Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* – production of extracellular enzymes and characterization of the major cellulases

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Piptoporus betulinus is a common wood-rotting fungus parasitic for birch (*Betula* species). It is able to cause fast mass loss of birch wood or other lignocellulose substrates. When grown on wheat straw, *P. betulinus* caused 65 % loss of dry mass within 98 days, and it produced endo-1,4- β -glucanase (EG), endo-1,4- β -xylanase, endo-1,4- β -mannanase, 1,4- β -glucosidase (BG), 1,4- β -xylosidase, 1,4- β -mannosidase and cellobiohydrolase activities. The fungus was not able to efficiently degrade crystalline cellulose. The major glycosyl hydrolases, endoglucanase EG1 and β -glucosidase BG1, were purified. EG1 was a protein of 62 kDa with a pI of 2.6–2.8. It cleaved cellulose internally, produced cellobiose and glucose from cellulose and cellobiosaccharides, and also showed β -xylosidase and endoxylanase activities. The K_m for carboxymethylcellulose was 3.5 g l⁻¹, with the highest activity at pH 3.5 and 70 °C. BG1 was a protein of 36 kDa with a pI around 2.6. It was able to produce glucose from cellobiose and cellobiosaccharides, but also produced galactose, mannose and xylose from the respective oligosaccharides and showed some cellobiohydrolase activity. The K_m for *p*-nitrophenyl-1,4- β -glucoside was 1.8 mM, with the highest activity at pH 4 and 60 °C, and the enzyme was competitively inhibited by glucose (K_i = 5.8 mM). The fungus produced mainly β -glucosidase and β -mannosidase activity in its fruit bodies, while higher activities of endoglucanase, endoxylanase and β -xylosidase were found in fungus-colonized wood.

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INTRODUCTION

Brown rot basidiomycetes cause the most destructive form of wood decay by rapidly depolymerizing the polysaccharidic component (Eriksson *et al.*, 1990). They also belong to the principal recyclers of lignocellulose in forest ecosystems (Gilbertson & Ryvarden, 1986). Although brown rot fungi can cause extensive lignin modification, little lignin is removed from the wood, probably due to the lack of ligninolytic peroxidases or oxidases. However, brown rot fungi rapidly cleave the cellulose and hemicelluloses in wood, and subsequently remove it so completely that extensively brown-rotted wood consists almost entirely of modified lignin (Eriksson *et al.*, 1990).

The enzymic cellulolytic system of brown rot fungi did not attract as much attention as that of other well-characterized

groups of cellulose-degrading micro-organisms. The white rot fungus *Phanerochaete chrysosporium* effectively degrades crystalline cellulose via a synergistic mechanism between endoglucanases and cellobiohydrolases (Eriksson *et al.*, 1990). Endoglucanases cleave within cellulose molecules, generating non-reducing ends. Cellobiohydrolases (exoglucanases) act processively from these free ends, remaining attached to the cellulose and releasing soluble cellobiose molecules, which are subsequently hydrolysed to assimilable glucose by β -glucosidases (Beguin & Aubert, 1994).

Endoglucanases have been isolated from several brown rot fungi, including *Gloeophyllum sepiarium* (Mansfield *et al.*, 1998), *Gloeophyllum trabeum* (Herr *et al.*, 1978a; Mansfield *et al.*, 1998), *Polyporus schweinitzii* (Bailey *et al.*, 1969), *Serpula incrassata* (Kleman-Leyer & Kirk, 1994) and *Tyromyces palustris* (Ishihara & Shimizu, 1984). In general, it appears that most endoglucanases of brown rot fungi are constitutively expressed and are not catabolically repressed by glucose (Cotoras & Agosin, 1992; Highley, 1973). Purified glucosidases have so far been reported only from *G. trabeum* (Herr *et al.*, 1978b) and *Poria vailantii* (Sison *et al.*, 1958).

Abbreviations: BG, 1,4- β -glucosidase; CMC, carboxymethyl cellulose; EG, endo-1,4- β -glucanase; HMM, high molecular mass; LMM, low molecular mass; PASC, phosphoric acid swollen cellulose; pNPC, *p*-nitrophenyl- β -D-cellobioside; pNPG, *p*-nitrophenyl- β -D-glucoside; pNPGa, *p*-nitrophenyl- β -D-galactoside; pNPM, *p*-nitrophenyl- β -D-mannoside; pNPX, *p*-nitrophenyl- β -D-xyloside.

While brown rot fungi do not produce cellobiohydrolase, with the exception of *Coniophora puteana* (Sethuraman *et al.*, 1998), and most of them have not been reported to degrade crystalline cellulose, there is an open question how they produce the substrate for their β -glucosidases if cellobiohydrolases are not involved. It was recently reported that the brown rot fungus *G. trabeum* produces a processive endoglucanase, an enzyme with the ability to cleave cellulose internally but also release soluble oligosaccharides (Cohen *et al.*, 2005). However, there is currently no information about similar activity in other brown rot fungi.

