Effect of L(–)Sorbitose on Cellulase Activity in *Trichoderma reesei* QM9414

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(Received 30 December 1985; revised 10 June 1986)

L(–)Sorbitose enhanced the extracellular levels of endoglucanase and filter paper unit activity in culture filtrates of *Trichoderma reesei* QM9414 cultivated on cellobiose or Avicel cellulose. Addition of sorbitose to the culture medium retarded the uptake of cellobiose by the organism; this uptake appeared to be dependent on the surface-bound β -glucosidase. A biochemical mechanism to explain the effect of sorbitose on cellulase induction, through its effect on surface-bound β -glucosidase and cellobiose uptake, is proposed.

INTRODUCTION

The cellulase enzyme system from *Trichoderma reesei* is favoured for rapid saccharification of crystalline cellulosic substances (Bisaria & Ghose, 1981; Mandels, 1982; Ladisch *et al.*, 1983). The normal extramycelial activity of β -glucosidase (EC 3.2.1.21), one of the components of the cellulase system, is however too low in *T. reesei* to be of much practical use (Allen & Sternberg, 1980). The addition of L(–)sorbitose, a ketohexose, to *T. reesei* QM9414 cultures growing on cellulase inducers such as cellobiose and Avicel cellulose enhances extracellular β -glucosidase activity (Bisaria *et al.*, 1986). At the same time, the activity of the cell-wall-bound fraction of β -glucosidase (Kubicek, 1981; Nanda *et al.*, 1982) is reduced by about 50% in the presence of sorbitose (Bisaria *et al.*, 1986). The observed increase in enzyme activity in sorbitose-supplemented cultures was accompanied by striking morphological changes such as growth in the form of tight pellets and increased branching of the mycelia (Bisaria *et al.*, 1986).

The cellulase enzyme system of *T. reesei* is a complex of endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) which can hydrolyse crystalline cellulose to soluble sugars. Cellulase activity is commonly expressed in terms of filter paper unit activity, measured as the combined action of both these enzymes on filter paper (Mandels *et al.*, 1976; Mandels, 1982; IUPAC, 1984). This paper presents the results of studies on the effect of sorbitose on the activity levels of cellulase in terms of endoglucanase and filter paper unit activities. Changes in cellobiose utilization in the presence of sorbitose were also studied in order to gain a better understanding of the mechanism of the observed increase in cellulase activity.

METHODS

Organism and culture conditions. *Trichoderma reesei* QM9414 was used. Growth media and culture conditions were as described previously (Bisaria *et al.*, 1986).

Enzyme assays. Since sorbitose (a reducing sugar) interferes with colorimetric estimation procedures based on reducing sugars, the cellulase enzyme from sorbitose-containing culture filtrates was acetone precipitated before assaying for cellulase activity. Chilled acetone (5.0 ml) was added to culture filtrate (1.0 ml) kept at 4 °C and protein precipitates were removed by centrifugation at 44000 g for 30 min at 4 °C. The precipitation recovered 90% of the protein in culture filtrates. The precipitate was dissolved in 1 ml 0.05 M-citrate buffer, pH 4.8, and assayed for filter paper unit activity and endoglucanase activity. Controls without sorbitose were also treated with acetone in a similar manner before enzyme assays. Appropriate controls were included to detect any residual sugars in enzyme samples. The assay procedures were as described by Mandels *et al.* (1976). Enzyme units are expressed as $\mu\text{mol glucose formed min}^{-1} (\text{ml undiluted enzyme solution})^{-1}$ under the assay conditions.

β -Glucosidase was measured by the method of Sternberg *et al.* (1977) using cellobiose as substrate. The enzyme was also found to have aryl- β -glucosidase activity as measured against *p*-nitrophenyl- β -D-glucopyranoside.

Fractionation of mycelium. Equal amounts of lyophilized mycelia obtained from cultures of *T. reesei* grown on 0.5% (w/v) cellobiose and 0.5% (w/v) cellobiose plus 5% (w/v) sorbose were fractionated by sonication into cell wall and cytoplasmic fractions as described previously (Bisaria *et al.*, 1986).

Estimation of cellobiose in the presence of sorbose. Since both cellobiose and sorbose are reducing sugars, cellobiose cannot be measured in the presence of sorbose by tests for reducing sugars. In such mixtures cellobiose was therefore hydrolysed with β -glucosidase to glucose which was then estimated by the glucose oxidase-peroxidase method (Bergmeyer & Brent, 1974). Sorbose, in the concentration range used, showed no interference with β -glucosidase or with glucose oxidase and peroxidase.

A sample (1.0 ml) containing 0.5–5 mg cellobiose was incubated with β -glucosidase (2000 IU mg⁻¹) solution (1.0 ml, 4000 IU, in 0.05 M-citrate buffer, pH 4.8) for 20 min at 50 °C. The large excess of β -glucosidase was used to ensure maximum hydrolysis of cellobiose. The reactions were stopped by immersing the test tubes in a boiling water bath for 10 min and the glucose content was determined in 0.5 ml samples.

