" layer. It reacted with goat anti-rabbit serum conjugated with fluorescein (Hyland Laboratories, Los Angeles, Calif.) which had been adsorbed with acetone-dried liver powder, either prepared according to the method of Weir (19) or purchased from Difco. Stained sites of invertase were then demonstrated by means of their green fluorescence with a fluorescence microscope (Carl Zeiss Standard Universal Microscope, excitation filter II, barrier filter 50/44).

Two kinds of control were used. First, normal rabbit serum without previous immunization against invertase was used. Second, no rabbit serum was used, and the goat anti-rabbit serum conjugate was applied directly onto the section.

RESULTS

Invertase associated with cell walls. The disrupted cell wall residue was found to retain 20 to

30% of the total intracellular invertase activity (Table 1). The exact percentage depended on the age of the mycelia used, which was about 16 hr in this particular experiment. Extensive washing of the cell wall residue overnight in PBS did not liberate significant amounts of the associated invertase.

TABLE 1. *Association of invertase with cell walls of Neurospora crassa*

Cell fraction	Units of invertase activity[a]	Glucosamine (μmoles per mg of dry wt)	Specific activity[b]	Per cent activity
Total mycelia....	1,028	0.061	59,217	100
Cell walls.....	234	0.384	13,933	23.5

[a] Unit of invertase activity is defined as the number of micromoles of glucose yielded by invertase acting on sucrose at 37 C per minute.

[b] Specific activity is defined as the units of invertase activity per micromole of glucosamine content.

Enzymatic staining for invertase. The glucose liberated from the hydrolysis of sucrose by invertase caused the deposit of a dark-brown insoluble form of DAB via a series of coupled reactions.

In a typical section of hyphae in late log-phase growth (Fig. 1b), there was a dark-brown color reaction on the plasma membrane and a lighter one on the cell wall. General light staining was observed in the cytoplasm, which had retracted a little from the cell wall in this preparation. The "lines" of dark color on the plasma membrane and the cell wall were not due to optical refraction effects at surfaces with different refractive indices. The plasma membrane did not show such "lines" if the cytoplasm was only uniformly stained with methylene blue. Since the cell wall had the same refractive index as its mounting medium, an im-

