

## Distribution of Wall-bound Invertase during the Asexual Life-cycle of *Neurospora crassa*

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### SUMMARY

The association of invertase with *Neurospora* wall was studied biochemically in six cytologically distinct stages of the *Neurospora* asexual life-cycle: (i) conidia, (ii) mid-log phase [10 h old], (iii) post-log phase hyphae [18 h old], (iv) aerial mycelium [4 days old], (v) submerged mycelium [4 days old], and (vi) mycelium [2 days old] induced to form conidiophores. The percentages of total invertase activity found in the wall fraction were 8, 18, 23, 8, 21 and 17, respectively. The specific activities of the wall fraction were 3, 85, 120, 40, 24, and 74 units of invertase activity per milligram protein, respectively. Histochemical localization of invertase with an indirect immunofluorescent technique also showed that conidiophores had strong activity in their walls as well as in the cytoplasm and that vacuoles were devoid of any activity.

### INTRODUCTION

Fungal development is usually accompanied by changes in enzyme activities (Zalokar, 1959; Nagasaki, 1968; Stine, 1969). A few mural exoenzymes in fungi are also present in amounts that vary with the age of the organism (Horikoshi & Ikeda, 1965; Mahadevan & Mahadkar, 1970). Since invertase [EC. 3.2.1.26,  $\beta$ -D-fructofuranoside fructohydrolase] is one of the mural exoenzymes found in *Neurospora* (Chang & Trevithick, 1972) this report records its changing distribution in the wall fraction during the *Neurospora* asexual life-cycle, as represented by six cytologically distinct stages. A histochemical technique using indirect immunofluorescent staining was also used to elucidate finer details of invertase localization.

### METHODS

*Organism.* Wild-type *Neurospora crassa* was grown on Fries minimal medium (Beadle & Tatum, 1945), 2.7% (w/v) galactose, and sodium succinate buffer (0.04 M, pH 5.2).

*Growth conditions.* Conidia in large quantities were obtained by the method of Zalokar (1959) from 7-day cultures grown in a Fernbach flask containing 500 ml of agar-solidified medium. Mid-log phase mycelium was obtained from cultures (2 ml of conidial suspension, extinction at 500 nm = 20, inoculated into 100 ml of growth medium in a 250 ml Erlenmeyer flask) grown at 30° and agitated at a speed of 250 rev./min in a gyratory shaker bath. It was harvested after 10 h by filtration through a membrane filter (type HA, Millipore Corp., Massachusetts, U.S.A.). Post-log phase mycelium (1 ml conidial suspension, extinction at 600 nm = 20, inoculated into 50 ml culture medium in a 125 ml Erlenmeyer flask) was similarly obtained after 18 h of incubation. Aerial and submerged mycelia were

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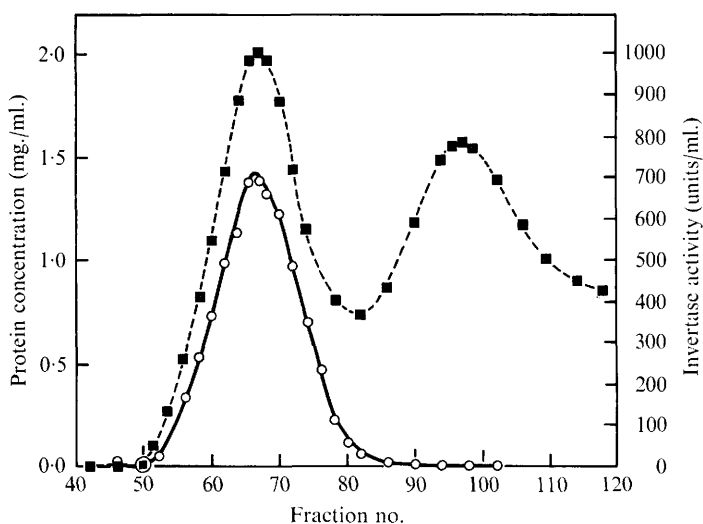


Fig. 1. Purification of invertase by Sephadex G-200 chromatography. The conditions of the experiment are described in the text. The protein concentration is calculated from the extinction at 280 nm using the extinction coefficient of invertase ( $\epsilon$  1%/1 cm = 18.6) given by Metzberg (1963*a*) and invertase activity was determined according to the method of Metzberg (1962). One unit of invertase activity is defined as the amount of enzyme releasing 1 micromole of glucose from sucrose/min at 37°. --■--, protein concentration; —○—, invertase activity.

obtained according to the method of Zalokar (1959) from 4-day stationary culture (200 ml). The mycelial mat on the surface of the culture medium in contact with air was designated as the aerial mycelium. The dispersed wisps of mycelium entirely submerged in the growth medium of the same flask were designated as the submerged mycelium. Mycelium from a 2-day shake culture (50 ml) was induced to form conidiophores by starving thin mats of mycelium on moist filter paper at room temperature for 8 h (Stine & Clark, 1967). About three mycelial mats, designated as 'conidiophores+mycelium', were obtained from 50 ml culture and pooled for each experiment.

