Enzymatic Properties and Intracellular Localization of the Novel *Trichoderma reesei* β-Glucosidase BGLII (Cel1A)

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This paper describes the characterization of an intracellular β-glucosidase enzyme BGLII (Cel1a) and its gene (*bgl2*) from the cellulolytic fungus *Trichoderma reesei* (*Hypocrea jecorina*). The expression pattern of *bgl2* is similar to that of other cellulase genes known from this fungus, and the gene would appear to be under the control of carbon catabolite repression mediated by the *cre1* gene. The BGLII protein was produced in *Escherichia coli*, and its enzymatic properties were analyzed. It was shown to be a specific β-glucosidase, having no β-galactosidase side activity. It hydrolyzed both cellotriose and cellotetraose. BGLII exhibited transglycosylation activity, producing mainly cellotriose from cellobiose and sophorose and cellobiose from glucose. Antibodies raised against BGLII showed the presence of the enzyme in *T. reesei* cell lysates but not in the culture supernatant. Activity measurements and Western blot analysis of *T. reesei* strains expressing *bgl2* from a constitutive promoter further confirmed the intracellular localization of this β-glucosidase.

Three categories of cellulases, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), are produced by saprophytic filamentous fungi for the degradation of insoluble cellulose into glucose. The endoglucanases mainly hydrolyze internal bonds in the cellulose polymer, producing new chain ends. The cellobiohydrolases, also known as exoglucanases, act processively from the chain ends, mainly producing cellobiose. This disaccharide and other short cello-oligomers are broken down to glucose by β-glucosidases. *Trichoderma reesei* possesses one of the best-known cellulase enzyme systems, and it has served as a model for fungal cellulose degradation (see chapters in the work cited in reference 10). The cellobiohydrolases and the endoglucanases from this fungus have been especially extensively characterized. The β-glucosidases of *T. reesei*, however, are less well characterized.

T. reesei has been reported to produce extracellular (5), cell-wall-bound (38), and intracellular (15) β-glucosidases. However, some data suggest that the extracellular and a major part of the cell-wall-bound activities could be due to the same enzyme (12, 26). The gene *bgl1* (3), encoding an extracellular β-glucosidase, has been isolated from *T. reesei*, and most probably it encodes the extracellular protein isolated previously (5). The corresponding BGLI enzyme has sequence similarity with other β-glucosidases belonging to family 3 of the glycosyl hydrolases (3). It has been shown that by increasing the copy number of *bgl1* and thus the amount of the BGLI enzyme in the cellulase mixture produced by *T. reesei*, the rate of reducing sugar formation from cellulose could be enhanced (3). This suggests that β-glucosidase activity is limiting for total hydrolysis of cellulose in the cellulase mixture. The *bgl1* gene has been deleted from *T. reesei* (8, 21), and a minor amount of extracellular β-glucosidase activity was still produced. This would indicate that there are other genes encoding extracellular β-glucosidases in the *T. reesei* genome. The disruption of the *bgl1* gene resulted in a delay in induction of the other cellulase genes by cellulose but not by sophorose, a β-1-2-linked disaccharide known as an efficient cellulase inducer (8). This suggests that BGLI is involved in formation of the soluble inducer of the cellulolytic enzymes. Results with similar implications were obtained with *bgl1* disruptants and multicopy strains by Mach et al. (21). The amount of BGLI present affected cellulase induction by cellulose or nonsaturating sophorose concentrations.

Information about a *T. reesei* gene (*bgl2*) encoding a second β-glucosidase belonging to glycosyl hydrolase family 1 was published recently (35). Some enzymatic properties of the corresponding BGLII (Cel1A) enzyme were reported. The same gene was cloned in our laboratory, and in this article we describe the analysis of the *bgl2* gene expression pattern and characterization of the BGLII protein. In contrast to the assumption of Takashima et al. regarding BGLII (35), data are presented in this paper indicating that BGLII is an intracellular enzyme.

MATERIALS AND METHODS

Strains. The *T. reesei* *bgl2* cDNA was cloned in the *Saccharomyces cerevisiae* strain H1152 (a, *sso2-1*, *leu2-3*, *trp1-1*, *ura3-1*, *sso1*::HIS3 [Markku Aalto and Sirkka Keränen, unpublished data]). The *Escherichia coli* strains TOP 10F′ (Invitrogen) and DH5α [F⁻, *endA1*, *hsdR17*(r_k^-, m_k^+), *supE44*, *thi-1*, λ⁻, *recA1*, *gyrA96*, *relA1*, Δ(*argFlacZYA*)*U169*, Φ80*lacZΔM15*] were used as plasmid hosts. BGLII was produced in the *E. coli* strain RV308 (su⁻, ΔlacX74, galISH, OP308, *strA*). The *T. reesei* strains QM9414 (23), Rut-C30 (27), and RutC-30, transformed with the *cre1* gene (13), were used in studies of *bgl2* regulation. The *ace2* disruptant strains and their parental strain ALKO2221 (2) were used when the effect of the *ace2* activator gene on *bgl2* induction was examined. The *T. reesei* strain RL-P37 (34) was used in Western analysis of BGLII localization. *T. reesei* QM9414 (23) was the host for constitutive *bgl2* expression.

