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Molecular Cloning and Expression of the Novel Fungal β-Glucosidase Genes from *Humicola grisea* and *Trichoderma reesei*[1]

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A novel fungal β-glucosidase gene (*bgl4*) and its homologue (*bgl2*) were cloned from the cellulolytic fungi *Humicola grisea* and *Trichoderma reesei*, respectively. The deduced amino acid sequences of *H. grisea* BGL4 and *T. reesei* BGL2 comprise 476 and 466 amino acids, respectively, and share 73.1% identity. These β-glucosidases show significant homology to plant β-glucosidases belonging to the β-glucosidase A (BGA) family. Both genes were expressed in *Aspergillus oryzae*, and the recombinant β-glucosidases were purified. Recombinant *H. grisea* BGL4 is a thermostable enzyme compared with recombinant *T. reesei* BGL2. In addition to β-glucosidase activity, recombinant *H. grisea* BGL4 showed a significant level of β-galactosidase activity, while recombinant *T. reesei* BGL2 showed weak β-galactosidase activity. Cellulose saccharification by *Trichoderma* cellulases was improved by the addition of recombinant *H. grisea* BGL4.

Key words: cellulase, cellulolytic fungi, β-glucosidase, *Humicola grisea*, *Trichoderma reesei*.

Endoglucanases (endo-1,4-β-D-glucanases; EG, EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-D-glucanases; CBH, EC 3.2.1.91), and β-glucosidases (1,4-β-D-glucosidase; BGL, EC 3.2.1.21) are three major cellulase components produced by cellulolytic fungi. Cellulose is efficiently hydrolyzed to cellooligosaccharides through the synergistic action of cellobiohydrolases and endoglucanases (*1*), and cellooligosaccharides are hydrolyzed to glucose by β-glucosidases. Among cellulolytic fungi, some *Trichoderma* species have very strong cellulose-degrading activities and their cellulase systems have been widely studied. The most highly produced cellulase component in *T. reesei* is CBHI, accounting for about 60% of the total secreted protein (*2*). CBHII and endoglucanases (mostly EGI) also account for about 20 and 10% of the total secreted protein, respectively (*2*). β-Glucosidases account for only 1% of the total secreted protein (*2*), and this is known to limit the saccharification of cellulose by *T. reesei* cellulases (*3*). This problem can be partially solved by adding β-glucosidases of other origin or isolating mutant strains of *T. reesei* that have increased levels of β-glucosidases by standard mutagenesis (*4*) or recombinant techniques (*5*).

The genus *Humicola* is known to produce many kinds of thermostable cellulases (*6, 7*). It is also known to be a good source of β-glucosidase activity (*6, 8, 9*), and thus is considered to be a candidate for supplementing the β-glucosidase activity of *T. reesei*. Although many endoglucanase and cellobiohydrolase genes have been cloned from *Humicola* species (*10-13*) and *T. reesei* so far (*14-16*), no β-glucosidase genes have been cloned from *Humicola* species, and only one β-glucosidase gene has been cloned from *T. reesei* (*5*).

In our previous study, we purified six extracellular β-glucosidases from *H. grisea* and characterized their enzymatic properties (*6*). *H. grisea* BGL1 is an oligomeric enzyme that shows a strong activity toward *p*-nitrophenyl-β-D-glucoside (PNPG). It also has β-xylosidase activity. *H. grisea* BGL2 and 3 are essentially the same enzyme, composed of four molecules each of a 94-kDa protein and a 21-kDa protein. *H. grisea* BGL4 is a monomeric enzyme with both β-glucosidase and β-galactosidase activities that shows the highest activity toward cellobiose among these purified β-glucosidases. *H. grisea* BGL5 is a heterodimeric enzyme comprising 46- and 49-kDa proteins, and has a strong activity toward cellooligosaccharides. *H. grisea* BGL6 is a monomeric enzyme showing strong activity toward PNPG. The saccharification of cellulose by *T. reesei* cellulases appears to be improved by supplementing the β-glucosidase activity of *T. reesei* cellulases. Among purified β-glucosidases, BGL4 is the best candidate for this purpose since it shows strong activity toward cellobiose, and, interestingly, it also has β-galactosidase activity. In this study, we report the isolation of the novel fungal β-

[1] The determined nucleotide sequences have been deposited in the DDBJ, GenBank, and EMBL databases under accession Nos. AB003109 and AB003110.

