β -Glucosidase and cellulase formation by a *Trichoderma reesei* mutant defective in constitutive β -glucosidase formation

JOSEF STRAUSS† and CHRISTIAN P. KUBICEK*

Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU Wien, Getreidemarkt 9, A-1060 Austria

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A mutant of *Trichoderma reesei* QM 9414 – *T. reesei* M8 – was isolated after γ -irradiation. It did not form β -glucosidase during growth on glycerol or glucose, but secreted β -glucosidase upon growth on cellobiose. The mutant was also able to grow on cellulose and secrete β -glucosidase, but with a longer lag. β -Glucosidase activity could be induced in the mutant in a replacement medium by cellobiose and β -methyl D-glucoside. Mycelia lacking β -glucosidase were able to take up both these inducers immediately, indicating the presence of a constitutive permease. The mutant produced cellulases upon growth on lactose and – after a lag – on cellulose. Mycelia of *T. reesei* M8 pregrown on glycerol or cellobiose could be induced by sophorose to produce cellobiohydrolase I in a replacement system. The findings show that (i) cellobiose and other β -linked disaccharides can be taken up by *T. reesei* without prior hydrolysis, and (ii) that cellobiose most probably induces β -glucosidase formation during growth on cellulose.

Introduction

The enzymology and genetics of the cellulase system secreted by the hypercellulolytic fungus *Trichoderma reesei* have been the subject of intense study because of the commercial potential of this system (Enari & Niku-Paavola, 1987; Knowles *et al.*, 1988). Four of the enzymes involved (cellobiohydrolase I and II, EC 3.2.1.91; endoglucanase I and III, EC 3.2.1.4) have already been well characterized; however, the enzyme catalysing the final hydrolysis of cellobiose to glucose, cellobiase (β -glucosidase, EC 3.2.1.21), has received less attention. Although its molecular properties have recently been established (Chirico & Brown, 1987; Schmid & Wandrey, 1987; Hofer *et al.*, 1989) and the molecular relationship between the extracellular, cell-wall-bound (Kubicek, 1981) and plasma-membrane-bound (Umile & Kubicek, 1986) forms have been assessed (Hofer *et al.*, 1989), little is known about the regulation of its formation. Sophorose – the most potent inducer of *T. reesei* cellulases (Mandels *et al.*, 1962) – does not concomitantly induce β -glucosidase and even represses its formation at high concentrations (Sternberg & Mandels, 1979, 1980). These authors also reported that

the enzyme is formed constitutively, and that only β -methyl D-glucoside promoted a fourfold induction (Sternberg & Mandels, 1982). Jackson & Talburt (1988) claimed that the β -glucosidase from *T. reesei* may be involved in cell-wall metabolism during conidiogenesis, and thus not be a true component of the cellulolytic enzyme system. On the other hand, the constitutive β -glucosidase appears to be involved in the induction of cellulase by cellulose (Kubicek, 1987).

In the present paper we report the isolation of a mutant of *Trichoderma reesei* QM 9414 that is defective in constitutive formation of β -glucosidase. We have used this mutant to investigate the regulation of β -glucosidase biosynthesis in this fungus and its role in cellulase biosynthesis and disaccharide uptake.

Methods

Organism and growth conditions. *Trichoderma reesei* QM 9414 and mutant M8 derived from it (see below) were used throughout this study. They were maintained on malt-agar slants. Inocula were prepared by harvesting conidia of 14-d-old cultures in sterile tap water containing 0.1% Tween 80. The conidia were added to 200 ml medium in wide-mouthed 1 litre Erlenmeyer flasks to a final concentration of 10^8 conidia l^{-1} . The fungi were grown in flasks on a rotary shaker (250 r.p.m.) at 28 °C in the medium described by Mandels & Andreotti (1978), except that the pH of the medium was kept at 5.0 with 50 mM-phosphate/citrate buffer (Labudova & Farkas, 1983). Carbon sources used are indicated for the respective experiments.

† Present address: Institut de Microbiologie, Université Paris Sud, 91405 Orsay, France.

Abbreviation: CBH I, cellobiohydrolase I.

Isolation of mutants defective in constitutive β -glucosidase formation. A suspension (5 ml) of 10^7 conidia ml^{-1} was sealed in an appropriate small glass vial and subjected to γ -irradiation for 25 s at 25 kW. Thereafter, the suspension was kept at 40 °C for 72 h to allow a decay in radioactivity before it was plated on cultivation medium (Mandels & Andreotti, 1978) containing glycerol as sole carbon source (5 g l^{-1}), solidified with 2.5% (w/v) agar. Since this treatment resulted in a killing rate of approximately 99% (optimized before), 0.1 ml samples (containing 10^4 putative survivors) were streaked on each plate. β -Glucosidase activity of individual colonies was assessed by overlaying 2–3-d-old plates (when defined colonies had just developed) with 10 ml 1% (w/v) agar in 50 mM-sodium citrate, pH 5, containing 10 mM-methylumbelliferyl β -D-glucoside. After 2 h incubation at 28 °C, plates were inspected under UV light. β -Glucosidase-negative mutants lacked the typical fluorescent halo around the colonies.

