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Molecular Sieving by *Neurospora* Cell Walls During Secretion of Invertase Isozymes

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ABSTRACT

TREVITHICK, JOHN R. (University of Wisconsin Medical School, Madison), AND ROBERT L. METZENBERG. Molecular sieving by *Neurospora* cell walls during secretion of invertase isozymes. J. Bacteriol. **92:** 1010–1015. 1966.—The secretion of invertase by young mycelia of *Neurospora* was studied. The process of secretion was found to be dependent upon growth. The results indicate that fractionation of light invertase, the monomer, from heavy invertase, the aggregated form, occurs at the cell wall. *Neurospora* strains *wild type*, *crisp*, *osmotic*, and the double mutant *crisp osmotic* were tested. An inverse relation exists between the fraction of the total invertase activity of the culture which the mold secretes into the medium and the degree of fractionation, defined as the ratio of the fraction of the invertase secreted into the medium that is light invertase to the fraction of the invertase remaining associated with the cells that is light invertase. The hypothesis is offered that the increased secretion of invertase and decreased degree of fractionation seen in *osmotic* mutants, and to a lesser extent in the other mutants, can be explained by an increased porosity of the cell wall.

It has long been known that many bacteria and fungi secrete a variety of enzymes into their growth media (26). Some of these extracellular enzymes are found only in filtrates of senescent cultures, where their presence can be attributed, at least tentatively, to autolysis. However, there are numerous examples of enzymes which are secreted during rapid growth of microorganisms. The mechanisms by which these enzymes are released or extruded remain unknown. A useful discussion of extracellular enzymes has been provided by Eberhart (9).

There is reason to believe that certain enzymes, most or all of which serve catabolic functions, are located outside the plasma membrane, either in or on the cell wall, or between the wall and the plasma membrane. Such enzymes will be referred to as mural and intramural, respectively. It has been convincingly shown that invertase, ascorbic oxidase, and a sulfhydryl oxidase of *Myrothecium verrucaria* are freely accessible to the external milieu, as judged by kinetic constants and by acid-inactivation behavior (17–19). Analogous results have been obtained with the invertase, cellobiase, trehalase, and maltase of *Aspergillus lichensis* (17); aminopeptidase (20), invertase (22, 29), nicotinamide adenine dinucleotide hydrolase (31), and β-glucosidases (2, 9) of *Neurospora crassa*; the invertase (3, 27) and acid phosphatase (11) of yeast; and various phosphomonoesterases and nucleases of *Escherichia coli* (16, 24). Interestingly, Cashel and Freese (5) suggested that cell walls of *Bacillus subtilis* are less retentive than those of *E. coli*, since alkaline phosphatase is extracellular in *B. subtilis*, but is intramural in *E. coli*. Davies and Elvin (Biochem. J. **93:**8P, 1964) have shown that treatment of yeast cells with β-mercaptoethanol will cause the release of invertase, which suggests that the "tightness" of the yeast cell wall may be maintained in part by disulfide bonds.

In vivo experiments involving inactivation by acid, affinity of enzymes for their substrates, or release of enzymes during protoplast formation by enzyme digestion of the cell wall, can provide evidence that the enzyme in question is mural or intramural, but do not allow a distinction between these two possibilities. Results obtained with fluorescent antibody suggest strongly that the α-amylase of *Aspergillus oryzae* is mural (28). By contrast, the solubilization of the invertase of *Myrothecium*, *Neurospora*, and yeast, by simple homogenization, suggests that at least some of the

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invertase is in free solution in the intramural space.

It has been noted (9) that *Neurospora* has an extracellular invertase that can be removed from conidia by washing, and an intramural invertase that can be liberated from conidia or mycelia by mechanical or enzymatic disruption of the cell wall (29). It seemed possible that intramural invertase might, at a very limited rate, pass through some of the larger pores in the cell wall and, thus, might be the source of extracellular invertase.

Recent findings in this laboratory (23) have shown that invertase can undergo a reversible transition between an active monomer and an aggregated active form (light and heavy invertase, respectively), and that this equilibration is quite slow in the physiological *p*H range. If, indeed, the pores in the cell wall permit the egress of invertase, it might be expected that light invertase would escape more rapidly than would the heavy form, and that light invertase would be enriched with respect to heavy form in the invertase which has become extracellular. Furthermore, the degree of this sieving would be greatest in the case of a cell wall with very small pores, so that only occasionally would a molecule of light invertase escape and never would a molecule of heavy invertase do so. Formally, a protoplast "cell wall" has infinitely large pores and can, therefore, perform no fractionation. It has been shown (29) that protoplasts secrete mainly heavy invertase. The fractionation pattern of intact *wild-type Neurospora* should lie somewhere between these idealized extremes.

