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ORIGINAL PAPER

A new β-glucosidase gene from the zygomycete fungus *Rhizomucor miehei*

Miklós Takó · Adél Tóth · László G. Nagy · Judit Krisch · Csaba Vágvölgyi · Tamás Papp

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Abstract In this study, a β-glucosidase coding gene (*bgl*) of the zygomycete fungus *Rhizomucor miehei* has been cloned and characterized. The gene comprises a total of 2,826 bp including the coding sequence of a 717 amino acids length putative protein and 10 introns dispersed in the whole coding region. The putative N-and C-terminal catalytic domains (aa 68 to aa 274 and aa 358–601, respectively) were identified; the two domains are connected with a 84-amino-acids linker. The catalytic region showed an extensive sequence homology with other fungal β-glucosidases classified as family 3 glycoside hydrolases. The isolated *Rhizomucor* gene was expressed in the related fungus *Mucor circinelloides*. Transformant *Mucor* strains maintained the introduced plasmid in an autoreplicative manner and showed significantly higher cellobiase activity than the recipient strain.

Keywords Family 3 glycoside hydrolase · Cellobiase · *Rhizomucor* · *Mucor* · Heterologous expression

M. Takó · A. Tóth · L. G. Nagy · C. Vágvölgyi · T. Papp (✉)
Department of Microbiology, Faculty of Science and Informatics, University of Szeged, 6726 Szeged, Közép fasor 52, Hungary
e-mail: pappt@bio.u-szeged.hu

J. Krisch
Department of Food Engineering, Faculty of Engineering, University of Szeged, Szeged, Hungary

Introduction

β-Glucosidases (β-D-glucoside glucohydrolases; 3.2.1.21) constitute a group of well-characterized and biologically important enzymes that catalyze the transfer of glycosyl group between oxygen nucleophiles. Their activity is fundamental in many biological pathways, such as degradation of structural and storage polysaccharides, host–pathogen interactions, cellular signalling and oncogenesis (Bhatia et al. 2002). These enzymes play a crucial role in large scale saccharification of cellulose by removing cellobiose that inhibits the exo- and endoglucanases. Filamentous fungi are known to be good producers of β-glucosidases and several fungal glucosidase genes have already been cloned and analyzed. However, Zygomycetes are rarely investigated from this aspect. Although a few enzymes have been characterized in this fungal group (Borgia and Mehnert 1982; Petruccioli et al. 1999; Takii et al. 2005), cloning and analysis of glucosidase genes have not been reported until to date.

The genus *Rhizomucor* comprises two well-established thermophilic fungal species, *R. pusillus* and *R. miehei* (Schipper 1978; Vágvölgyi et al. 1999). In particular, *R. miehei* is interesting from a biotechnological aspect in consequence of its extracellular enzyme production. Commercially produced *R. miehei* aspartic protease is widely used in industrial cheese making to substitute calf chymosin (Outtrup and Boyce 1990; Rao et al. 1998) and its lipase is one

of the most studied fungal lipases (Maheshwari et al. 2000). Despite their significance, members of the genus *Rhizomucor* have remained genetically relatively poorly characterized.

In a carbon source assimilation study (Vastag et al. 1998), *R. miehei* was able to utilize cellobiose and other disaccharides as sole carbon sources. An early publication reports on the cellulolytic activity of *R. pusillus*, where it produced hydrolytic enzymes that attacked native cellulose, acid-swollen cellulose, carboxymethylcellulose and cellobiose (Somkuti et al. 1969). The same study suggested the presence of a multiple cellulolytic enzyme system in this fungus. However, the literature lacks further information on the hydrolytic breakdown of cellulose and its derivatives by the members of the genus *Rhizomucor*.

The aim of the present study was to clone and characterize the β-glucosidase gene and its adjacent element from *R. miehei*. With this object, the isolated gene was introduced into the related fungus *Mucor circinelloides* and expression of the gene and induction of the promoter were examined in this heterologous system.

Materials and methods

Strains

The wild-type *R. miehei* NRRL 5282 was used for the gene cloning and the *M. circinelloides* strain MS12 (Velayos et al. 1997) a *leuA*$^-$, *pyrG*$^-$ mutant derived from the wild-type CBS 277.49 served as a recipient in the transformation experiments. *Escherichia coli* DH5α strain was used in all cloning experiments and plasmid amplifications cultivating on Luria–Bertani medium at 37°C; if necessary, ampicillin was added to a final concentration of 50 μg ml^{-1}.