Radical-generating reactions have been confirmed to participate in cellulose degradation by several brown rot fungi (Goodell, 2003; Hammel *et al.*, 2002). These include the involvement of quinone cycling (Jensen *et al.*, 2001) and cellobiose dehydrogenase-catalysed reactions (Hyde & Wood, 1997). However, production of reactive radicals has not been found in all brown rot species tested (Kleman-Leyer & Kirk, 1994). The enzymic system of these fungi is thus of primary importance for the utilization of cellulose and hemicelluloses. Only scant information is available about the enzymic activity during wood decay, i.e. in colonized wood and fungal fruit bodies.

The aim of this study was to estimate the activity of glycosylhydrolases of the brown rot fungus *Piptoporus betulinus*, detect these activities in the natural substrate and characterize the main cellulolytic enzymes. *P. betulinus* is a hardwood-specific parasite of birch (*Betula* species) trees in northern temperate forests and causes a very fast wood decay. It is also one of the most common brown rot species in central Europe (Baldrian & Gabriel, 2002), and has been reported to depolymerize cellulose (Bell & Burnett, 1966). The fungus exhibits a high rate of wheat straw degradation accompanied by a high production of hydrolytic enzymes, which makes it interesting for potential biotechnological use.

METHODS

Reagents. Chemicals were obtained from Sigma-Aldrich/Fluka. Phosphoric acid swollen cellulose (PASC) was prepared from Avicel, as described previously (Wood, 1988).

Organism and maintenance. The wood-rotting basidiomycete *P. betulinus* CCBAS585 was obtained from the Culture Collection of Basidiomycetes (CCBAS, Institute of Microbiology Academy of Sciences of the Czech Republic). For the preparation of inocula, the fungus was grown on malt extract (ME) agar plates (15 g malt extract l⁻¹, 20 g agar l⁻¹) at 28 °C for 7 days. Mycelium agar plugs 7 mm in diameter (cut along the edge of an actively growing colony) were used as inocula.

Degradation of lignocellulose. For enzyme activity estimations, the cultivation of *P. betulinus* proceeded in 100 ml Erlenmeyer flasks containing 5 g air-dried milled wheat straw. The straw was moistened with 15 ml distilled water (final water content 75%). The flasks were stoppered, autoclaved (2 × 25 min at 121 °C) and inoculated with two agar plugs with mycelium. The cultures were

incubated at 28 °C in the dark (Baldrian & Gabriel, 2003). Three flasks were collected on each sampling day (days 14, 28, 42, 56, 70 and 98).

Enzyme activity in fruit bodies and colonized wood. Fresh fruit bodies of *P. betulinus* were collected from dead birch trees (*Betula pendula*) from several sites in central Bohemia, Czech Republic. Samples of wood immediately adjacent to the fruit bodies with apparent fungal growth were also collected. Natural samples were stored at 4 °C until analysed (less than 48 h). pH was determined in water extracts of all samples.

Enzyme extraction. Buffers of different composition (pH 5–7, 100 mM phosphate buffer, 160 mM acetate buffer) and distilled water were compared for the maximum enzyme activity recovery from straw, fruit bodies and wood. The highest recovery from wheat straw was achieved with 160 mM sodium acetate buffer (pH 5.0), while distilled water was the most effective extractant for both fruit bodies and wood. Each sampling day, straw cultures in Erlenmeyer flasks were cut into small pieces and soaked with 40 ml sodium acetate buffer (160 mM, pH 5.0), while the fresh fruit bodies and wood were homogenized using a laboratory blender and soaked with 40 ml distilled water. The homogenized substrates were extracted at 4 °C for 2 h on a shaker. Extracts were filtered and the filtrates were kept at 4 °C until analysed (less than 24 h). Low molecular mass (LMM) and high molecular mass (HMM) fractions of culture extract from day 56 were prepared by ultrafiltration using a 10 kDa-cutoff membrane with an Amicon ultrafiltration cell (Millipore) and desalting on PD10 columns (Amersham).

The solid residues of the straw substrate, fruit bodies or wood were collected after filtration, dried at 105 °C to constant mass and weighed. The loss of dry mass in the straw cultures was calculated as the difference against the dry mass at day 0.

Assays. Activities of endo-1,4- β -glucanase (EG), endo-1,4- β -xylanase and endo-1,4- β -mannanase were routinely measured with azo-dyed carbohydrate substrates [carboxymethyl cellulose (CMC), birchwood xylan and galactomannan, respectively], using the protocol of the supplier (Megazyme). The reaction mixture contained 0.2 ml 2% dyed substrate in 200 mM sodium acetate buffer (pH 5.0), and 0.2 ml sample. The reaction mixture was incubated at 40 °C for 20–60 min and the reaction was stopped by adding 1 ml ethanol followed by 10 s vortexing and 10 min centrifugation (Baldrian *et al.*, 2005). The amount of released dye was measured spectrophotometrically at 595 nm and the enzyme activity was calculated according to standard curves that correlated the dye release with the release of reducing sugars.