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Bgl2 expression studies. To examine the effect of the carbon source on the expression of *bgl2*, the strain QM9414 was grown in shake flasks (200 rpm, 28°C) in minimal medium (29) with 3% glucose, 3% lactose, 2% cellobiose, or 3% cellulose (Solka Floc) for 2 (glucose) or 3 days. In experiments in which induction by sophorose was tested, QM9414 was grown in a minimal medium with 2% sorbitol or glycerol or 5% glucose for 72 or 57 h (glucose) and 1 mM sophorose was added at that time and 10 h later. The growth was continued for another 15 h, and the cells were harvested. Cultures, carried out with no sophorose additions for the total length of the experiment, were grown as controls of the induction. The glucose-depleted sample was derived from a glucose batch fermentation reaction mixture at 125 h, 50 h after the glucose in the fermentation had run out (14). The *cre1-1* mutant strain RutC-30 and the same strain transformed with the wild-type *cre1* gene (13) were cultivated in minimal medium with 2% glucose for 3 days. The *ace2* disruptant and its parental strain were grown in minimal medium with glycerol as previously described (2), and the mycelium was transferred into a medium with Solka Floc cellulose. The induction of *bgl2* was studied by Northern hybridization from samples harvested 9, 12, 15, 18, and 32 h after the medium change. The mycelia were collected by filtering, washed by 0.9% NaCl, and stored frozen. Total RNA was isolated as previously described (4) or by the Trizol reagent (Life Technologies). Northern hybridization was performed with the ^{32}P-labeled *bgl2* cDNA on Hybond N nylon filters as instructed by the manufacturer. The digital images were created by scanning films or Western filters with a Hewlett Packard Scanjet 4c scanner and processed with the Microsoft Powerpoint program.

Production of BGLII in *E. coli*. A DNA fragment with the *bgl2* protein-coding region of the cDNA fused with a C-terminal addition of six histidine codons was made by PCR with the primers TAGGATGAATTCATGTTGCCCAAGGACT TTCAG (forward primer with an *Eco*RI site) and ATACGAAAGCTTTCAGT GGTGGTGGTGGTGGTGCGCCGCCGCAATCAGCTCGT (reverse primer with six histidine codons, a stop codon, and a *Hin*dIII site). The PCR product was cloned between the *Eco*RI and *Hin*dIII sites of the pKKtac vector (36). Strain RV308 with the *bgl2* expression construct was grown at 37°C to an optical density at 600 nm of 0.9, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added as an inducer, and growth was continued for 4 h at 30°C. The BGLII protein was detected by Western blotting with anti-His$_6$ antibody (Roche) and purified with Ni-nitrilotriacetic acid-agarose (Qiagen) according to the instructions of the manufacturer of the QIAexpressionist kit (Qiagen).

Activity measurements. Both substrate analogs (*p*-nitrophenyl [PNP]-α-D-galactopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-xylopyranoside, PNP-α-L-arabinofuranoside, PNP-β-L-arabinopyranoside, PNP-β-D-glucopyranoside [Sigma], 4-methylumbelliferyl-β-D-glucoside [MUG, Sigma], 5-bromo-4-chloro-3-indolyl-β-D-glucoside [X-Glu; Sigma], and 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-Gal; Sigma]) and native oligosaccharides (cellobiose, cellotriose, cellotetraose, lactose [Merck], and sophorose [Serva]) were used to test the substrate specificity of BGLII. The activity towards the substrate analogs was determined by incubating 100 μl of the substrate (the PNP substrates [10 mM], 0.75 mg of X-Glu and X-Gal/ml, 1 mM of MUG) and 10 nkat of enzyme (as measured with PNP-β-D-glucopyranoside) at 45°C for 24 h in 50 mM Na-phosphate buffer, pH 7.0. The activity was measured spectrophotometrically at 405 nm (PNP substrates) or detected visually in UV light for MUG and in daylight for X-Glu and X-Gal. The activity towards native substrates was determined by incubating 1 ml (0.5% wt/vol) of the substrate and 2.5 nkat of purified BGLII in 50 mM sodium phosphate buffer, pH 7.0, at 45°C for 24 h. The samples were boiled for 5 min and analyzed by using high-pressure liquid chromatography (HPLC) as previously described (37).

The transglycosylation activity of BGLII was measured by incubating 1 ml of 20% or 40% glucose or 1 ml of 10% or 20% cellobiose with 100 nkat of purified BGLII/g of substrate in 50 mM Na-phosphate buffer (pH 7.0) at 45°C for 24 h. The samples were boiled for 5 min and analyzed using HPLC.