Sugar uptake by *T. reesei*. Washed exponential-phase mycelium (10 mg dry weight) grown on either glucose or cellobiose, or spores (prepared as described below) were suspended in Vogel salt solution (10 ml) (Vogel, 1956) containing specified amounts (see Results) of various sugars (glucose, cellobiose and/or sorbose). Removal of sugars was studied by measuring their residual concentrations in the medium over a period of time. For experiments comparing the removal of glucose and cellobiose in the presence of sorbose, residual glucose was measured by the glucose oxidase-peroxidase method and cellobiose as described above. Sorbose was estimated by the method of Dische & Devi (1960). For experiments to study the utilization patterns of glucose and cellobiose in the absence of sorbose, residual sugar concentrations were estimated by the Nelson (1944) and Somogyi (1952) methods.

Inactivation of conidial β -glucosidase. Conidia were harvested from 7-d-old malt extract agar slants of *T. reesei*, suspended in distilled water and filtered through glass wool. The concentration of spores was determined by measuring the OD₅₄₀ of the spore suspension: 1 unit of conidial concentration was defined as that giving an OD₅₄₀ of 1.0.

To inactivate the surface-bound conidial β -glucosidase, conidia from 10 ml suspension (concentration 1 unit) were sedimented by centrifugation, suspended in chilled 0.1 M-HCl (10 ml) and incubated at 4 °C for 5 min according to the method of Mandels (1953). The acid-treated conidia were then centrifuged and resuspended in 0.05 M-citrate buffer, pH 4.8 (10 ml), containing cellobiose or glucose at specified concentrations. Regeneration of surface β -glucosidase was examined by periodically assaying the spore suspension for enzyme activity. No regeneration took place up to 24 h. The acid-treated spores were therefore used as β -glucosidase-negative spores.

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

Chemicals. L(–)Sorbose and cellobiose were from Merck, Avicel cellulose from Serva, and glucose oxidase (type V), peroxidase and β -glucosidase from Sigma.

RESULTS AND DISCUSSION

Effect of sorbose on extracellular cellulase activity

T. reesei was cultivated on 0.5% (w/v) cellobiose alone and on the same concentration of cellobiose in the presence of increasing (1–5%, w/v) concentrations of sorbose. Higher endoglucanase activity was observed in the cultures containing sorbose, and the increase in enzyme activity was greater, the higher the concentration of sorbose added (Fig. 1). With 5% sorbose a seven-fold increase in endoglucanase activity over the control was observed on day 10.

The growth pattern of *T. reesei* on cellobiose in the presence and absence of sorbose was reported by Bisaria *et al.* (1986). Very poor growth and no enzyme activity were observed in culture media where sorbose was used as the sole carbon source.

A relatively high ratio (1:10) between concentration of inducing substrate (cellobiose) and non-inducing sugar (sorbose) led to a marked increase in extracellular endoglucanase activity (Fig. 1). The effect of sorbose on extracellular cellulase 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 that made on day 2, sorbose additions led to an increase in extracellular endoglucanase activity; the addition of sorbose on day 4 had most effect (Fig. 2). Similar results were obtained for filter paper unit activity (results not shown). While sorbose addition on day 4 resulted in a 15% increase in extracellular endoglucanase

Fig. 1

Fig. 1. Effect of addition of sorbose to cellobiose medium on extracellular endoglucanase activity in *T. reesei*. ●, 0.5% Cellobiose (control); ○, 0.5% cellobiose + 1% sorbose; ▲, 0.5% cellobiose + 2.5% sorbose; △, 0.5% cellobiose + 3.25% sorbose; ■, 0.5% cellobiose + 5% sorbose.

Fig. 2

Fig. 2. Effect of addition of sorbose (arrows) to Avicel cellulose medium on extracellular endoglucanase activity during different growth phases of *T. reesei*. ○, Control (1% Avicel cellulose); ●, test (1% Avicel cellulose + 5% sorbose).

activity (Fig. 2), it gave an increase of 40% in filter paper unit activity, as measured on day 10.