Endoglucanase activity was also assayed in 10–30 min reactions, using a 1% (w/v) solution of CMC in 50 mM citrate/phosphate buffer (pH 5.0) at 40 °C. Xylanase and mannanase were assayed likewise with 1% (w/v) birchwood xylan or galactomannan as the substrates. Activities against Avicel and PASC were determined in 250 μ l reactions that contained 1% (w/v) substrate in 50 mM citrate/phosphate (pH 5.0) and 0.02% (w/v) Na₂N₃. The capped mixtures were shaken at 40 °C for 1–24 h. Reducing sugars were determined as glucose equivalents by the ferricyanide method (Sethuraman *et al.*, 1998). For all glycosylhydrolase assays, one unit of activity was defined as the amount of enzyme that converted one nanomole of substrate per minute.

The activity of 1,4- β -glucosidase (BG) was determined using *p*-nitrophenyl- β -D-glucoside (pNPG) in 50 mM sodium acetate buffer (pH 5.0) in 20–60 min reactions at 40 °C. The release of *p*-nitrophenol from the substrate was determined by the increase in absorbance at 400 nm (ϵ = 18.3 mM⁻¹ cm⁻¹) after addition of Na₂CO₃ to the reaction mixtures (Sadana & Patil, 1988). Activities of cellobiohydrolase,

1,4- β -xylosidase and 1,4- β -mannosidase were assayed using *p*-nitrophenyl- β -D-cellobioside (pNPC), *p*-nitrophenyl- β -D-xyloside (pNPX) and *p*-nitrophenyl- β -D-mannoside (pNPM), respectively, using the same method.

Cellobiose dehydrogenase was assayed by monitoring 2,6-dichlorophenolindophenol-reducing activity in 50 mM sodium acetate buffer (pH 5.0) (Temp & Eggert, 1999).

Laccase activity was measured using 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid in citrate/phosphate (100 mM citrate, 200 mM phosphate) buffer (pH 5.0). Activities of peroxidases, including Mn-peroxidase were assayed using 3,3-dimethylaminobenzoic acid and 3-methyl-2-benzothiazolinone hydrazine in succinate/lactate buffer (100 mM, pH 4.5), as described previously (Ngo & Lenhoff, 1980; Bourbonnais & Paice, 1990; Baldrian *et al.*, 2000). All spectrophotometric measurements were done in a microplate reader (Sunrise, Tecan) or a UV-VIS spectrophotometer (Lambda 11, Perkin-Elmer).

Statistical analyses were performed by the Statistica 7.0 software package (StatSoft). Differences at $P < 0.05$ were regarded as statistically significant unless otherwise stated. For better comparison of values obtained in a system with changing water content and dry mass, enzyme activities during lignocellulose degradation were expressed per gram of original dry mass of straw.

Purification of EG. The water extract of a 42-day culture grown on milled wheat straw was filtered and concentrated by ultrafiltration using a 10 kDa-cutoff membrane with an Amicon ultrafiltration cell (Millipore). The concentrate was loaded onto a DEAE-Sepharose HR 10/10 column (Pharmacia LKB) equilibrated with 20 mM phosphate buffer, pH 6.0. Proteins were eluted with a gradient of 0–0.5 M NaCl in 20 min at a flow rate of 0.5 ml min⁻¹. The EG fractions were pooled, concentrated and applied to a Superdex 200 HR 10/30 column (Pharmacia LKB). Elution was performed with 20 mM phosphate buffer, pH 6.0, containing 0.15 M NaCl, at a flow rate of 0.5 ml min⁻¹. Desalted EG fractions were applied to MonoQ HR 5/5 anion-exchange column (Pharmacia LKB) equilibrated with 20 mM phosphate buffer, pH 6.0, and eluted with a gradient of 0–0.5 M NaCl in 20 min at a flow rate of 0.5 ml min⁻¹. In the next step, EG fractions were desalted and chromatographed again on MonoQ equilibrated with 20 mM phosphate buffer, pH 7.5, under the conditions described above. The purity of the enzyme was checked using SDS-PAGE. The concentrated EG fractions were desalted to deionized water and stored frozen (–18 °C).

Purification of BG. The water extract of a 42-day culture grown on milled wheat straw was filtered and concentrated by ultrafiltration using a 10 kDa-cutoff membrane with an Amicon ultrafiltration cell (Millipore). The concentrate was loaded onto a DEAE-Sepharose HR 10/10 column (Pharmacia LKB) equilibrated with 20 mM phosphate buffer, pH 6.0. Proteins were eluted with a gradient of 0–0.5 M NaCl in 20 min at a flow rate of 0.5 ml min⁻¹. Desalted BG fractions were applied to a MonoQ HR 5/5 anion-exchange column (Pharmacia LKB) equilibrated with 20 mM phosphate buffer, pH 6.0, and eluted with a gradient from 0 to 0.5 M NaCl in 20 min at a flow rate of 0.5 ml min⁻¹. The BG fractions were pooled, concentrated and applied to a Superdex 200 HR 10/30 column (Pharmacia LKB). Elution was performed with 20 mM phosphate buffer, pH 6.0, containing 0.15 M NaCl, at a flow rate of 0.5 ml min⁻¹. In the next step, BG fractions were again desalted and chromatographed, employing MonoQ equilibrated with 20 mM phosphate buffer, first at pH 6.0 and then at pH 7.5, and 50 mM sodium acetate buffer, pH 4.5, respectively. Purity of the enzyme was checked using SDS-PAGE. The concentrated BG fractions were desalted to deionized water and stored frozen (–18 °C).

