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Cloning of Genes Encoding α-L-Arabinofuranosidase and β-Xylosidase from *Trichoderma reesei* by Expression in *Saccharomyces cerevisiae*

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A cDNA expression library of *Trichoderma reesei* RutC-30 was constructed in the yeast *Saccharomyces cerevisiae*. Two genes, *abf1* and *bxl1*, were isolated by screening the yeast library for extracellular α-L-arabinofuranosidase activity with the substrate *p*-nitrophenyl-α-L-arabinofuranoside. The genes *abf1* and *bxl1* encode 500 and 758 amino acids, respectively, including the signal sequences. The deduced amino acid sequence of ABFI displays high-level similarity to the α-L-arabinofuranosidase B of *Aspergillus niger*, and the two can form a new family of glycosyl hydrolases. The deduced amino acid sequence of BXLI shows similarities to the β-glucosidases grouped in family 3. The yeast-produced enzymes were tested for enzymatic activities against different substrates. ABFI released L-arabinose from *p*-nitrophenyl-α-L-arabinofuranoside and arabinoxylans and showed some β-xylosidase activity toward *p*-nitrophenyl-β-D-xylopyranoside. BXLI did not release L-arabinose from arabinoxylan. It showed α-L-arabinofuranosidase, α-L-arabinopyranosidase, and β-xylosidase activities against *p*-nitrophenyl-α-L-arabinofuranosidase, *p*-nitrophenyl-α-L-arabinopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside, respectively, with the last activity being the highest. It was also able to hydrolyze xylobiose and slowly release xylose from polymeric xylan. ABFI and BXLI correspond to a previously purified α-L-arabinofuranosidase and a β-xylosidase from *T. reesei*, respectively, as confirmed by partial amino acid sequencing of the *Trichoderma*-produced enzymes. Both enzymes produced in yeasts displayed hydrolytic properties similar to those of the corresponding enzymes purified from *T. reesei*.

L-Arabinose residues in furanose form are widely distributed in plant tissue heteropolysaccharides, such as arabinans, arabinogalactans, and arabinoxylans. Arabinan is a polymer of β-1,5-linked arabinofuranose residues and can be substituted mainly at position O-3 but also at O-2 with one or two arabinose residues (1). In arabinoxylan, α-L-arabinofuranosyl residues appear as side groups attached to the β-1,4-xylopyranosyl backbone by α-1,3 or α-1,2 linkages (1). The presence of arabinose as side groups can restrict enzymatic hydrolysis of hemicelluloses in various industrial applications, such as the improvement of the digestibility of ruminant feed (5). Enzymes cleaving α-L-arabinofuranosidic linkages can act synergistically with xylanases in the hydrolysis of arabinoxylans (14, 28).

Enzymes hydrolyzing L-arabinose linkages have been purified from several bacteria and fungi. Depending on the substrate specificity, they have been classified as α-L-arabinofuranosidases (EC 3.2.1.55) that hydrolyze terminal nonreducing α-L-1,2-, α-L-1,3-, and α-L-1,5-arabinofuranosyl residues and as endo-1,5-α-L-arabinases (EC 3.2.1.99) that hydrolyze endo-1,5-α-L-arabinofuranosidic linkages from arabinans containing L-arabinose in the backbone chain but have no activity toward terminal nonreducing L-arabinofuranosyl residues (18). On the basis of the specific activities of the enzyme purified from *Streptomyces purpuracens* (21) and one of the enzymes purified from *Aspergillus niger*, α-L-arabinofuranosidase B (ABF B) (32, 40), the α-L-arabinofuranosidases have been further classified into two groups. The *A. niger*-type enzymes are active on small synthetic substrates and arabino-oligosaccharides and are able to hydrolyze arabinosyl side groups of arabinans, arabinogalactans, and arabinoglucuronoxylans. The *Streptomyces purpuracens*-type enzymes act only on α-L-arabinosides of low molecular mass and oligosaccharides containing arabinose (18).

Several genes encoding α-L-arabinofuranosidases have been isolated from bacteria, such as *Butyrivibrio fibrisolvens* (*xylB*) (42), *Streptomyces lividans* (*abfA*) (24), *Prevotella ruminicola* B1(4) (12), *Clostridium stercorarium* (*arfA* and *arfB*) (36), and *Pseudomonas fluorescens* (*xynC*) (19). However, genes encoding enzymes hydrolyzing L-arabinose linkages of fungal origin have been isolated only from *A. niger*. The following are the three genes coding for nonrelated protein sequences: α-L-arabinofuranosidase A (*abfA*) (11), ABF B (*abfB*) (10), and an endo-1,5-α-L-arabinase (9). Based on protein sequence similarities, the endo-1,5-α-L-arabinase of *A. niger* (ABN A) and α-L-arabinofuranosidase of *B. fibrisolvens* (XYLB) have been grouped in family 43 and the α-L-arabinofuranosidases of *A. niger* (ABF A) and *Streptomyces lividans* have been grouped in family 51 in the general classification of glycosyl hydrolases (16). No similarities with any other sequences have been reported for any of the α-L-arabinofuranosidases, except that the *P. fluorescens* and *B. fibrisolvens* enzymes seem to contain a cellulose binding domain of the bacterial type and a starch binding domain, respectively (19, 42).