Western detection of BGLII. Polyclonal antibodies were raised against BGLII produced in *E. coli*. Two rabbits were immunized with 400 μg of the protein, and two booster injections of 200 μg were given at 3-week intervals. The antiserum was used at a 1:10,000 dilution in Western blotting. To detect the BGLII protein in *T. reesei* and to study its localization, the fungus was grown for 3 days in minimal medium with 0.2% peptone and 2% glucose, 2% lactose, or 2% Solka Floc cellulose as the carbon source. The mycelia were washed with 0.9% NaCl, and lysates were prepared by grinding them into fine powder under liquid nitrogen and resuspending into 50 mM Na-phosphate buffer (pH 7.0) with protease inhibitor cocktail (Roche). Cell debris was removed by centrifugation at 14,000 × *g* for 10 min. The total protein concentrations of the lysates were measured as previously described (20). Sodium dodecyl sulfate (SDS) gels from the cell lysate samples containing 20 μg of total protein and culture medium samples of 30 μl were processed as previously described (18), and Western detection was carried out with the alkaline phosphatase-secondary antibody conjugate (Bio-Rad).

Constitutive *bgl2* expression in *T. reesei*. The *bgl2* cDNA was released from the yeast expression vector pAJ401 (31) by an *Eco*RI-*Ssp*I digestion and cloned into the pAN52-1NotI vector (30), digested with *Nco*I, and treated with mung bean nuclease. A hygromycin resistance marker cassette was cloned into the *Not*I site of the resulting plasmid. The final expression vector, pJKP4, was transformed into *T. reesei* QM9414 as previously described (29). The transformants were selected on medium with 100 μg of hygromycin B/ml and purified through single-spore cultures before further analysis. Two transformants and the parental strain were grown in minimal medium (29) with 0.5% peptone and either 2% glucose or 2% cellobiose as the carbon source, and samples from the mycelia and supernatants were collected after 2 and 3 days of growth. Genomic DNA was isolated from the parental strain and the two transformants, and Southern hybridization with the *bgl2* cDNA from the DNA as a probe was performed as previously described (32). Cell-wall-associated β-glucosidase activity was measured from the culture grown for 3 days with glucose as the carbon source. After the growth, the mycelia were harvested immediately from 1 ml of the cultures by centrifugation at 6,000 × *g* for 3 min and washed twice with 0.9% NaCl. The cells were then resuspended into 50 mM Na-phosphate buffer (pH 7.0) with 10 mM PNP-β-D-glucoside substrate, and β-glucosidase activity was measured. Total protein lysates were prepared from the mycelia for Western detection of BGLII as described above. The protein content of each lysate was measured as previously described (20), and β-glucosidase activity was measured with PNP-β-D-glucoside from samples containing 5 μg of total protein. Using the same substrate, activity was also measured for 100 μl of samples taken from the culture supernatants. All the activity measurements were carried out from triplicate samples. Western detection of BGLII was carried out as described above from 40 μl of culture supernatant samples and cell lysate samples containing 20 μg of total protein.

RESULTS

Isolation of the *bgl2* gene. An attempt was made to clone components of the *T. reesei* secretory pathway by complementation in yeast of the *sso2* gene involved in exocytosis (1). The temperature-sensitive *sso2-1* yeast strain was transformed with a *T. reesei* expression library in a yeast vector (24), and several clones supporting growth of the strain in the restrictive temperature were found. One of these contained a cDNA encoding a novel β-glucosidase belonging to family 1 of the glycosyl hydrolases. Both the cDNA and the chromosomal copy were sequenced. The isolated gene was identical to the *T. reesei* β-glucosidase gene *bgl2*, whose sequence was published by Takashima et al. (35).

Expression pattern of the *bgl2* gene. The cellulase and hemicellulase genes of *T. reesei* are regulated according to the carbon source and are under carbon catabolite repression mediated by the *cre1* gene (13, 14, 25). Therefore, *bgl2* expression was studied by Northern hybridization using total RNA samples derived from cultures performed with different carbon sources and using strains with mutant and wild-type *cre1* genes. An activator gene, *ace2*, affecting the induction of the *T. reesei* cellulase gene, has been cloned recently (2), and the effect of *ace2* disruption on *bgl2* activation was also studied in this work by Northern hybridization. In addition to the *bgl2* experimentation, the Northern blots were probed with the actin-encoding *act1* gene as a loading control. Because *act1* mRNA levels are relatively variable on different carbon sources, a staining of the RNA gel with acridine orange is shown in Fig. 1 as an additional loading control.

No *bgl2* mRNA could be detected in samples derived from cultures carried out with glucose, sorbitol, or glycerol (Fig. 1, lanes 1, 4, and 6). The other *T. reesei* cellulases have been found to be induced in glucose-grown cultures in late stages
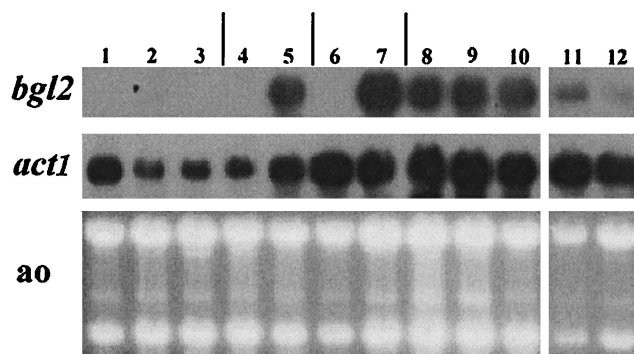
FIG. 1. Northern analysis of the *bgl2* gene expression. *T. reesei* was grown on minimal medium with glucose (lanes 1 to 3 and 11 and 12), sorbitol (lanes 4 and 5), glycerol (lanes 6 and 7), lactose (lane 8), cellobiose (lane 9), or cellulose (lane 10) as the carbon source. The effect of sophorose on the *bgl2* mRNA level is shown in lanes 3, 5, and 7. Lane 2 contains a sample from a carbon-source-depleted stage of a glucose culture. The involvement of the *cre1* gene in *bgl2* regulation was studied in the *cre1-1* mutant strain RutC-30 (lane 11) and in RutC-30 transformed with the wild type *cre1* (lane 12). A control probing with the actin gene (*act1*) and a staining of the gel with acridine orange (ao) are shown.