Characterization of purified enzymes. SDS-PAGE was performed using a 10% polyacrylamide gel. Analytical IEF was carried out with a Multiphor II electrophoresis system (Pharmacia LKB). The IEF gel (7.5%) was prepared using ampholines of pI 2.5–5.0 and pI 3.5–10.0 (Pharmacia LKB). A Low pI protein calibration kit, pI 2.5–6.5 (Pharmacia LKB), was used for the estimation of isoelectric point. Gels were stained using a Silver Stain Plus kit (Bio-Rad) and BG gels were activity-stained with pNPG. Protein concentrations were determined with the Bio-Rad Protein Assay kit by the method of Bradford, with BSA as the standard (Bradford, 1976). The molecular mass of the enzymes was estimated by gel filtration on a Superdex 200 HR 10/30 column (Pharmacia LKB) with gel filtration standards (Pharmacia LKB), and by SDS-PAGE with Sigma CK molecular mass markers.

The effect of pH on enzyme activity was examined in the pH range 2.25–8.0 in 50 mM citrate/phosphate buffer. CMC was used as substrate for EG, and pNPG, pNPX, pNPM and *p*-nitrophenyl- β -D-galactoside (pNPGa) were used as substrates for BG. Microcal Origin Professional 7.0 software was used for curve fitting. The effect of temperature on the enzyme activity and stability was determined in the range 10–80 °C. Substrate specificity for EG and BG was tested in 50 mM citrate/phosphate buffer, pH 5.0, with a range of substrates (Table 3). For EG, the K_m and k_{cat} were determined for CMC in 50 mM citrate/phosphate buffer (pH 2.5), and for pNPC, pNPG and pNPX in 50 mM citrate/phosphate buffer (pH 5.0); for BG, the K_m and k_{cat} were determined for pNPG, pNPM and pNPX in 50 mM citrate/phosphate buffer at the respective optimal pHs. The inhibitory effect on pNPG cleavage by BG was tested for glucose, cellobiose, mannose, galactose and xylose in 50 mM citrate/phosphate buffer (optimal pH) and respective K_i values were calculated. The kinetic parameters K_m , k_{cat} and K_i were obtained by a non-linear least-square fitting procedure using the ordinary Michaelis-Menten equation (Lineweaver-Burk plots) and using a non-linear regression with Microcal Origin Professional 7.0 software.

Oligoglucoside analysis. TLC of the oligoglucosides released from Avicel, PASC, cellobiose, cellotriose and cellotetraose was done on silica gel (Merck 60 GF₂₅₄). Samples of EG1, BG1 or HMM fraction of 56-day *P. betulinus* culture were incubated with the respective substrates for 18 h at 40 °C, concentrated, and aliquots containing 2.5 μ g total sugars were applied. The plate was developed with *n*-propanol/water/concentrated ammonia (70:20:10, v/v), as the solvent system, and the spots were visualized by charring with 1 M H₂SO₄ in ethanol (Dvořáková *et al.*, 2001).

RESULTS

Degradation of lignocellulose

P. betulinus colonized the whole volume of wheat straw substrate in the first 2 weeks after inoculation. The initial rate of substrate degradation was relatively low and reached 11.4% after 21 days of cultivation. The highest rate of dry mass loss was observed during the subsequent weeks (52.1% on day 56). At the end of the experiment on day 98, the loss of the straw substrate dry mass reached 65.3%.

P. betulinus cultures exhibited all seven tested cellulose and hemicellulose-degrading activities: EG, endo-1,4- β -xylanase, endo-1,4- β -mannanase, cellobiohydrolase, BG, 1,4- β -xylosidase and 1,4- β -mannosidase. The activity of ligninolytic enzymes, laccase and peroxidases, was not detected, nor was the activity of cellobiose dehydrogenase.