Reported effects of sorbose on fungal morphology (Tatum *et al.*, 1949) and cell wall composition (De Terra & Tatum, 1961; Mahadevan & Tatum, 1965) led us to assume initially that sorbose would primarily affect the release of β -glucosidase, which is cell-wall bound in *T. reesei* (Kubicek, 1981; Nanda *et al.*, 1982). Our previous observations on sorbose-induced changes in cell wall composition were able to explain the increase in extracellular β -glucosidase activity (Bisaria *et al.*, 1986). However, these observations could not explain the increase in extracellular cellulase activity, as cellulases show very little association with cell walls and their synthesis and secretion are closely associated or concurrent events in *T. reesei* (Vaheri *et al.*, 1979; Sternberg & Mandels, 1979). The morphological changes in the mycelium observed in the presence of sorbose may possibly provide an explanation. *T. reesei* grew as tight, fine pellets with highly branched mycelium in the presence of sorbose (Bisaria *et al.*, 1986). Further, Mukhopadhyay & Ghose (1977) reported that the growth of *T. reesei* as tight pellets enhanced cellulase yields. An increase in hyphal tips due to branching of mycelium could therefore be responsible for increase in cellulase production, as these are known to be the site for secretion of extracellular enzymes in fungi (Chang & Trevithick, 1974).

Effect of sorbose on subcellular distribution of endoglucanase

Endoglucanase activity was estimated in extracellular, intracellular and cell-wall fractions of mycelia grown on cellobiose (0.5%) in the presence and absence of sorbose (5%) (Fig. 3). The extracellular activity in sorbose-supplemented cultures was enhanced over that in control cultures. There was no detectable activity associated with cell walls in either of the cultures. While the endoglucanase activity in the intracellular fraction (released after sonication) of control cultures was negligible, considerable endoglucanase activity was found in sorbose-supplemented cultures. It was maximal on day 4 and then decreased progressively, although at all stages it was higher than that in control cultures. The observed increase in extracellular and intracellular endoglucanase suggested that increased synthesis, rather than increased secretion of cellulase occurred in the presence of sorbose.

Effect of sorbose on cellobiose uptake

Equal amounts of exponential-phase mycelia obtained from 0.2% (w/v) glucose medium were washed and suspended in 10 ml volumes of Vogel salt solution containing 0.5% cellobiose and

Fig. 3. Subcellular distribution of endoglucanase activity in *T. reesei* cultivated on cellobiose in the presence and absence of sorbose. —, Control (0.5% cellobiose); ---, test (0.5% cellobiose + 5% sorbose); ●, extracellular activity; ▲, intracellular activity.

0.5% cellobiose plus 5% sorbose. Removal of cellobiose from the medium was monitored as described in Methods (Fig. 4). In the absence of sorbose, 0.5% cellobiose was completely removed from the medium in 24 h, but when 5% sorbose was present the cellobiose was not exhausted until 48 h. Growth, as measured by dry weight estimation, was slightly retarded in the presence of sorbose. In contrast to the results with cellobiose, the presence of sorbose did not affect the rate of removal of glucose from the medium: 0.5% glucose was completely removed by the mycelium in 24 h in both the presence and the absence of 5% sorbose (results not shown). Sorbose was poorly taken up by the mycelium: when supplied as the sole carbon source its concentration remained virtually constant over 48 h incubation.

The effect of sorbose on cellobiose uptake provided an important clue to its possible effect on increased cellulase activity. It has been reported that conditions which reduce the rate of uptake of cellulase inducers are conducive to higher cellulase yields. For example, Sternberg & Mandels (1979) obtained maximum cellulase induction in *T. reesei* at 28 °C and pH 2.8, conditions under which the rate of uptake of sophorose (a powerful inducer) was much below the maximum attainable at 39 °C and pH 5.0. The high inducer activity of sophorose has been ascribed to its slower rate of uptake, about 10–20% of that of cellobiose. It seemed possible, therefore, that the increased cellulase activity observed in sorbose-supplemented cultures was due to a reduced rate of uptake of cellobiose, which resulted in relief of catabolite repression.

Investigation of the mechanism of sorbose uptake in T. reesei

In order to elucidate how sorbose was reducing the rate of cellobiose uptake, it was essential to know more about the mechanism of cellobiose uptake in *T. reesei*. Two possible mechanisms of disaccharide uptake were investigated: (i) uptake by an inducible, specific cellobiose permease (Ng & Zeikus, 1982), and (ii) hydrolytic cleavage of the molecule by the cell-wall-associated β -glucosidase and subsequent uptake of the monomers (Haliwell, 1979). Phosphotransferase systems are widely distributed among prokaryotes but have not yet been found in eukaryotes (Postma & Lengeler, 1985). In cellulolytic bacteria such as '*Cellvibrio gilvus*' (Swisher *et al.*, 1964), *Ruminococcus flavefaciens* (Ayers, 1959), *Clostridium thermocellum* (Sih & McBee, 1966; Ng & Zeikus, 1982) and *Cellulomonas* sp. (Schimz & Decker, 1985), inorganic-phosphate-dependent phosphorylation of cellobiose by cellobiose phosphorylase (EC 2.4.1.20) instead of

Fig. 4

Fig. 4. Effect of sorbose on cellobiose uptake by *T. reesei*. ●, Cellobiose (0.5%); ○, cellobiose (0.5%) + sorbose (5%).