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Abbreviations: BGL, β-glucosidase; CBH, cellobiohydrolase; EG, endoglucanase; PNPC, *p*-nitrophenyl-β-D-cellobioside; PNPG, *p*-nitrophenyl-β-D-glucoside; PNPGal, *p*-nitrophenyl-β-D-galactoside.

glucosidase gene (*bgl4*) encoding BGL4 from *H. grisea*. This is the first report of the cloning of a gene encoding an enzyme with both β-glucosidase and β-galactosidase activities from *H. grisea*. We also isolated the gene homologous to the *H. grisea bgl4* gene from *T. reesei* (designated *bgl2*). The enzymatic properties of recombinant *H. grisea* BGL4 and recombinant *T. reesei* BGL2 produced by *Aspergillus oryzae* were investigated.

MATERIALS AND METHODS

Strains, Plasmids, and Media—*H. grisea* var. *thermoidea* IFO9854 and *T. reesei* QM9414 was used for DNA isolation. Stock cultures were stored on agar (1.5%) slants of MY medium (2% malt extract, 0.2% yeast extract). For total RNA preparation, the spores of *H. grisea* were inoculated into 1 liter of cellulase-inducing medium [5% Avicel, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% urea, 0.03% $CaCl_2 \cdot 2H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.1% Bacto peptone, 1% yeast extract, 0.1% Tween 80, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.00016% $MnSO_4 \cdot 4\text{-}5H_2O$, 0.00014% $ZnSO_4 \cdot 7H_2O$, 0.0002% $CoCl_2$, pH 6.8] and grown for 5 days at 37°C with shaking; mycelia were harvested by filtration. *A. oryzae* M-2-3 (*argB*$^-$) was used as a host for the expression of the cloned β-glucosidase genes and the stock culture was stored on DPY medium (*17*). For fungal transformation, the expression vector pAMYB118 (*12*) and its derivatives, and the *argB* containing plasmid pSal23 (*18*) were used. Czapek-Dox medium was used for fungal transformation (*17*). For the expression of β-glucosidase genes, *A. oryzae* transformants were cultivated in CD-P medium for 4 days at 30°C (*17*). For general DNA manipulation, *Escherichia coli* JM109 and *E. coli* MV1184 were used as hosts for the cloning vector, pUC118 (*19*).

Amino Acid Sequence Analysis—*H. grisea* BGL4 was purified as described previously (*6*). Trypsin-digested peptides of *H. grisea* BGL4 were purified by HPLC on C-4 or C-18 columns (Senshu Pak VP-304 or Senshu Pak VP-318, respectively, Senshu, Tokyo), and their amino acid sequences were analyzed by Edman degradation using an automated protein sequencer (Model 470A protein sequencer, Applied Biosystems).

Genomic DNA Cloning and Sequencing—Unless otherwise stated, standard procedures were used for recombinant DNA techniques (*19*). Fungal chromosomal DNA was prepared as described previously (*11*). Partial amino acid sequences of *H. grisea* BGL4 (Tyr-Gly-Met-Asn-His-Tyr and Tyr-Val-Asp-Tyr-Ala-Asn) were used to design two degenerate primers, 5′-TA(CT)GG(AGCT)ATGAA(CT)CA(CT)TA-3′ (sense) and 5′-TT(AGCT)GC(AG)TA(AG)TC(AGCT)AC(AG)TA-3′ (antisense), respectively. PCR was carried out using these primers with *H. grisea* chromosomal DNA as the template, and a fragment of about 450 bp was amplified and confirmed to encode a portion of the β-glucosidase gene. The amplified fragment was isolated and radiolabeled with [α-^{32}P]dCTP and a random primed DNA labeling kit (Boehringer Mannheim), and used as a probe for the detection of fungal β-glucosidase genes. Southern hybridization of *H. grisea* and *T. reesei* chromosomal DNA digested with *Pst*I was performed as described previously (*11, 12*). The hybridizing fragments were cloned into pUC118 using the colony hybridization technique (*19*). DNA sequencing was performed on both strands by the dideoxy sequencing method (*20*) using a single strand-nested deletion kit (TaKaRa) and a *Bca*BEST sequencing kit (TaKaRa).

