JOURNAL OF BACTERIOLOGY, Oct. 1973, p. 295-303

Vol. 116, No. 1
Printed in U.S.A.

Induction of β-Glucosidases in *Neurospora crassa*

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Received for publication 29 March 1973

The induction of β-glucosidases (EC 3.2.1.21) was studied in *Neurospora crassa*. Cellobiase was induced by cellobiose, but other inducers had little effect on this enzyme. Cellobiase activity was very low in all stages of the vegetative life cycle in the absence of di-β-glucoside inducer. Aryl-β-glucosidase was semiconstitutive at late stages of culture growth prior to conidiation. At early stages, aryl-β-glucosidase was induced by cellobiose, laminaribiose, and gentiobiose, and weakly induced by galactose, amino sugars, and aryl-β-glucosides. The induction properties of the β-glucosidases are compared with those of the other disaccharidases of *Neurospora*. The induction of β-glucosidases was inhibited by glucose, 2-deoxy-D-glucose, and sodium acetate. Sodium phosphate concentrations between 0.01 and 0.1 M stimulated induction of both enzymes, while concentrations above 0.1 M were inhibitory. The optimal condition for induction of both β-glucosidases was pH 6.0. Cellobiase induction was relatively more inhibited than aryl-β-glucosidase in the range of pH 6.0 to 8.0.

Earlier work with the β-glucosidase system of *Neurospora* concerns the mechanism whereby this primitive eukaryote regulates enzymes both as a biochemical genetic system (5, 6, 17, 18) and in terms of spatial orientation within the cell (7).

This report is concerned with the response of β-glucosidases to a variety of inducers to determine whether these two enzymes differ in induction pattern and how these results can be generalized in terms of other disaccharidases that have been studied in *Neurospora* (22).

The induction properties of the aryl-β-glucosidase and cellobiase of *Neurospora* suggest that they represent two fundamentally different classes of disacchardiases. Aryl-β-glucosidase represents the broadly inducible (or derepressible) class of enzymes, while cellobiase represents enzymes with a highly specific induction requirement. The differences in the induction patterns of these β-glucosidases reported here support earlier conclusions that they are induced as separate systems (7).

MATERIALS AND METHODS

Chemicals. *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, D-arabinose, arbutin, cellobiose, galactose, indoxyl-β-D-glucoside, maltose, mannose, β-methyl-D-glucoside, *p*-nitrophenyl-β-D-glucopyranoside (PNP-G), phloridzin, quercetin, and D-xylose were obtained from Calbiochem, Los Angeles, Calif. Aurothioglucose, 2-deoxy-D-glucose, D-fructose, glucono-δ-lactone, melibiose, phenyl-β-D-glucopyranoside, and salicin were products of Nutritional Biochemicals Corp., Cleveland, Ohio. L-Fucose, D-fucose, 6-deoxy-D-glucose-β-methyl-glycoside, melezitose, and methyl-β-D-thioglucopyranoside were purchased from Mann Research Laboratories, subsidiary of B-D Laboratories, Inc., New York, N.Y. Gentiobiose and trehalose were products of Sigma Chemical Co., St. Louis, Mo. D-Glucose and lactose were obtained from Matheson Coleman & Bell, Norwood, Ohio. Phenethyl alcohol (PEA) was purchased from Eastman Organic Chemicals, Rochester, N.Y. We are grateful to Elwyn T. Reese for the donation of laminaribiose.

Growth and harvest of conidia. Conidia were grown in Erlenmeyer flasks containing modified glycerol-complete agar medium for 7 days at 25 C (6). For harvesting, sterile, glass-distilled water was added to the growth flask, and the conidial suspension was then filtered through glass wool into centrifuge tubes and centrifuged for 5 min at 3,000 × *g*. The conidia were washed twice more with water and centrifuged after each wash. Aseptic techniques were used throughout the growth, harvest, and induction procedures.

Standard induction procedure. Washed conidia were suspended in 10 ml (final volume) of 0.1 N HCl and shaken gently for 5 min. This treatment destroyed all patent β-glucosidase activity without impairing cell viability. This was done to minimize the possibility that the wall-bound β-glucosidase could alter the β-glucoside inducers by transglucosidation (3). The conidia were then centrifuged, and the pellet was suspended in 10 ml of 0.1 M potassium phosphate buffer, pH 6.0. Conidia were inoculated into 125-ml Erlenmeyer flasks containing 40 ml of standard induction medium with an inoculum that had an optical

density reading of 0.050 to 0.100 at 600 nm (equivalent to 3×10^6 to 6×10^6 cells per ml of induction medium) in a Beckman model 151 spectrocolorimeter. In the initial experiments, 1 mM cellobiose was selected as an inducer based on studies of β-glucosidase induction in yeast (4) and cellulase induction in other fungi (19).

The inoculated flasks were placed in a reciprocal shaker-water bath set at 25 C and a speed of 160 cycles per min. The flasks were removed from the bath after 5 to 6 h and chilled in an ice bath. The contents of the flasks were then centrifuged at 3,000 × *g* for 20 min at 5 C. The supernatant fraction was discarded, and the pellet was either refrigerated at 5 C prior to further treatment of frozen at −25 C.