The major α-L-arabinofuranosidase of the filamentous fungus *Trichoderma reesei* has been purified and characterized. This enzyme is able to release terminal L-arabinosyl residues from small synthetic substrates and polysaccharides such as arabinoxylan (28). During purification, fractions containing minor α-arabinosidase activities were also detected (28). For instance, the β-xylosidase of *T. reesei* has been reported to have clear α-L-arabinosidase activities against small synthetic model

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substrates (29), which is also the case with many other characterized β-xylosidases. Furthermore, it is possible that *T. reesei* produces additional α-L-arabinosidase activities. The identification of such proteins and isolation of the corresponding genes could be laborious and time-consuming by traditional methods. We previously developed a simple method for the isolation of hydrolase genes on the basis of their expression in the yeast *Saccharomyces cerevisiae* (33). In this report, we describe the cloning of two genes from *T. reesei* RutC-30 on the basis of α-L-arabinofuranosidase activity by this methodology.

MATERIALS AND METHODS

Strains, vectors, and media. *Escherichia coli* JS4 [F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)X*74 galN galK hsdR2* ($r_K^-m_K^-$) *mcrBC rpsL* (Str^r) *thi recA1*] (Bio-Rad) was used to prepare the cDNA library. *S. cerevisiae* DBY746 (α *his3Δ1 leu2-3 leu2-112 ura3-52 trp1-289 cyh*^{R}) of D. Botstein (Stanford University) was used as a host for the expression library. The cDNA library was constructed in the yeast expression plasmid pAJ401 (33), which contains the selective marker *URA3*, the 2μm origin of replication, and the yeast *PGK* promoter and terminator. *S. cerevisiae* DBY746 was grown in yeast-peptone glucose medium. Synthetic complete medium minus uracil was used to propagate plasmid-carrying yeast strains (37).

Construction of the *T. reesei* cDNA expression library in *S. cerevisiae*. Poly(A)$^+$ mRNA isolated from *T. reesei* RutC-30 cultivated on medium containing several plant polysaccharides was previously prepared (38). cDNA was synthesized by using a ZAP-cDNA synthesis kit (Stratagene) and was ligated to plasmid pAJ401 cut with the restriction enzymes *Eco*RI and *Xho*I. *E. coli* JS4 was transformed by electroporation according to the manufacturer's (Bio-Rad) instructions, and a library of 1.3×10^6 independent clones was obtained. Forty micrograms of plasmid DNA isolated from a pool of *E. coli* clones was transformed into *S. cerevisiae* DBY746 by electroporation (4), yielding a library of 6.5×10^5 independent yeast transformants.

Screening of the yeast expression library. Yeast transformants (5.3×10^5) were suspended to a cell density of 1,000 cells per ml in synthetic complete medium minus uracil containing 1 mM the substrate *p*-nitrophenyl-α-L-arabinofuranoside (PNPA) (N-1381; Sigma). Aliquots of 300 μl (approximately 300 cells) were distributed into 96-well microtiter plates (Cell-Cult; Sterilin Limited, Hounslow, United Kingdom) and incubated for 5 days at 30°C without agitation. From each well, 100 μl of growth medium was transferred together with 50 μl of 1 M Na_2CO_3 to new microtiter plates, and α-L-arabinofuranosidase activity was detected as described below. Yeast clones from wells producing α-L-arabinofuranosidase activity were further purified in two successive rescreening steps, using cell densities of approximately 30 and 10 cells per well, respectively. Pure clones were finally isolated by plating on selective media and detecting activities of single colonies. Plasmids were recovered from yeast clones by isolating total yeast DNA and transforming it into *E. coli* by electroporation for restriction enzyme analysis.

DNA sequencing and sequence analysis. A series of random transposon insertions (39) were made with a TN 1000 kit (Gold Biotechnology, Inc.), and the clones were sequenced with a Pharmacia Autoread sequencing kit and run on an automated A. L. F. DNA sequencer (Pharmacia). The sequences were assembled by using the StadenPackage program (8) on a Sun workstation. Sequencing work was carried out at the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki, Helsinki, Finland. DNA and protein sequences were analyzed by using the programs contained in PC/GENE (release 6.5; IntelliGenetics, Inc.) and Genetic Computer Group Sequence Analysis Software Package version 8.