Isolation of RNA and Synthesis of cDNA—Mycelia were ground in liquid nitrogen, and total RNA was extracted by the guanidine thiocyanate-CsCl method (*19*). The polyadenylated RNA was purified with an mRNA purification kit (Pharmacia). The synthesis of cDNA was done with 5 μg of the purified mRNA and a cDNA synthesis system plus kit (Amersham).

Construction of Expression Plasmids—To construct the expression vector of the *H. grisea bgl4* gene, an approximately 2.4 kb *Bst*1107I (nucleotide position −48)-*Hin*dIII fragment containing the full length *H. grisea bgl4* gene was excised and inserted into the *Sma*I-*Hin*dIII sites of the fungal expression vector pAMYB118, giving rise to pAMYB-HgBGL4. To construct the expression vector of the *T. reesei bgl2* gene, an approximately 1.8 kb blunt-ended *Sph*I-*Eco*T22I fragment containing the full length *T. reesei bgl2* gene was sub-cloned into the *Sma*I site of pUC118. Then an approximately 1.6 kb *Spl*I-*Hin*dIII fragment containing the region encoding the putative catalytic domain of *T. reesei* BGL2 (Tyr 15-Ala 466) was excised from this plasmid and inserted into the *Spl*I-*Hin*dIII sites of pAMYB-HgBGL4, giving rise to pAMYB-TrBGL2 (Fig. 4).

Fungal Transformation—Transformation of *A. oryzae* was performed according to the method of Gomi *et al.* (*18*).

Enzyme Assay—Enzyme activities were measured by incubating 0.27 ml of 1 mg/ml PNPG solution in 50 mM sodium phosphate buffer (pH 6.0) with 0.03 ml of enzyme solution at the optimal temperature for the reaction of each enzyme (*H. grisea* BGL4, 55°C; *T. reesei* BGL2, 40°C) for 10 min. The reaction was stopped by adding 0.3 ml of 2% Na_2CO_3 and the absorbance at 410 nm was measured. *p*-Nitrophenyl-β-D-cellobioside (PNPC) and *p*-nitrophenyl-β-D-galactoside (PNPGal) were also used as substrates instead of PNPG. One unit of enzyme was defined as the activity producing 1 μmol of *p*-nitrophenol per min under these assay conditions.

Measurement of the Saccharification of Cellulose—The saccharification of cellulose by *Trichoderma* cellulases was measured by incubating 10 μl of 1% Avicel suspension in 50 mM sodium phosphate buffer (pH 6.0) with 1 μl of 5 mg/ml *Trichoderma* cellulase (Cellulase Onozuka R-10) solution in the presence and absence of 1 μl of 0.1 mg/ml recombinant *H. grisea* BGL4 solution at 37°C for an appropriate time. The released glucose produced by these reactions was then measured by the mutarotase-glucose oxidase method (GlucoseCII-Testwako, Wako).

Protein Purification—Recombinant β-glucosidases produced by *A. oryzae* transformants were purified by cultivating for 4 days in CD-P medium, after which the culture supernatants were obtained by filtration. Solid ammonium sulfate was added to the filtrates to 40% saturation and the precipitates were removed by filtration. The samples were loaded onto a column of Phenyl Toyopearl 650M (2.5×5.0 cm, Tosoh), equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 40% saturated ammonium sulfate. The enzymes were eluted with a linear gradient of 40 to 0% saturated ammonium sulfate in the same buffer. Fractions showing β-glucosidase activity were collected and ultrafiltrated with Centriprep 30. The desalted samples were

buffered by adding 1/10 volume of 100 mM Tris-HCl buffer, pH 7.5, and loaded onto a column of MonoQ 10/10 (Pharmacia), pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.5. The enzymes were eluted with a linear gradient of 0 to 0.5 M of NaCl in the same buffer, and purified β-glucosidases were obtained. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (*21*). Protein content was measured by a dye-binding assay kit (Protein assay kit, Bio-Rad) using γ-globulin as the standard.

