Effect of L(−)Sorbose on the Release of β-Glucosidase by *Trichoderma reesei* QM9414

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L(−)Sorbose, a sugar known to cause paramorphogenesis in fungi, was tested for its effect on morphology and release of cell-wall bound β-glucosidase (EC 3.2.1.21) in the cellulolytic fungus *Trichoderma reesei* QM9414. Sorbose caused an increase in branching and septation in the growing mycelium. Extracellular β-glucosidase activity was enhanced when cellobiose or cellulose growth medium was supplemented with sorbose. In sorbose-supplemented cultures, the β-glucosidase activity associated with the wall fraction was less than half that in unsupplemented cultures. The intracellular activity was also lower in the sorbose-supplemented cultures than in unsupplemented controls. The glucosamine/glucose ratio of wall hydrolysates from sorbose-supplemented cultures was about twice that of control hydrolysates. Since β-glucosidase is closely associated with 1,3-β-glucan in the walls of *T. reesei*, a decrease in wall glucan content, and the resulting weakened association of the enzyme with the walls, is probably responsible for its increased release into the culture medium in the presence of sorbose.

INTRODUCTION

L(−)Sorbose, a ketohexose, was first shown to induce colonial morphology in *Neurospora crassa* by Tatum *et al.* (1949). When supplemented with sorbose, wild-type *N. crassa* forms isolated, well-defined colonies on solid medium and grows as fine, tight pellets in liquid culture medium, resembling in this respect its colonial mutants. This environmentally induced change in morphology without any corresponding genetic alteration is termed 'paramorphogenesis' (Tatum *et al.*, 1949). Hyphae of *N. crassa*, which normally grow as long, sparsely branched filaments, exhibit a marked increase in number of branches and septa per unit length in the presence of sorbose. This paramorphogenic change is associated with an increase in the glucosamine/glucose ratio in cell wall hydrolysates (DeTerra & Tatum, 1961). Incorporation of glucose into the 1,3-β-glucan fraction of *N. crassa* walls decreases in the presence of sorbose (Mahadevan & Tatum, 1965; Crocken & Tatum, 1968), and sorbose inhibits the activities of 1,3-β-glucan synthase and glycogen synthase in *N. crassa* when added to growth medium or to an *in vitro* system (Mishra & Tatum, 1972).

The β-glucosidase from *Trichoderma reesei* is particularly important since the cellulase enzyme system from this source is so far the cellulase of choice for practical saccharification of cellulose (Bisaria & Ghose, 1981; Mandels, 1982; Woodward & Wiseman, 1982; Gilbert & Tsao, 1983). However, the β-glucosidase activity in the culture filtrate of *T. reesei* is too low for practical saccharification of cellulose and has to be supplemented from other sources (Allen & Sternberg, 1980). β-Glucosidase is reported to be a cell-wall-bound enzyme in *T. reesei*, associated with 1,3-β-glucan and chitin wall polymers (Kubicek, 1981; Nanda *et al.*, 1982). Given the effect of sorbose on wall glucan synthesis in *N. crassa*, it was of interest to investigate its effect on the release of wall-bound β-glucosidase in *T. reesei*. The reported increase in number of hyphal branches in the presence of sorbose is also relevant to studies of cellulase secretion, because in fungi, hyphal tips are important in enzyme secretion (Chang & Trevithick, 1974). Therefore, the effect of sorbose on the degree of branching of mycelia of *T. reesei* was also studied.

METHODS

Organism and growth conditions. *Trichoderma reesei* QM9414 (formerly *T. viride* QM9414) was used. It was maintained on malt agar slants at 4 °C, and subcultured every month.