The two strains, 74-OR8-1a and 33(2-6)A, used throughout the investigation are both nutritionally autotrophic, and 33(2-6)A contains the yellow and *cot-1* mutations. Many of the induction experiments in this report were repeated with a different system. Strain 33(2-6)A was grown at 33 C for 40 h to achieve uniform colonies. The colonies were washed, induced as above at 25 C, harvested by filtration on Whatman no. 1 paper, washed twice with water, and frozen at −25 C.

For extraction, the mycelial colonies were removed from the freezer and placed in 30 ml of 0.01 M potassium phosphate buffer (pH 6.0) and 10 g of glass homogenizing beads in the 50-ml chamber of a Sorvall Omni-Mixer no. 155 for 10 min at a setting of 8. The resulting slurry was then treated with a Branson Sonifier for 1 min at 5 C at a setting of 6.3 A and allowed to extract for 1 h at 5 C with frequent stirring prior to centrifugation for 1 h at 13,000 × *g* at 5 C. This induction gave essentially the same results shown with conidia, thus confirming the equivalence of the induction systems for the β-glucosidases in conidia and mycelia.

Assay methods. β-Glucosidase activity in cell-free extracts was assayed by using a discontinuous method (5) with PNP-G as substrate.

β-Glucosidase activity in intact cells and modified cells was also assayed by the discontinuous PNP-G method with the following modifications. Each cell sample was evenly suspended, and a small fraction was removed for a cell count (hemocytometer). Cell samples of 0.1 ml were pipetted into test tubes (10 by 75 mm); 0.9 ml of standard buffer containing 1 mg of PNP-G was added to each tube to start the reaction. This mixture was shaken gently for 10 min, and the enzyme reaction was stopped by the addition of 0.5 ml of 1 M tris(hydroxymethyl)aminomethane. Cells were removed by centrifugation at 3,000 × *g* for 10 min. After induction, cells were exposed to 1 mM PEA (final concentration) which altered their permeability, thus releasing the cryptic cellobiase (7). Both β-glucosidases were then assayed directly. The ratio of aryl-β-glucosidase to cellobiase was determined by comparing two samples, one of which had been heated for 1 min at 60 C. Cellobiase activity was destroyed by this treatment, and the activity remaining after heat treatment was attributed to aryl-β-glucosidase. The heated samples were immediately cooled in an ice bath for 15 min and then returned to 25 C for assay.

Calculations of activity. One enzyme unit is defined as 1 μmol of PNP released per min at 25 C as measured at 410 nm in a Beckman 151 spectrocolorimeter. Specific cell activity is expressed as units per 10^8 cells.

RESULTS

Induction with pregerminated conidia. Both strains showed induction of aryl-β-glucosidase and cellobiase during a 7-h period (Fig. 1). Aryl-β-glucosidase production became apparent first while cellobiase activity lagged by about an hour and then paralleled the rate of aryl-β-glucosidase production. The lack of further induction beyond 7 h is probably due to the limitation of metabolic reserves since there was no carbon source other than 1 mM cellobiose in the medium. The amount and rate of enzyme synthesis varied in other strains and in different batches of conidia, but the results generally paralleled those shown in Fig. 1. Cellobiase activity often showed a decline after it has reached a maximum, while aryl-β-glucosidase activity reached a stable plateau. This difference may be due to the location of aryl-β-glucosidase in the mural space that seems to stabilize this enzyme (7, 26).

Effect of pH on induction. Conidia were induced for 6 h under standard conditions by using 0.1 M potassium phosphate buffer with pH adjusted in a range from 5.0 to 8.0. Induced cells were centrifuged and resuspended in 0.1 mM phosphate buffer, pH 6.0, prior to the standard assay. Both β-glucosidases possessed an induction optimum at pH 6.0 (Fig. 2). Aryl-β-glucosidase induction was less affected by changes of pH, while cellobiase induction was greatly reduced above pH 7.0. If the pH effect is limited to the exterior of the cell membrane, this may suggest that either the uptake or the utilization of cellobiose (at the surface of the cell) is different in the cellobiase induction system than in the aryl-β-glucosidase induction system. There is some reason to believe that the changes due to pH shown here do not go beyond the membrane into the interior of the cell (7, 26). One explanation is that there are two transport systems for cellobiose that may lead, respectively, to the induction of either aryl-β-glucosidase or to cellobiase.

Effect of buffer molarity on induction. Conidia were induced in the standard way while varying the concentration of potassium phosphate buffer (pH 6.0) from 0 to 500 mM. The induction of both enzymes was greatly inhibited at concentrations of buffer greater than 100 mM (Fig. 3). Cellobiase induction was more inhibited at these high concentrations than was