RESULTS AND DISCUSSION

Cloning of the H. grisea bgl4 Gene—*H. grisea* BGL4 was purified as described previously (*6*). The protein was digested with trypsin and the proteolytic fragments were purified by reverse phase HPLC. Amino acid sequence analysis revealed the sequences of the five internal peptides.

Degenerate oligonucleotide primers corresponding to the sequences of two peptides (Tyr-Gly-Met-Asn-His-Tyr and Tyr-Val-Asp-Tyr-Ala-Asn) were synthesized for use in PCR to amplify the DNA fragment encoding a portion of the *bgl4* gene. A specific fragment of about 450 bp was amplified by PCR, and then isolated and used as a probe to screen for a genomic clone of the *bgl4* gene. Southern hybridization of *H. grisea* chromosomal DNA digested with *Pst*I was performed, and a 9.4 kb fragment was found to hybridize uniquely with the probe; this fragment was cloned into pUC118 using the colony hybridization technique. Restriction and partial sequence analysis suggested that a part of the DNA, a 4.0 kb *Hin*dIII fragment, contained the *bgl4* gene, and this fragment was sequenced on both strands by the dideoxy sequencing method (Fig. 1). The sequence contained a coding region of 1,532 nucleotides interrupted by an intron of 104 nucleotides, both of which were confirmed by cDNA sequencing. The translation product of the coding region should contain 476 amino acids with a molecular mass of 54,061 Da. The sequences of all five peptides were found in the deduced amino acid sequence shown in Fig. 1. The codon usage of this gene is similar to that of *H. grisea* cellulase genes (*11, 12*), with cytosine and guanine preferred in the third position of codons, and a strong bias against the use of adenine in the third position.

Cloning of the T. reesei bgl2 Gene—So far, only one β-glucosidase gene (*bgl1*) has been cloned from *T. reesei* (*5*), but its sequence shows no significant homology to the *H. grisea bgl4* gene cloned in the present work. To examine whether *T. reesei* has a gene homologous to the *H. grisea bgl4* gene, Southern hybridization of *Pst*I-digested *T. reesei* chromosomal DNA was performed using the *H. grisea bgl4* gene as a probe, and the membrane was washed under conditions of low stringency (*11*). A 7.0 kb fragment was found to hybridize uniquely with the probe, and this fragment was cloned as described above and sequenced on both strands by the dideoxy sequencing method (Fig. 2). The sequence contained a putative coding region of 1,472 nucleotides interrupted by an intron of 74 nucleotides located in an identical position to that in the *H. grisea bgl4* gene. The translation product of the putative coding region should contain 466 amino acids with a molecular mass of 52,239 Da. The deduced amino acid sequence shows 73.1% identity to that of *H. grisea* BGL4 (Fig. 3). The sequence homology and enzymatic properties of the translation product (see below) suggest that this putative coding region encodes a β-glucosidase gene homologous to the *H. grisea bgl4*, and we designated it the *T. reesei bgl2* gene.

Sequence Homology—β-Glucosidases can be divided into two enzyme subfamilies, β-glucosidase A (BGA) and β-glucosidase B (BGB), on the basis of their structure similarities (*22, 23*). The BGA family includes bacterial β-glucosidases, archaebacterial β-galactosidase, plant thioglucosidases, and human lactase-phlorizin hydrolase, while the BGB family includes fungal and rumen bacterial β-glucosidases (*24*). It is also known that the BGA family is related to cellulase family A (*22, 23, 25*). Interestingly, a homology search revealed that *H. grisea* BGL4 and *T. reesei* BGL2 show significant similarity to β-glucosidases of plant origin, which belong to the BGA family (Fig. 3), rather than to β-glucosidases of the BGB family. *H. grisea* BGL4 and *T. reesei* BGL2 show 46.3 and 43.8% identity to *Costus specious* furostanol glycoside 26-*O*-β-glucosidase (*24*), respectively; 41.4 and 41.6% identity to *Sorghum bicolor* cyanogenic β-glucosidase (dhurrinase) (*26*), respectively; 38.7 and 38.5% identity to *Trifolium repens* cyanogenic β-glucosidase (linamarase) (*27*), respectively.