Vogel (1956) salt solution as modified by Montenecourt & Eveleigh (1977) was used as the basal growth medium. For preparation of inoculum, glucose (0·2%) was added to the basal medium. Batches of this medium (100 ml in 500 ml flasks) were inoculated with *T. reesei* and incubated at 28 °C on a gyratory shaker at 250 r.p.m. These cultures were used to inoculate cellobiose or cellulose medium after 24 h growth. Cellobiose (0·5%) and Avicel cellulose (1%) were used as cellulase-inducing substrates. Sorbose was added to the medium at different concentrations as indicated. Cellobiose and sorbose stock solutions were filter-sterilized and added to the autoclaved basal medium. Cultures were incubated at 28 °C on a gyratory shaker at 250 r.p.m.

To study the effect of sorbose addition at various stages of growth of *T. reesei* in Avicel cellulose medium on extracellular activity of β-glucosidase, the fungus was cultivated on medium with 1% Avicel cellulose and on days 2, 4, 6 and 8 the contents of one flask were divided into two. Sorbose (5%, w/v) was added to one portion of the culture while the other served as a control. Samples were withdrawn on subsequent days and assayed for β-glucosidase activity.

Precipitation of extracellular enzyme and isolation of mycelial components. Mycelia from control (0·5% cellobiose) and test (0·5% cellobiose plus 5% sorbose) cultures were harvested at specified intervals and centrifuged. Chilled acetone (5 ml) was added to 1 ml culture filtrate kept at 4 °C and the protein precipitate was removed by centrifugation at 44000 *g* for 30 min at 4 °C. The precipitate, which recovered 90% of the protein as determined by the Lowry method, was dissolved in 1 ml 0·05 M-sodium citrate buffer, pH 4·8, and used for activity measurement.

The mycelial pads obtained after centrifugation of the culture broth were washed five times with distilled water and lyophilized. Portions (10 mg) of lyophilized control and test mycelia were subjected to ultrasonication for 25 min at 4 °C. The conditions for ultrasonication were standardized to obtain near complete disintegration of hyphae (as observed microscopically) and maximum protein release (checked by protein analysis of supernates). The suspension was centrifuged at 11 000 *g* for 10 min. The supernate was analysed for intracellular enzyme. The residue (cell wall fragments) was washed three times with 0·05 M-citrate buffer, pH4·8, and was finally suspended in citrate buffer to an OD_{540} of 0·80.

β-Glucosidase assay. β-Glucosidase (EC 3.2.1.21) activity in acetone-precipitated culture supernates was measured by using *p*-nitrophenyl β-D-glucosidase (pNPG) as substrate (Berghem & Pettersson, 1974). Appropriately diluted enzyme sample (0·2 ml) was incubated with 1·8 ml 1 mM-pNPG in citrate buffer (0·05 M, pH 4·8) and incubated at 50 °C for 10 min. The reaction was stopped by adding 1 ml 1 M-sodium carbonate. The absorbance of *p*-nitrophenol liberated was measured at 410 nm. One unit of activity (U) was defined as 1 μmol *p*-nitrophenol liberated min^{-1} (ml undiluted enzyme solution)$^{-1}$. The enzyme also had activity against cellobiose. Sorbose did not interfere with the estimation of β-glucosidase activity in the concentration range used in the experiments.

Estimation of cell dry weight. Culture samples were filtered on a dried and preweighed glass filter and washed thoroughly with distilled water. The filter with mycelium was then dried at 80 °C for 24 h (to a constant weight) and weighed.

Gross chemical analysis of walls. The procedure followed was a modification of those of DeTerra & Tatum (1961) and Mahadevan & Tatum (1965). The 4-d-old mycelium harvested from cellobiose and sorbose-supplemented cellobiose cultures was washed repeatedly with distilled water and treated with 10% (w/v) SDS for 16 h at 4 °C with constant stirring. The SDS treatment caused release of intracellular components, leaving behind the wall debris. Cell breakage was nearly complete as seen by microscopy. The walls were washed with distilled water until free of SDS, and lyophilized. Lyophilized walls (50 mg) were hydrolysed in 3 M-HCl for 3 h at 100 °C, and the hydrolysates were neutralized with NaOH. Total hexosamine was estimated by a modification of the Elson and Morgan method (Davidson, 1966) using glucosamine as standard. Glucose concentration was determined by the glucose oxidase–peroxidase method (Bergmeyer & Brent, 1974).