EG was the major endo-cleaving polysaccharide hydrolase, accounting for more than 85 % of the total activity, with a mean activity of 6400 U g^{-1} compared to 720 U g^{-1} for endo-1,4- β -xylanase and 120 U g^{-1} for endo-1,4- β -mannanase (Fig. 1). Peak activity was reached on day 56 for EG ($11\,300 \text{ U g}^{-1}$), while it was earlier for endo-1,4- β -xylanase (day 42, 1450 U g^{-1}) and endo-1,4- β -mannanase (day 21, 345 U g^{-1}). BG was the major exo-cleaving hydrolase, with a mean activity of $810\,000 \text{ U g}^{-1}$, accounting for approximately 67 % of the total activity of this group of enzymes. The activity of 1,4- β -mannosidase was higher than that of 1,4- β -xylosidase ($199\,000$ and $106\,000 \text{ U g}^{-1}$, respectively), and a lower yet significant cellobiohydrolase activity was detected ($88\,000 \text{ U g}^{-1}$). The activity of BG increased rapidly during the first 4 weeks of the experiment, reaching a peak value of $1.4 \times 10^6 \text{ U g}^{-1}$ on day 28 (Fig. 1). The activity of 1,4- β -mannosidase peaked on day 42 at $380\,000 \text{ U g}^{-1}$, and both 1,4- β -xylosidase and cellobiohydrolase activities were

Fig. 1. Time-course of enzyme activities during the growth of *P. betulinus* on wheat straw. (a) Endo-cleaving hydrolases: EG ($\square$), endo-1,4- β -xylanase ($\triangle$) and endo-1,4- β -mannanase ($\circ$). (b) Exo-cleaving hydrolases: BG ($\blacksquare$), 1,4- β -xylosidase ($\blacktriangle$), 1,4- β -mannosidase ($\bullet$) and cellobiohydrolase ($\diamond$). Means of three replicates and standard errors are shown.

relatively stable during the whole cultivation period. The hydrolytic activity towards all glycosylhydrolase substrates was associated exclusively with the HMM fraction of *P. betulinus* culture extract, and no activity was detected with the LMM fraction or the LMM fraction plus 0.1 mM FeCl_3 and H_2O_2 .

Purification and characterization of EG

The course of purification is summarized in Table 1. Three separate peaks of EG activity were detected during the purification process, accounting for 77, 17 and 6 % of the total activity. The major isoenzyme EG1 was purified to electrophoretic homogeneity. The molecular mass of the protein was 62 kDa by SDS-PAGE and 69 kDa by gel filtration. IEF showed a major band at pH 2.6 and a minor one at 2.8 (Fig. 2). Although the enzyme was active in a broad pH range from 2.5 to 6.0, the highest activity was detected at low pH (Fig. 3). EG1 exhibited the highest substrate specificity for CMC (Table 3), while it cleaved xylan, galactomannan and PASC at low rates (26, 10 and 1 %, respectively, compared to CMC), and its activity against Avicel was extremely low. The enzyme exhibited high activity against pNPC and, interestingly, also against pNPX. The K_m and k_{cat} were 3.5 g l^{-1} and 46.7 s^{-1} for CMC, 9.9 g l^{-1} and 1.2 s^{-1} for pNPG, 16 g l^{-1} and 7.7 s^{-1} for pNPC, and 57 g l^{-1} and 23.1 s^{-1} for pNPX. The highest rate of the enzyme reaction was detected at 70°C (Fig. 4).

Purification and characterization of BG

Two separate peaks of BG activity were detected during the purification process, accounting for 81 and 19 % of the total activity (Table 2). The major isoenzyme BG1 was purified to electrophoretic homogeneity. The molecular mass of the protein was 36 kDa by SDS-PAGE and 42 kDa by gel filtration. IEF showed a diffuse band at pH 2.6 (Fig. 2). In addition to pNPG, BG1 also performed hydrolytic cleavage of pNPGa, pNPM and pNPX with relatively high activities: 110, 42 and 25 % compared to pNPG at pH 5.0 (Table 3). The activity with the disaccharide-containing glycosides pNPL and pNPC was low (2.8 and 4.2 %). The pH profile for BG1 activity showed different optima for individual substrates tested. The most acidic optimum, at pH 3.25, was found for pNPGa, while it was pH 3.50–4.25 for pNPG, pNPM and pNPX (Fig. 3). The lowest K_m was found for pNPG (1.8 mM); the K_m values for pNPM and pNPX were 6–8 times higher (11.3 mM for pNPM and 15.3 mM for pNPX). The k_{cat} values were 96 s^{-1} for pNPG, 850 s^{-1} for pNPM and 30 s^{-1} for pNPX. The enzyme was also active at a low rate against CMC and PASC, while the activity against Avicel was negligible. Glucose, mannose, xylose, galactose and cellobiose were tested for their inhibitory effect on BG1. Glucose and mannose were found to inhibit the enzyme competitively, with K_i values 5.8 and 82.5 mM , respectively. The monosaccharides galactose and xylose, and the disaccharide cellobiose, did not affect the activity of the

Table 1. Purification of EG from the wheat straw culture of *P. betulinus*

Purification step	Total protein (μg)	Total activity (10^3 U)	Specific activity ($\text{U } \mu\text{g}^{-1}$)
Culture filtrate	4226	729.6	170
DEAE-Sepharose, pH 6.0	3690	726.5	200
Superdex 200, pH 6.0	1105	324.4	290
MonoQ, pH 6.0	570	58.2	100
EG1*	—	44.6	—
EG2*	—	9.94	—
EG3*	—	3.60	—
EG1: MonoQ, pH 7.5	20	40.4	2020

*Purified enzyme.

enzyme. The highest rate of enzyme reaction was detected at 60°C (Fig. 4).