BGA family enzymes are considered to share a common mechanism for the hydrolysis of β-glycosidic bonds (*24*), and have two highly conserved amino acid stretches involved in their putative active sites; the Ile/Val/Leu-Thr/Ser-Glu-Asn-Gly motif has been shown to contain an active nucleophilic center (*28, 29*), and the Asn-Glu-Pro motif has been shown to contain an acid-base catalyst (*29, 30*). In addition to the amino acid sequence homology with BGA family enzymes, both *H. grisea* BGL4 and *T. reesei* BGL2 contain these putative active site motifs, suggesting that these β-glucosidases belong to the BGA family. The main function of these fungal β-glucosidases should be to hydrolyze cellobiose to glucose (*6*), while the functions of plant β-glucosidases are related to saponin metabolism (*24*) or cyanogenesis (*26, 27*). Thus, the substrates of plant β-glucosidases are structurally more complex than those of fungal β-glucosidases. It should be noted that *H. grisea* BGL4 has significant β-galactosidase activity (*6*). *H. grisea* BGL4 may have broader substrate specificity and be able to hydrolyze more complex substrates.

Expression of β-Glucosidase Genes in Aspergillus oryzae—In our previous study, we expressed *H. grisea* and *T. reesei* cellulase genes in *A. oryzae* and reported their enzymatic properties (*11, 12, 31*). In this expression system, the introduced *H. grisea* and *T. reesei* cellulase genes were correctly transcribed, indicating that splicing signals in introns of these fungal cellulase genes are recognized by *A. oryzae*. In order to characterize and compare the enzymatic properties of *H. grisea* BGL4 and *T. reesei* BGL2, we also used an *A. oryzae* expression system (*17*) and constructed expression vectors, pAMYB-HgBGL4 and pAMYB-TrBGL2. Our first attempt to express the *T. reesei bgl2* gene in *A. oryzae* was unsuccessful for unknown reasons, but when the fusion gene between the *H. grisea bgl4* and the *T. reesei bgl2*, consisting of two portions encoding the putative signal peptide of *H. grisea* BGL4 and the catalytic domain of *T. reesei* BGL2, was used in pAMYB-TrBGL2 (Fig. 4A), expression was successful. A comparison of the putative signal peptides of *H. grisea*