The invertase activity was determined by the method of Metzberg (1962).

*Determination of protein.* Folin phenol reagent was used according to the method of Lowry, Rosebrough, Farr & Randall (1951). After the reaction was completed, samples with insoluble residues, e.g. walls, were centrifuged at 6000 rev./min for 10 min in a GLC Sorvall Centrifuge before the supernatant fractions were taken for colorimetric readings. Bovine serum albumin dried overnight in a vacuum desiccator over NaOH was used as the standard.

#### *Immunofluorescent staining.*

(a) *Preparation of antigen.* Invertase was prepared from *Neurospora crassa* according to the method of Metzberg (1963*a*). The final purification step with carboxymethyl cellulose column chromatography was replaced by G-200 Sephadex gel filtration which allowed almost 100% recovery of enzyme activity. A 3 × 100 cm column of G-200 Sephadex was equilibrated with buffer B (sodium succinate buffer, 0.005 M, pH 5.0, containing 0.001 M-EDTA) for over 24 h. The column was charged with about 100 mg of protein dissolved in 1.5 ml of buffer A (sodium succinate buffer, 0.1 M, pH 5.0, containing 0.001 M-EDTA). Elution with buffer B proceeded at a flow rate of 0.2 ml/min and 3 ml fractions were collected. The whole operation was performed below 4°.

Extinction at 280 nm and invertase activity of the eluted fractions were measured (Fig. 1). All of the invertase activity was recovered from fractions 50 to 82. Disc-gel electrophoresis for detecting protein components and invertase activity was performed on some fractions by the method of Metzberg (1964). A predominant single band of protein, coincident with invertase activity, was detected in fractions 59 to 67. Faster moving bands of impurities appeared in fraction 76.

(b) *Preparation of anti-invertase serum.* Electrophoretically pure invertase (3 mg in 1.8 ml of buffer B) dispersed in 1.8 ml Freund's complete adjuvant was injected subcutaneously into each of three rabbits, followed after eight weeks by a second injection of 1 mg invertase in 2 ml phosphate buffered saline (PBS: 0.9 % sodium chloride in 0.01 M-sodium phosphate buffer at pH 7.1). Rabbit serum was collected six days later by cardiac puncture and processed according to the method of Campbell, Garbey, Cremer & Sussdorf (1963). The serum gave a single precipitin line against pure invertase by Ouchterlony's two-dimensional diffusion test (Campbell *et al.* 1963). Samples were frozen and diluted 15-fold with PBS before use.

(c) *Staining procedure.* The 'Sandwich technique' according to Nairn (1969) was used. Sections of mycelium (2  $\mu$ m thick) were cut in a cryostat at  $-20^{\circ}$  and allowed to react with rabbit-anti-invertase serum. The antigen (invertase) -antibody (rabbit-anti-invertase immunoglobulin) complex so formed was allowed to react with a fluorescein-labelled goat-anti-rabbit immunoglobulin preparation. The invertase:rabbit antibody:goat antibody 'sandwich' complex was made visible by the fluorescein label when a u.v. light source was applied

Table 1. *Invertase distribution in Neurospora crassa of different physiological stages\**

Neurospora cultures grown under different conditions were harvested. Mycelia were collected by filtration, washed with distilled water, blotted dry with filter paper to give the wet weight, and disrupted by sonic treatment. A sample of the mycelial homogenate was taken for total cellular activity determination. The rest of the sample was used for wall preparation by washing with PBS, 1% (v/v) Triton X-100, M-NaCl (twice) and PBS (twice) successively. The wall fractions were recovered by centrifugation at  $100 \times g$  for 15 min each time.

	Conidia	Mid-log (100 ml)	Post-log (50 ml)	Stationary culture-4 days (200 ml)				Conidio- phores + mycelium
				Aerial mycelium		Submerged mycelium		
				Flask†	Bottle‡	Flask†	Bottle‡	
<b>A. Total cellular activity</b>								
(i) Wet wt (g)	3.46	0.35	0.29	0.69	1.32	0.26	0.39	1.30
(ii) Total activity§	144	203	179	617	963	429	671	1500
(iii) Specific activity	0.63	6.5	8.7	10.7	11.0	14.5	14.5	16.2
<b>B. Wall fraction</b>								
(i) Total activity§	11.7	37.6	41.7	43.7	89.6	96.1	137	260
(ii) Specific activity	2.93	85.4	120	40.8	40.7	22.1	26.2	74
(iii) % of A:	8.15	18.5	23.4	7.0	9.3	22.4	20.5	17.4
$\frac{B(i)}{A(ii)} \times 100\%$								

\* All entries are the means of duplicate experiments except †, ‡, and those pertaining to 'conidia'. Samples in each experiment were assayed in duplicate. Deviations of experimental data from the mean values were generally less than 10%.