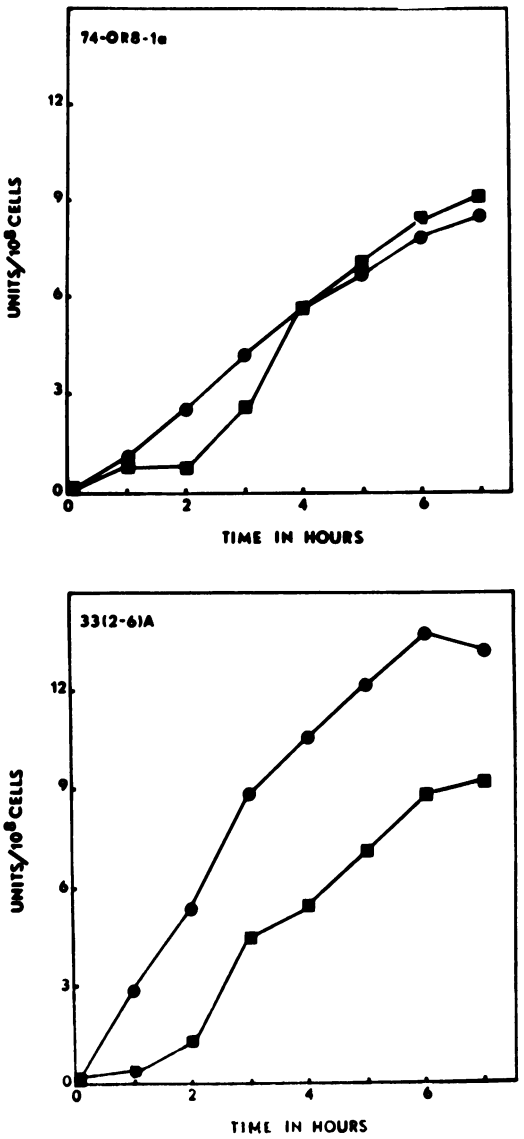

FIG. 1. *Induction of β-glucosidases in Neurospora strains. Induction conditions and whole-cell assay were standard (see Materials and Methods). Inducer was 1 mM cellobiase. Enzymes were:* ●, *aryl-β-glucosidase;* ■, *cellobiase.*

aryl-β-glucosidase induction. In strain 74-OR8-1a, the induction of both enzymes at lower concentrations was similar. In strain 33(2-6)A, induction of aryl-β-glucosidase was relatively greater at concentrations below 100 mM. We are presently investigating the possibility that high molar phosphate buffer blocks the entry of the inducer. The results shown in Fig. 3 may reflect an inhibition of cellobiose permeation imposed by potassium or phosphate ions. Again, the differential response of the induction systems (thought to be internal to the membrane) at high phosphate molarities suggests two "paths" that the cellobiose may follow through the membrane to the two respective induction systems.

Effect of cellobiose concentration on induction. The effect of increasing concentration of

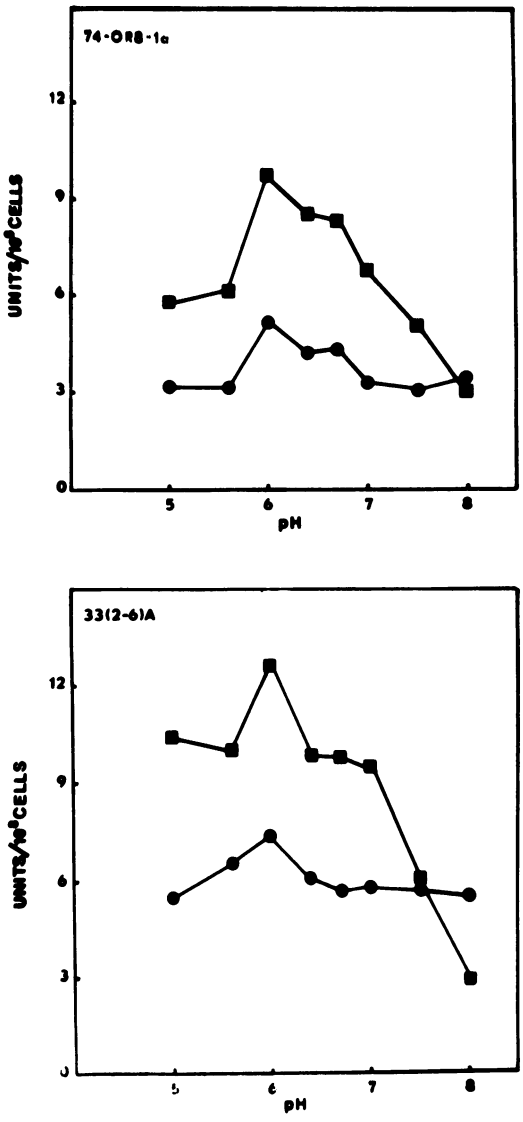

FIG. 2. *Effect of pH on the induction of β-glucosidases. Standard 6-h induction procedure was used with 1 mM cellobiose at 0.1 M concentration. Assays were by standard intact cell method. Enzymes were:* ●, *aryl-β-glucosidase;* ■, *cellobiase.*