Morphological mutants of *Neurospora* are available which show evidence of an alteration in the structure of the cell wall (15; S. Galsworthy, Ph.D. Thesis, Univ. Wisconsin, Madison, 1965.) For example, the strain *osmotic* (10) produces exudates on solid medium and has been presumed to have a weak or defective cell wall. In the accompanying paper (30), it is shown that the pores of *osmotic* walls are larger than those of *wild type*. Such a strain should secrete a higher steady-state proportion of its total invertase and should show a decreased degree of fractionation, when extracellular and intramural pools are compared with those of *wild type*. The results reported below show that such a light-heavy fractionation does occur, and that the degree of this molecular sieving is inversely related to the proportion of extracellular invertase in various strains. It should, however, be emphasized that the hypothesis of secretion through pores in the cell wall is tentative; many types of independent evidence will be required before it can be accepted.

MATERIALS AND METHODS

Chemicals. Phenazine methosulfate, nitro blue tetrazolium dye, and phosphodiesterase from *Crotalus adamanteus* were purchased from Sigma Chemical Co., St. Louis, Mo. Reagents used in polyacrylamide-gel electrophoresis were obtained from the Eastman Chemical Products, Inc., Kingsport, Tenn. Pancreatic deoxyribonuclease was a product of the Worthington Biochemical Corp., Freehold, N.J. Calf thymus deoxyribonucleic acid (DNA) was isolated as described by Zamenhof et al. (32).

Invertase determination. Invertase was determined by the method of Metzenberg (21), with 0.05 M buffer substituted for the 0.02 M buffer; the reaction was stopped by heating (1 min at 100 C). The glucose released was estimated by the glucose oxidase method.

To determine the proportions of the different isozymes of invertase, the appropriate solutions were analyzed by estimating the activity of each isozyme, after separation by electrophoresis on polyacrylamide gels (6, 25). The activity of the isozymes was measured by staining the gels for invertase activity, according to the method of Metzenberg (23), with the following modifications. The lower gel contained five times the concentration of glucose oxidase used previously (23). Concentrated upper buffer and glucose oxidase were added directly to the sample. The sample was placed in the gel tube, and G-200 Sephadex (Pharmacia, Uppsala, Sweden) was added to the sample, while the tube was sharply tapped, until a thick gel was obtained. Ornstein-Davis working buffer (half strength) of the same glucose oxidase concentration as the spacer and sample gels was carefully added above the jelled sample, and the samples were subjected to electrophoresis at about 10 C in an ice-cooled buffer reservoir. Electrophoresis was stopped when the band of bromophenol blue dye reached within 1 to 4 mm of the end of the 75 mm of gel.

The reagent for development of the gels (23) was modified by the addition of glucose oxidase to the same concentration used in the working buffer. The reagent at 0 C was deoxygenated by gassing with nitrogen, which had first passed through alkaline pyrogallol. The reagent was then carefully added, without turbulence, to each gel-containing tube. The tubes were sealed so that no air was trapped under the stopper, and the reagent was allowed to diffuse into the gels for 4.5 hr at 0 C in the dark. For development of color, the tubes were placed in a water bath at 37 C in the dark for 20 min, then were again placed in an ice bath for 5 min or more to stop the color development. Preliminary destaining was carried out in the dark with 7% acetic acid at 0 C; subsequent treatment was with 7% acetic acid at room temperature.

The amount of color produced by each isozyme was measured by the integrator attachment of a model E densitometer (Canal Industrial Co., Bethesda, Md.). When the number of integrator counts was plotted against the known amount of the particular isozyme applied to the gel, smooth standard curves were obtained. These standard curves had average errors of less than 7.5%, when the analysis was performed on quadruplicate standards. By use of these standards,

the percentage of light invertase present in each experimental sample was determined.

Neurospora strains. Several strains of *Neurospora crassa* were used in these experiments. In addition to *wild type* containing the Emerson genetic background (R. W. Barratt, *unpublished data*), the strains *osmotic* (10), *crisp* (14), and the double mutant *crisp osmotic* were used. The latter three strains were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H. The properties of these strains suggested that abnormalities in the porosity of their cell walls were present. For example, the *osmotic* strain is characterized by its inability to grow on media of high osmotic strength and by its poor formation of conidia.