Induction of cellulase and β -glucosidase formation in mycelia of *T. reesei*. Mycelia were pregrown in the medium described above with glycerol as carbon source, and induced to form cellulases or β -glucosidase in a replacement medium as described by Sternberg & Mandels (1982) using sophorose or β -methyl D-glucoside as inducers. Mycelia were cultivated in replacement medium in 100 ml Erlenmeyer flasks containing 10 ml of culture suspension, agitated on a rotary shaker at 200 r.p.m. at 28 °C for the appropriate time. Mycelial density in the replacement medium at the beginning of the experiment was 1 g dry wt l^{-1} . The initial inducer concentrations in the medium were 1 and 3 mM for sophorose and β -methyl D-glucoside, respectively.

During some experiments, uptake of the inducer was followed throughout the incubation. For this purpose, samples (0.25 ml) of the culture broth were collected at intervals, centrifuged in an Eppendorf centrifuge (15 min), and the supernatant analysed for sophorose or β -methyl D-glucoside by the phenol/sulphuric acid method (Dubois *et al.*, 1956).

Determination of biomass concentration. Biomass concentration was determined by filtering appropriate samples from the culture broth through G1 sintered funnels, washing the mycelial mat with a twofold volume of tap water, followed by a twofold volume of distilled water, and then drying the mat to constant weight at 105 °C. For cellulose-containing media, intracellular protein was extracted and measured as a biomass equivalent essentially as described previously (Kubicek, 1981).

Assay of enzyme activities. Enzyme activities were assayed either in the culture supernatant [obtained by filtering the culture broth through a G1 sintered funnel, followed by centrifugation (5 min) in an Eppendorf centrifuge] in the case of endoglucanase, or in unfiltered culture broth to measure the total amount of secreted (i.e. cell-membrane-bound, cell-wall-bound and truly secreted) β -glucosidase. Endoglucanase and β -glucosidase were assayed as described by Kubicek (1981), with carboxymethylcellulose (Serva) and *p*-nitrophenyl β -D-glucoside, respectively, as substrates. One unit (1 U) of enzyme activity is that which released 1 μmol glucose equivalent min^{-1} . Specific activities were expressed as units of activity (mg protein) $^{-1}$. Protein was estimated by the Coomassie Blue binding method (Bradford, 1976).

Electrophoretic techniques. For qualitative demonstration of individual cellulases in the culture supernatant, samples from the culture broth were subjected to SDS-PAGE, followed by Western blotting to nitrocellulose, and immunological detection as described previously (Kubicek *et al.*, 1987). Monoclonal antibodies CH-6 and BG-1 (Mischak *et al.*, 1989; Hofer *et al.*, 1989) were used to detect cellobiohydrolase I and β -glucosidase, respectively, on the blots.

Results

Mutant isolation procedure.

By the outline in Methods, we attempted to isolate *T. reesei* mutants devoid of β -glucosidase activity when grown on glycerol or glucose as a carbon source. However, yield was very poor: such mutants appeared with a frequency of 0.03% among the survivors. Various strategies to enrich the desired mutants were tried: however, the fact that the conidia of the fungus already contain β -glucosidase (Kubicek *et al.*, 1988), which cannot be released by surface active agents (unpublished results), impaired the use of filtration enrichment on cellobiose as a carbon source. Also, the use of β -D-glucosides of potentially toxic aromatic compounds, e.g. *p*-nitrophenyl β -D-glucoside, was unsuccessful. Although *p*-nitrophenol severely inhibited growth of *T. reesei*, selection on medium containing this compound led to an exclusive enrichment of *p*-nitrophenol-resistant mutants without enrichment of β -glucosidase-negative colonies. Therefore, visual inspection after overlaying with methylumbelliferyl β -D-glucoside was the only successful selection strategy during this work.

From several potential β -glucosidase-defective isolates, after several subcultivations only a single stable mutant (M8) was obtained. This was used for further investigations.