FIG. 2. SDS gel analysis of BGLII produced in *E. coli*. Using Western blotting, the protein was detected by anti-HisTag antibodies (lane 1). The purified BGLII protein is shown in a Coomassie-stained gel (lane 2).

when the carbon source has run out (14), but *bgl2* was not induced at this stage (lane 2). The *bgl2* gene was strongly expressed on lactose, cellobiose, and cellulose (lanes 8, 9, and 10). When sophorose, a disaccharide capable of inducing the other cellulase genes, was added to cultures grown on sorbitol or glycerol, the *bgl2* gene was strongly induced. This is in agreement with the findings presented in a previous paper (19), in which it was shown that intracellular β-glucosidase activity is induced by sophorose. In our experiment, sophorose did not induce *bgl2* when it was added to a glucose-grown culture. One explanation for this could be inhibition of sophorose uptake by a glucose-inhibited β-diglucoside permease (17).

In the strain RutC-30, which is a *cre1* mutant (13), the *bgl2* gene was expressed in a culture performed on glucose (Fig. 1, lane 11). In the derivative of RutC-30 that was transformed with the wild-type *cre1*, the expression level of *bgl2* was lower than that in RutC-30 in glucose cultures (Fig. 1, lane 12). The apparent involvement of *cre1* in *bgl2* regulation is in agreement with the fact that two putative binding sites of the CREI protein can be found in the *bgl2* promoter region (data not shown). In an *ace2* disruption strain, *bgl2* induction by cellulose was delayed and was not as strong as that in the parental strain (data not shown), in similarity to findings for the cellulase genes *cbh1*, *cbh2*, and *egl1* and the xylanase gene *xyn2* (2). In conclusion, the *bgl2* gene is regulated in a manner largely similar to that of other *T. reesei* cellulase genes.

The hydrolytic properties of BGLII. In order to be able to purify the *T. reesei* BGLII protein, the protein-coding region of *bgl2* was expressed in *E. coli* from the *tac* promoter as a C-terminal histidine tag fusion. Clear activity towards β-glucosidase substrates was detected in lysates of *E. coli* cells carrying the *bgl2* expression plasmids, whereas control cells with the vector alone did not produce any activity (data not shown). The BGLII-HisTag protein was purified from *E. coli* lysate by Ni-affinity chromatography, with a yield of about 5 mg per liter of culture. After purification, the protein was 50 to 60% pure
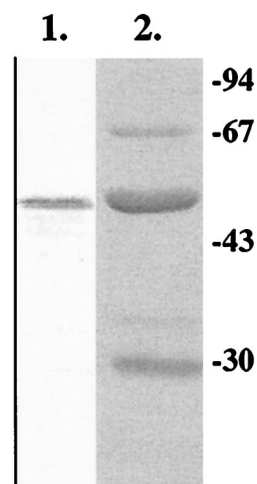
(Fig. 2). Because no β-glucosidase activity was detected in the host strain, not even in prolonged incubations, the decision was made to use the partially purified BGLII protein in activity assays.

The pH profile of BGLII was measured with PNP-β-D-glucoside as the substrate in the pH range of 3 to 8. The enzyme had a broad pH optimum in the range from 5.5 to 7 (data not shown).

The substrate specificity of BGLII was analyzed by hydrolysis tests with chromogenic substrates in which glucose, galactose, mannose, arabinose, or xylose was linked to the chromogenic group by a β linkage. These were used to test whether BGLII would have activity towards lactose or disaccharides derived from the degradation of different hemicelluloses. PNP-α-D-galactopyranoside and PNP-α-L-arabinofuranoside were used to test whether BGLII would have α-galactosidase or α-arabinofuranosidase side activities, respectively. The only substrates hydrolyzed by BGLII were those with glucose linked to the chromogenic group via a β-1-4 linkage, i.e., methylumbelliferyl-β-D-glucoside, PNP-β-D-glucopyranoside, and X-Glu (Table 1). Thus, according to this experiment, BGLII is a specific β-glucosidase. That BGLII does not have β-xylosidase activity was a somewhat unexpected finding, since PNP-β-D-xylopyranoside closely resembles PNP-β-D-glucopyranoside. D-Xylopyranoside has the same hydroxyl configuration as D-

TABLE 1. Hydrolysis of different chromogenic substrates by BGLII

Substrate	Hydrolysis
Methylumbelliferyl-β-D-glucoside (MUG)	+++
PNP-α-D-galactopyranoside	−
PNP-α-L-arabinofuranoside	−
PNP-β-D-glucopyranoside	+++
PNP-β-D-mannopyranoside	−
PNP-β-D-xylopyranoside	−
PNP-β-L-arabinopyranoside	−
X-Gal	−
X-Glu	+++