DNA manipulation techniques

R. miehei was grown in yeast extract—glucose liquid medium (YEG; 5 g yeast extract, 20 g glucose per litre) under continuous shaking (200 rpm) at 37°C for 3 days. Genomic DNA of *R. miehei* was prepared from mycelia disrupted with pestle and mortar in liquid nitrogen and purified in a 0.5 mg ml^{-1} bisbenzimide-CsCl density gradient (Iturriaga et al. 1992). Plasmid DNA preparation, cloning and transformation of *E. coli*, as well as electrophoresis of nucleic acids, were performed following standard procedures (Sambrook et al. 1989). Nucleic acids for hybridization were labelled with the digoxigenin-based "PCR DIG Probe Synthesis Kit" (Roche), following the instructions of the manufacturer. Immunological detection of nucleic acid blots was performed using the DIG Nucleic Acid Detection Kit (Roche), according to the conditions recommended by the supplier.

Cloning of the *R. miehei bgl* gene

Analysing of known fungal β-glucosidase sequences, two degenerated primers, BGL1 and BGL2, were designed that correspond to the amino acid sequence motifs GLDM and FPYLV, respectively. Using this primer pair, a fragment of 742 bp was amplified by PCR from the genomic DNA of *R. miehei* and its sequence was determined. Table 1 presents the nucleotide sequences of the primers used in the study.

The entire gene together with the upstream and downstream flanking regions was determined by the inverse PCR (IPCR) method (Ochman et al. 1988). Figure 1 summarizes the PCR strategy used to clone the *Rhizomucor bgl* gene. To obtain DNA templates for the IPCR experiments, four sets of fragments were prepared digesting 10 μg aliquots of the genomic DNA with the enzymes *Xba*I, *Hind*III, *Pst*I and *Xho*I (Fermentas), each in a total volume of 120 μl. The digested DNA was purified with phenol:chloroform:isoamyl alcohol (PCI; 25:24:1) followed by a subsequent extraction with chloroform:isoamyl alcohol (CI; 24:1). After ethanol precipitation of the aqueous phase, samples were air dried and resuspended in 17 μl sterile distilled water. Resulting DNA fragments were self-ligated with T4 ligase (Fermentas) for 18 h at 4°C and the circularised DNA was extracted again with PCI and CI. After ethanol precipitation and air drying, samples were resuspended in 10 μl distilled water, from which 50 ng were used in the subsequent IPCR reactions.

IPCR primers were designed in opposite orientation to that of normal PCR (Table 1). PCR reactions were carried out under the following parameters: a denaturation was done at 94°C for 2 min, followed by 10 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 3 min, followed by 20 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 3 min with a cycle

Table 1 Oligonucleotide primers designed for this study

Primer	Sequence (5′–3′)
BGL1	GGCTCGAGGGYYTNGAYATG
BGL2	GGCTGCAGACNARRTANGGRAA
BGL3	GGCTCAGGCACTGTCGAC
BGL4	GCCTTCGTCTTGTCCCAACTTGTA
BGL5	GACGTCCTCTTTGGTGATGTCAAC
BGL6	AGCTGGTTGTAGCTACACATAATG
BGL7	GTCTTTCCTACACCACCTTTGAGT
BGL8	GTTACCAATGTAGTGTTTGGCAGT
BGL9	AGTACACTTTGCATATTGGTGCTA
BGL10	CGGCTACGCCTTGCATAGCTGAC
BGL17	CTCGCGGCCGCTTAGTAAAGATAGCTACGGCGCT
BGL19	GGATCGATATGTTTGCAAAGACTGCGTTG

Restriction sites designed in the primers are underlined

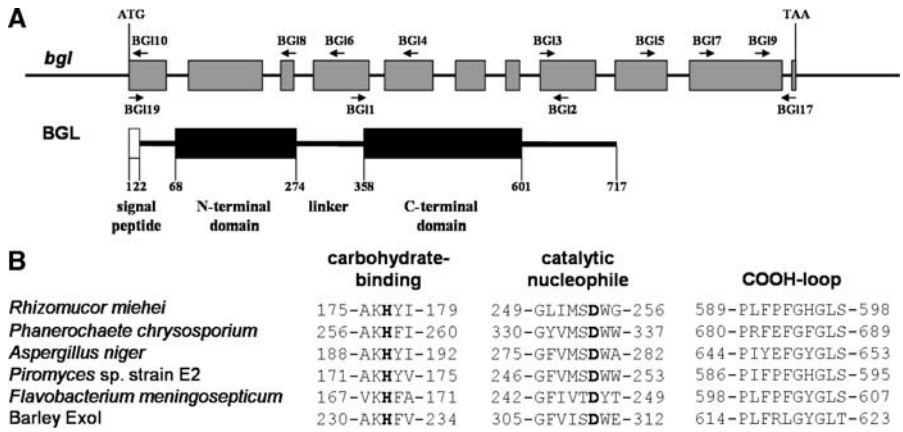

Fig. 1 The *R. miehei* β-glucosidase gene and protein. **a** Schematic representation of the *bgl* gene and the main regions of the encoded protein. *Arrows* indicate the positions of the primers for the IPCR amplifications and cloning. **b** Comparison of conserved sequence motifs of some family 3 glycoside hydrolases. *Bold* characters indicate the putative H^+ donor and the catalytic nucleophile

