Isolation and Characterization of a Mutant of *Trichoderma reesei* Showing Reduced Levels of Extracellular β -Glucosidase

By SAROJ MISHRA,*† S. RAO AND J. K. DEB

Biochemical Engineering Research Centre, Indian Institute of Technology, Hauz Khas,
New Delhi 110016, India

(Received 21 March 1989; revised 27 July 1989; accepted 9 August 1989)

A mutant (D6-13) of *Trichoderma reesei* that produced low levels of extracellular β -glucosidase was physiologically and biochemically characterized. The mutant was unable to grow and produced very little cellulase activity on Solka-Floc, Avicel, micro-crystalline cellulose and acid-swollen cellulose. Whereas growth was nearly normal on cellobiose, lactose and glucose, cellulase production was slightly decreased on cellobiose and lactose. The mutant produced nearly normal levels of FPA (filter-paper activity) and endoglucanase on sophorose. A distinct lag and a lowered specific sugar consumption rate was observed for the mutant on 0.3% cellobiose; this appeared to be due to lowered wall-bound β -glucosidase activity. The multiplicity and role of different β -glucosidases in *T. reesei* is discussed.

INTRODUCTION

The cellulase and β -glucosidase systems from *Trichoderma reesei* have been extensively studied to understand the mechanism of cellulase induction by cellulose (Merivuori *et al.*, 1984; Kubicek, 1987; Kubicek *et al.*, 1988). The cellulase enzymes are inducible by cellulose, cellobiose, cellobionolactone, lactose and sophorose (Sternberg & Mandels, 1979, 1982; Bruchmann *et al.*, 1987), whereas glucose and glycerol are repressors of cellulase biosynthesis (Montenecourt & Eveleigh, 1979). Since cellulose is insoluble it has been suggested that the true inducer may be sophorose, resulting from the degradation of cellulose by the combined action of endoglucanase and β -glucosidase (Gritzali & Brown, 1979; Vaheri *et al.*, 1979*a,b*). In *T. reesei* inhibition of plasma-membrane-bound β -glucosidase by nojirimycin and glucono- δ -lactone prevents endoglucanase induction by cello-oligosaccharides but not by sophorose, implicating β -glucosidase in *de novo* cellulase induction (Kubicek, 1987).

Direct evidence for the role of β -glucosidase in cellulase induction would be provided by the characteristics of *T. reesei* mutants negative for or low in β -glucosidase production. This paper contains the first report of the isolation of a mutant of *T. reesei* low in extracellular β -glucosidase production. The mutant was physiologically and biochemically characterized in terms of its ability to grow and to produce cellulases on insoluble and soluble cellulase inducers. The presence of β -glucosidases in other subcellular fractions was also investigated to understand the origin and multiplicity of the enzyme.

METHODS

Organism and isolation of mutant. *Trichoderma reesei* D-1/6 (Ghose *et al.*, 1982) was the parent strain. The low- β -glucosidase mutant D6-13 used in this study was isolated as a routine auxotrophic mutant from D-1/6 using the UV-irradiation method described for isolation of amino acid auxotrophs in *T. reesei* by Toyama *et al.* (1984). The rate of survival of treated conidia was 1%. D6-13 is a lysine-requiring auxotroph.

† Present address: Unite de Physiologie Cellulaire, Department des Biotechnologies, Institut Pasteur, 28 Rue du Dr Roux, 75724 Paris Cedex 15, France.

Abbreviation: FPA, filter-paper activity; M CCP, micro-crystalline cellulose powder.

Growth conditions. *T. reesei* D-1/6 and D6-13 were maintained on potato-dextrose agar (PDA) slants at 4 °C and subcultured once every 2 weeks. Growth was in Vogel's minimal medium (Vogel, 1964) containing (w/v) 0.1% peptone, 0.2% Tween 80 and different cellulase inducers as described below. The initial pH was adjusted to 5.3 unless otherwise specified and left uncontrolled thereafter.