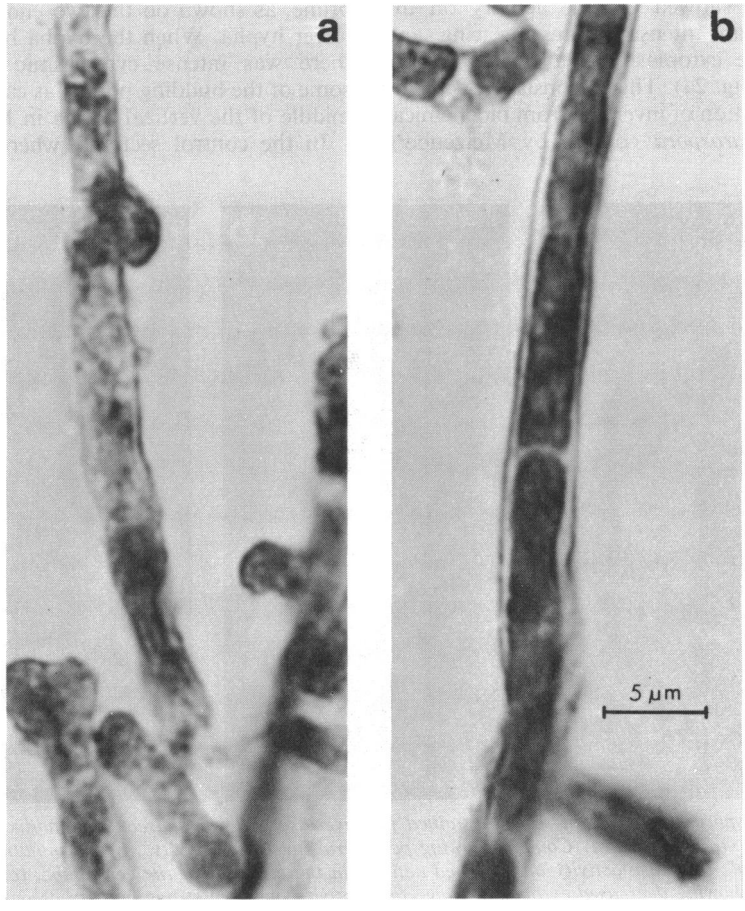

FIG. 1. *Neurospora crassa (2-μm section) hyphae in log phase, enzymatically stained for invertase with DAB as described in text. × 2,700. (a) Intense peripheral stain on the two budding points obliquely facing each other. (b) Hyphae showing invertase activity on the cell wall and cytoplasm, and intense activity on the plasma membrane.*

mersion oil, it was almost invisible under the microscope when no cell wall staining occurred.

In each of the two longer hyphae (Fig. 1a), a branching point was jutting out toward the center of the photograph. Intense color reactions were found on the peripheries of their tips.

At a shorter time of incubation, i.e., 30 min, the two control sections invariably showed no visible reaction, whereas the section incubated with the normal substrate showed a light general cytoplasmic stain and a very intense color on the plasma membrane, with an intensity of about half of that shown in Fig. 1. A color reaction occurred in the cell walls when the incubation time was extended to 90 min. By this time, the control sections did show a visible nonspecific staining in the cytoplasm and plasma membrane, but never on the cell walls.

Indirect immunofluorescence staining for invertase. The invertase distribution during growth from conidial germination through log phase to the stationary phase was followed.

The conidia showed enzyme activity on the periphery as an intense fluorescing ring and throughout the cytoplasm as a weaker diffuse fluorescence (Fig. 2a). This is consistent with the intramural location of invertase from biochemical studies on *Neurospora* conidia by Metzenberg (13). During germination, as shown in Fig. 2b, invertase fluoresence was found at the growing tip and was markedly increased in the conidial cytoplasm. Only the cell wall at the growing tip showed fluorescence; the rest of the conidial cell wall had lost the discrete peripheral fluoresence. In the early log phase, the young hyphal cell wall had become strongly fluorescing, and the hyphal cytoplasm was weakly so (Fig. 2c). In the meantime, the exhausted conidial portion had lost most of its enzyme activity and was only faintly fluorescing.

As the hyphae passed from the log phase into the stationary phase of growth, enzyme activity was found associated not only with the cell wall but even more strongly with the cytoplasmic content. This can be clearly seen at the left end of the lower hypha in Fig. 3 where part of the cytoplasm was lying outside the cell wall envelope. There were some intensely fluorescing scattered dots, some of which were actually attached just on the outside of the plasma membrane, as shown on the two end portions of the lower hypha. When the hypha began to branch, there was intense cytoplasmic fluorescence at some of the budding points, as can be seen on the middle of the vertical hypha in Fig. 3.