Fig. 5

Fig. 5. Cellobiose uptake by glucose- and cellobiose-grown inocula of *T. reesei*. ●, Glucose-grown inoculum; ○, cellobiose-grown inoculum.

hydrolysis has been reported. The action of this enzyme results in conservation of the β -1, 4 bond energy during activation of glucose with inorganic phosphate. Cellulolytic species that contain this enzyme prefer cellobiose to glucose for growth. We did not investigate the presence of cellobiose phosphorylase, and therefore our conclusions about the effect of sorbose on cellobiose uptake and cellulase production remain tentative.

To test the inducibility of cellobiose-transporting enzymes, *T. reesei* grown separately on 0.2% glucose and 0.2% cellobiose media was harvested after 24 h growth. Equal amounts (on a dry weight basis) were inoculated into two portions of growth medium containing 0.1% cellobiose, and the uptake of cellobiose was measured (Fig. 5). The carbon source used for the initial inoculum did not affect the rate of uptake of cellobiose. Growth in the two cases also followed similar trends. Since initial growth on cellobiose did not lead to increased uptake of cellobiose from the medium, it appears that *T. reesei* lacks an inducible permease for this sugar. The presence of cellobiose-induced permeases has been reported in other cellulolytic organisms, such as *Sporotrichum thermophile* (Canevascini *et al.*, 1979) and non-cellulolytic organisms such as *Clostridium thermohydrosulfuricum* (Ng & Zeikus, 1982).

To test the possible involvement of surface-bound β -glucosidase in cellobiose uptake, cellobiose uptake rates were compared in normal spores (showing surface-bound β -glucosidase activity) and acid-treated spores (in which the enzyme was inactivated: see Methods). As shown in Fig. 6(a), glucose uptake continued in spores after acid treatment, albeit at a slower rate ($17 \mu\text{g h}^{-1}$ per concentration unit of spores, versus $22 \mu\text{g h}^{-1}$ for normal spores). Cellobiose uptake, on the other hand, did not occur in acid-treated spores (Fig. 6b). Addition of 0.4 IU β -glucosidase (adjusted to 10 times the β -glucosidase activity associated with 1 unit of spore suspension) to acid-treated spores resulted in partial recovery of uptake activity (Fig. 6b), increasing cellobiose uptake to $9 \mu\text{g h}^{-1}$.

Cellobiose uptake in acid-treated mycelia could not be studied because acid treatment, while inactivating mycelium-bound β -glucosidase, also completely inhibited the glucose transport system. Glucose transport showed no recovery until 24 h after acid treatment. Although these results do not elucidate the mechanism of cellobiose uptake in *T. reesei*, they do indicate the involvement of surface-bound β -glucosidase in the process, and suggest that a pre-transport hydrolysis of cellobiose is carried out by this enzyme. This tentative inference is supported by the results of Sternberg & Mandels (1979), who observed that nojirimycin, an inhibitor of β -glucosidase, completely inhibited respiration of *T. reesei* on either sophorose or cellobiose, but had no effect on endogenous respiration. The failure of added soluble β -glucosidase to restore cellobiose uptake to the normal level also suggests a stereospecific action of surface-bound β -glucosidase on cellobiose uptake. Pre-transport hydrolysis of disaccharides has been reported for

Fig. 6. Glucose (a) and cellobiose (b) uptake by normal spores (●), acid-treated (β -glucosidase-negative) spores (○), and acid-treated spores in the presence of externally added β -glucosidase (□).

other fungi, e.g. in sucrose transport by *Neurospora crassa* (Marzluf & Metznerberg, 1967) and in maltose transport by *Mucor rouxii* (Reyes & Renz-Herrera, 1972).

As discussed in a previous paper (Bisaria *et al.*, 1986) the cell-wall-bound β -glucosidase activity in sorbose-supplemented cultures of *T. reesei* was reduced to half the control levels. The cytoplasmic activity of the enzyme was also markedly reduced in the presence of sorbose. The reduction in cell-wall-associated β -glucosidase was attributed to chemical changes in the cell-wall glucan fraction caused by sorbose. We therefore propose that sorbose enhances extracellular cellulase activity in *T. reesei* by decreasing the cell-wall-associated component of β -glucosidase. The sorbose-induced decrease in the levels of cell-wall-bound β -glucosidase may slow down the uptake of cellobiose, a condition known to enhance cellulase yields (Sternberg & Mandels, 1979). Furthermore, reduced levels of intracellular β -glucosidase in the presence of sorbose could also contribute to an increase in cellulase synthesis by ensuring higher stability of the intracellular inducer that is synthesized by transglycosylation as suggested by Gritzalli & Brown (1979). Our hypothesis to explain the effect of sorbose in increasing cellulase activity remains tentative, however, and further studies are needed to elucidate the mechanism of this effect.

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