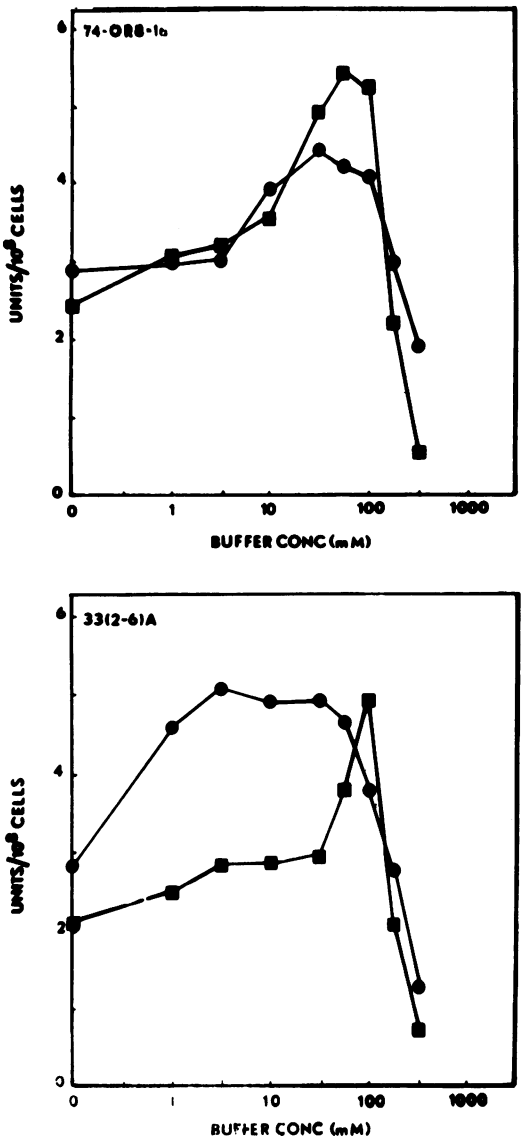

FIG. 3. *Effect of increasing molarity of potassium phosphate buffer on the induction of β-glucosidases. Cells were prepared and induced by standard procedure. Standard intact cell assay was used. Potassium phosphate levels varied as indicated. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiase. Strain number is shown on the graph.*

cellobiose on the standard induction of β-glucosidase is shown in Fig. 4. Both enzymes in both strains were induced increasingly in the range from 0 to 5 mM cellobiose. Beyond a concentration of 5 mM, the induction effect decreased. The decreased induction at 10 mM may be due to low levels of glucose present in commercial cellobiose or to the conversion of cellulose to glucose by the fungus at these concentrations.

Induction by di-β-glucosides. The relative induction efficiency of three di-β-glucosides was tested by exposing conidia from strain 74-OR8-1a to increasing concentrations of cellobiose, laminaribiose, or gentiobiose. The induction of cellobiase is shown in Fig. 5. Laminaribi-

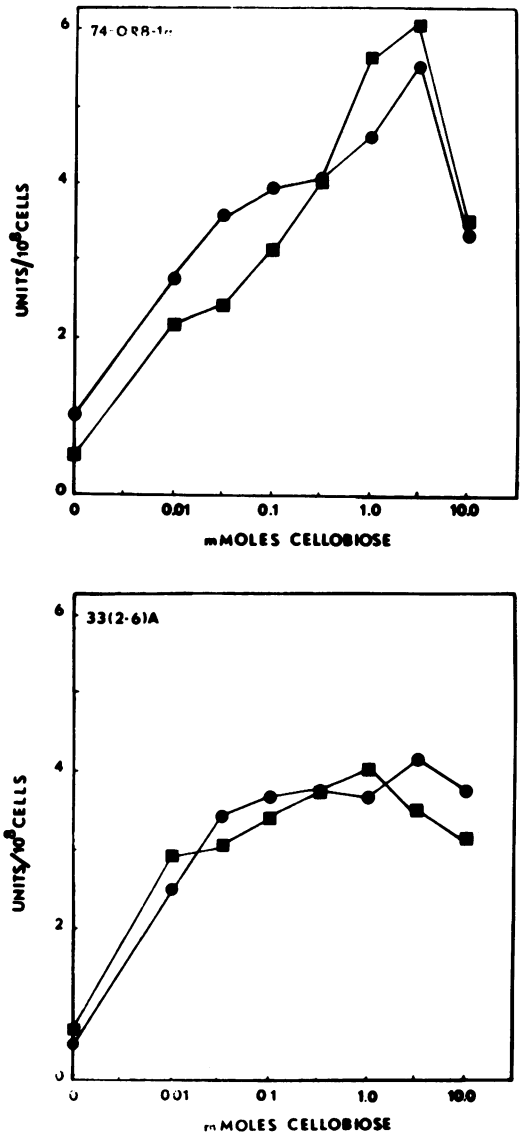

FIG. 4. *The effect of cellobiose on the induction of β-glucosidases. Cells were prepared by standard 6-h induction procedure with cellobiose concentrations indicated. Standard cell assay was used. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiase.*