In the control sections, where normal rabbit

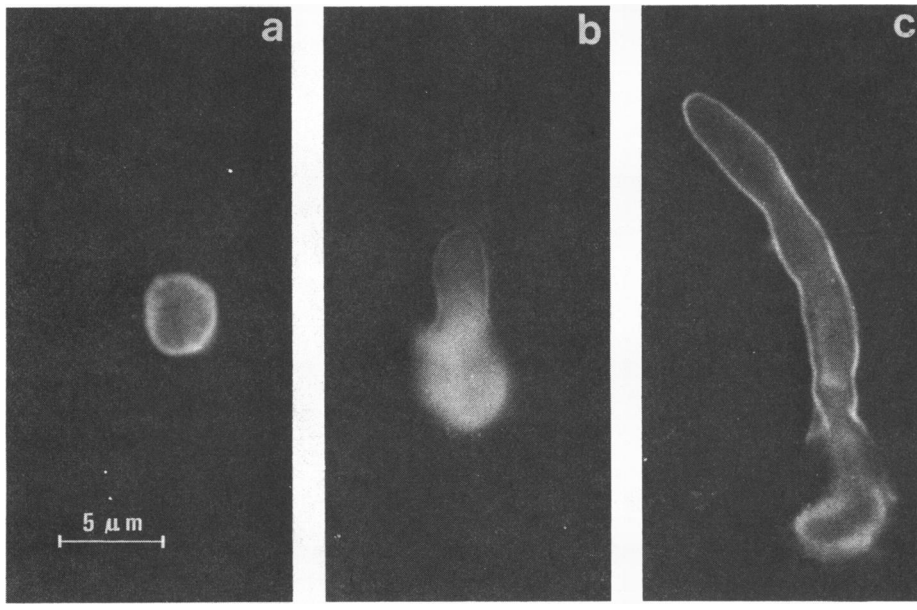

FIG. 2. *Neurospora crassa (2-μm section) stained for invertase with an indirect immunofluorescent technique as described in text.* × *2,700. (a) Conidia showing peripheral invertase activity. (b) Germinating conidia with a general cytoplasmic invertase activity and marked activity in the cell wall of the germ tube. (c) Young hypha in early log phase, showing decreased cytoplasmic invertase activity but very strong activity associated with the cell wall.*

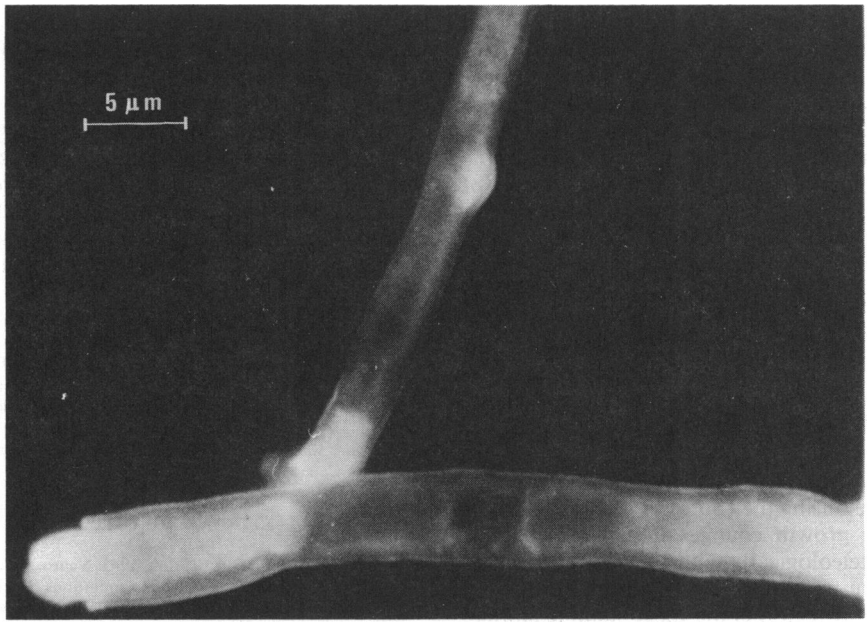

FIG. 3. *Neurospora crassa hyphae (2-μm section) in early stationary phase, stained for invertase with an indirect immunofluorescent technique. × 2,700. Strong activity is associated with the budding point in the vertical hypha. In the horizontal hypha, the cytoplasm shows intense fluorescence, especially where it has emerged from the cell wall envelope on the left. Fluorescing dots are seen most clearly in this hypha on the plasma membrane and in the space between the plasma membrane and the cell wall.*

serum or no rabbit serum was used, no significant green fluorescence was observed.

DISCUSSION

Although it was found that a major portion of invertase activity is localized between the plasmalemma and cell wall in *Neuraspora* conidia (13) and that 95% of the external enzyme is removed during cell wall digestion in yeast (8), the question of whether it is just physically trapped between the two barriers or actively bound to them has never been answered. In this work, it was shown that 20 to 30% of the total invertase was bound to the cell wall of *N. crassa*.