Growth of strains. Conidial suspensions of all strains, except *osmotic*, were prepared as described by Metzenberg (22), except that the conidia were harvested by scraping them off the petri plate (for wild type) or by teasing them into suspension in sterile water. Heavy suspensions of conidia were inoculated into the medium of Fries (1), supplemented with fructose (0.15 M) as a carbon source, and with sodium succinate buffer (*p*H 5.3, except where otherwise specified) to give a final concentration of 0.04 M with respect to total succinate. The cultures were shaken (200 cycles per min) at 30 C for approximately 8 hr on a New Brunswick Gyrorotatory Shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

For growth of the *osmotic* strain, petri plates containing Fries' minimal medium, solidified with agar, were inoculated and incubated for 2 days at 25 C. The wispy, young mycelia were scraped from the plates and were suspended in the above medium, with care being taken to avoid large clumps. Initially and during early hours of growth, the *osmotic* culture was passed up and down repeatedly in a large bore pipette to break the mycelia into small clumps; these cultures were grown for 18 to 24 hr.

The young mycelia of all strains were harvested by centrifugation and were washed twice with fresh medium. Finally, the preparations were taken up in a small volume of medium (ca. 10 ml each); during incubation with shaking at 30 C, invertase release was followed.

In most experiments the medium was buffered at a *p*H of 5.3. Dropping slowly from this original *p*H value, the *p*H of the medium reached about 5.0 after 3 hr of incubation.

Samples, taken initially and at intervals throughout the incubation were stored at 0 C. Portions were taken for analysis of the total invertase activity of the suspension and of the supernatant solution obtained when the young mycelia were separated by centrifugation. The activity of the supernatant solution was usually expressed as a percentage of the total activity of the suspension. The residue from the centrifugation was washed once with buffer and was ground with sand for 3 min to release most of the intracellular invertase; the cellular debris and sand were removed by centrifugation for 5 to 10 min at 700 $\times$ *g*. The ratio, light invertase-total invertase in this intracellular fraction, and the corresponding ratio for the invertase secreted into the medium during growth, were determined by the polyacrylamide gel electrophoresis technique described above.

RESULTS AND DISCUSSION

When young mycelia of wild-type *Neurospora* were resuspended in fresh medium and were allowed to keep growing, they continued to produce invertase. The total invertase and the secreted invertase both showed steady increases as the experiment progressed (Fig. 1), whereas the intramural invertase (by difference) reached a plateau after 150 min. An identical culture to which cycloheximide, a well-known inhibitor of protein synthesis (12), was added, was used as a control. This culture continued to release invertase at a very slow rate throughout the incubation period.

Previous experience had shown that light and heavy invertase were stable for several days in dilute solution at 0 C. However, it was deemed necessary to show that the two forms of invertase, likewise, were not rapidly interconverted under the actual conditions of the incubation, i.e., during vigorous shaking at 30 C. Thus, in both incubation mixtures, dialysis bags containing mostly heavy and mostly light invertase were included as internal controls. No significant changes in the percentage of light and heavy invertase were found in these samples. In the incubation mixture inhibited by cycloheximide, the light invertase made up an average 59.3% of the secreted invertase in the samples taken at 150, 225, and 300 min, and 12.6% of the intramural invertase in the samples taken at 0, 75, and 150 min. In the incubation mixture without cycloheximide, light invertase made up 46% of the secreted and 15.3% of the bound invertase, for the four samples taken after the initial sample. Thus, in the samples from inhibited and uninhibited cultures, the percentage of light invertase in the extracellular medium was increased over the percentage in the intramural fraction by factors of 4.7 and 3.1, respectively. These results indicate that there is significant fractionation of light invertase from heavy invertase by the cell wall.

Additional experiments, similar to that described above, were carried out on different strains of *Neurospora*. After 3 hr of incubation, the *p*H was still higher than 5; controls established that, in similar media buffered at several *p*H values pure invertase lost only 20% of its activity during 6 hr of incubation, or about 10% after 3 hr. Solutions containing mostly heavy and mostly light invertase behaved similarly in this respect. The percentage of either isozyme present did not vary more than 7% during a 6-hr incubation. Hence, the proportions of light and heavy invertase found in the medium were a fair repre-