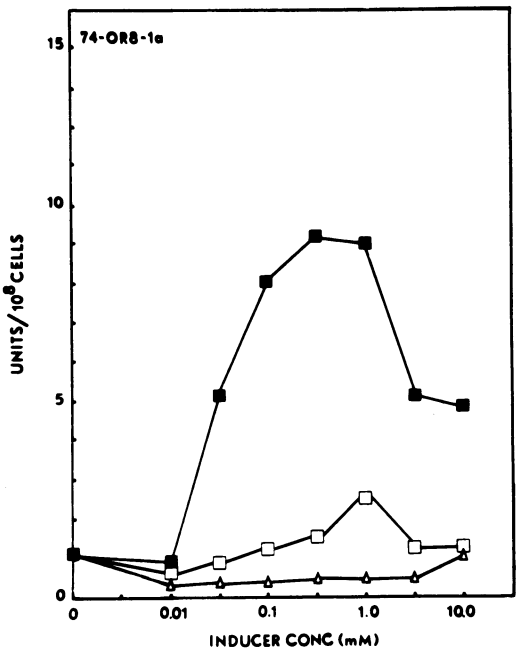

FIG. 5. *Induction of cellobiase by β-diglucosides. Induction (6 h) and assay were standard. Inducers were at 1 mM concentrations:* ■, *cellobiose;* □, *laminaribiose;* ▲, *gentiobiose.*

ose and gentiobiose were relatively ineffective inducers compared to cellobiose. A marked inhibition by higher concentrations of cellobiose in this experiment occurred above 10 mM concentration.

The responses of the aryl-β-glucosidase induction system in the same experiment are shown in Fig. 6. A maximal induction was reached at 0.1 mM with cellobiose and laminaribiose, while gentiobiose continued to increase in effectiveness up to a concentration of 10 mM. Cellobiose again was the most effective inducing agent, followed by gentiobiose and laminaribiose. Higher concentrations of cellobiose were only slightly inhibitory to aryl-β-glucosidase induction.

Other β-glucosidase inducers. A variety of compounds were tested for their ability to induce both β-glucosidases using the standard 6-h conidial induction. The results shown in Tables 1 and 2 are calculated as a percentage of standard cellobiose induction. Among the monosaccharides, xylose and galactose induced aryl-β-glucosidase slightly, while galactose had a slight positive effect on cellobiose induction.

The disaccharide maltose induced both β-glucosidases to a limited extent. There is no uniform induction pattern among the oligo-β-glucosides. Aryl-β-glucosidase was slightly induced by arbutin, salacin, and phenyl-β-D-

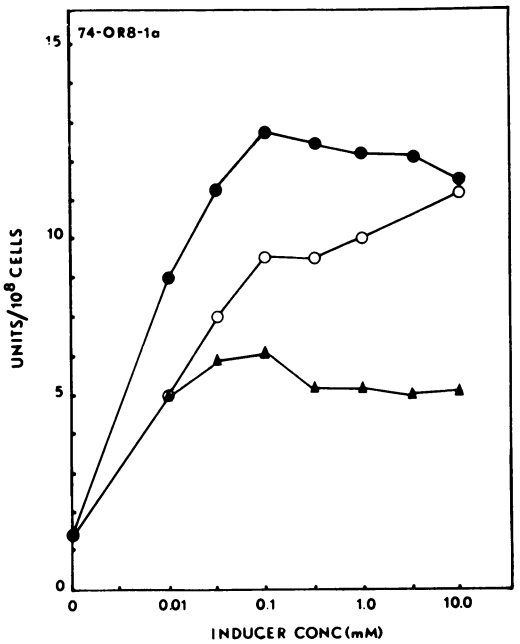

FIG. 6. *Induction of aryl-β-glucosidase by β-diglucosides. Induction (6 h) and assay were standard. Inducers were at 1 mM concentrations:* ●, *cellobiose;* ○, *laminaribiose;* ▲, *gentiobiose.*

TABLE 1. *Induction of β-glucosidases by monosaccharides and disaccharides*[a]

Inducers	74-OR8-1a		33(2-6)A	
	Cellobiase	Aryl-β glucosidase	Cellobiase	Aryl-β glucosidase
Control	5	8	4	6
D-Arabinose	5	12	8	13
D-Xylose	6	20	5	23
D-Fucose	0	−8	−6	0
L-Fucose	0	0	2	8
D-Fructose	8	9	7	5
D-Galactose	14	43	21	45
D-Glucose	13	11	12	4
Mannose	10	12	7	5
Cellobiose	100	100	100	100
Maltose	25	30	23	45
Mellibiose	0	0	−4	−5
Melezitose	5	0	13	12
Lactose	5	6	1	6
Trehalose	6	2	6	9

[a] Cultures were induced 6 h under standard conditions. Values represent percentages of the cellobiose induction. Each inducer was at 1 mM concentration.

TABLE 2. *Induction of β-glucosidases by heteroglucosides and sugar derivatives*[a]

Inducers	74-OR8-1a		33(2-6)A	
	Cellobiase	Aryl-β glucosidase	Cellobiase	Aryl-β glucosidase
Control	5	8	4	6
Arbutin	10	15	15	14
Indoxyl-β-D-glucoside	6	7	2	5
β-methyl-D-glucoside	2	0	7	8
Methyl-β-D-thiogluco-pyranoside	0	0	2	−6
PNG-G	5	14	8	5
Phenyl-β-D-glucopy-ranoside	4	17	3	19
Phloridzin	1	6	−3	3
Quercetin	7	0	5	−1
Salicin	7	25	0	24
6-Deoxy-D-glucose β-methyl-glucoside	1	3	5	−2
N-acetyl-D-galactosa-mine	10	30	17	22
N-acetyl-D-glucosa-mine	1	38	2	26
N-acetyl-D-mannosa-mine	1	30	0	20
Aurothioglucose	1	31	2	16
Glucono-δ-lactone	10	12	13	12

[a] Conditions were identical to those in Table 1. Values represent percentage of induction by mM cellobiose standard.

glucopyranoside but not by aliphatic or sulfur glucosides. Cellobiase induction was not significantly stimulated by these oligo-β-glucosides.