Protein sequencing. The amino acid sequences of one purified peptide produced by trypsin digestion of the β-xylosidase of *T. reesei* (29) and the N terminus of the α-L-arabinofuranosidase of *T. reesei* (28) were determined at the University of Kuopio, Kuopio, Finland, by using an Applied Biosystems 477A protein sequencer equipped with a 120-A analyzer (Applied Biosystems, Inc., Foster City, Calif.).

Enzyme production in yeasts. *S. cerevisiae* strains transformed with plasmids p17SA (*abf1*), p18SA (*bxl1*), and pAJ401 (control) were cultivated on synthetic complete medium minus uracil in a laboratory fermentor (CMF Mini Fermentor; Chemap AG) with a total working volume of 1,100 ml, including 100 ml of inoculum. Cultivation conditions were as follows: temperature, 30°C; pH, controlled between 4.9 and 5.1 (or adjusted to pH 5.0 ± 0.1) by the automatic addition of 1 M NaOH and 1:5 diluted H_3PO_4 (85%); partial O_2 pressure, ≥20% (controlled by agitation); aeration, 48 liters h^{-1}. Approximately 30 ml of fresh medium containing 40% glucose was added after 18 and 24 h when glucose was consumed, as determined by using Glukotest sticks (Boehringer Mannheim). After 42 h, cells were separated by centrifugation (Heraeus Sepatech Cryofuge 8000; 5,350 × *g*, 20 min, 4°C). The supernatants were filtered (Whatman glass microfiber filter GF/F 1825 090) and concentrated 50-fold by ultrafiltration (Amicon Diaflo 13142 PM10 membrane).

Activity assays. α-L-Arabinosidase activity was determined by using 1 mM *p*-nitrophenyl-α-L-arabinofuranoside (PNPA; N-1381; Sigma) in 50 mM sodium citrate buffer, pH 4.0. Twenty microliters of the enzyme sample was incubated with 180 μl of preheated substrate solution at 50°C for 10 min. The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. The liberated *p*-nitrophenyl was measured at 405 nm with a Multiskan MCC/340 spectrophotometer. α-L-Arabinopyranosidase and β-L-arabinopyranosidase activities were determined similarly by using 1 mM *p*-nitrophenyl-α-L-arabinopyranoside (N-3512; Sigma) and 1 mM *p*-nitrophenyl-β-L-arabinopyranoside (N-0520; Sigma), respectively.

β-Mannosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-xylosidase activities were determined in a similar manner by using 1 mM *p*-nitrophenyl-β-D-mannopyranoside (N-9383; Sigma), 1 mM *p*-nitrophenyl-β-D-glucopyranoside (N-7006; Sigma), 1 mM *p*-nitrophenyl-α-galactopyranoside (N-0877; Sigma), 1 mM *p*-nitrophenyl-β-D-galactopyranoside (N-1252; Sigma), and 5 mM *p*-nitrophenyl-β-D-xylopyranoside (N-2132; Sigma), respectively, in 50 mM sodium citrate buffer, pH 5.0.

Hydrolysis experiments. Arabinoxylans from wheat and rye were purchased from MegaZyme (North Rocks, Australia). Arabinoglucuronoxylan from oat spelts (X-0376) was from Sigma, and arabinoglucuronoxylan from pine kraft pulp was kindly provided by Jan Jansson (Finnish Pulp and Paper Research Institute, Espoo, Finland). 4-*O*-Methylglucuronoxylan from beechwood was purchased from Roth (Karlsgruhe, Germany), and unsubstituted xylan from beechwood was purchased from Lenzing AG (Lenz, Austria). The substrates (5 or 10 g/liter) in 0.1 M sodium citrate buffer, pH 4, were hydrolyzed with concentrated yeast culture filtrates at 40°C. The dosages (in nanokatals) of yeast-produced α-L-arabinosidase and β-xylosidase varied (see text) and were based on activities toward PNPA and *p*-nitrophenyl-β-D-xylopyranoside, respectively. The endoxylanase (pI 9; 500 nkat/g of substrate) previously purified from *T. reesei* (41) was used in some experiments. The reactions were stopped by boiling for 3 m, and the L-arabinose liberated was determined with a commercial assay kit (Boehringer test combination 176 303). Other sugars were analyzed by high-performance liquid chromatography (HPLC) coupled with pulsed amperometric detection (Dionex Corp.) as described previously (15).

Other methods. Southern blot analysis of genomic DNA of *T. reesei* QM 9414 digested with four restriction enzymes was performed under stringent conditions by standard procedures (35).