Reproducibility. All experiments were done in triplicate and the results were reproducible. The data points presented represent mean values, which were within ±5% of the individual values.

Chemicals. L(−)Sorbose and cellobiose were from Merck, Avicel cellulose from Serva, glucosamine hydrochloride from Fluka, glucose oxidase and peroxidase from Koch-Light, and *p*-nitrophenyl β-D-glucoside from Sigma.

RESULTS AND DISCUSSION

Effect of sorbose on morphology and growth of T. reesei

Visual examination of 2-d-old cultures of *T. reesei* in 0·5% cellobiose medium (control) and 0·5% cellobiose medium containing 2% sorbose showed growth in the latter medium to be in the

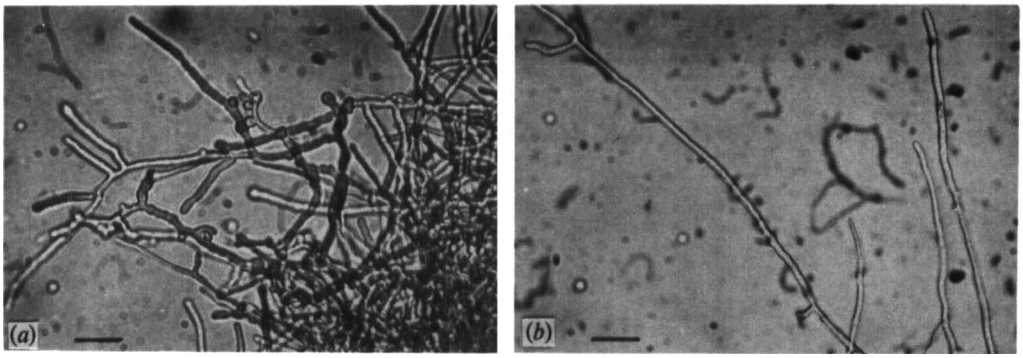

Fig. 1. Hyphae from mycelium of *T. reesei* grown in (*a*) 0·5% cellobiose medium plus 2% sorbose, and (*b*) 0·5% cellobiose medium. Bars, 20 μm.

form of fine, tight pellets with a greater tendency to settle than the more homogeneous growth on cellobiose alone. Microscopic observations revealed clumps of densely packed mycelium in sorbose-supplemented cultures as compared with loose, dispersed mycelium in control cultures. Peripheral hyphae of the mycelial clumps in sorbose-supplemented cultures were highly branched, with stubby tips and short internodal distances (Fig. 1*a*). In contrast, in cellobiose medium *T. reesei* mycelium grew as long, tapering hyphae with sparse branching (Fig. 1*b*).

Growth patterns of *T. reesei* in medium with 0·5% cellobiose and 0·5% cellobiose plus 5% sorbose are compared in Fig. 2. For the first 2 d, there was less growth in the presence of sorbose than in the control culture, but whereas growth in the control culture started declining after 2 d, an increase in biomass continued until the 4th day in the sorbose-supplemented culture. The same growth patterns were observed when the experiment was done at a controlled pH of 4·5 (results not shown), indicating that a change in pH was not responsible for the change in growth pattern in the presence of sorbose. When sorbose (1–5%) was used as the sole carbon source, growth was very poor and extracellular β-glucosidase activity was negligible.

The gross and microscopic changes observed in *T. reesei* mycelium grown in the presence of sorbose corresponded closely with the classical description of sorbose-induced paramorphogenesis in other fungi (Tatum *et al.*, 1949).

Effect of sorbose on extracellular β-glucosidase activity

β-Glucosidase activity in culture filtrates was compared when *T. reesei* was cultivated in medium containing 0·5% cellobiose alone or plus increasing concentrations (1–5%) of sorbose. Sorbose enhanced the extracellular β-glucosidase activity, the effect increasing with increase in the concentration of sorbose (Fig. 3). In 8–10-d-old cultures supplemented with 5% sorbose, the β-glucosidase activity was four times that in unsupplemented cultures of the same age. The protein content of culture filtrates was also about four times greater in 5% sorbose-supplemented cultures than in control cultures (1·5 mg ml^{-1}) (data not shown).