† Culture grown in a 2 l Erlenmeyer flask.

‡ Culture grown in a 500 ml Roux bottle.

§ Total activity is expressed in units of invertase activity, defined as  $\mu$ mole glucose liberated from sucrose/min at  $37^{\circ}$ .

|| Specific activity is expressed as units of invertase activity/mg protein.

to the tissue section under a microscope. The procedure and modifications of this method have been described in detail by Chung & Trevithick (1970). Controls using (i) normal rabbit serum, (ii) anti-invertase rabbit serum previously adsorbed with purified invertase for 48 h, and (iii) goat anti-rabbit serum directly on the tissue sections, showed negligible fluorescence under similar conditions.

#### RESULTS

The specific activity and percentage of invertase associated with the wall fraction varied significantly in the different phases of the *Neurospora* life-cycle. (Table 1).

Both the percentage of total cellular activity and the specific activity of invertase in the wall fraction were lowest in conidia. During the log phase of growth lasting 12 to 14 h, two general trends of invertase distribution were evident: an increasing proportion of the cellular activity became incorporated into the wall fraction; specific activity of the wall-bound (mural) invertase also increased. Hence, the percentages of total cellular activity found in the wall fraction rose from 8 % in the conidia to 18 % in the mid-log phase mycelium and then to 23 % in the post-log phase mycelium; the specific activity of the mural invertase also increased 30-fold, and then 40-fold, respectively, over that in the conidial wall fraction.

The effect of ageing on the distribution of invertase can be inferred from a comparison of mycelia in or near the log phase with mycelia in a 4-day stationary culture. Specific activity of total cellular invertase showed an almost twofold increase in the stationary cultures, whereas the specific activity of the wall fraction showed a two- to fivefold decrease in these older cultures.

The effects of differentiation were demonstrated by the two populations of mycelia of the same age, i.e. the aerial mycelium and the submerged mycelium in the 4-day stationary culture. According to Zalokar (1959), morphological and biochemical differences occur as a result of such differentiation. Besides having different enzymic characteristics, the submerged or deep mycelium generally possessed more moribund features, such as vacuoles and fat globules, than the aerial or surface mycelium. The invertase distribution also varied in these mycelia. Specific activity increased (to 1.5 times) in total cellular invertase and decreased (to half) in the wall fraction of the more senescent submerged mycelium. Vacuoles were shown by indirect immunofluorescent staining to be devoid of invertase activity (Fig. 2).

In the 'conidiophores + mycelium' culture, invertase of high specific activity was found in the total mycelial homogenate as well as the wall fraction (Table 1). However, since the major portion of the sample material consisted of undifferentiated hyphae rather than differentiated conidiophores, it was difficult to ascribe the high activities to the conidiophores *per se* on biochemical grounds alone. Histochemical staining by an indirect immunofluorescent technique showed that strong invertase activity was indeed apparent both in the cytoplasm and the walls of conidiophores (Fig. 3), thus giving some qualitative support to the gross biochemical data.

#### DISCUSSION

In *Neurospora* conidia, Eberhart (1961) showed that part of the invertase was truly extracellular, i.e. washed away from the conidia with water. Metzberg (1963*b*) also showed that most of the conidial invertase was cell-bound exoenzyme, i.e. completely accessible to substrate, H<sup>+</sup>, and inhibitors in the medium. The peripheral localization of this enzyme was confirmed by Chung & Trevithick (1970) with immunofluorescent staining on conidial sections. However, only 8 % of the total cellular activity was found in this report to be in the