Nucleotide sequence accession numbers. The complete nucleotide sequences of *abf1* and *bxl1* can be obtained from the GenBank data library under accession no. Z69252 and Z69257, respectively.

RESULTS

Isolation of genes encoding α-L-arabinofuranosidase activity from the expression library. In this work, a yeast cDNA expression library was created by using mRNA isolated from *T. reesei* RutC-30 cultivated in medium containing several plant polysaccharides (38) to induce hydrolase production. The yeast library was cultivated in microtiter plates in medium containing the substrate PNPA, and the release of *p*-nitrophenol in the medium was assayed. The screening of the library resulted in 20 yeast clones secreting α-L-arabinofuranosidase activity. Restriction analysis and partial sequencing of the plasmids recovered from yeasts showed two groups, 16 clones containing cDNA inserts representing the same gene and 4 clones representing another gene. The plasmids, p17SA and p18SA, respectively, from these two groups were chosen for further analysis.

The genes in p17SA and p18SA were called *abf1* and *bxl1*, respectively, on the basis of further biochemical characterizations (see below). The complete nucleotide sequences of *abf1* and *bxl1* can be obtained from the GenBank data library under accession numbers Z69252 and Z69257, respectively. On the basis of Southern blot analysis of genomic DNA of *T. reesei*, the genes present in p17SA and p18SA appear to be single-copy genes (results not shown).

Amino acid sequence analysis. The deduced protein sequence of ABFI, encoded by the *abf1* gene, is 500 amino acids long and has a putative signal sequence of 21 amino acids with a predicted cleavage site after Ala-21 (43) (Fig. 1). The mature protein has a length of 479 amino acids, with a calculated molecular mass of 49.1 kDa. A single potential N-glycosylation site is present in the amino acid sequence (Fig. 1). These features are in good agreement with the reported 53-kDa molecular mass determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for an α-L-arabinofuranosidase purified from *T. reesei* (28). The N-terminal

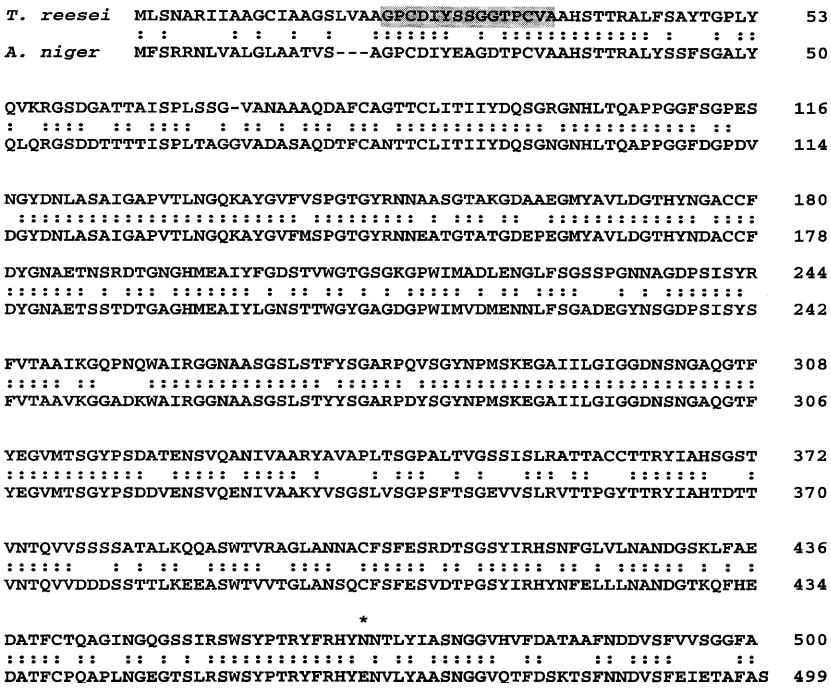

FIG. 1. Alignment of the deduced amino acid sequences of the α-L-arabinofuranosidase ABFI of *T. reesei* and *A. niger* ABF B (10). The one-letter amino acid code is used. Identical amino acids are indicated by colons. The N-terminal amino acids determined from the purified *T. reesei* α-L-arabinofuranosidase are shaded. The putative N-glycosylation site in ABFI is shown by an asterisk. Hyphens indicate gaps.

amino acid sequence of this purified enzyme was determined, and it corresponds to that of the deduced ABFI sequence (Fig. 1), indicating that most likely *abf1* is the corresponding gene for the previously purified *T. reesei* α-L-arabinofuranosidase.