Effect of carbon source on growth and β -glucosidase formation by *T. reesei* QM 9414 and M8

T. reesei QM 9414 and the mutant M8 were compared for growth and β -glucosidase formation on various carbon sources (Table 1): both strains grew in a comparable manner on all carbon sources tested, the only exception

Table 1. Growth and β -glucosidase formation by *T. reesei* QM 9414 and M8 on various carbon sources

The concentration of all carbon sources was 0.5% (w/v). β -Glucosidase activity is given as total (i.e. both mycelium-associated and released) extracellular activities per ml of culture volume. Numbers in parentheses indicate the respective amount of mycelial protein present at the time of harvest (in mg per litre of culture volume). All samples were harvested after 30 h growth except for M8 on cellulose, which was harvested after 80 h. Values are given for a single experiment only, but repetition yielded consistent results.

Carbon source	β -Glucosidase activity	
	QM 9414	M8
Glucose	0.080 (152)	<0.010 (124)
Glycerol	0.080 (143)	<0.010 (118)
Lactose	0.060 (86)	0.030 (132)
Cellobiose	0.300 (162)	0.370 (154)
Cellulose	0.230 (65)	0.140 (45)

Fig. 1. Growth (●) and β -glucosidase (■) formation by *T. reesei* QM 9414 (filled symbols) and M8 (open symbols) on crystalline cellulose (5 g l⁻¹). The medium was prepared according to Mandels & Andreotti (1978), but peptone was omitted. Growth is given in mg mycelium-associated protein per litre of culture volume.

Fig. 2. Uptake of β -methyl D-glucoside by *T. reesei* QM 9414 (○) and M8 (●) during induction of β -glucosidase in a replacement system, using glycerol-pregrown mycelium.

being that M8 exhibited a significantly longer lag on cellulose. The specific β -glucosidase activity of M8 was significantly lower than that of QM 9414 on glucose, glycerol and lactose, but β -glucosidase was clearly present upon cultivation on cellobiose. Growth of M8 on cellulose (in the absence of peptone) exhibited a considerable lag but was subsequently comparable, and β -glucosidase was also formed (Fig. 1). These results indicate that we have apparently isolated a mutant of *T. reesei* with a defect in a regulatory region rather than a β -glucosidase structural gene.

Interestingly, we found that the β -glucosidase formed by QM 9414 and M8 was exclusively cell-wall and cell-membrane-bound during cultivation on all carbon sources except lactose and cellulose. During growth on these two compounds, 10–30% of the total external β -glucosidase activity appeared in the culture supernatant (unpublished data).

Induction of β -glucosidase formation in *T. reesei* QM 9414 and M8

β -Methyl D-glucoside has been reported to be an inducer of *T. reesei* β -glucosidase (Sternberg & Mandels, 1982). Since M8 still produced β -glucosidase during growth on cellobiose, we precultivated the two strains of *T. reesei* on glycerol, and investigated the induction of β -glucosidase by transferring them to medium containing either β -methyl D-glucoside or cellobiose. Both inducers promoted β -glucosidase formation, albeit at a lower rate with cellobiose (31.5 and 11.7 versus 54.2 and 18.3 U ml⁻¹ for β -methyl D-glucoside and cellobiose as inducers with strains QM 9414 and mutant M8, respectively). Although the mycelium of *T. reesei* M8 contained virtually

no β -glucosidase activity, it took up β -methyl D-glucoside without a lag (Fig. 2). The same kinetics were also observed with cellobiose (C. Fritscher & C. P. Kubicek, unpublished data). These results therefore demonstrate that M8, although defective in constitutive β -glucosidase formation, can be induced to form this enzyme. The fact that the two inducers used are apparently taken up by the β -glucosidase-devoid mycelium indicates the presence of a constitutive permease for these sugars in *T. reesei*.

Regulation of formation of cellobiohydrolase I by *T. reesei* M8

T. reesei QM 9414 secretes a cellulase enzyme system into the culture supernatant when it is grown on cellulose or on lactose as inducing (cellulose) and carbon-catabolite-derepressing (lactose) carbon sources, respectively (Kubicek *et al.*, 1990). Since β -glucosidase has been implicated in the formation of the true inducer during cellulose degradation (Gritzali & Brown, 1979; Vaheri *et al.*, 1979; Kubicek, 1987), we investigated whether mutant M8 can produce cellulases on both these carbon sources. Cellobiohydrolase I (CBH I), which accounts for up to 60% of the secreted cellulase protein, was measured as a 'marker' of cellulase formation during these experiments. Fig. 3 shows Western blots of culture filtrates from M8, indicating that lactose led to apparently normal CBH I formation; the pattern of CBH I secretion during growth of M8 on cellulose paralleled that of growth and β -glucosidase formation, exhibiting a similar lag.

In order to find out whether mutant M8 can be induced to form CBH I by sophorose in a normal manner, resting cell experiments with glycerol- and

Fig. 3

Fig. 3. Immunodetection of CBH I in culture broth of *T. reesei* QM 9414 and M8, after growth on lactose (L) and cellulose (C) for 60 h (M8 on cellulose for 120 h). Samples (10 μ l of culture broth, 2–4 μ g protein) were subjected to SDS-PAGE and Western blotting as described in Methods, and stained by using monoclonal antibody CH-6 (Mischak *et al.*, 1989). The positions of prestained marker proteins (MP) are indicated.