Of the glucose derivatives, the D-acetyl amino sugars stimulated aryl-β-glucosidase production while glucono-δ-lactone had only a slight stimulus for both β-glucosidases.

Synergistic effects with mixed inducers. Those saccharides that showed a slight positive induction effect were tested in mixtures with cellobiose to determine whether the effects were additive. Most combinations showed only slight or no additive effect. When a mixture of xylose and cellobiose was used for induction (Fig. 7), cellobiase was apparently induced increasingly by greater xylose concentrations, while added xylose did not change significantly the induction of aryl-β-glucosidase. This apparent synergistic effect of xylose is due to a modification of the cellobiase assay system rather than to a direct effect on cellobiase induction. We have found that xylose accelerates the hydrolysis of PNP-G by purified cellobiase. This is probably due to the stimulation of transferase activity by cellobiase (3), but as yet a stable transferase product with xylose has not been found. A similar stimulatory effect by ethylene glycol and related compounds has been shown on the

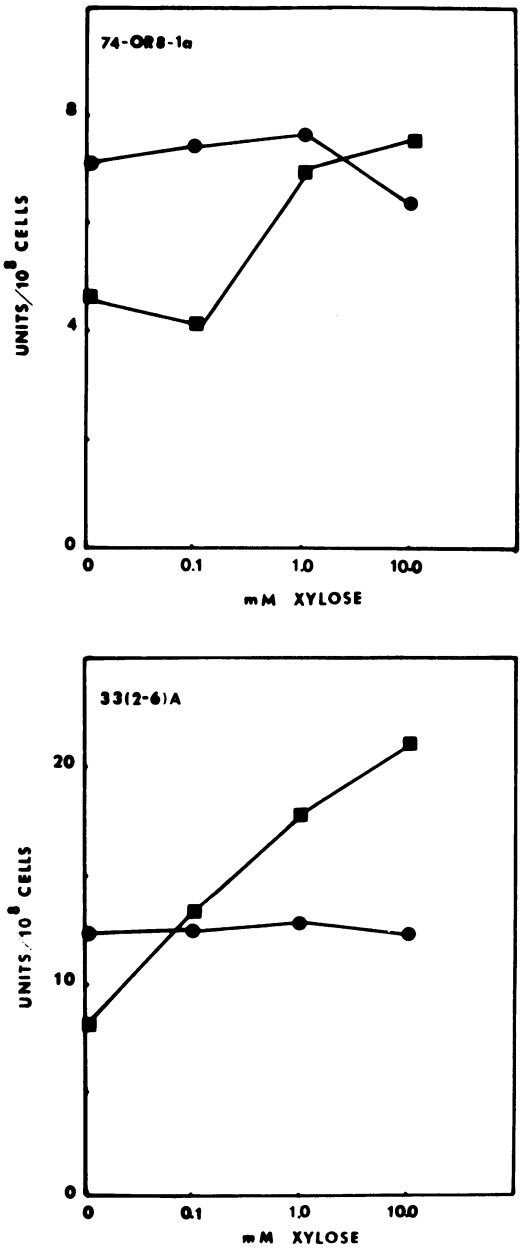

FIG. 7. *Effect of xylose on the induction of the β-glucosidases in two wild-type strains. Standard 6-h induction was used. All cells were induced with 1 mM cellobiose and with increasing concentrations of xylose added at the beginning of the induction. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiase.*

assay of β-galactosidase of *Neurospora* (14). In this connection, cells grown on xylose for 6 h have very high cellobiase activity which can be abolished 3 h after xylose is washed from the medium.

The effect of mixtures of glucose and cellobiose on induction is shown in Fig. 8. Increasing glucose concentrations inhibit the positive effect that 1 mM cellobiose has on the induction of both β-glucosidases.

A similar experiment with 1 mM cellobiose and 2-deoxy-glucose is shown in Fig. 9. This deoxy sugar is not metabolized by *Neurospora* (20, 24), but it is nearly as effective as glucose in blocking induction. This suggests that the effect of both sugars may be an inhibition of cellobiose permeation rather than a direct effect on transcription or translation of the messenger ribonucleic acid for either β-glucoside. Without cellobiose uptake studies, this point remains unsettled.

Sodium acetate also inhibits the induction of both β-glucosidases by 1 mM cellobiose (Fig. 10). It is possible that, in this case, acetate blocks the uptake of cellobiose in *Neurospora* as it blocks glucose uptake (20). Since acetate also alters the crypticity of surface enzymes (26), it is not clear what the mechanism of action may be involved in blocking β-glucosidase induction.

Production of aryl-β-glucosidase in aging cultures. As in the case of several disaccharidases (12, 27), aryl-β-glucosidase is produced late in the vegetative life cycle of *Neurospora*. The presence of this enzyme in conidia is probably carried over from pre-conidial cultures (3, 7). The occurrence of aryl-β-glucosidase seems correlated with the initiation of conidiation (Fig. 11). By contrast, cellobiase activity remains low throughout the entire vegetative cycle unless an inducer, such as cellobiose, is added to the medium.