Computer analysis carried out with the ABFI sequence showed no amino acid sequence similarities to any of the bacterial α-L-arabinofuranosidases, and no similarity to the ABF A (11) or endo-1,5-α-L-arabinase (ABN A) (9) of *A. niger* was found. However, protein alignment of *T. reesei* ABFI with *A. niger* ABF B (10) shows more than 70% identity (Fig. 1).

The predicted amino acid sequence of BXLI, encoded by the *bxl1* gene, is 758 amino acids long and contains a putative signal sequence of 20 amino acids with a predicted cleavage site after Ala-20 (43) (Fig. 2). The mature protein would thus be 738 amino acids long, with a calculated molecular mass of 80.4 kDa. Ten potential N-glycosylation sites are scattered along this protein sequence (Fig. 2).

Computer analysis carried out with the deduced amino acid sequence of BXLI revealed that it has no similarity to ABFI or any reported α-L-arabinofuranosidase sequence. Interestingly, it shows significant similarities to the protein sequences of the β-glucosidases classified in family 3 in the general classification of glycosyl hydrolases (16), which also includes a β-glucosidase (BGLI) previously isolated from *T. reesei* (2). The most conserved regions in BXLI are found between amino acids 69 and 314 and between 487 and 643 compared with the protein sequences of the β-glucosidases most similar to it (Fig. 2). The rest of BXLI shows low-level or no similarity with the sequences of β-glucosidases.

Asp-311 in BXLI appears conserved in all the sequences aligned in Fig. 2. The corresponding Asp has been suggested to be part of the active site of the β-glucosidases from *T. reesei* and other organisms (2, 3). Next to this region is a conserved motif (Ala/Ser-Gly-Leu-Asp-Met/Leu) in β-glucosidases which contains the other conserved Asp residue, which has been suggested to be the second carboxylic residue involved in the catalytic activities of β-glucosidases (2) (Fig. 2). This motif does not appear in BXLI, and the position of the second possible active-site carboxylic residue is not evident (Fig. 2).

Hydrolytic properties of the yeast-produced enzymes. In order to clarify the enzymatic activity of the protein encoded by *bxl1* and to further characterize ABFI, yeast strains transformed with plasmids p17SA (*abf1*), p18SA (*bxl1*), and pAJ401 (control) were cultivated in a fermentor. The growth medium was concentrated and used for the determination of enzymatic activities and hydrolytic properties toward several substrates.

The concentrate containing ABFI showed mainly α-L-arabinofuranosidase activity and some β-xylosidase activity when *p*-nitrophenyl derivatives were used as substrates (Table 1). No other significant activities were detected. Similar results have been reported for the α-L-arabinofuranosidase purified from *T. reesei*, whose β-xylosidase activity toward *p*-nitrophenyl-β-D-xylopyranoside was 1 to 2% of the α-L-arabinofuranosidase activity toward PNPA (28). ABFI liberated L-arabinose from arabinoxylans from different sources (Table 2). No other mono- or oligosaccharides were released, as determined by HPLC (data not shown). The addition of *T. reesei* endoxylanase to hydrolysis experiments did not affect significantly the action of ABFI at the enzyme dosage used (Table 2). The α-L-arabinofuranosidase purified from *T. reesei* was included in some hydrolysis experiments, and the amounts of L-arabinose released were comparable to the amounts released by the enzyme produced in yeasts (Fig. 3).

Interestingly, although the concentrate containing BXLI showed α-L-arabinofuranosidase activity toward PNPA, the main activity detected was β-xylosidase (Table 1). It also showed clear α-L-arabinopyranosidase activity which was higher than the α-L-arabinofuranosidase activity. The fact that BXLI has higher β-xylosidase activity is in accordance with its preference for L-arabinose in pyranoside form since xylose