The importance, and the inherent disadvantages, of histochemical techniques have been thoroughly discussed in an excellent review by Glenner (4). The only two problems considered in this series of enzymatic and immunological staining techniques were the retention of enzymes in the sections and the specificity of the stain. By a separate biochemical assay, a major fraction of the invertase on freshly cut sections of mycelia was washed away by vigorous mechanical stirring. Consequently, methods of retaining this washable portion of the enzyme were investigated with the enzymatic staining procedure. Imposing a molecular physical matrix on the cellular component by polymerizing acrylamide which had previously permeated the hyphal cells reduced the proportion of the washable fraction of invertase by one-half. Diffusion of soluble components from the tissue sections was also reduced by using a very viscous incubation medium containing 8% gelatin. These two methods were found to reduce the overall enzymatic stain intensity, presumably because of a decreased rate of diffusion of substrates in the acrylamide gel or gelatin. In a preliminary experiment, fixation of the cut sections with 10% Formalin followed by 5% glutaraldehyde reduced both the proportion of the washable fraction as well as the enzyme activity. However, the pattern of DAB stain deposited in the sections treated with these three different methods did not seem to differ appreciably from that obtained with the fresh untreated sections. This indicated that the total invertase had a distribution similar to that of the fraction which was not washed away. Hence, no further attempt was made to retain the soluble cellular components on the tissue sections.

The negative results of the controls used in both the enzymatic and the immunofluorescence methods confirmed the specificity of reactions involved.

In the enzymatic staining method, the oxida-

tion of glucose by glucose oxidase can be coupled to deposition of an insoluble dye complex by two routes: (i) peroxidase plus DAB and (ii) phenazine methosulfate plus NBT, which is normally used (2). The new stain with DAB is about an order of magnitude more sensitive because the reaction color begins to appear after 10 min of incubation, as compared with more than 1 hr in the case of NBT. With DAB, 90 min is required for development of an intense dark-brown stain, whereas the NBT stain requires at least 3 hr to develop a less intense but detectable color. The definition and pattern of localization obtained with the DAB stain is similar to that obtained by immunofluorescence, whereas that demonstrated by means of NBT staining is rather poorly defined.

The invertase distribution detected by immunofluorescence during the course of germination and logarithmic growth changes in a way which is correlated teleologically with the changing physiology of the mycelium. In the dormant conidia, the peripheral region is the most suitable storage place for an exoenzyme so that it is ready to act on the external substrates once conditions prove favorable for germination. In the germinating hypha, the growing tip is continuously contacting new substrate areas, and its cell wall might be expected to be well supplied with the exoenzyme that may either be newly synthesized or supplied from the conidial store (which consequently becomes enzymatically depleted).

Strong enzymatic activity associated with the cell walls in early log phase, the plasma membrane in late log phase, and the cytoplasm in early stationary phase may correspond to a gradually decreasing egress and an increasing accumulation of exoenzymes inside the cell.

Just as with growing hyphal tips, branch points are actively growing regions coming into contact with new substrates. Thus, they are also expected to be well supplied with the same exoenzyme.

It is interesting to note that, in the mature hypha, intense localized enzyme activity appearing as dots can be seen in the immunofluorescence staining. These dots are found in the intramural space and also next to the plasma lemma. These may possibly be analogous to the secretion packets of another kind of exoenzymes, the proteases demonstrated by Matile (11).

Ultrastructural studies by Grove (5) and Girbardt (3) have demonstrated that, in a wide variety of fungi, the hyphal tip and periphery characteristically contain a large number of different kinds of vesicles. It is likely that many of these vesicles are involved in the formation of new cell wall and cell membrane and the discharge of secretory material (6). Our histochemical data would indicate that these vesicles may also contain a major portion of the exoenzymes secreted by the cell.

The histochemical localization of invertase and the biochemical demonstration of its association with the cell walls presented in this work will serve as a foundation from which we hope to delineate the process of exoenzyme secretion with further biochemical and ultrastructural studies.

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