elongation (5 s) for each successive cycle; the final cycle was followed by an extension step of 68°C for 7 min. Each amplification product was cloned into the plasmid pTZ57R/T using the InsT/Aclone PCR Product Cloning Kit (Fermentas) according to the instructions of the manufacturer and sequenced in both directions.

Investigation of the *bgl* copy number

For Southern hybridization analysis, 5 μg *R. miehei* genomic DNA was digested with the appropriate restriction enzyme. After gel electrophoresis (0.8% agarose/TAE), DNA was transferred onto nylon membranes; prehybridization (1 h) and hybridization (16 h) were carried out under low stringency (55°C) and high stringency (65°C) conditions. For hybridization, the same digoxigenine labelled probe was used as in the screening of the genomic library. After hybridization, membranes were washed twice in 2× SSC and 0.1% SDS at room temperature for 5 min and then twice with 0.5× SSC and 0.1% SDS at 55°C (for low stringency) or with 0.1× SSC and 0.1% SDS at 65°C (for high stringency) for 15 min, respectively.

Sequence analysis

Blast searches (Altschul et al. 1997) in the EMBL and GenBank databases were performed for prediction of the coding sequence. The programs ProtParam

(Gasteiger et al. 2005) and SignalP (Bendtsen et al. 2004) were used to characterize the proposed protein and to predict the signal peptide, respectively. Domain search and prediction were performed using the Motif Scan (MyHits) program (Pagni et al. 2007). Structure prediction was made by the Swiss-Modell (Arnold et al. 2006) using the barley ExoI (NCBI accession number: AAD23382) as template; for 3D-visualization and comparison of protein sequences and structures, the Structure based Sequences Alignment Program (STRAP; http://www.charite.de/bioinf/strap/) was used. All programs were accessed trough the Swiss Expasy Server (www.expasy.ch) and used with default parameters.

To perform phylogenetic analyses, amino acid sequences of several β-glucosidases were downloaded from the NCBI GenBank; raw sequence data of Zygomycetes hypothetical proteins were obtained from the genome sequence databases of *M. circinelloides* (DoE Joint Genome Institute; *M. circinelloides* CBS277.49 v1.0; http://genome.jgi-psf.org/Mucci1/Mucci1.home.html); *Phycomyces blakesleeanus* (DoE Joint Genome Institute *P. blakesleeanus* v1.1; http://genome.jgi-psf.org/Phybl1/Phybl1.home.html) and *Rhizopus oryzae* (Broad Institute; *R. oryzae* Database; http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae). Outgroups were selected so as to represent the major groups within the glycoside hydrolase family 3 recognized by Harvey et al. (2000). Alignment of sequences utilized the following strategy: first, larger groups were identified based on a NJ tree computed from a rough alignment obtained with ClustalW. Then, these groups were aligned separately in ProbCons, a probabilistic multiple alignment tool which outperforms ClustalW in many respects (Do et al. 2005). A protein substitution matrix was computed from the data by running the algorithm for ten pre-training replicates. To refine the alignments, 25 iterative refinement steps were performed. Subsequently, the resulting multiple alignments were aligned to each other by the profile option in Muscle (Edgar 2004). The resulting alignments were used to infer trees using Bayesian MCMC algorithm implemented in MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). Best fit substitution models were estimated by the Reversible-Jump MCMC approach, which does not require a priori selection of substitution model, but samples different models during the MCMC run in proportion to their posterior probabilities. Markov Chains were run for three million generations sampling every 100th generations. The first 10,000 trees were discarded as burn-in, and the remaining trees were used to compute a 50% Majority Rule consensus tree. Bayesian Posterior Probabilities (BPP) $\geq$ 0.95 were considered significant.