TABLE 2. Hydrolysis experiments of different oligosaccharides with BGLII[a]

Hydrolyzer	Concn (mg/liter) of:					
	Glucose	Cellobiose	Cellotriose	Cellotetraose	Lactose	Sophorose
Control cellotriose	0	0	3,800	32.5	0	0
BGLII cellotriose	57	132.5	3,650	70.5	0	0
Control cellotetraose	0	0	305	4,300	0	0
BGLII cellotetraose	45	0	380	4,050	0	0
Control lactose	0	0	0	0	4,300	0
BGLII lactose	0	0	0	0	4,250	0
Control sophorose	14	0	0	0	0	5,050
BGLII sophorose	180	0	0	0	0	4,750

[a] The controls were incubated without enzyme addition in a manner similar to that for the samples containing BGLII. The concentrations of the indicated sugars in the reaction mixtures after the incubations are shown.

glucopyranoside but without the primary hydroxyl group at C-5. This suggests that the primary hydroxyl is necessary for substrate recognition.

In the next experiment, we tested whether BGLII is active on longer cello-oligosaccharides, cellotriose, and cellotetraose or the disaccharide sophorose that has two glucose units linked by a β-1-2 linkage. As mentioned above, sophorose is a potent inducer of the cellulase genes. A further hydrolysis test was made with lactose, to confirm the negative hydrolysis result obtained with X-Gal in the previous experiment. The di- or oligosaccharides were incubated in 0.5% concentrations in the presence of BGLII, and the hydrolysis products were analyzed by HPLC. To reveal possible impurities or instability in the substrates, the incubations were also done without BGLII and the reaction mixtures were analyzed similarly. The results (Table 2) show that BGLII has activity towards both cellotriose and cellotetraose. The formation of the expected products, glucose and cellobiose, could be detected from cleavage of cellotriose. Interestingly, a small amount of cellotetraose was also produced by BGLII from cellotriose, showing that BGLII has transglycosylation activity. Glucose and cellotriose were formed from cellotetraose, suggesting that BGLII can hydrolyze only a terminal linkage in the substrate and not the central one. Analysis of the reaction products by HPLC does not reveal whether the enzyme cleaves the glucose from the reducing or the nonreducing end. Lactose was not hydrolyzed at all by BGLII in this experiment. Sophorose appeared to be a substrate for BGLII, since a small amount of glucose was formed from it during incubation.

Transglycosylation studies. To examine more thoroughly the transglycosylation activity of BGLII, the enzyme was incubated with 10% or 20% cellobiose and 20% and 40% glucose. After the incubations, the reaction mixtures were analyzed by HPLC. Samples treated similarly but having no BGLII were prepared as controls. With 10% cellobiose, about equal amounts of glucose and cellotriose were produced (Fig. 3). With 20% cellobiose, the final concentration of cellotriose was higher than that of glucose, indicating that transglycosylation had a higher rate than hydrolysis. Small amounts of cellotetraose and sophorose were also detected as end products of the incubation of 20% cellobiose with BGLII. From 20% glucose, a small amount of sophorose was formed by BGLII. When incubated with 40% glucose, BGLII produced substantial amounts of sophorose and cellobiose by transglycosylation (Fig. 3).

Localization of BGLII in *T. reesei*. The BGLII protein purified from *E. coli* was used as an antigen in the immunization of rabbits. An antiserum reacting with the purified BGLII was obtained (Fig. 4, lane 1). The *T. reesei* strain was grown in minimal medium with glucose, lactose, or cellulose as the carbon source. As shown in Fig. 1, the *bgl2* gene is not expressed in a culture grown using glucose but the mRNA is present in mycelia grown on lactose or cellulose. Samples from cell lysates and supernatants of cultures grown on these carbon sources were analyzed by Western blotting with the BGLII antiserum. Consistent with the Northern hybridization results, no signal was obtained from either the cell lysate or the supernatant of the glucose-grown culture (Fig. 4, lanes 6 and 7). In the cell lysates derived from the lactose and cellulose cultures, a protein with about the same mobility as that demonstrated by BGLII in *E. coli* was detected (Fig. 4, lanes 2 and 4). No signal, however, was obtained from the supernatants of the same cultures (Fig. 4, lanes 3 and 5). These results would indicate that BGLII is not secreted to the culture medium by *T. reesei*.

Constitutive expression of *bgl2* in *T. reesei*. The *bgl2* cDNA was expressed constitutively from the *Aspergillus nidulans gpdA* promoter in *T. reesei* strain QM9414. The transformants were first analyzed by Southern hybridization to detect the transformed construct. Two transformants that had one copy of the construct and the endogenous *bgl2* locus intact (data not shown) were chosen for further studies.