Activity against pNPC did not appear as a separate peak but coincided with the peaks of EG or BG activity. It is thus probable that there is no specific cellobiohydrolase enzyme, and the activity appears as a side-activity of other enzymes, mainly BG (Table 3).

Oligoglucosides produced from cellulase substrates

Analysis of oligoglucoside cleavage products confirmed that no detectable amounts of reducing oligosaccharides were produced by *P. betulinus* culture extract from Avicel. Both glucose and cellobiose were produced from PASC, and glucose was the only product of oligosaccharide cleavage (Fig. 5). EG1 released cellobiose and glucose from PASC and cellotriose, and cellobiose alone from cellotetraose. BG1 released glucose as the reaction product from cellobiose, cellotriose and cellotetraose, and also from PASC.

Enzyme activities in fruit bodies and fungus-colonized wood

The activity of extracellular polysaccharide hydrolases was also estimated in the extracts from *P. betulinus* fresh fruit

bodies and fungus-colonized wood collected in the fruiting season during summer–autumn of 2004 and 2005. Although the fungus-colonized wood was collected in the immediate vicinity of fruit bodies and isolation on agar media confirmed that the mycelium present in wood was *P. betulinus*, a contribution by other micro-organisms present in wood to the measured values of enzyme activity cannot be completely ruled out. All tested enzymes were detected at least in some natural samples of both the fruit bodies and the fungus-colonized wood. There was, however, high variability of enzyme activities in these natural substrates, probably connected with the development stage of the fungus and the extent of mycelial colonization of wood.

Exo-cleaving glycosylhydrolases were the dominant enzymes in fungal fruit bodies. Among them, BG activity was the highest, with tens to hundreds of thousands of units per gram dry mass, while the activities of 1,4- β -mannosidase and cellobiohydrolase ranged from below ten to tens of thousands of units per gram dry mass, and 1,4- β -xylosidase activity was even lower (Table 4). The activity of endo-cleaving hydrolases was often not detected in the fruit bodies at all, and ranged from below ten to a few tens of units per gram dry mass. Endo-1,4- β -mannanase activity was much lower than that of the other two enzymes (Table 4). Within a fruit body, enzyme activities of monosaccharide hydrolases

Fig. 2. SDS-PAGE (a) and IEF (b) of purified BG1 and EG1. The gels were stained by silver staining; lanes M contain molecular mass markers (a) and pH markers (b). Numbers indicate molecular mass in kDa (SDS-PAGE) and pH (IEF).

Fig. 3. Effect of pH on the activity of EG and BG of *P. betulinus*. Open symbols, EG; filled symbols, BG. Substrates: pNPG (squares), pNPM (circles), pNPX (triangles), pNPGa (inverted triangles). Data points represent means of three replicate estimations, and are expressed as a percentage of the highest activity; relative standard errors did not exceed 5%.

were usually higher in the hymenium compared to the trama, but the differences were not statistically significant. The size (mass) of the fruit bodies as an approximate indication of their developmental stage did not show any correlation with enzyme activities.

EG and endo-1,4- β -xylanase activities were much higher in *P. betulinus*-colonized wood than in the fruit bodies, ranging in from below ten to a few tens of units per gram dry mass, while the activity of endo-1,4- β -mannanase was low (Table 4). BG was the most active exo-cleaving enzyme. Only three out of 13 samples exhibited BG activity higher than 10 000 U g⁻¹, while it was less than 1000 U g⁻¹ in seven samples.

The activities of individual enzymes in wood and fruit bodies were significantly different, both when the whole sets

Fig. 4. Effect of temperature on the activity of EG1 and BG1 of *P. betulinus*. □, EG; ■, BG. The data are expressed as a percentage of the highest activity; relative standard errors did not exceed 5%.

of data or the pairs of fruit body/adjacent wood were compared. The difference was significant at a probability level of 0.01 in all cases except endo-1,4- β -mannosidase, the enzyme with low activity in both fruit bodies and wood. Also, the pH values of fruit bodies and wood were significantly different, with more acidic values of around 3.0–3.5 in wood and higher values of 4.5–5.5 in the fruit bodies (Table 4).

DISCUSSION

The brown rot basidiomycete *P. betulinus* exhibited activities of all tested glycosylhydrolases and caused a rapid degradation of wheat straw during its growth. The mass loss after 98 days was comparable to that of the white rot fungus *Pleurotus ostreatus*, while the activities of

Table 2. Purification of BG from the wheat straw culture of *P. betulinus*

Purification step	Total protein (μ g)	Total activity (10^3 U)	Specific activity (U μ g ⁻¹)
Culture filtrate	5289	18 660	3530
DEAE-Sepharose, pH 6.0	4700	15 910	3390
Superdex 200, pH 6.0	413	5910	14 300
MonoQ, pH 6.0	127	2790	22 000
BG1*	—	2260	—
BG2*	—	530	—
BG1: MonoQ, pH 7.5	60	740	12 300
BG1: MonoQ, pH 4.5	13	250	19 200

*Purified enzyme.