Miscellaneous induction effects. The addition of yeast extract (0.1%) to the induction medium stimulates the induction of cellobiase by cellobiose by almost threefold without strongly affecting aryl-β-glucosidase induction. The active component is not known.

Sodium citrate (0.1 mM) inhibits both the induction of cellobiase and aryl-β-glucosidase by over 50% when cellobiose is the inducer. Induction of both enzymes is completely inhibited by (10 mM) sodium citrate.

Under standard induction conditions, sodium azide (10 mM) inhibits cellobiose induction almost completely while aryl-β-glucosidase is inhibited 60%. In a similar manner, when 0.5M sodium arsenate (pH 6.0) is used in the absence of phosphate, cellobiase synthesis is almost completely inhibited, while 25% of expected aryl-β-glucosidase is produced. The same differential inhibition is shown with mM cyclic

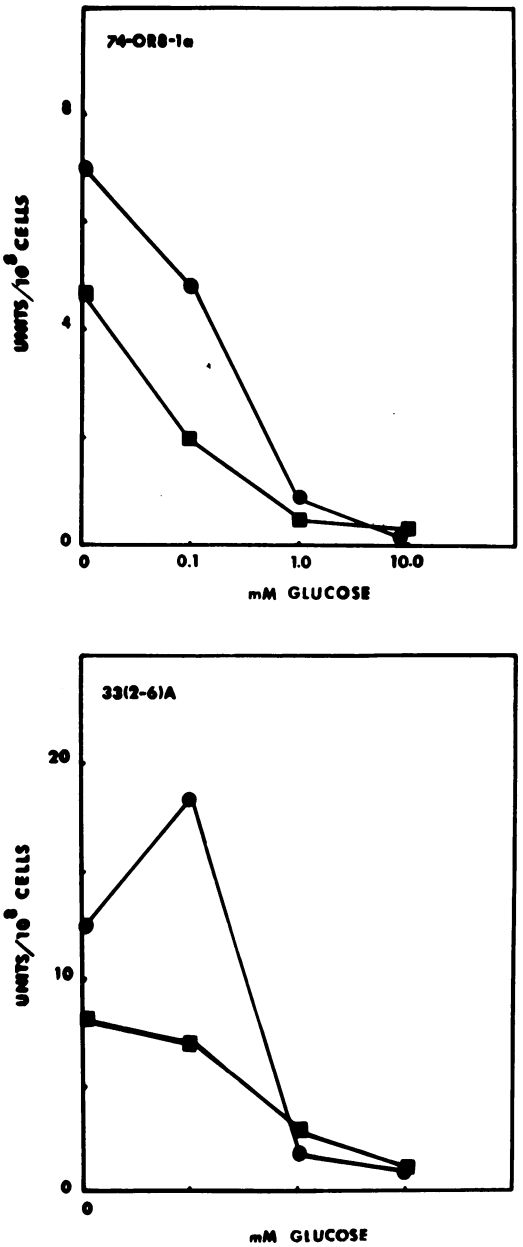

FIG. 8. *Effect of glucose on the induction of the β-glucosidases in two wild-type strains. Standard 6-h induction was used. All cells were induced with 1 mM cellobiose and with various concentrations of glucose added at the beginning of the induction. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiose.*

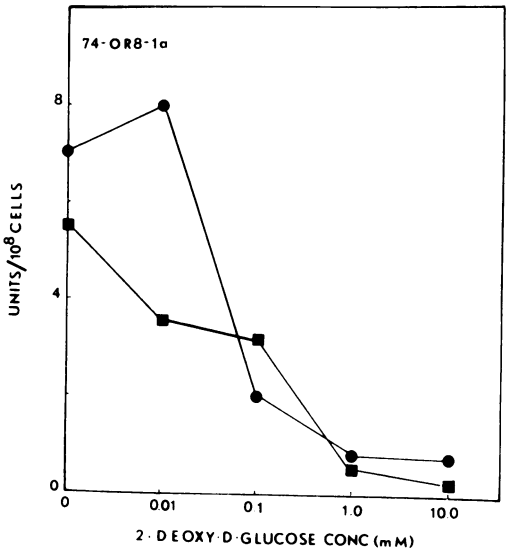

FIG. 9. *Effect of 2-deoxy-D-glucose on induction of β-glucosidase activity. Cells of strain 74-OR8-la were prepared by the standard 6-h induction and assay methods. Inducer was 1 mM cellobiose. 2-Deoxy-D-glucose was added at the start of induction. β-glucosidases are: ●, aryl-β-glucosidase; ■, cellobiase.*

adenosine-3′,5′-monophosphate where cellobiase is inhibited 90%, while aryl-β-glucosidase is inhibited only 50%. Cyclic adenosine 3′,5′-monophosphate (1 mM) does not overcome the glucose inhibition of induction or cause increases in either β-glucosidase induction by itself.