Batch cultures. These were grown in 100 ml of culture medium in 500 ml flasks at 28 °C with shaking as described previously (Mishra *et al.*, 1982). The cultures were inoculated with a 10% (v/v) inoculum of a 24 h seed culture grown from conidia on 0.5% glucose. Samples were removed every 24 h (for cultures growing on insoluble cellulosic materials) or 6 h (for cultures growing on soluble inducers) during the exponential growth phase. The supernatant was assayed for sugars, soluble protein and enzyme activities. The biomass of D-1/6 and D6-13 grown on soluble sugars was determined as described previously (Sahoo *et al.*, 1986).

Estimation of residual sugars. Residual sugars in the growth medium were assayed by the dinitrosalicylic acid method (Sumner & Somers, 1949). The specific consumption rate for each sugar, defined as sugar consumed ($\text{mg ml}^{-1} \text{ h}^{-1}$)/amount of biomass at the time of assay (mg dry wt ml^{-1}) was calculated as described previously (Sahoo *et al.*, 1986).

Subcellular fractionation. For measurement of β -glucosidase activity in various subcellular fractions, D-1/6 and D6-13 were cultivated on 0.3% cellobiose medium. Samples were removed every 5–6 h and sonicated under conditions described previously (Sahoo *et al.*, 1986) to give extracellular, wall-bound and intracellular fractions. These were stored at 4 °C.

Enzyme assays. Filter-paper activity (FPA) and endoglucanase activities were measured according to the method of Mandels *et al.* (1976) using 50 mg Whatman no. 1 paper and 1% (w/v) carboxymethylcellulose as substrates respectively. Reducing sugars produced were measured by the dinitrosalicylic method (Sumner & Somers, 1949). β -Glucosidase activities were measured using *p*-nitrophenyl β -D-glucoside (3 mg ml^{-1}) as the substrate as recommended by the IUPAC (1984). The FPA and endoglucanase activity of cultures grown on insoluble cellulase inducers were measured in the extracellular culture filtrate obtained after centrifugation of the whole cell broth at 700 *g* for 20 min. For samples cultivated on soluble cellulase-inducers, enzyme activities were measured on acetone-precipitated samples as described previously (Sahoo *et al.*, 1986). Enzyme assays were done in triplicate on four independently grown cultures. The values for replicate assays differed from the mean by less than 7%.

Chemicals. Solka-Floc SW 40 (spruce-wood, mesh 40) was from Fluka; Avicel cellulose was from Serva; MCCP (micro-crystalline cellulose powder; 70–75 μm particle size) was from Cellulose products of India; phosphoric-acid-swollen cellulose was prepared from MCCP as recommended by the IUPAC (1984).

RESULTS

Cellulase production and growth on insoluble cellulase inducers

The parent D-1/6 and its mutant derivative D6-13 were cultivated for 10 d on a variety of insoluble cellulase inducers (1% concentration). The initial pH of the medium was adjusted to 5.3 or 3.5 and left uncontrolled thereafter. The final pH attained in both cases was 6.2. D-1/6 produced high levels of FPA and endoglucanase on all the substrates, with production mostly being greater at an initial pH of 3.5 (Table 1). Maximum production of FPA (2.59 IU ml^{-1}) and of endoglucanase (5.92 IU ml^{-1}) was obtained on 1% Solka-Floc. Similarly, D-1/6 produced β -glucosidase activities on all the substrates; however, enzyme activity was lower at an initial pH of 3.5 except on phosphoric-acid-swollen cellulose (Table 1).

The mutant D6-13 produced low levels of FPA (1.25–18.5% of parental levels), endoglucanase (5.4–40% of parental levels) and β -glucosidase (1.6–30% of parental levels) on all the substrates irrespective of the initial pH of the medium (Table 1). Interestingly, MCCP gave slightly better production of endoglucanase activity by D6-13. Very poor growth was observed for D6-13 compared to D-1/6 on these substrates (data not shown). Repeated attempts to cultivate the seed inoculum (grown on 0.5% glucose) of D6-13 on insoluble cellulose led to heavy sporulation in the medium.

Cellulase synthesis on soluble inducers

Cultivation of D-1/6 and D6-13 on soluble cellulase inducers yielded FPA, endoglucanase and β -glucosidase activities as shown in Table 2. The FPA activity of D6-13 on cellobiose and sophorose was comparable to values obtained with D-1/6, although maximum activities on cellobiose were obtained for D6-13 after 12 h growth. The endoglucanase activities of D-1/6 and D6-13 were comparable on sophorose, while the mutant produced only 40% of parental activities on cellobiose. The enzyme activities on lactose followed a slightly different pattern.