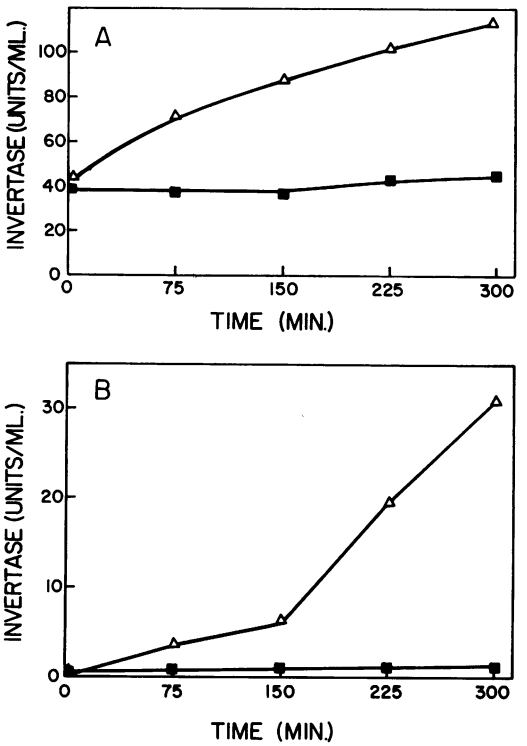

FIG. 1. *Invertase activity of wild type as a function of time of incubation. (A) Total invertase; (B) secreted invertase; △, no inhibitor; ■, cycloheximide present at a concentration of 20 μg/ml.*

sentation of the proportions at the time of secretion.

If morphological mutants have cell walls with altered properties, it might be expected that these would fractionate heavy invertase from light in a manner distinguishable from that of *wild type*. In an ideal case, the mutants would differ mainly in pore size, as was suggested above. Another possibility is that the cell walls would differ in thickness. In this case, an enzyme would be subjected to molecular sieving in a manner analogous to fractional distillation, with a variable number of theoretical plates. The efficiency of the sieving would depend strongly on the length of the pathway of diffusion, and thus a thicker cell wall would be expected to show both more efficient molecular sieving and a decreased rate of release of solute. That variations in both pore size and wall thickness are possible is suggested by the study of the properties of the cell walls of the *wild type* and *osmotic* strains (30).

The morphological mutants *crisp*, *osmotic*, and the *crisp osmotic* double mutant were compared with *wild type*. The strains all secreted into the medium invertase, which contained a higher percentage of light invertase than that present in the intramural fraction. In addition, there seemed to be an inverse relation between s (the amount of invertase secreted into the medium in 3 hr, expressed as a fraction of the total activity of the culture), and the degree of fractionation d. The degree of fractionation d was defined as (light invertase in the medium/total invertase in the medium)/(light invertase in the bound fraction/total invertase in the bound fraction). To express this relation in a more meaningful form, the value of s obtained was plotted against $1/d$ [a plot which might theoretically approach the origin of the graph (Fig. 2)]. As may be seen, the results seem to indicate that such a relation exists, although the points show considerable scatter. The error due to inactivation of invertase during the period of the incubation was insignificant in comparison with that involved in estimating the ratio (assuming an average error of 7.5% for the determination of light and heavy invertase).

Experiments performed at a higher *p*H (5.9), with a larger number of strains, indicated a similar relation between the percentage of secretion of invertase and the value $1/d$. Some difficulty was experienced in these cases, because higher percentages of intracellular light invertase gave lower ratios and rendered the results less meaningful.

If cytolysis were to contribute, in a major way, to the release of invertase into the medium, no fractionation of invertase could be expected in cells that lyse. Such cells would have a value of $1/d = 1.0$. Thus, the altered fractionation pattern of *osmotic* could be explained as a trivial consequence of cytolysis. However, in this case, it would be expected that intracellular constituents, such as DNA, would also appear in the medium. To test this possibility, young mycelia were prepared by inoculating conidia into the usual medium (10 ml), modified by reducing the level of KH_2PO_4 to 0.001 M, and by adding carrier-free inorganic P^{32}-phosphate to give a final specific activity of 5 μc/μmole. The young mycelia were suspended in nonradioactive medium containing thymus DNA (0.5 mg/ml) to act as a "trap" for any DNA released during subsequent growth. The cultures were shaken, in the usual manner, for 3 hr (30 C); the mycelia were quickly isolated by filtration, were washed, and were defatted, as described by Lehman et al (13). The mycelial DNA was estimated by the method of Burton (4). The specific activity of the mycelial fraction was determined by wet-ashing a sample for assay of radioactivity and phosphorus (8). The culture filtrate was treated with 3 volumes of 95% ethyl alcohol, and the precipitate, containing the thymus DNA "trap," was saved and was redissolved in buffer. It was obvious from the greatly reduced viscosity of the recovered DNA