As is evident from Fig. 3, a relatively high ratio (1:10) between concentrations of inducing substrate (cellobiose) and non-inducing sugar (sorbose) led to a marked increase in extracellular β-glucosidase activity. The effect of sorbose on extracellular β-glucosidase activity in *T. reesei* cultured on 1% Avicel cellulose was studied by adding 5% sorbose at various stages of growth, the rationale being that the cellulose would be progressively consumed during growth, resulting in the desired high ratio between sorbose and residual cellulose. Except for the addition made on day 2, sorbose additions led to an increase in extracellular β-glucosidase activity (Fig. 4). The addition of sorbose on day 4 was optimal for stimulating extracellular production of β-glucosidase by *T. reesei* growing on cellulose, resulting in an 88% increase in extracellular β-glucosidase activity over the control value by day 10.

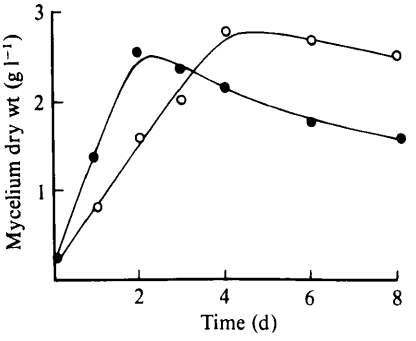

Fig. 2

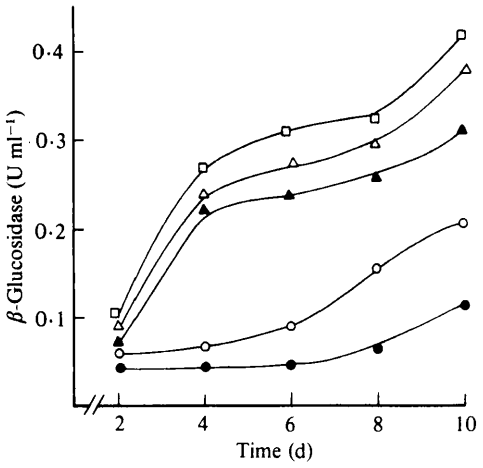

Fig. 3

Fig. 2. Growth characteristics of *T. reesei* in medium containing 0·5% cellobiose (●) or 0·5% cellobiose + 5% sorbose (○).

Fig. 3. Effect of addition of sorbose to 0·5% cellobiose medium on extracellular β-glucosidase activity of *T. reesei*. Concentration of sorbose added: ●, 0% (control); ○, 1%; ▲, 2·5%; △, 3·25%; □, 5%.

Changes in the subcellular distribution of β-glucosidase in the presence of sorbose

To investigate the mechanism of sorbose-induced enhancement of β-glucosidase activity in culture supernates of *T. reesei*, the subcellular distribution of β-glucosidase in mycelia grown in the presence and absence of sorbose was compared. Equal amounts of lyophilized mycelia obtained from cultures cultivated on 0·5% cellobiose and 0·5% cellobiose plus 5% sorbose were fractionated as described. The β-glucosidase activity associated with the various fractions is shown in Fig. 5. In sorbose-supplemented cultures, at all stages of growth, the enzyme activity associated with sonicated cell debris (putative wall-associated enzyme component) was less than half the activity present in the same fraction from control cultures. The intracellular activity (released by sonication) was also lower in the sorbose-supplemented cultures. As observed before (Fig. 3), the extracellular activity was higher in the sorbose-supplemented culture than in the control. These observations further substantiate our hypothesis that sorbose causes dissociation of wall-bound β-glucosidase in *T. reesei*.