Plasmid construction and transformation

For heterologous expression in *M. circinelloides*, a vector designated as pTM8 (Fig. 2) was constructed as follows. The *bgl* gene of *R. miehei* was amplified by PCR using *Not*I- and *Cla*I-modified specific primers (BGL17 and BGL19; Table 1), and cloned into pBluescript II SK+ (Stratagene). Then, *bgl* gene was cut out from this construct with the enzymes *Cla*I and *Xba*I and it was transferred into the plasmid pPT43 (Papp et al. 2006). The resulting plasmid was digested with *Pvu*II to obtain the gene flanked with the promoter and terminator regions of the *M. circinelloides gpd1* gene (Wolff and Arnau 2002). The fragment was then ligated into the plasmid pTM7 (Lukács et al. 2009). This plasmid contain the orotidine-5-phosphate-decarboxylase gene (*pyrG*) of *M. circinelloides* (Benito et al. 1992) inserted into the pSP73 (Fermentas).

For the transformation experiments, protoplasts of *M. circinelloides* were prepared as described earlier (Nagy et al. 1994). Polyethylene glycol mediated transformations of protoplasts were performed according to van Heeswijck and Roncero (1984).

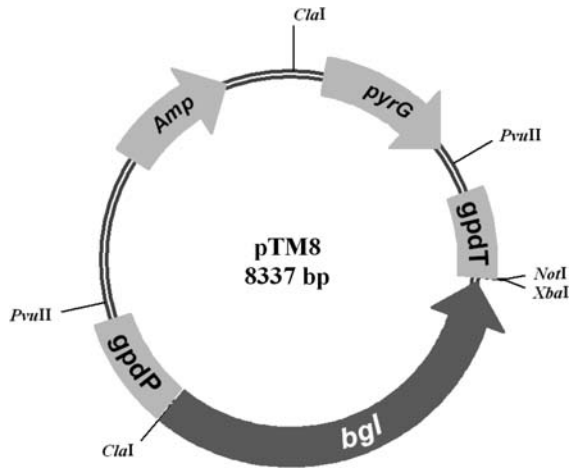

Fig. 2 The plasmid pTM8 used for the expression of the *R. miehei bgl* gene in *M. circinelloides*

Transformants were selected on the basis of the complementation of uracil auxotrophy of the recipient strain. Selection and further culturing of the transformants were performed on solid minimal medium (YNB; 10 g glucose, 0.5 g yeast nitrogen base without amino acids, 1.5 g $(NH_4)_2SO_4$, 1.5 g glutamic acid and 20 g agar per litre), supplemented with leucine and/or uracil (200 μg ml^{-1}, respectively) as required.

Testing of the β-glucosidase activity

To test β-glucosidase activity of the transformants in liquid culture, 5×10^5 spores were inoculated in 15 ml YNB containing 0.15 g wheat bran instead of glucose and supplemented with 200 μg ml^{-1} leucine. For culturing of the original MS12 strain, medium was supplemented with 200 μg ml^{-1} uracil also. Strains were cultivated under continuous shaking at 25°C for 6 days. Each day, 500 μl samples were collected and filtered to remove insoluble particles. The filtrates were centrifuged at 16,200*g* for 30 min and the supernatant was used for enzyme activity measurements (diluted 1:10 with distilled water if necessary).

To test β-glucosidase activity in solid-state fermentation, 5 g wheat bran and 5 ml distilled water were mixed and supplemented with 200 μg ml^{-1} leucine (and with the same amount of uracil in the case of the recipient strain). After inoculation this medium with 10^6 spores, cultures were incubated at 25°C for 6 days. An Erlenmeyer flask was daily taken and extracted with 30 ml distilled water at 4°C for 3 h. After filtration of the extract, 1 ml filtrate was centrifuged at 16,200*g* for 10 min. The supernatant was diluted to 1:1,000 with distilled water and analyzed for total protein concentration and β-glucosidase activity.

β-Glucosidase activity was determined by *p*-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma). Twenty microliter of 7 mM PNPG was given to 180 μl diluted extract, and incubated for 30 min at 50°C. The reaction was stopped by 50 μl of 100 mM sodium carbonate, and the *p*-nitrophenol release was measured at 405 nm. One enzyme activity unit was equal to 1 μmol *p*-nitrophenol released in 1 min. Enzyme activities were measured in 96-well microtiter plates using an ASYS Jupiter HD (ASYS Hitech) microplate reader.