Fig. 4

Fig. 4. Immunodetection of CBH I in the culture broth of sophorose-induced mycelia of *T. reesei* QM 9414 and M8, pregrown on cellobiose (C) and glycerol (G). Samples (10 μ l of culture broth, 0.5–1 μ g protein) were taken from cultures that had been exposed to the inducer for 20 h and processed as described in the legend to Fig. 3.

cellobiose-pregrown mycelia were done. Fig. 4 indicates that CBH I was induced in mycelia by growth on both carbon sources. Also the same time-course of induction was observed with both mycelia (data not shown). These findings are in accordance with those reported earlier (Kubicek, 1987) and show that β -glucosidase is not necessary for cellulase formation when sophorose is used as an inducer.

Discussion

The prime goal of the present investigation was the isolation of a β -glucosidase-negative mutant of *T. reesei*. However, we observed that such mutants apparently occur at a very low frequency. We do not know whether this is due to a very low level of expression of the β -glucosidase gene or to an as yet unknown essential function of β -glucosidase for *T. reesei*, such as an involvement in morphogenesis (Jackson & Talburt, 1988) which would render a mutation in the β -glucosidase gene lethal. Cloning of β -glucosidase from this fungus is now in progress in at least two laboratories (T. Berges, C. Barreau & J. Begueret, conference poster,

Vienna, 1989; R. L. Mach & C. P. Kubicek, unpublished), which should provide an answer to this important question.

One mutant, defective in constitutive formation of β -glucosidase, was isolated. While the properties of the mutant are consistent with the existence of two different β -glucosidase genes in *T. reesei*, one constitutive and one inducible, recent results comparing the identity of the plasma-membrane-bound (constitutive) β -glucosidase with the cell-wall-bound and the extracellular β -glucosidase from this fungus contradict this idea (Hofer *et al.*, 1989). At present, no proof is available for the existence of a second secreted β -glucosidase from *T. reesei*. Hence we conclude that we have isolated a mutant altered or defective in the regulation of β -glucosidase formation.

Studies on the induction of cellulase formation in the mutant M8 showed that it grew only poorly on cellulose and also secreted little cellulase into the medium. In contrast, cellulase levels comparable to strain QM 9414 were secreted in a replacement medium upon induction by sophorose. These findings stress the importance of β -glucosidase for inducing cellulases in *T. reesei* during growth on cellulose – most probably by forming the inducer – as shown recently (Kubicek, 1987); further-

more, they show that *T. reesei* apparently possesses a transport system for β -linked disaccharides. Our studies on the uptake of cellobiose and β -methyl D-glucoside by mycelia of M8 pregrown on glucose or glycerol (such mycelia lack β -glucosidase) indicate that this transport system is constitutive and has apparently not been altered in M8; hence the slow growth of the mutant on cellulose may be due to uptake of cellobiose instead of extracellular hydrolysis. Recent studies from our laboratory show that the *in vivo* activity of the permease is less than one-tenth of that of total extracellular β -glucosidase (C. Fritscher, R. Messner & C. P. Kubicek, unpublished results).

The permease may also be involved in regulating β -glucosidase activity: in the present paper, we have provided evidence that both cellobiose and β -methyl D-glucoside act as physiological inducers of β -glucosidase. In view of the existence of a constitutive uptake system, this may provide a mechanism by which the cell avoids overaccumulation of cellobiose under conditions which the intracellular β -glucosidase (Inglin *et al.*, 1980) cannot handle. Hence elevated intracellular cellobiose levels may induce increased extracellular cellobiose hydrolysis.

β -Glucosidase has often been considered not to be a component of the cellulase system of *T. reesei* because it is differentially regulated. In contrast, the present results suggest that cellulases and β -glucosidase are both components of a regulatory network appropriate for economic cellulose hydrolysis: the constitutive β -glucosidase may function in the induction of cellulases by formation of sophorose from cellobiose (supplied by the constitutive cellulases; Kubicek *et al.*, 1988), whereas additional β -glucosidase may become induced upon increased cellobiose formation by the induced cellulases. Such a model also suggests that the amount of β -glucosidase secreted by *T. reesei* may depend on the activity of the intracellular β -glucosidase described by Inglin *et al.* (1980). Repression of formation of extracellular β -glucosidase (Sternberg & Mandels, 1980) and induction of the intracellular β -glucosidase (Loewenberg, 1984) by sophorose is in accordance with this view. This may offer strategies for finding β -glucosidase-overproducing mutants of *T. reesei* in the future.

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