Effect of sorbose on the chemical composition of walls

Wall fractions obtained from mycelia grown on cellobiose in the absence and presence of sorbose were subjected to acid hydrolysis. Only gross changes in the total glucose and total glucosamine content were studied. The results are presented in Table 1. An increase of about 50% in total glucosamine content and a decrease of about 26% in total glucose content were observed in cell walls of sorbose-supplemented cultures as compared with the control. The glucosamine/glucose ratio of wall hydrolysates from sorbose-supplemented cultures was about twice that of the control.

Although we did not completely fractionate the wall polymers some inferences may be drawn from the gross chemical analysis. As most of the glucose in *T. reesei* walls is present as 1,3-β-glucan and 1,6-α-glucan (Benitez *et al.*, 1976) a decrease in glucose content implies a decrease in wall glucan content in the presence of sorbose. The observed increase in total glucosamine content similarly presumably reflects an increase in wall chitin, since most glucosamine exists in its *N*-acetyl form in this fraction (Benitez *et al.*, 1976; Burnett, 1976). Such an increase in chitin content might be a mechanism to preserve wall integrity when the glucan content is decreased in the presence of sorbose. As a mechanism for the observed sorbose-induced increase in extracellular β-glucosidase activity in *T. reesei*, we propose that alterations in the relative

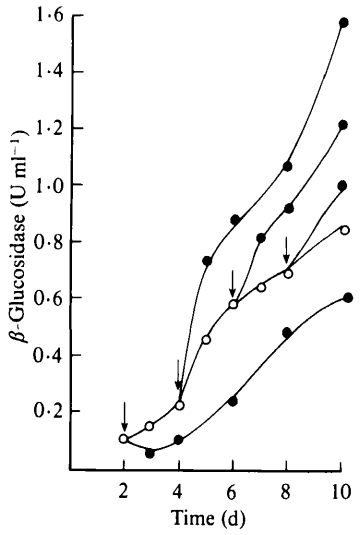

Fig. 4

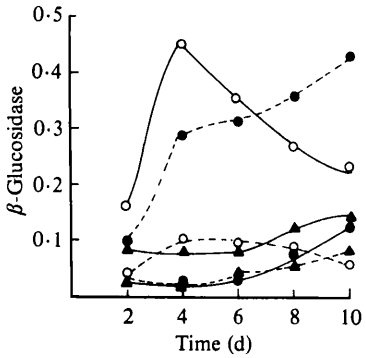

Fig. 5

Fig. 4. Effect of addition of sorbose to Avicel cellulose medium during different growth phases of *T. reesei* on extracellular β-glucosidase activity. ○, 1% Avicel (control); ●, 1% Avicel + 5% sorbose added on days 2, 4, 6 and 8 (arrows).

Fig. 5. Subcellular distribution of β-glucosidase in *T. reesei* cultivated in 0·5% cellobiose medium in the absence (——) and presence (- - - -) of 5% sorbose. ●, Extracellular activity (U ml^{-1}). ○, Cell-wall-bound activity; ▲, intracellular activity [both U (mg dry wt mycelium)$^{-1}$].

Table 1. *Glucose and glucosamine contents of cell walls of T. reesei grown on cellobiose in the presence and absence of sorbose*

Cell walls from 4-d-old mycelia were hydrolysed with HCl. The hydrolysates were neutralized with NaOH and used for determination of glucosamine and glucose content. The values presented are means of triplicate experiments. Variation about the means was within ±5%.

Carbon source	Percentage of cell wall (dry wt)		Glucosamine glucose
	Glucosamine	Glucose	
Cellobiose (0·5%)	7·0	22·6	0·31
Cellobiose (0·5%) + sorbose (5%)	11·2	16·7	0·67

proportions of glucan and chitin brought about by sorbose decrease the retention of β-glucosidase in the walls. As reported earlier (Nanda *et al.*, 1982) the β-glucosidase of *T. reesei* is closely associated with the 1,3-β-glucan fraction of the walls. A decrease in the wall glucan content, and the resulting weakened association of the enzyme with the walls, is probably responsible for its increased release into the culture medium.

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