Purification of the *Rhizomucor* BGL enzyme

R. miehei (10^6 spores) was inoculated in 130 g wheat bran supplemented with 130 ml distilled water and incubated at 40°C for 6 days. All steps of the purification processes were carried out at 4°C. The culture medium was extracted with 800 ml of 100 mM acetate buffer (pH 5.0) and left to stand for 12 h at 4°C. After filtration, the crude extract was centrifuged at 5,040*g* for 15 min, and then, the protein of the supernatant was precipitated by ammonium sulphate. Precipitates from the fractions of 50–85% saturation were collected by centrifugation at 5,040*g* for 15 min and re-dissolved in the smallest possible volume of 100 mM acetate buffer (pH 5.0). Precipitate of the fraction having saturation between 75 and 85% showed the highest PNPG-hydrolyzing activity. This concentrated enzyme solution was loaded onto a Sephadex G-100 (Sigma) column equilibrated with 50 mM acetate buffer (pH 6.0); the exclusion range was 4–150 kDa (27 × 400 mm). Elution was performed with the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions containing PNPG-hydrolyzing activity were collected and applied to a Macro-Prep High Q anion exchange column (12.6 × 40 mm; Bio-Rad) equilibrated with 50 mM acetate buffer (pH 6.0). The enzyme was eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 1 ml min^{-1}. Final purification of β-glucosidase was performed on a Sephacryl S-200 HR column (exclusion range 5–250 kDa; 16 × 60 mm; GE Healthcare) equilibrated with 50 mM acetate buffer (pH 5.0) containing 150 mM NaCl, and eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. The purity and the molecular weight of the purified protein were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), which was performed on NuPAGE Bis–Tris gel (Invitrogen) using NuPAGE MES running buffer (Invitrogen) according to the manufacturer's instructions. Protein bands were detected by staining the gel with 0.0025% Coomassie Brilliant Blue R-250. To identify the purified protein, tryptic peptides were generated (Hellman et al. 1995) and analysed by HPLC-ESI ion trap mass spectrometry. The LC–MS analysis was carried out by the Proteomics Research Group at the Biological Research Center of the Hungarian Academy of Sciences.

Results and discussion

The *bgl* gene of *R. miehei*

Using the IPCR method, a segment of 4,063 bp containing the entire β-glucosidase gene of *R. miehei* together with its flanking regions was isolated and sequenced. The sequence was deposited to the EMBL Nucleotide sequence database (accession no.: AM922334). The coding sequence was predicted by similarities to other known fungal β-glucosidase genes found in sequence databases. The *bgl* gene comprises a total of 2,826 bp including the coding sequence of a putative 717 amino acids length protein and 10 introns (70, 64, 68, 60, 76, 68, 77, 73, 82 and 34 bp in length) dispersed in the whole coding region (Fig. 1).

The 578 nucleotides-long promoter region contains a typical putative TATA box (TATAAAA) locating at the expected -69 position from the start codon and two pyrimidine-rich stretches at the positions -49 and -385. The putative CAAT motif was found 357 nucleotides upstream from the start codon. Three possible motifs corresponding to the consensus sequence (SYGGR) of the binding sites of carbon catabolite repressor proteins (Suto and Tomita 2001) were observed at the positions -126 (CCG-GAG), -135 (CCCCAC) and -248 (CCGGGG). In the terminator region, two putative polyadenylation sequence (AATAAA) were identified 20 and 37 nucleotides downstream from the stop codon.

Based on the PCR amplification experiments, it was suspected that the *R. miehei* genome contains only a single copy of the *bgl* gene. Southern hybridization analysis contradicted to this suggestion and indicated that the gene is present in two copies in the *R. miehei* genome (Fig. 3).

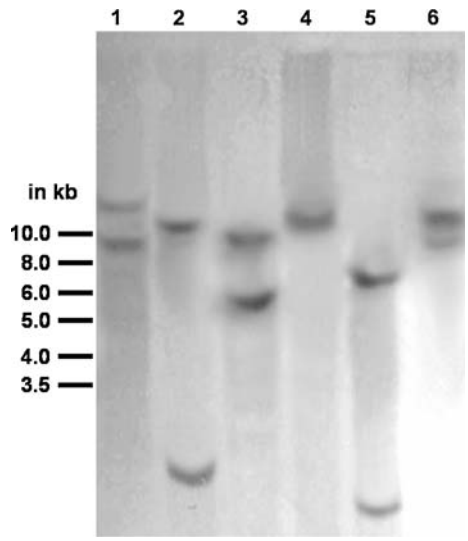

Fig. 3 Southern hybridization analysis of *R. miehei* genomic DNA under low stringency condition; DNA was digested with different restriction enzymes (lanes 1–6: *Sma*I, *Sca*I, *Pvu*II, *Xba*I, *Kpn*I and *Apa*I, respectively) and probed with a *bgl* fragment. The enzymes used do not cut the probe