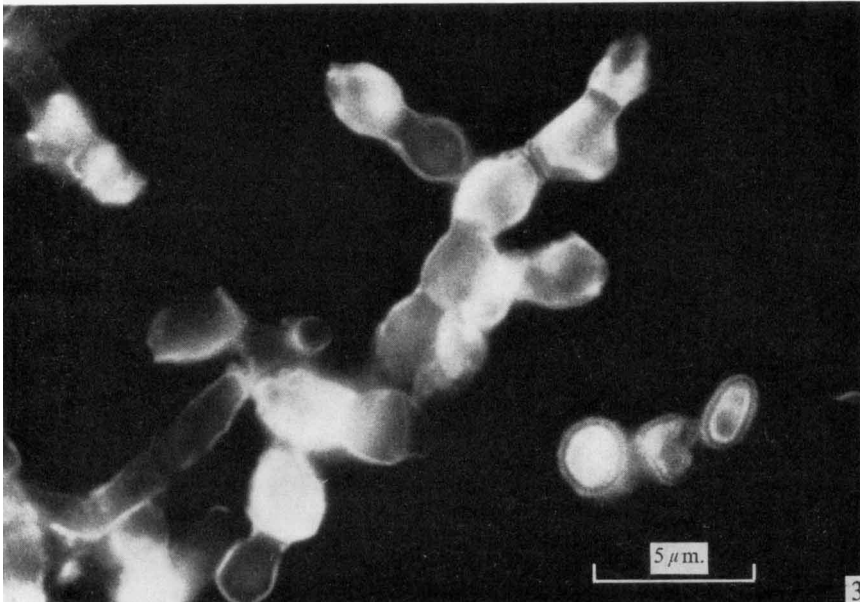
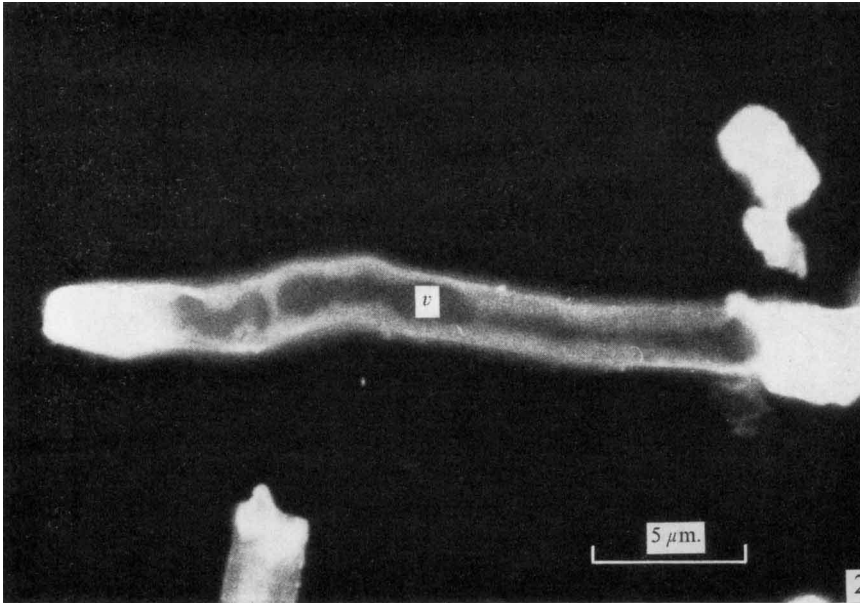


Fig. 2. *Neurospora* hypha ( $2\ \mu\text{m}$  thick) from submerged mycelium stained for invertase by an indirect immunofluorescent technique. Large vacuoles (v) were devoid of invertase activity.

Fig. 3. *Neurospora* conidiophores ( $2\ \mu\text{m}$  thick) stained for invertase with an indirect immunofluorescent technique. Strong invertase activity occurred generally in the cytoplasm and the walls.

wall fraction, i.e. a mural exoenzyme. Therefore, most of the invertase activity must be free within the periplasmic space and/or associated with the outside of the plasma membrane.

The transition of *Neurospora* to a highly active metabolic state during germination of conidia and subsequent logarithmic growth is often accompanied by increased enzymatic activities in biosynthetic pathways (Stine, 1968), in respiratory activities (Zalokar, 1959; Weiss, 1963), and in various hydrolytic reactions (Hill & Sussman, 1964; Scott & Metzberg, 1970). The increase in specific activity of total cellular invertase as well as wall-bound invertase observed here probably reflects the active synthesis and secretion of this enzyme during the initial stages of the *Neurospora* life-cycle.

In the later stages of the *Neurospora* life-cycle, when senescence was brought about either by ageing or differentiation, specific activity of the total cellular invertase also increased. Since invertase is a derepressible enzyme (Metzberg, 1962), such elevation of activity may have been caused by a relief from intracellular catabolite repression due to depletion of exogenous sugar supply, or a general reduction of metabolic activity.

The absence of invertase activity in *Neurospora* vacuoles (Fig. 2) contrasts with its presence in yeast vacuoles (Beteta & Gascon, 1971). It has been suggested that the yeast vacuoles function similarly to the zymogen granules in the mammalian pancreas (Palade, Siekevitz & Caro, 1962). If so they would correspond more closely to 'secretory vacuoles' and would be quite different in nature from the moribund vacuoles in *Neurospora* hyphae.

In conclusion, significant changes of invertase activity were observed during the *Neurospora* life-cycle. Transition from dormancy to active growth was accompanied by increased specific activity of the total cellular invertase as well as of the wall-bound invertase. In cytologically older mycelia, either from ageing or differentiation, specific activity of the total cellular invertase was also higher than in the younger mycelia but their wall fractions did not show a concomitant increase.

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