Table 1. Cellulase production after 10 d by *T. reesei* D-1/6 and D6-13 on insoluble cellulase inducers

	Carbon source (1%, w/v)	Initial pH	Enzyme activity (IU ml ⁻¹)*		
			FPA	Endoglucanase	β -Glucosidase
D-1/6	Solka-Floc	5.3	1.2	2.07	0.56
		3.5	2.59	5.92	0.22
		5.3	0.05(4.1%)	0.41(19.8%)	0.009(1.6%)
D6-13		3.5	0.14(5.4%)	0.35(5.91%)	0.05(22.7%)
D-1/6	Avicel	5.3	1.11	3.01	0.3
		3.5	2.4	3.7	0.27
		5.3	0.074(6.6%)	0.6(19.9%)	0.02(6.6%)
D6-13		3.5	0.03(1.25%)	0.2(5.4%)	0.015(5.55%)
D-1/6	MCCP	5.3	0.60	1.48	0.21
		3.5	1.11	1.48	0.10
		5.3	0.02(2.7%)	0.6(40.5%)	0.07(33%)
D6-13		3.5	0.09(8.1%)	0.37(25.0%)	0.01(10%)
D-1/6	Acid-swollen cellulose	5.3	0.45	1.85	0.10
		3.5	0.77	1.48	0.23
		5.3	0.07(18.5%)	0.50(27.0%)	0.03(30%)
D6-13		3.5	0.016(4.1%)	0.4(21.6%)	0.04(17.3%)

* Numbers in parentheses are percentages of parental values obtained under the same conditions.

Table 2. Cellulase production by *T. reesei* D-1/6 and D6-13 on soluble cellulase inducers

	Carbon source	Enzyme activity (IU ml ⁻¹)*		
		FPA	Endoglucanase	β -Glucosidase
D-1/6	Cellobiose (0.3%)	0.5 [4 h]	2.5 [4 h]	0.25 [8 h]
D6-13		0.47(94) [12 h]	1.0(40) [12 h]	0.06(24) [8 h]
D-1/6	Lactose (1%)	1.29 [72 h]	4.4 [72 h]	0.24 [72 h]
D6-13		0.66(51) [72 h]	2.5(57) [72 h]	0.017(7) [72 h]
D-1/6	Sophorose (0.3%)	0.45 [8 h]	1.02 [8 h]	0.24 [8 h]
D6-13		0.35(77) [8 h]	0.96(94) [8 h]	0.01(4) [8 h]

* Numbers in parentheses are percentages of parental values obtained under the same conditions. Times given in square brackets are when the reported activities reached a maximum in the extracellular medium.

While the extracellular β -glucosidase activities of D-1/6 on cellobiose, lactose and sophorose were similar (0.25 IU ml⁻¹), D6-13 produced very low extracellular β -glucosidase activities on these three substrates (Table 2).

Specific growth rate, maximum biomass and specific consumption rates of sugars

In an attempt to evaluate and compare growth-associated properties of D6-13 with those of D-1/6, the strains were cultivated on cellobiose, lactose and glucose. The specific growth rate of D6-13 was slightly less on cellobiose (76% of the parental value) although no significant

Fig. 1. Uptake of cellobiose (a), lactose (b), sophorose (c) and glucose (d) by *T. reesei* D6-13 (●) and D-1/6 (○).

Table 3. Specific growth rate, maximum biomass and specific sugar consumption rates of *T. reesei* D-1/6 and D6-13 on soluble sugars

Carbon source		Lag (h)	Specific growth rate (h ⁻¹)*	Maximum biomass (mg ml ⁻¹)†	Specific sugar uptake rate [mg h ⁻¹ (mg dry wt) ⁻¹]
D-1/6	Cellobiose (0.3%)	<4	0.25	2.6	0.025
				[20 h]	
D6-13		8	0.19(76)	2.3	0.017(68)
				[20 h]	
D-1/6	Lactose (1%)	<4	0.23	5.5	0.050
				[24 h]	
D6-13		8	0.15(65)	3.2	0.025(50)
				[24 h]	
D-1/6	Glucose (1%)	No lag	0.13	7.5	0.070
				[20 h]	
D6-13		No lag	0.11(84)	7.4	0.062(89)
				[24 h]	

* Numbers in parentheses are percentages of parental values obtained under the same conditions.