Table 3. Substrate specificities of *P. betulinus* EG1 and BG1

The data represent means of three replicate estimations; relative standard errors did not exceed 5%.

Substrate	Specific activity (U μg^{-1})	
	EG1	BG1
Avicel	0.38	0.11
PASC	204	7.3
CMC	2020	10.2
Xylan	521	ND*
Galactomannan	17.8	ND
pNPC	333	1460
pNPL	ND	538
pNPG	53.1	19 200
pNPM	ND	8080
pNPX	998	2230
pNPGa	ND	21 100

*ND, Not determined.

glycosylhydrolases were higher by up to two orders of magnitude (Baldrian *et al.*, 2005).

The participation of reactive oxygen species and reactive radicals in brown rot fungi has repeatedly been demonstrated (Hyde & Wood, 1997; Jensen *et al.*, 2001; Hammel *et al.*, 2002; Goodell, 2003). *P. betulinus* did not produce cellobiose dehydrogenase, nor was there a detectable hydrolytic activity of the LMM (< 10 kDa) fraction of the culture extracts after the addition of Fe(III), and 2,5-dimethoxybenzoquinone was not found in the cultures (data not shown). However, the involvement of quinone cycling in the radical reactions (Jensen *et al.*, 2001) cannot be ruled out.

Like several other brown rot fungi (Ishihara & Shimizu, 1984; Kleman-Leyer & Kirk, 1994; Mansfield *et al.*, 1998; Cohen *et al.*, 2005), *P. betulinus* produced more enzymes with endoglucanase activity. The molecular mass of the major endoglucanase EG1 (62 kDa) was higher than that of the endoglucanases of most other brown rot fungi. With the

exception of the LMM enzymes Cel 25 from *S. incrassata* (Kleman-Leyer & Kirk, 1994) and Cel 12a from *G. trabeum* (Herr *et al.*, 1978a; Cohen *et al.*, 2005), the molecular mass is typically between 35 and 50 kDa (Keilich *et al.*, 1969; Ishihara & Shimizu, 1984; Kleman-Leyer & Kirk, 1994; Clausen, 1995; Mansfield *et al.*, 1998; Cohen *et al.*, 2005). Larger enzymes (Cel 57) have been found only in fungi that produce a greater number of isoenzymes: *Gl. sepiarium* (Bhattacharjee *et al.*, 1993) and *S. incrassata* (Kleman-Leyer & Kirk, 1994). The isoelectric point of EG1 was more acidic than the 3.1–4.9 reported for other enzymes (Kleman-Leyer & Kirk, 1994; Mansfield *et al.*, 1998; Cohen *et al.*, 2005), and the pH optimum was also more acidic: 3.5 compared to the 4.0–4.5 for *Gl. sepiarium*, *G. trabeum* and *P. schweinitzii* endoglucanases. However, all of these enzymes are highly active at pH < 4.0 (Keilich *et al.*, 1969; Herr *et al.*, 1978a; Mansfield *et al.*, 1998). The low pH optimum of endoglucanase corresponds with the low pH of wood colonized by *P. betulinus* (Table 4).

EG1 exhibited high affinity towards its best substrate: the K_m for CMC was 3.5 g l^{-1} . EGS endoglucanase from *Gl. sepiarium*, EGT from *G. trabeum* and a 29 kDa endoglucanase from *G. trabeum* had K_m values of 7.6, 6.3 and 13.1 g l^{-1} , respectively. EG1 also exhibited detectable activity towards a range of other substrates. Interestingly, it also cleaved pNPX with high activity, but showed little activity with galactomannan and pNPG. The activity with Avicel was extremely low. With respect to the oligosaccharide hydrolysis, EG1 showed a combination of EGT and Cel5A activities from *G. trabeum*: like EGT it yielded glucose and cellobiose from PASC, but unlike EGT, EG1 also cleaved cellotriose (Mansfield *et al.*, 1998; Cohen *et al.*, 2005). Although a low activity with pNPG was detected, EG1 was unable to release glucose from cellobiose. Like both EGT and Cel5A, EG1 is also a processive enzyme able to cleave oligosaccharides from HMM cellulose (although not crystalline cellulose).

Although all brown rot fungi probably produce β -glucosidase activity (Goodell, 2003), only the enzymes from *Por. vailantii* and *G. trabeum* have been purified, and the latter is the only well-characterized β -glucosidase (Sison *et al.*, 1958; Herr *et al.*, 1978b). The *G. trabeum*

Fig. 5. TLC showing products released from Avicel, PASC and oligoglucosides. The standard lanes (Std) include glucose (G1), cellobiose (G2), cellotriose (G3) and cellotetraose (G4). The other lanes show, from left to right, products released by *P. betulinus* culture extract, purified EG1 and purified BG1 from Avicel (Avic.), PASC and oligoglucosides G2–G4.