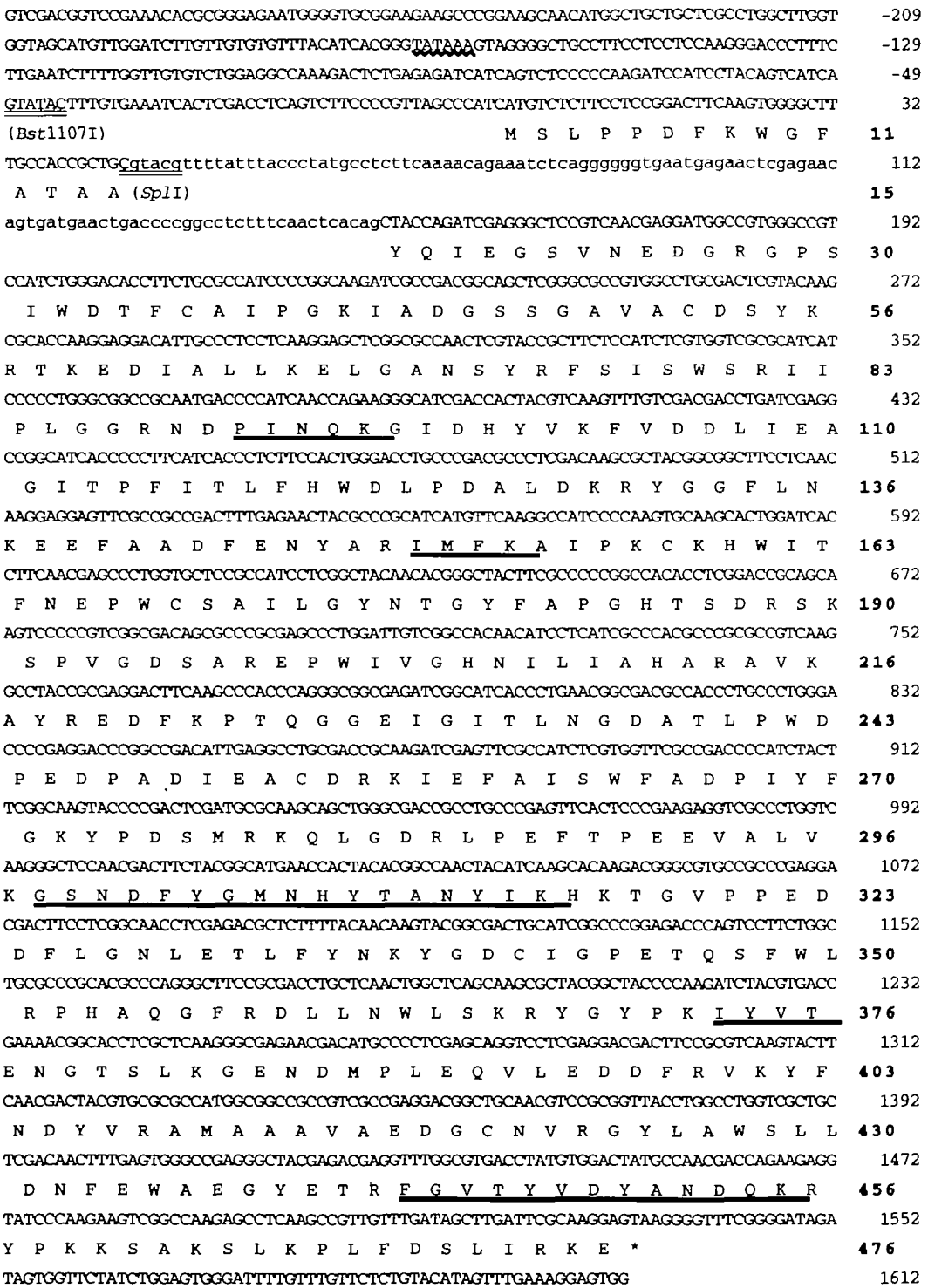

Fig. 1. **Nucleotide sequence of the *H. grisea bgl4* gene and the deduced amino acid sequence.** Nucleotide numbering begins with the translational initiation site. Intron sequences are shown in lowercase letters. A putative TATA sequence is shown by the waved underline. The peptide sequences determined by Edman degradation are underlined. The restriction enzyme sites used to construct the expression vector are double-underlined.

BGL4 and *T. reesei* BGL2 revealed three differences between them (Fig. 3): (i) there is no amino acid residue in the *T. reesei* BGL2 signal peptide corresponding to Ser 2 of the *H. grisea* BGL4 signal peptide; (ii) Pro 5 and (iii) Lys 8 in the *H. grisea* BGL4 signal peptide are replaced by Lys and Gln, respectively, in the *T. reesei* BGL2 signal peptide. Probably because these differences are critical, the signal peptide of *T. reesei* BGL2 might not work well in *A. oryzae*.

pAMYB-HgBGL4 and pAMYB-TrBGL2 (20 μg, each) were introduced into *A. oryzae* M-2-3, an arginine-auxo-