† Times given in square brackets are when the biomass values reached a maximum.

Fig. 2. Subcellular distribution of β -glucosidase in extracellular (a), intracellular (b) and wall-bound fractions (c) of *T. reesei* D6-13 (●) and D-1/6 (○) cultivated on 0.3% cellobiose.

difference was noticed when the two strains were cultivated on glucose (Table 3). The maximum biomass attained on either glucose or cellobiose was similar for both mutant and parent; however, on lactose the mutant attained only 58% of the parental value. Interestingly, a lag of 8 h was noticed for D6-13 before growth commenced on cellobiose and lactose.

The specific sugar uptake rates of the strains are also shown in Table 3. While there was no significant difference in the uptake rates of glucose, uptake rates of cellobiose and lactose in D6-13 were 68% and 50% of the parental values respectively.

Time-course of sugar uptake

The uptake of soluble cellulase inducers by D-1/6 and D6-13 is shown in Fig. 1(a-c). On cellobiose and lactose the lag in sugar uptake was much more pronounced in the case of the mutant than it was with the parent (Fig. 1a, b). The rate of uptake of cellobiose was faster in D-1/6 than in D6-13 (Fig. 1a). After the initial lag, uptake rates for lactose were similar in both cases (Fig. 1b). No difference in sophorose or glucose uptake rates was observed between D-1/6 and D6-13 (Fig. 1c, d).

Distribution of β -glucosidase activities in subcellular fractions

The distribution of β -glucosidase activity in subcellular fractions of D-1/6 and D6-13 was monitored during the first 24 h of cultivation on cellobiose to determine whether other β -glucosidases (namely, intracellular and wall-bound enzymes) are produced by D6-13 in addition to extracellular β -glucosidase, and to understand the possible relationship amongst the enzymes. As shown in Fig. 2(a-c), D6-13 produced very low extracellular β -glucosidase activity during the first 20 h cultivation, after which there was a slight increase (Fig. 2a). Similarly, low intracellular activities were detected in D6-13 compared to D-1/6 (Fig. 2b). This contrasted with the β -glucosidase activity of the wall-bound fraction which contained almost 65% of the parental value (Fig. 2c).

DISCUSSION

The exact sequence of events involved in *de novo* induction of cellulase by insoluble cellulosic material is not clearly understood although there is strong evidence supporting the role of 'membrane-bound' β -glucosidase in the induction phenomenon. Recent reports (Kubicek, 1987; Kubicek *et al.*, 1988) indicate that while initial attack on crystalline cellulose is mediated by conidial-bound endoglucanase to release short-chain celloextrins, the next step appears to be the formation of the 'true inducer' from celloextrins by a plasma-membrane-bound β -glucosidase (Umile & Kubicek, 1986). Inhibition of the 'membrane-bound' β -glucosidase by known inhibitors of β -glucosidase, viz. nojirimycin and glucono- δ -lactone, prevented *de novo* induction of cellulase synthesis by celloextrins but not by sophorose (Kubicek, 1987).

The characteristics of *T. reesei* D6-13, a low β -glucosidase mutant that is unable to grow and produce a complete array of cellulases on insoluble cellulosic inducers, demonstrates the involvement of β -glucosidase in the induction phenomenon. D6-13 appears to be a regulatory β -glucosidase mutant as a change in the nature of the cellulosic material (as with phosphoric-acid-swollen cellulose or micro-crystalline cellulose, considered to be more amorphous materials) led to slightly better production of β -glucosidase. The elevated levels of β -glucosidase on MCCP and acid-swollen cellulose (33 and 30% respectively) may also partly explain the higher endoglucanase activities obtained on these substrates. As expected, the mutant was not impaired in its ability to produce FPA and endoglucanase on sophorose. Thus β -glucosidase is not required for cellulase synthesis by *T. reesei* cultivated on sophorose. Similar conclusions were made by Kubicek (1987).