DISCUSSION

The disaccharidases of *N. crassa* fall into two classes based on their production in response to specific inducers or various conditions of growth. The first class of enzyme, illustrated by invertase, trehalase, and acid β-galactosidase, can be induced either by disaccharides that are usually substrates or induced equally well by certain monosaccharides that may be neither normal substrates nor combine with the enzyme itself (1, 10, 11, 13, 14, 21). This class of enzyme becomes constitutive late in the vegetative life cycle prior to conidiation (12, 27). Induction in the presence of monosaccharides and spontaneous production associated with conidiation seem to be reversals of catabolite repression (8, 9, 15). This agrees with observations that enzyme is not produced when a significant level of glucose is present in the induction medium.

The range of increase for both β-glucosidases is well below that for similar enzyme systems in bacteria (2, 25) but comparable to other disaccharidases of *Neurospora*. Both cellobiase and aryl-β-glucosidase seem to be exceptions to the general situation that disaccharide substrates are not the best inducers of specific disaccharidases in *Neurospora* (1, 11, 21). It is significant

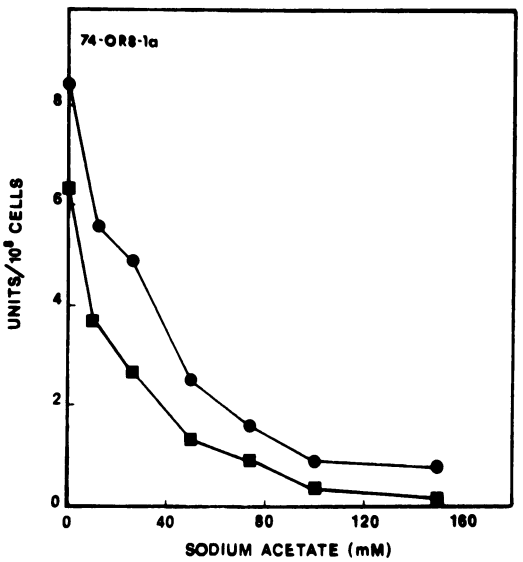

FIG. 10. *Induction of β-glucosidases by 1 mM cellobiose with added sodium acetate. Conditions of induction were standard using strain 74-OR8-la. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiase.*

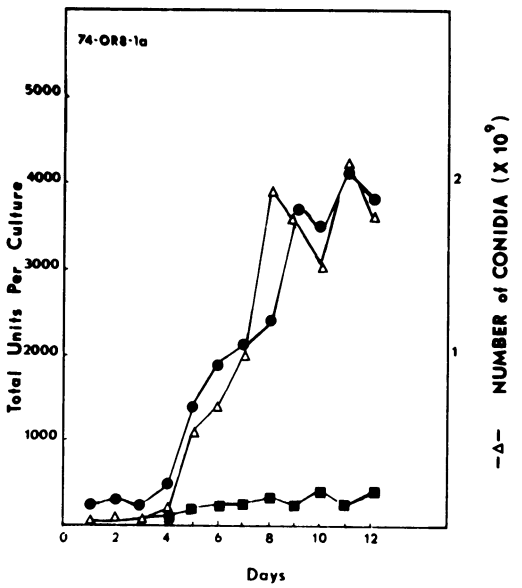

FIG. 11. *Production of β-glucosidases and conidia by standing cultures of strain 74-OR8-la. Cultures contained 25 ml of Vogel media plus 2% sucrose in 250-ml Erlenmeyer flasks. The total contents were filtered and assayed by standard methods. Enzymes were: ●, aryl-β-glucosidase; ■, cellobiose; Δ, conidia.*

that both enzymes are produced most effectively by cellobiose induction. Aryl-β-glucosidase is also induced by several aryl or alkyl β-glucosides and some non-β-glucosides. It is apparently sensitive to derepression and is normally produced just prior to conidiation. As in the case of trehalase, this enzyme seems to be under a general control system that is activated by conditions that produce less than maximal cell growth rates (11, 22).

Cellobiase represents a second class of disaccharidase which is relatively specific in its requirements for induction. Cellobiase is only slightly affected by monosaccharides or other inducers and is not significantly produced in the vegetative life cycle without application of cellobiose. This specificity places cellobiase at one extreme of the spectrum of *Neurospora* disaccharidase types.

The distinctive induction properties of each β-glucosidase suggest that the two β-glucosidases are indeed products of different systems. This is not unexpected because of the physical and genetic differences in these enzymes (7, 16, 24).

Induction studies may be complicated by the presence of an enzyme in the cell wall that can destroy the inducer before effective entry into the cell. Even though normal levels of aryl-β-glucosidase in conidial walls do not prohibit a significant induction of both β-glucosidases, induction can be enhanced (25%) by prior removal of mural aryl-β-glucosidase activity by acid treatment. We propose that wall-bound disaccharidases may form new transglucosidation products with unknown induction capabilities. As yet this transglucosidation effect has only been demonstrated for aryl-β-glucosidase in vitro (3). Murzluf and Metzenberg (20) used the term "cytotropic" to describe an orienting effect that a mural enzyme might have on the entry of compounds such as sugars into a cell. The wall-bound aryl-β-glucosidase (7) may have a second kind of quasi regulatory role in the enzyme induction of both β-glucosidases by alteration or destruction of inducer molecules.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant G.B.8453.

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