The transformants expressing BGLII constitutively were used to show more conclusively the localization of this enzyme. The two transformants and the parental strain were grown in three parallel cultures in minimal medium with either glucose or cellobiose as the carbon source. The glucose cultures were performed to allow study of the β-glucosidase activity derived solely from the transformed construct, because the endogenous *bgl2* gene and presumably most of the other β-glucosidase genes are not expressed on this carbon source. Using cell lysate and culture supernatant samples and intact mycelium samples, β-glucosidase activity was examined to reveal secreted, intracellular, and cell-wall-associated activity. The localization of BGLII was also studied by Western blotting using the cell lysates and supernatants.

In the cell lysates from the glucose-grown parental strain, very low β-glucosidase activity was found (Fig. 5). The two transformants produced much more activity, presumably originating from the transformed *bgl2* construct. The transformants also had much more activity than the parental strain in
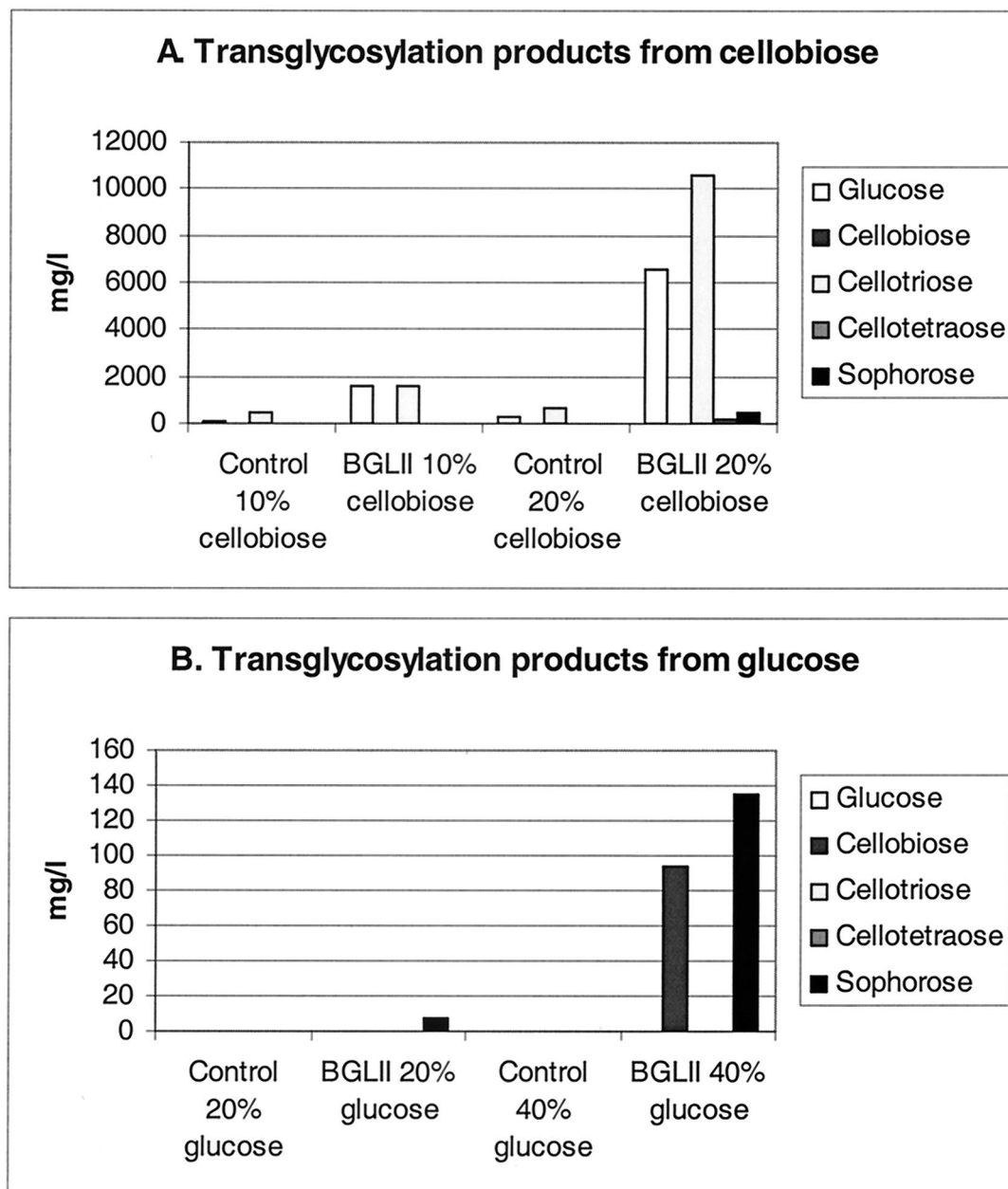
FIG. 3. The transglycosylation activity of BGLII was studied with 10% and 20% cellobiose (A) and 20% and 40% glucose (B). The incubations were done with or without (control) the BGLII enzyme.

the lysates of mycelia grown on cellobiose. This suggests that in these conditions, the *A. nidulans gpdA* promoter reaches a higher expression level than the promoter of the endogenous *bgl2* gene. In the supernatant samples of the cultures performed on glucose, practically no β-glucosidase activity was detected from the parental strain or the transformants. A very low level of activity was found in the measurements made with intact mycelia from glucose cultures, but in this assay, the three strains had about equal levels of β-glucosidase (data not shown). This activity could be due to the presence of a constitutive cell-wall-associated β-glucosidase enzyme described earlier (38). Still more proof for the localization of BGLII was

obtained from Western blot analysis, carried out using the cell lysate and culture supernatant samples from the glucose culture. The band corresponding to BGLII could be clearly detected in the cell lysate samples from the two transformants but not in those from the parental strain. This band could not be detected in any of the culture medium samples (data not shown).