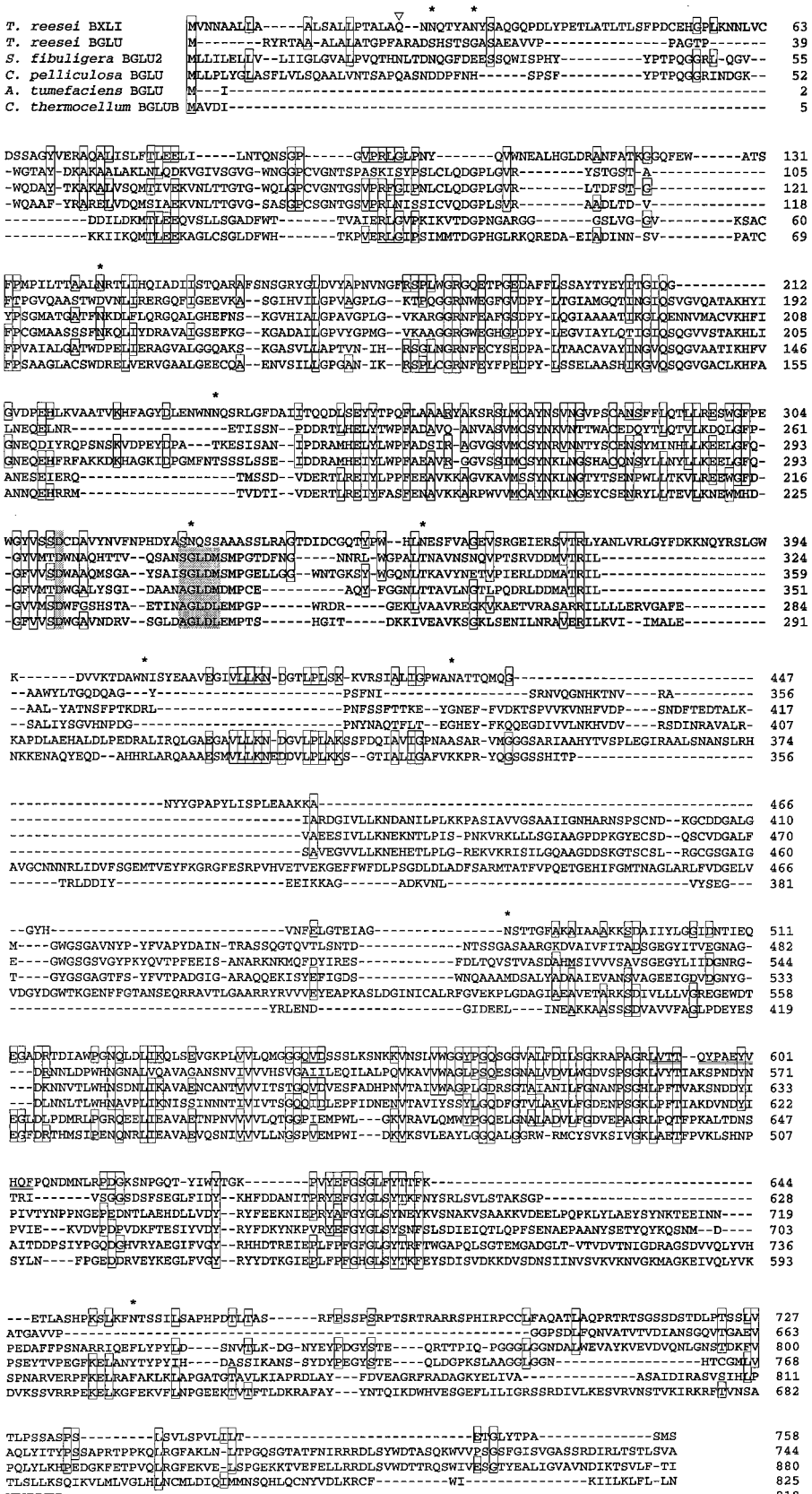

FIG. 2. Alignment of the deduced protein sequence of *T. reesei* BXLI with those of the β-glucosidases of *T. reesei* (2), *Saccharomycopsis fibuligera* (23), *Candida pelliculosa* (20), *Agrobacterium tumefaciens* (6), and *Clostridium thermocellum* (13) by using the CLUSTAR program version 1.20. The one-letter amino acid code is used. Hyphens indicate gaps. Amino acids identical between BXLI and two or more of the other sequences are boxed. The conserved active site D and motif (A/S-G-L-D-M/L) in β-glucosidases are shaded. The putative first amino acid Q of the mature BXLI is marked with an open triangle, and the amino acid sequence determined for a peptide of the purified *T. reesei* β-xylosidase is double underlined. The putative N-glycosylation sites in BXLI are shown by asterisks.

TABLE 1. Extracellular enzymatic activities determined from culture filtrates of yeast strains producing ABFI or BXLI[a]

Strain	Activity (nkat ml^{-1})[b]							
	αGal	αAraf	αArap	βArap	βXyl	βGlu	βGal	βMan
Control	0.0	0.0	0.0	0.00	2.5	6.5	0.3	0.0
ABFI	0.5	171.1	0.0	0.01	7.1	6.6	0.2	0.2
BXLI	0.0	2.4	4.0	0.03	16.3	5.2	0.2	0.0

[a] The activities produced by the host yeast strain are shown as the control and are comparable to the background activities detected in other, similar fermentor cultivations (25).