The *R. miehei* β-glucosidase

The predicted molecular mass of the putative BGL protein is 78.7 kDa. Analysis of the protein using SignalP predicted the presence of a 22-amino-acid signal peptide (MFAKTALALLTAWSAMQGVA GG) suggesting the extracellular nature of the enzyme. The predicted mature BGL protein has a calculated molecular mass of 76.5 kDa and a pI of 5.0. Comparison of the *R. miehei* BGL with database sequences showed it highly similar to fungal β-glucosidases classified as family 3 glycoside hydrolases. These enzymes have a characteristic two-domain globular structure (Varghese et al. 1999). Accordingly, the putative N-terminal domain forming the $(\alpha/\beta)_8$ barrel (aa 68–274) and the C- terminal $(\alpha/\beta)_6$-sandwich domain (aa 358–601) connected with a 84-amino-acid linker could be identified in the BGL protein (Fig. 1). Glycoside hydrolase family 3 enzymes are retaining glycohydrolases, which require amino acids in the active site that act as the nucleophile and the general acid/base, respectively in the reaction mechanism. Analysis of the *Rhizomucor* BGL aligned with known fungal β-glucosidases showed the residue D254 as the catalytic nucleophile, situated in a conserved motif SDW and the residue H177 as the proposed H^+ donor in the motif KHY (Fig. 1). The conserved COOH-terminal antiparallel loop sequence (Harvey et al. 2000) was also identified (PLFPFGHGLS). The putative catalytic acid/base residue is difficult to deduce from sequence comparison because of the lack of homology at the appropriate part of the sequence. It is very likely that different sub-families may have unique general acid/base catalysts, which have been identified in case of only a few far related enzymes, such as the barley β-glucan exohydrolase (ExoI; Varghese et al. 1999; Hrmova et al. 2001) or

the β-glucosidase of *Flavobacterium meningosepticum* (Chir et al. 2002). Comparing the barley ExoI and the *R. miehei* BGL, the motif PYTET (aa 488–492) of ExoI containing the catalytic acid was aligned with the motif IVVDG (aa 472–476), where D475 can be a potential acid/base catalyst. The structure model predicted using the barley ExoI as a template shows the putative nucleophile D254 and the residue D475 being in appropriate positions (Fig. 4), although this result must be interpreted with precaution because of the relatively low similarity between ExoI and BGL, and experimental identification of the acid/base catalyst is needed to prove this suggestion.

To purify the extracellular β-glucosidase enzyme in high amount, *R. miehei* was grown on wheat bran medium for 6 days at 40°C. The enzyme was purified from the crude extract to homogeneity by ammonium sulphate precipitation and gel filtration followed by anion exchange and size-exclusion chromatographies. The protein was homogenous in terms of its behaviour on SDS–PAGE, as shown in Fig. 5. The molecular weight of the purified enzyme estimated

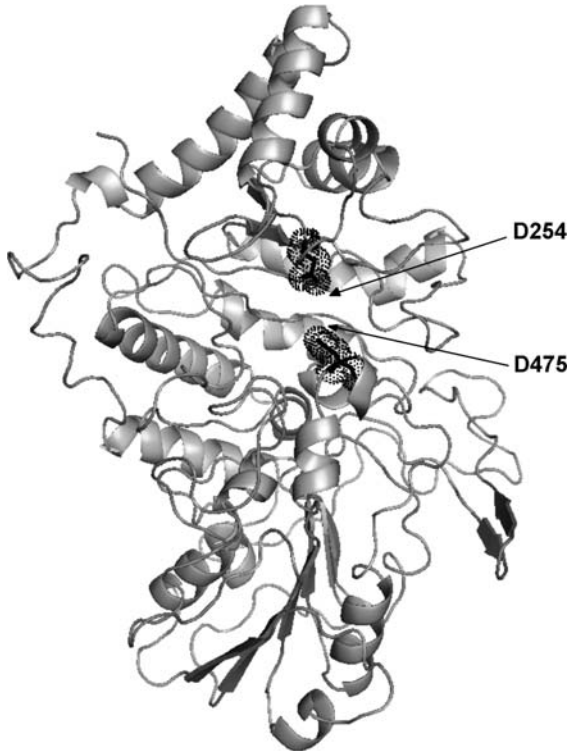

Fig. 4 Ribbon representation of the structure prediction for the *R. miehei* β-glucosidase. The barley β-glucan exohydrolase isoensyme ExoI was used as template for the prediction