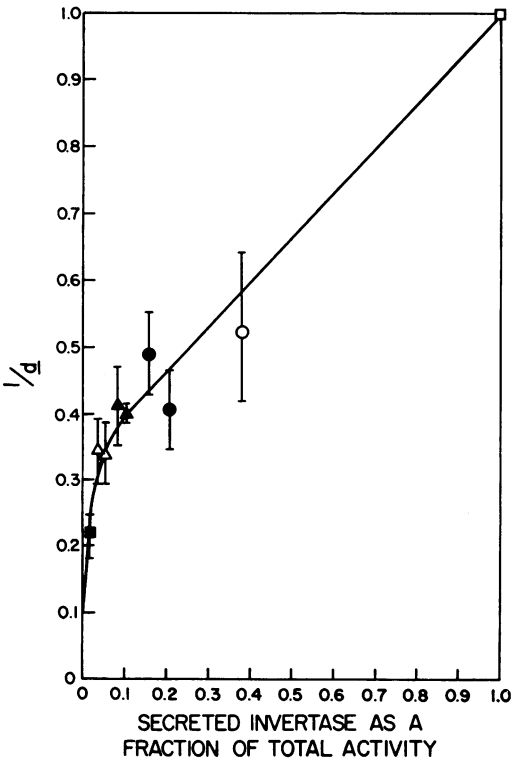

FIG. 2. *Sieving of isozymes as a function of the fraction of the total invertase secreted. The ordinate, 1/d, is the reciprocal of the degree of fractionation of light from heavy invertase, as defined in the text. The symbols are as follows:* △, *wild type;* ▲, *crisp;* ●, *crisp osmotic;* ○, *osmotic;* □, *wild-type protoplasts;* ■, *wild type with cycloheximide 10 μg/ml. This figure is a composite of several experiments.*

that an extensive depolymerization had occurred. By the absorbancy at 260 mμ, it was possible to correct for the less-than-quantitative recovery of the DNA.

Evidence that the recovered material was DNA includes the following. Treatment of the putative recovered DNA with pancreatic deoxyribonuclease and phosphodiesterase (13) gave rise to digestion products which showed an elution pattern from Dowex-1-formate similar to that obtained with a digest of authentic DNA. No such materials were recoverable from the culture filtrate of a flask to which no DNA "trap" had been added. The recovery of the DNA "trap" was 80% of the added material in the case of *osmotic* and was quantitative in *wild type*. Of the alcohol-insoluble material recovered from the culture filtrates of *wild type* and *osmotic*, 57 and 56% of the radioactivity, respectively, was adsorbable by Norite and was judged to be associated with DNA and oligodeoxynucleotides. From these data on the intra- and extracellular DNA-P^{32}, it was calculated that during the standard 3-hr incubation, 0.6 and 0.9% of the DNA of *wild type* and *osmotic*, respectively, appeared in the medium. Presumably, these figures also represent the upper limits of cytolysis. Such values are many times lower than would be required to explain the secretion of invertase. It is possible that even the release of this small proportion of the DNA does not represent lysis, but rather secretion. At least in the case of ribonucleic acid, it is clear that a nucleic acid can be secreted by *Bacillus subtilis* without cell lysis (7). Hence, it seems that the increased secretion and decreased fractionation of invertase in *osmotic*, and presumably in the strains of intermediate properties, are attributable to altered sieving at the cell wall.

Two crude physical models of the alteration in cell walls may be offered: (i) the strains might differ in the cross-sectional area of the pores in their cell walls and (ii) the strains might differ in the thickness of their walls and, thus, in the length of the channels through which the invertase may presumably be fractionated during the process of secretion into the medium. These hypotheses are obviously not mutually exclusive, and the true situation may involve both of these considerations. In the accompanying paper (30), evidence is presented that purified cell walls of *osmotic* have more space available to high molecular weight polymers and show striking differences in chemical composition when compared with those of *wild type*. Since the volume of distribution technique is an equilibrium measurement rather than a kinetic one, the first model suggested appears to provide a more satisfying explanation of the results.

M. S. Manocha and J. R. Colvin (*personal communication*) have studied the ultrastructure of the *Neurospora* cell wall by electron microscopy combined with enzymatic dissection. They have found direct evidence of the existence of microchannels, with diameters in the range of 40 to 70 A. These microchannels constitute a branching, three-dimensional system; Manocha and Colvin have independently suggested on the basis of this and other evidence that "they serve as conduits for movement of macromolecular substances, perhaps in both directions."

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