[b] Activities were measured, as described in Materials and Methods, in 1 ml of 50-fold-concentrated culture medium. αGal, α-galactosidase; αAraf, α-L-arabinofuranosidase; αArap, α-L-arabinopyranosidase; βArap, β-L-arabinopyranosidase; βXyl, β-xylosidase; βGlu, β-glucosidase; βMan, β-mannosidase.

appears in this form. BXLI did not liberate L-arabinose from any of the arabinoxylans tested, not even when used together with *T. reesei* endoxylanase (results not shown). A β-xylosidase which has high-level α-L-arabinofuranosidase activity has been previously purified from *T. reesei* (29). This β-xylosidase produces xylose from xylo-oligosaccharides of different lengths (29) and from polymeric xylan (17). The concentrated yeast culture filtrate containing BXLI clearly hydrolyzed xylobiose better than did the control yeast culture filtrate (results not shown). It was also able to slowly release xylose from polymeric xylan (Fig. 4), which the control yeast concentrate was not able to do.

To investigate whether BXLI corresponded to the previously purified β-xylosidase of *T. reesei*, the purified enzyme was subjected to amino acid sequencing. The N terminus of this enzyme appeared blocked. This is consistent with the fact that the predicted mature BXLI sequence starts with a Gln (Fig. 2) which can be spontaneously cyclized, making it unreactive to most amino acid sequencing procedures (31). This enzyme was therefore digested with trypsin, and the amino acid sequence of an isolated peptide was determined. The same sequence can be found in the predicted protein sequence of BXLI (Fig. 2). In addition, the calculated molecular mass of BXLI (80.4 kDa) is in rough agreement with the molecular mass (100 kDa) estimated by SDS-PAGE for the glycosylated enzyme purified from *T. reesei* (29). These results indicate that most likely BXLI corresponds to the β-xylosidase previously characterized from *T. reesei.*

DISCUSSION

The cloning of genes by expression in yeasts has proven to be a good strategy to isolate in a quick manner genes encoding desired enzyme activities (7, 33). We have used this method for the isolation of novel genes encoding extracellular enzymes of *T. reesei*, for example, the cellulase EGV (33), a β-glucanase

TABLE 2. Release of L-arabinose from arabinoxylans of different origins

Origin	Free arabinose (% of theoretical)[a]	
	ABFI alone	ABFI + xylanase
Oat spelts	37	44
Rye	23	24
Wheat	31	31

[a] L-Arabinose released from arabinoxylan (5 g/liter) by ABFI produced by yeasts (5,000 nkat/g of substrate) in 24 h, alone and together with *T. reesei* endoxylanase (500 nkat/g of substrate).

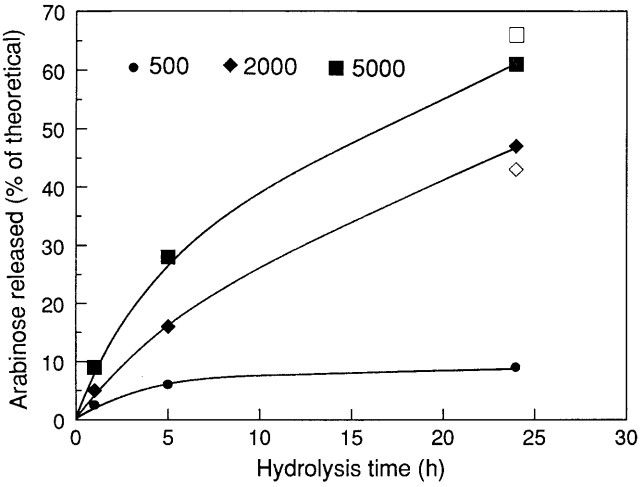

FIG. 3. Liberation of L-arabinose from arabinoglucuronoxylan (10 g/liter) isolated from pine kraft pulp by the indicated dosages of ABFI (in nanokatals per gram of substrate) produced by yeasts. Open symbols indicate hydrolysis by the purified enzyme produced by *T. reesei.*

(34), and α-galactosidases (25), which were previously unknown at the protein and gene levels. The present work describes the isolation of two *T. reesei* genes, *abf1* and *bxl1*, encoding α-L-arabinofuranosidase activity by the same methodology. In this case, the genes turned out to code for enzymes already previously purified from *T. reesei*, an α-L-arabinofuranosidase (ABFI) (28) and a β-xylosidase (BXLI) (29), respectively. The fact that the major activity encoded by *bxl1* is β-xylosidase, not arabinofuranosidase, shows that the methodology is also suitable for detecting minor activities.