DISCUSSION

According to the activity measurements carried out in this work, BGLII would appear to be a specific β-glucosidase,
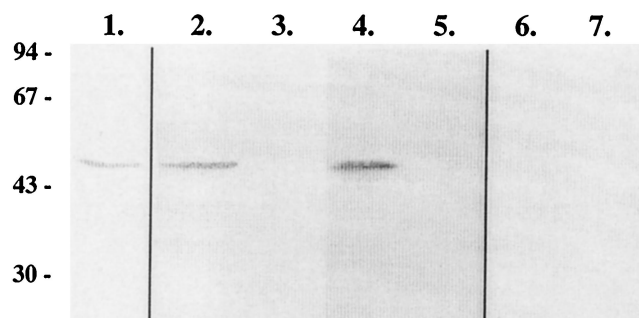
FIG. 4. Localization of BGLII by Western analysis. Samples of BGLII produced in *E. coli* (lane 1) or *T. reesei* (lanes 2, 4, and 6) cell lysates or culture supernatants (lanes 3, 5, and 7) are shown. The *T. reesei* samples were derived from liquid cultures performed with either lactose (lanes 2 and 3), cellulose (lanes 4 and 5), or glucose (lanes 6 and 7) as the carbon source.

cleaving only a β-1-4-bond next to a glucose unit. BGLII had no β-mannosidase, β-xylosidase, β-arabinosidase, or α-arabinofuranosidase activity. Furthermore, no activity was detected towards X-Gal (Table 1) or lactose (Table 2). However, Takashima et al. (35) reported that BGLII has weak activity towards PNP-β-D-galactoside, a substrate analog of lactose. In that study, *Humicola grisea* BGL4 was found to be clearly more active on this substrate than *T. reesei* BGLII. Many of the β-glucosidases of glycosyl hydrolase family 1 have been reported to have some side activities, towards lactose, for example (7, 28).

Several of the family 1 β-glucosidases have been shown to possess transglycosylation activity, and research into their use

in oligosaccharide synthesis is being carried out (for examples, see the work cited in references 6, 9, 11, and 16). *T. reesei* BGLII also showed clear transglycosylation activity in our experiments, which were performed with high concentrations of cellobiose and glucose (Fig. 3). The trials carried out in this work did not include any optimization; therefore, the yields of the transglycosylation products were clearly lower than those obtained with, e.g., thermostable bacterial β-glucosidases (7, 9). The reaction products produced by *T. reesei* BGLII from 40% glucose are of special interest. In these conditions, BGLII produced substantial amounts of sophorose and cellobiose. Thus, it appears that in high glucose concentrations, two glucose units can bind to the BGLII active site and the enzyme can form a glycosidic linkage between them by reverse hydrolysis in a manner previously described (22). The binding of at least one of the glucose units to the enzyme would appear to be flexible, since both β-1-2 and B-1-4 linkages can be formed between the glucose units.

The localization of *T. reesei* BGLII was studied in this work using Western detection and activity measurements from cell lysates and culture supernatants from an untransformed strain and a strain expressing BGLII constitutively from the *A. nidulans gpdA* promoter. The protein could not be detected in the supernatants of any of the cultures performed, but it could be clearly detected, both by its activity (Fig. 5) and by Western blot analysis (Fig. 4), in cell lysates. This indicates that BGLII is not secreted by *T. reesei*. We have also shown that BGLII expressed in *S. cerevisiae* is not secreted (data not shown). The activity measurements performed with intact *T. reesei* mycelia did not show any difference between the parental strain and the transformants, in which *bgl2* is expressed constitutively,

FIG. 5. β-Glucosidase activity in cell lysates of *T. reesei* transformants expressing BGLII constitutively (119a and 145a) and their parental strain (QM 9414). The strains were cultivated with either glucose or cellobiose as the carbon source, and samples are shown for 2- and 3-day-old mycelia.

```
BGLII      MLPKDFQWGFATAAYQIEGAVDQDGR...
BGL4       MSLPPDFKWGFATAAYQIEGSVNEDGR...
BglA       MTIFGPQDFMWGTATAAYQIEGAYQEDGR...
           * ** ** ********* .***
```

FIG. 6. Alignment of the N-terminal amino acid sequences of *T. reesei* BGLII, *Humicola grisea* BGL4, and *Bacillus polymyxa* BglA. Identical amino acids are indicated by asterisks, and similar ones are indicated by dots. The lysine and aspartate residues mentioned in the Discussion section are in bold.

even though the transformants had clearly higher activity in the cell lysate samples than the parental strain (Fig. 5). This would suggest that BGLII is not bound to the cell wall and resides neither in the periplasmic space nor at the plasma membrane, since PNP-β-D-glucoside, which was used as a substrate, most probably can diffuse through the cell wall. Thus, it is very likely that BGLII is a cytoplasmic protein. This is also consistent with the pH profile of BGLII. Unlike the secreted *T. reesei* cellulases that have acidic pH optima, BGLII is most active at levels of pH close to neutral.