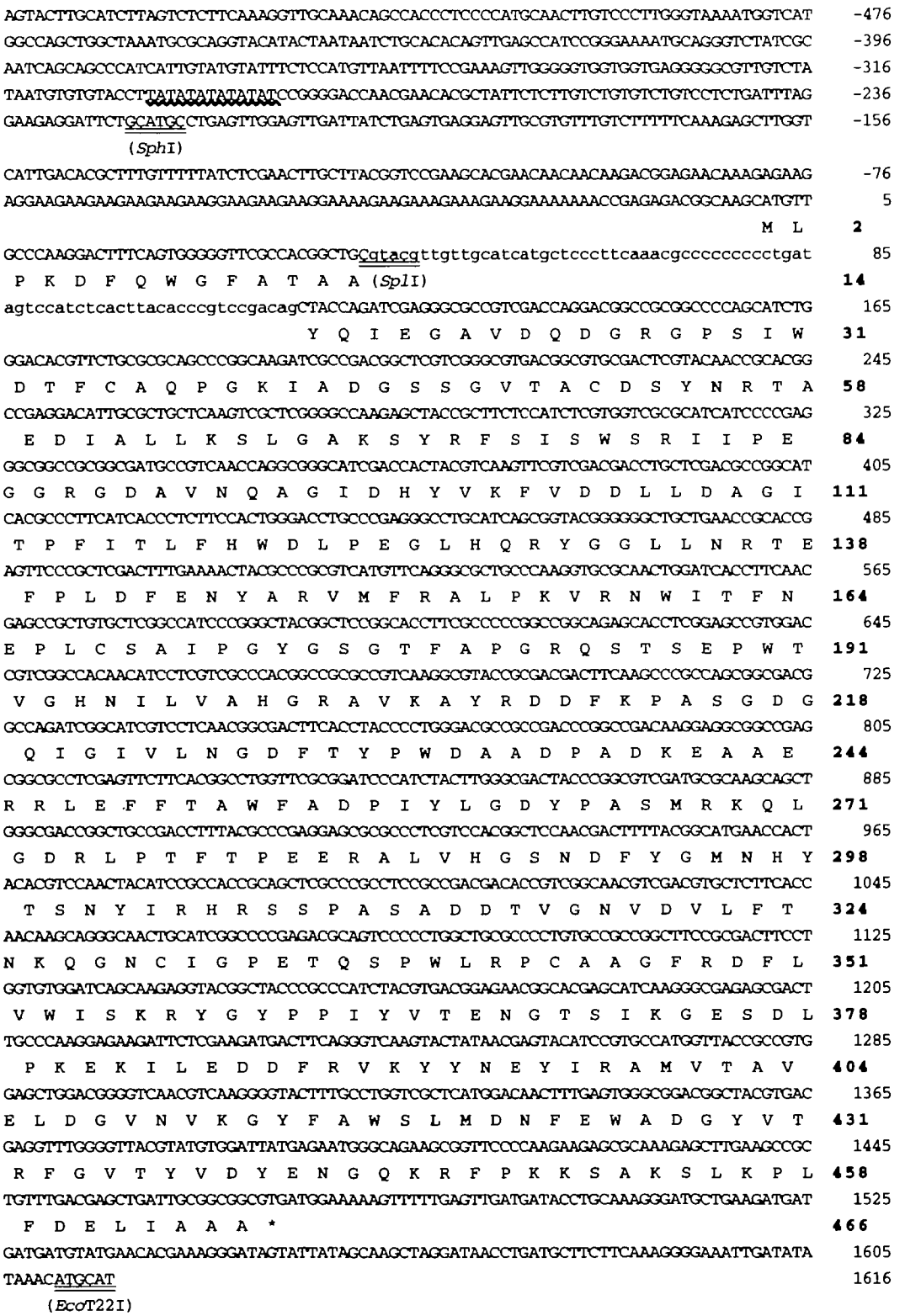

Fig. 2. **Nucleotide sequence of the *T. reesei bgl2* gene and the deduced amino acid sequence.** Nucleotide numbering begins with the translational initiation site. Intron sequences are shown in lower-case letters. A putative TATA sequence is shown by the waved underline. The restriction enzyme sites used to construct the expression vector are double-underlined.

troph, by co-transformation with 20 μg of an *argB* containing plasmid, pSal23 (*18*). The expression of these β-glucosidase genes is regulated by the Taka-amylase promoter, and both are inducible by the addition of maltose as a carbon source. The *arg*$^+$ transformants were isolated and grown in 10 ml of CD-P medium (containing maltose as a