The constitutive cellulase levels of D6-13 and D-1/6 on 2% glucose were the same (about 7% of the fully induced levels of D-1/6 grown on 1% Solka-Floc). Thus D6-13 still produces basal levels of endoglucanase and FPA. The low levels of endoglucanases produced by D6-13 on Solka-Floc and Avicel are most likely the conidial-associated endoglucanases. As mentioned earlier, heavy sporulation was observed for D6-13 on these substrates. The endoglucanases and several other glycanases and glycosidases have been shown to be associated with the conidia in *T. reesei* (Kubicek *et al.*, 1988) as well as in some other fungi (for review, see Bisaria & Mishra, 1989).

Two other important conclusions can be made regarding the multiplicity and origin of β -glucosidases in *T. reesei*. The presence of the enzyme in the wall-bound fraction and its absence in the intracellular compartment at all times of cultivation on cellobiose suggests that the two are genetically independently determined. Second, the major source of the extracellular enzyme may not be the wall-bound enzyme, believed to be released on autolysis of the mycelium (Vaheri *et al.*, 1979a; Kubicek, 1981) but either the intracellular enzyme or the 'plasma-membrane-bound' enzyme. The multiplicity of β -glucosidases has been suggested by several authors (Sternberg & Mandels, 1980; Kubicek, 1987; Schmid & Wandrey, 1987; Jackson & Talburt, 1988) but the function of the various enzymes is still not clear. Sternberg & Mandels (1980) proposed the existence of a constitutive and an inducible form of the enzyme in *T. reesei*. Based on an intensive purification procedure, Schmid & Wandrey (1987) demonstrated two distinct β -glucosidases in the extracellular culture filtrates of *T. reesei* and the properties of one of the purified enzymes appears to be similar to the plasma-membrane-bound enzyme reported by Umile & Kubicek (1986). Recently, based on the similar M_r and pI values of the membrane-bound enzyme and an extracellular β -glucosidase, Jackson & Talburt (1988) made similar conclusions. Two immunologically distinct extracellular β -glucosidases have been demonstrated in *T. reesei* (C. P. Kubicek, personal communication) strongly indicating that the multiplicity exists at the genetic level and that it should be possible to isolate mutants for the different forms of the enzyme.

In the light of this and of our results with D6-13, it is proposed that there are at least two genetically distinct β -glucosidases in *T. reesei*: a high-affinity plasma-membrane-bound β -glucosidase (absent in D6-13) involved in the formation of the inducer and *de novo* cellulase synthesis on crystalline substrates, and a low-affinity enzyme (present in D6-13) secreted predominantly into the cell-wall and involved in the uptake of cellobiose. The role of the wall-bound enzyme in cellobiose uptake has been reported earlier (Sternberg & Mandels, 1982; Bisaria *et al.*, 1986; Sahoo *et al.*, 1986). We are currently investigating the presence/absence of the plasma-membrane-bound enzyme in the mutant.