The screening of the expression bank was done by using the substrate PNPA, which is very sensitive to most α-L-arabinofuranosidases which release terminal L-arabinose residues. The screening resulted in the isolation of a single true α-L-arabinofuranosidase gene (*abf1*), and this gene codes for an enzyme similar to *A. niger* ABF B. *A. niger* produces another α-L-arabinofuranosidase active on PNPA, ABF A (32). It cannot be excluded that *T. reesei* also produces additional enzymes hydrolyzing PNPA which are expressed at very low levels and have so far escaped purification procedures based on PNPA activity (28). If this is the case, screening a larger number of yeast clones would be needed to isolate the corresponding gene. On the other hand, polymeric substrates would be needed to screen for genes encoding other types of activities, for example, endo-1,5-α-L-arabinases (18) and activities similar to that of the *Aspergillus awamori* (1,4)-β-D-arabinoxylan arabinofuranosidase (22), which can release terminal L-arabinose residues but only from long-chain arabinoxylan.

Some α-L-arabinosidases have been classified in family 43 or 51 in the general classification of glycosyl hydrolases (16). However, the α-L-arabinofuranosidase ABF B from *A. niger* (10), for instance, is not related in amino acid sequence to any other hydrolytic enzyme previously characterized and thus could not be classified. Here it was found that *T. reesei* ABFI has more than 70% amino acid identity with *A. niger* ABF B; thus, it could be considered that these two enzymes, which also have similar substrate specificities (28, 32), can form a new family of glycosyl hydrolases.

Interestingly, BXLI shows no amino acid similarity to any of the reported β-xylosidases classified in families 39, 43, and 52. However, despite the lack of β-glucosidase activity, BXLI has

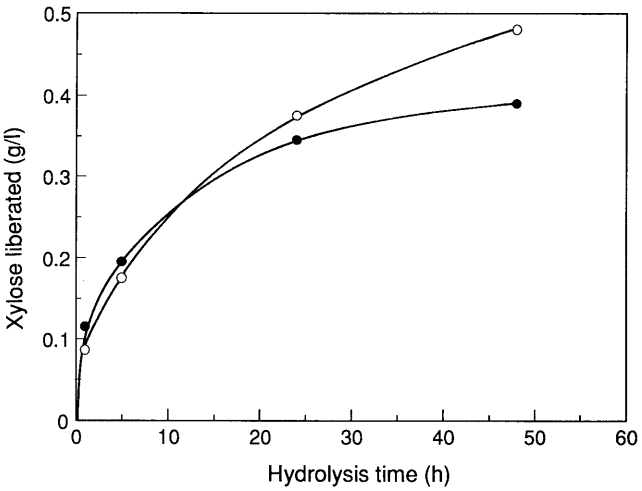

FIG. 4. Liberation of xylose from polymeric beechwood 4-*O*-methylglucuronoxylan (●) (10 g/liter) and unsubstituted beechwood xylan (○) (10 g/liter) by yeast-produced BXLI (750 nkat/g of substrate).

significant similarities to the enzymes of family 3, which is very conserved by including only β-glucosidases. One putative active-site carboxylic residue of the β-glucosidases appears to be conserved in BXLI also (Asp-311) and could take part in the catalytic activity. Unlike with the β-glucosidases, the second putative catalytic Asp cannot be pointed out in the BXLI sequence. Nevertheless, the overall level of similarity between BXLI and the β-glucosidases is high, and this enzyme can be assigned to family 3 as the only member with a different enzymatic activity.

Both ABFI and BXLI showed α-L-arabinofuranosidase and β-xylosidase activities against the substrates PNPA and *p*-nitrophenyl-β-D-xylopyranoside, respectively. This dual activity against synthetic model substrates has been reported for several α-L-arabinosidases and β-xylosidases of different origins. However, ABFI and BXLI produced by yeasts showed their actual activities when natural oligomeric or polymeric substrates were used. ABFI released only L-arabinose from different arabinoxylans, and BXLI released xylose from xylobiose and xylan but did not release L-arabinose from arabinoxylans.

Heterologous proteins expressed in the yeast *S. cerevisiae* can become subject to posttranslational modifications, such as hyperglycosylation; this has been observed for several *T. reesei* cellulases (26, 27) and a mannanase (38). Nevertheless, all enzymes were secreted in enzymatically active form by yeasts. The specific catalytic activities of cellobiohydrolases I and II of *T. reesei* were reduced, but the substrate specificities were not affected (26, 30). Apparently, the possible posttranslational modifications did not affect significantly the activities of ABFI and BXLI produced in yeasts; they displayed hydrolytic properties similar to those of enzymes purified from the natural host, *T. reesei*. The advantages of *S. cerevisiae* are that it produces practically no hydrolases attacking polymeric (hemi)cellulosic substrates and that possible background activities against oligosaccharides can be easily checked. These features increase the usefulness of yeasts in the isolation of novel hydrolase genes. Despite the rather low production levels, yeasts can provide sufficient amounts of enzyme for preliminary biochemical characterization with no need of purification of the enzyme or expression of the gene in another host.

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