Table 4. Activities of cellulose- and hemicellulose-degrading enzymes and pH in the fruit bodies of *P. betulinus* and in the wood colonized by the fungus

For fruit bodies, $n=39$; for wood, $n=13$. P , probability of the hypothesis that the means are the same (t test). Enzyme activity is expressed in U (g dry mass)⁻¹. Ranges and medians are shown.

Enzyme activity	Fruit bodies		Wood		P
	Range	Median	Range	Median	
EG	0–42	7.7	13–167	46	<0.00001
Endo-1,4- β -xylanase	0–46	15.4	5–87	49	<0.00001
Endo-1,4- β -mannanase	0–15	1.2	0.0–5.8	1.8	0.10445
BG	10 000–669 000	139 000	0–74 000	3000	<0.00001
1,4- β -Xylosidase	0–7700	2000	200–5100	600	0.00842
1,4- β -Mannosidase	1000–75 000	12 000	0–6600	600	0.00052
Cellobiohydrolase	0–86 000	10 000	0–7300	500	0.00002
pH	3.54–5.65	4.95	2.83–3.63	3.23	<0.00001

β -glucosidase is a large enzyme with a molecular mass of 320 000 Da, active with glucose and cellooligosaccharides, but inactive with CMC or crystalline cellulose. Its K_m values for pNPG and pNPX are 0.4 and 3.3 mmol l⁻¹, respectively (Herr *et al.*, 1978b). BG1, the enzyme responsible for most of the *P. betulinus* β -glucosidase activity, is much smaller, with a molecular mass even smaller than that of β -glucosidases from white rot fungi (Deshpande *et al.*, 1978; Smith & Gold, 1979; Copa-Patino & Broda, 1994; Cai *et al.*, 1998). The K_m value of *P. betulinus* BG1 for pNPG is lower than that of *G. trabeum*, but is well within the relatively broad range (0.1–5.3 mmol l⁻¹) found in wood-rotting fungi (Smith & Gold, 1979; Copa-Patino & Broda, 1994; Lyman *et al.*, 1995; Wei *et al.*, 1996; Morais *et al.*, 2002). In addition to its activity against cellobiose and cellooligosaccharides, BG1 also exhibits low activity against PASC. The wide range of substrates hydrolysed by BG1, with no preference for one principal substrate, makes this enzyme a unique molecule among cellulases of wood-rotting basidiomycetes (Deshpande *et al.*, 1978; Herr *et al.*, 1978b; Smith & Gold, 1979; Copa-Patino & Broda, 1994; Cai *et al.*, 1998).

From the overlapping substrate specificities of *P. betulinus* enzymes it seems obvious that the classification of glycosylhydrolases into endo- and exo-cleaving enzymes cannot be strict while 'processive' endoglucanases show oligosaccharide-releasing activity. Also, the cellulolytic and hemicellulolytic enzymic systems cannot be separated, since several enzymes show activity against more than one substrate (Copa-Patino & Broda, 1994; Cohen *et al.*, 2005). The classification according to the major activity can thus be misleading with respect to the actual physiological role.

Although growth in wood and the production of fruit bodies are the most ecologically relevant modes of the existence of wood-rotting fungi, relatively little attention has been paid to the production of enzymes under these conditions, and there are no reports about cellulolytic enzymes in the fruit bodies of brown rot fungi growing under natural conditions.

Poria placenta produces several endo-cleaving glycosylhydrolases and 1,4- β -glycosidases during growth in *Liquidambar styraciflua* (sweetgum) wood, with xylanase as the main enzyme (Highley, 1973). Production of xylanase and BG has also been detected in wood colonized by the white rot fungus *Ceriporiopsis subvermispora*. All of the hydrolytic activities detected in *P. betulinus*-colonized straw were also present in the colonized wood, despite the great range of detected activities. Activities of endo-1,4- β -xylanase, EG and 1,4- β -xylosidase were detected in all samples of wood colonized by *P. betulinus*, while not all samples exhibited detectable endo-1,4- β -mannanase, BG or 1,4- β -mannosidase activity. The pH in the wood was low, probably due to the production of organic acids (Takao, 1965) that formed a favourable environment for endoglucanase. This enzyme was possibly also responsible for at least some 1,4- β -xylosidase activity detected in wood. Interestingly, fruit bodies of *P. betulinus* with pH values more favourable for BG activity frequently contained no activity of endo-cleaving hydrolases. It seems that the endo-cleavage of polysaccharides by *P. betulinus*, along with the processive production of cellobiose, is preferentially performed in wood, while the fruit bodies contain mostly disaccharide and oligosaccharide hydrolases.

This work shows that *P. betulinus* is able to perform fast lignocellulose hydrolysis due to its hydrolytic enzymes with relatively wide substrate specificities. The enzymes are produced both during fungal colonization of wood and on wheat straw, the latter fact being of possible use for the fast production of cellulolytic enzymes for biotechnology purposes.

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