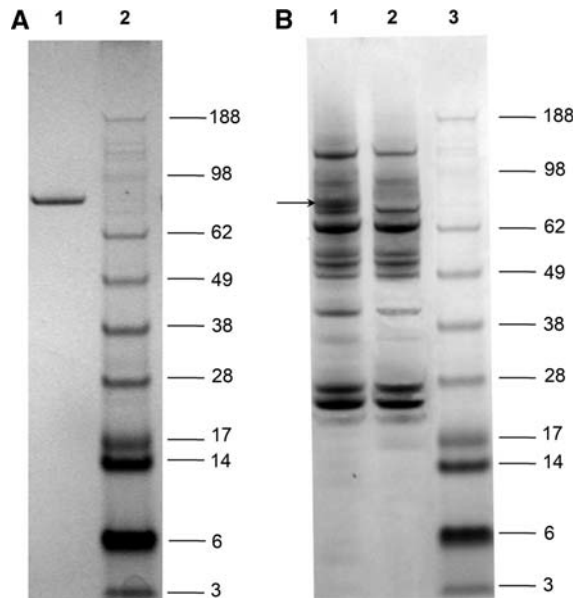

Fig. 5 SDS–PAGE analysis of the purified *R. miehei* BGL enzyme (**a**) and partially purified protein samples extracted from the recipient *M. circinelloides* MS12 strain and one of the transformants (**b**). In the latter case, culturing, extraction and purification on Sephadex G100 were performed as described for the *R. miehei* BGL in Sect. "Materials and methods". **a** Lane 1, the purified β-glucosidase (4 µg); lane 2, SeeBlue Plus 2 Standard (Invitrogen) as molecular weight marker. **b** Lanes 1 and 2, partially purified protein samples from the transformant MS12 + pTM8/1 and the recipient MS12, respectively; lane 3, SeeBlue Plus 2 Standard (Invitrogen)

by SDS–PAGE (about 75–80 kDa) corresponded well to that predicted on the basis of the putative amino acid sequence. Moreover, LC–MS analysis of the peptides generated by trypsin digestion identified the isolated protein as the predicted *R. miehei* BGL and confirmed that the purified enzyme is encoded by the *bgl* gene.

Evolution of fungal β-glucosidases

Blast searches with the amino acid sequence of the *R. miehei* BGL revealed the existence of four, two and five highly similar hypothetical proteins in the databases of the *M. circinelloides*, *P. blakesleeanus* and *R. oryzae* genomes, respectively. Within the glycosyde hydrolase family 3, all these sequences showed highest similarity to members of subfamily 4 containing fungal enzymes with β-glucosidase activity as circumscribed by Harvey et al. (2000). These Zygomycetes sequences were involved in

phylogenetic analyses carried out to address the evolution of fungal β-glucosidases of the group 4; as outgroups, some glucosidases representing the other subfamilies were also involved into the analysis. Members of the Reversible-Jump MCMC analysis selected the WAG model implemented in MrBayes as the best fit model to the data, with a posterior probability of 1.00. The resulting consensus tree is presented on Fig. 6. Almost all clades received high BPPs ($\geq$0.95), and the topology is largely congruent with that obtained by Harvey et al. (2000). Our results support monophyly of zygomycetes β-glucosidases. It is noteworthy that sequences labelled as *Phycomyces* 17174, *Mucor* 74361 and *Rhizopus* RO3G_09386.1 formed a separate, well-supported clade (BPP = 1.00) in a sister position to all other zygomycete sequences. Based on the inferred topology it is most parsimonious to assume that the above mentioned three sequences emerged by an early duplication, preceding the speciation events giving rise to *Mucor*, *Rhizopus* and *Phycomyces* taxa, because two subclades of the zygomycete clade correspond to the phylogeny of the species themselves (Voigt et al. 1999). Because the duplication happened before divergence of *Rhizomucor miehei*, it also suggests that *R. miehei* should have another hitherto unrecognised copy of the *bgl* gene.

Heterologous expression of the *R. miehei bgl* in *M. circinelloides*

The plasmid pTM8 contained the entire *bgl* gene of *R. miehei* fused with the promoter and terminator regions of the *M. circinelloides gpd1* gene. This plasmid was introduced into *M. circinelloides* by PEG-mediated protoplast transformation. Transformation frequency was low: only three transformants were gained from the experiment. Presence of the plasmid in the transformants was proven by PCR amplification and sequencing (results not shown).