The partial purification of an intracellular β-glucosidase enzyme from *T. reesei* has been reported (15). The purified enzyme was not characterized thoroughly, but its pH profile matches very well with the one we obtained for BGLII. The molecular mass of the isolated enzyme was determined by gel filtration as 98 kDa. The molecular mass of BGLII, as determined in this work by SDS-gel electrophoresis, was 50 kDa (Fig. 2 and 4). We have no proof of dimerization for BGLII, but if it is dimeric, it could be the same enzyme as the one isolated previously (15).

In previous work on BGLII, Takashima et al. (35) first isolated the gene encoding a secreted *Humicola grisea* β-glucosidase BGL4 and then used this gene as a hybridization probe and isolated the *T. reesei* bgl2 gene, assuming that this gene product would also be secreted. Both of the isolated genes were expressed in *Aspergillus oryzae*. *T. reesei* BGLII was not secreted from *A. oryzae* when its protein-coding region was solely in the expression construct. Only when *T. reesei* BGLII was linked to the *H. grisea* BGL4 signal sequence was it secreted. This is consistent with our finding that BGLII is intracellular in *T. reesei*. The N-terminal amino acid sequences of *T. reesei* BGLII and *H. grisea* BGL4 are highly similar (Fig. 6), but neither of them is recognized as a signal sequence by a signal sequence prediction program (39). This could be due to the presence of an aspartate residue found close to the N terminus. Acidic residues are not commonly found in the amino termini of signal sequences; instead, they usually have one or two positively charged amino acids. Both *T. reesei* BGLII and *H. grisea* BGL4 have a lysine close to the aspartate. In *T. reesei* BGLII, the lysine is immediately before the aspartate, and in *H. grisea*, it is positioned two residues after the aspartate (Fig. 6). The di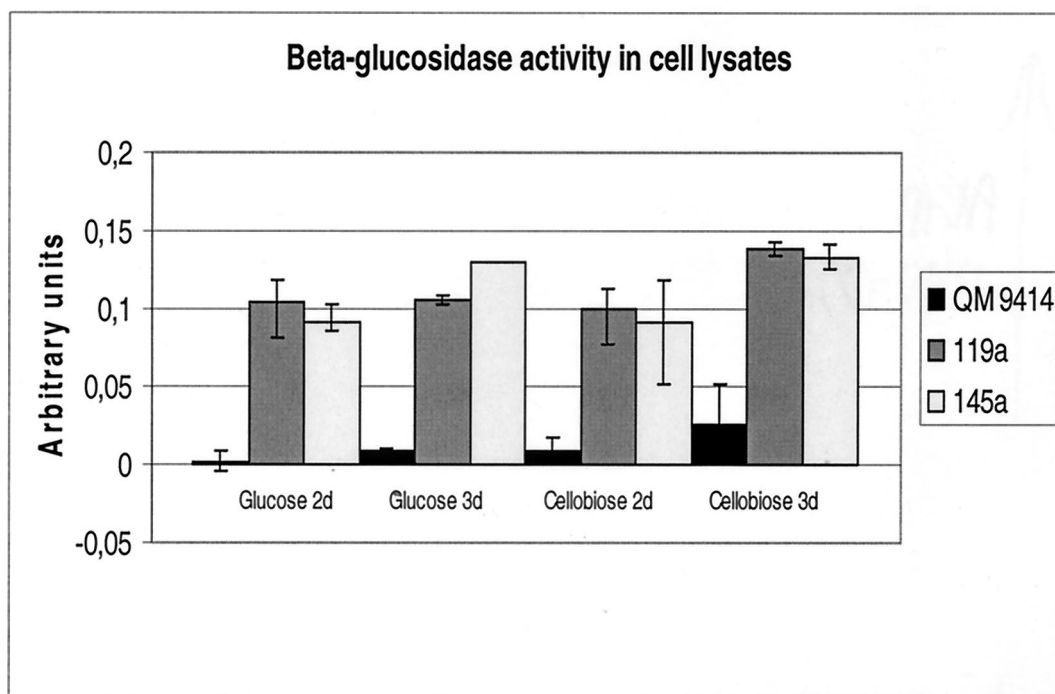fference in position of the lysine with respect to that of the aspartate could explain the fact that this sequence from *H. grisea* works as a signal sequence and the one in *T. reesei* BGLII does not.

It is interesting that the putative signal sequence region of *H. grisea* BGL4 shows clear amino acid similarity to those of other β-glucosidases of family 1 from plants and bacteria (an example is shown in Fig. 6). This was an unexpected finding, because signal sequence regions are not conserved between species

although they have some common features. Furthermore, this conserved region in *Bacillus polymyxa* BglA and other enzymes with known crystal structures is a part of the active enzyme and forms a β-strand and an α-helix into the structure (Fig. 6) (33) (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Therefore it would seem likely that *H. grisea* BGL4 was also originally an intracellular enzyme and that a mutation at its N-terminal region would have turned this region into a signal sequence.

ACKNOWLEDGMENTS

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