REFERENCES

- BISARIA, V. S. & MISHRA, S. (1989). Regulatory aspects of cellulase biosynthesis and secretion. *CRC Critical Reviews in Biotechnology* **9** (in the Press).
- BISARIA, V. S., NANDA, M. & GHOSE, T. K. (1986). Effect of L(-)-sorbitol on release of β -glucosidase by *Trichoderma reesei* QM 9414. *Journal of General Microbiology* **132**, 973–978.
- BRUCHMANN, E.-E., SCHACH, H. & GRAF, H. (1987). Role and properties of lactonase in a cellulase system. *Biotechnology and Applied Biochemistry* **9**, 146–159.
- GHOSH, V. K., GHOSE, T. K. & GOPALKRISHNAN, K. S. (1982). Improvement of *Trichoderma reesei* strain through mutation and selective screening technique. *Biotechnology and Bioengineering* **24**, 241–244.
- GRITZALI, M. & BROWN, R. D., JR (1979). The cellulase system of *Trichoderma*: relationship between purified extracellular enzymes from induced and cellulose grown cells. *Advances in Chemistry Series* **181**, 237–260.
- IUPAC (1984). *Measurement of Cellulase Activities. Recommendations of the Commission of Biotechnology, International Union of Pure and Applied Chemistry*. New Delhi: Indian Institute of Technology.
- JACKSON, M. A. & TALBUT, D. E. (1988). Purification and partial characterization of an extracellular β -glucosidase of *Trichoderma reesei* using cathodic run, polyacrylamide gel electrophoresis. *Biotechnology and Bioengineering* **32**, 903–909.
- KUBICEK, C. P. (1981). Release of carboxymethyl cellulase and β -glucosidase from cell walls of *Trichoderma reesei*. *European Journal of Applied Microbiology and Biotechnology* **13**, 226–231.
- KUBICEK, C. P. (1987). Involvement of a conidial endoglucanase and a plasma-membrane-bound β -glucosidase in the induction of endoglucanase synthesis by cellulose in *Trichoderma reesei*. *Journal of General Microbiology* **133**, 1481–1487.
- KUBICEK, C. P., MUHLBAUER, G., ROTZ, M., JOHN, E. & KUBICEK-PRANZ, E. M. (1988). Properties of a conidial-bound cellulase enzyme system from *Trichoderma reesei*. *Journal of General Microbiology* **134**, 1215–1222.
- MANDELS, M., ANDREOTTI, R. A. & ROCHE, C. D. (1976). Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium* **6**, 21–33.
- MERIVUORI, H., SIEGLER, K. M., SANDS, J. A. & MONTENECOURT, B. S. (1984). Regulation of cellulase biosynthesis and secretion in fungi. *Biochemical Society Transactions* **13**, 411–414.
- MISHRA, S., GOPALKRISHNAN, K. S. & GHOSE, T. K. (1982). A constitutively cellulase producing mutant of *Trichoderma reesei*. *Biotechnology and Bioengineering* **24**, 251–254.
- MONTENECOURT, B. S. & EVELEIGH, D. E. (1979). Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. *Advances in Chemistry Series* **181**, 289–301.
- SAHOO, D. K., MISHRA, S. & BISARIA, V. S. (1986). Influence of L-sorbitol on growth and enzyme synthesis of *Trichoderma reesei* C-5. *Journal of General Microbiology* **132**, 2761–2766.
- SCHMID, G. & WANDREY, CH. (1987). Purification and partial characterization of a celloextrin glucosylase (β -glucosidase) from *Trichoderma reesei* strain QM 9414. *Biotechnology and Bioengineering* **30**, 571–585.
- STERNBERG, D. & MANDELS, G. R. (1979). Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. *Journal of Bacteriology* **139**, 761–769.
- STERNBERG, D. & MANDELS, G. R. (1980). Regulation of the cellulolytic system in *Trichoderma reesei* by sophorose: induction of cellulase and repression of β -glucosidase. *Journal of Bacteriology* **144**, 1197–1199.
- STERNBERG, D. & MANDELS, G. R. (1982). β -Glucosidase induction and repression in the cellulolytic fungus *Trichoderma reesei*. *Experimental Mycology* **6**, 115–124.
- SUMNER, J. B. & SOMERS, G. F. (1949). Dinitrosalicylic method for glucose. In *Laboratory Experiments in Biological Chemistry*, 2nd edn, pp. 38–39. New York: Academic Press.
- TOYAMA, H., YAMAGUCHI, K., SHINMYO, A. & OKADA, H. (1984). Protoplast fusion of *Trichoderma reesei*, using immature conidia. *Applied and Environmental Microbiology* **47**, 363–368.
- UMILE, C. & KUBICEK, C. P. (1986). A constitutive, plasma-membrane bound β -glucosidase in *Trichoderma reesei*. *FEMS Microbiology Letters* **34**, 291–295.
- VAHERI, M. P., VAHERI, M. P. O. & KAUPINEN, V. S. (1979a). Formation and release of cellulolytic enzymes during growth of *Trichoderma reesei* on cellobiose and glycerol. *European Journal of Applied Microbiology and Biotechnology* **8**, 73–81.
- VAHERI, M. P., LEISOLA, M. & KAUPINEN, V. S. (1979b). Transglycosylation products of the cellulase system of *Trichoderma reesei*. *Biotechnology Letters* **1**, 41–46.
- VOGEL, H. J. (1964). Distribution of lysine pathways in fungi: evolutionary implications. *American Naturalist* **98**, 2105–2115.