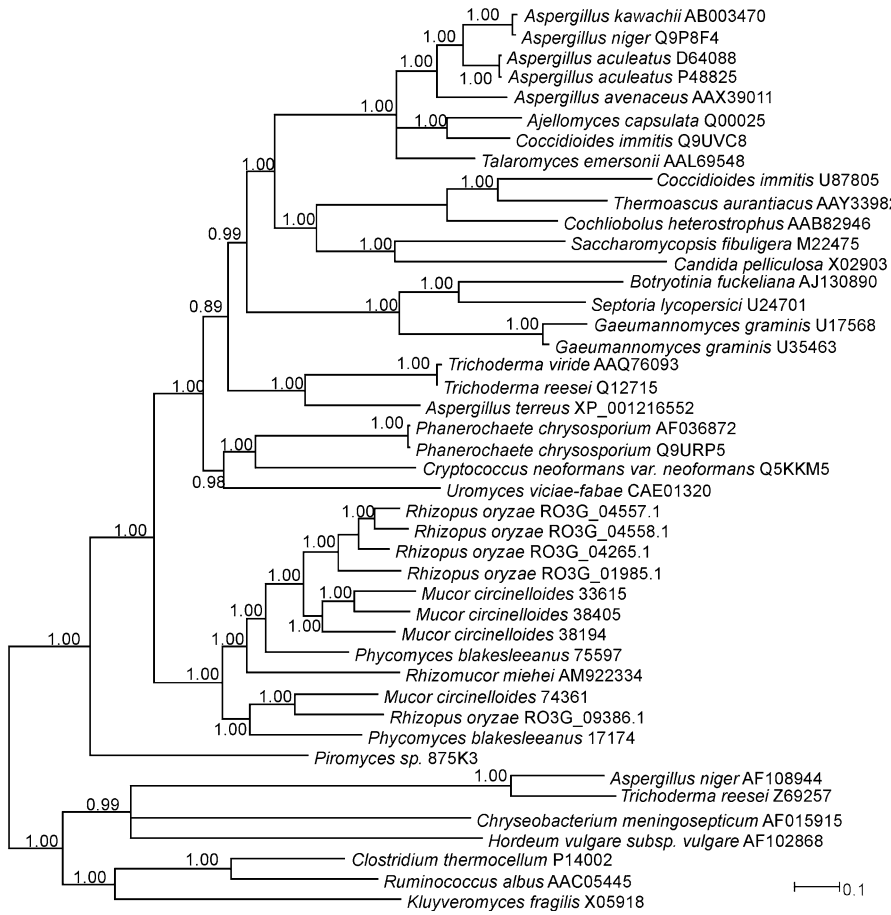

Fig. 6 Phylogenetic analysis of group 4 fungal family 3 glycoside hydrolases: Bayesian 50% Majority Rule consensus phylogram; numbers indicate posterior probabilities (BPP). Amino acid sequences of *M. circinelloides*, *P. blakesleeanus* and *R. oryzae* were obtained by similarity search in genome databases (protein IDs and designations of the corresponding database are indicated on the tree). Other sequences were obtained from the NCBI/EMBL databases (accession numbers are indicated on the tree)

Maintenance of the selective conditions was necessary that the transformants retain the plasmid indicating that the plasmid did not integrate into the genome.

After culturing the recipient MS12 strain and the transformants on wheat bran, their proteins were extracted from the culture filtrates and partially purified on a Sephadex G100 column; SDS–PAGE analysis of these protein samples revealed the presence of an extra band in the protein pattern of the transformants (Fig. 5). The size of this band corresponded well to that of the *Rhizomucor* BGL suggesting that the introduced gene had been expressed in the transformants.

Extracellular β-glucosidase activity of the recipient MS12 strain and some of the transformants was determined after culturing the strains in both liquid and solid-state cultures. Table 2 shows the volumetric activities of the protein extracts purified from the culturing media of the strains at the sixth culturing day.

R. miehei is a good glucosidase producer and enzyme extracts of the NRRL 5282 strain also had remarkable activity. In spite of the presence of four copies of hypothetical β-glucosidase genes in the *M. circinelloides* genome, this fungus showed relatively low glucosidase activity under the applied culturing conditions. Although transformants showed much higher enzyme activities than the original MS12 strain, their values did not reach that of the *R. miehei* enzyme. Maybe the *Mucor* strain produces and/or secretes the heterologous enzyme with somewhat lower efficiency than *R. miehei* or the latter fungus produces another active β-glucosidase enzyme. Anyway, introduction of the *Rhizomucor bgl* gene significantly increased the enzyme activity of the transformants in both liquid and solid-state cultures indicating the functionality of the gene and the effectiveness of the corresponding enzyme.

Table 2 β-Glucosidase activity of the original and the transformed *M. circinelloides*, and the *R. miehei* strains

Strain	Volumetric activity (U ml^{-1})[a]	
	Liquid culture	Solid-state culture
M. circinelloides MS12	0.11	7.18
M. circinelloides MS12 + pTM8/1	0.37	23.3
M. circinelloides MS12 + pTM8/2	0.31	18.1
M. circinelloides MS12 + pTM8/3	0.34	19.6
R. meiehei NRRL 5282	3.7	38.3

[a] Averages of three measurements

This paper reports on the isolation of a gene encoding a β-glucosidase from *R. miehei*. As we know, this is the first β-glucosidase gene from a zygomycete fungus that has been characterized. The corresponding protein has also been purified; detailed characterization of the enzyme is in progress.

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