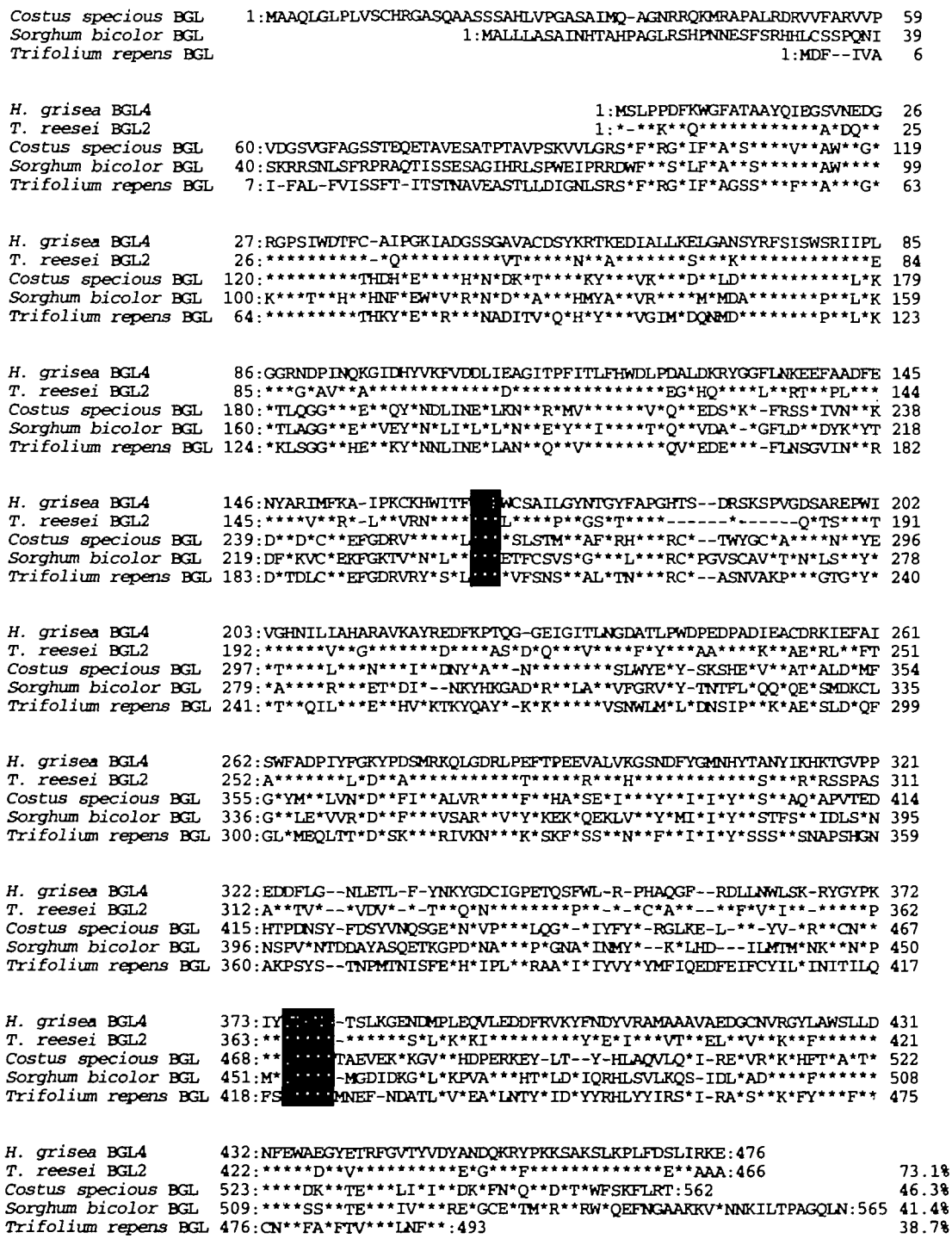

Fig. 3. **Sequence comparison of *H. grisea* BGL4, *T. reesei* BGL2, and other β-glucosidases of plant origin.** Asterisks denote residues identical to those in *H. grisea* BGL4. Highly conserved motifs containing a putative active nucleophilic center are shown as white characters on a black background.

carbon source) for 4 days at 30°C and assayed for enzyme activities toward PNPG. The control strain transformed with pSal23 and pAMYB118 showed no activity toward PNPG under these assay conditions, while some transformants transformed with pSal23 and pAMYB-HgBGL4 or pAMYB-TrBGL2 showed apparent activity toward PNPG. Clones showing the highest activity of each enzyme were selected and used for enzyme production. The clones were cultured for four days in CD-P medium, after which the enzymes were purified from the culture supernatants by Phenyl Toyopearl and MonoQ column chromatographies (Fig. 4B). Under these culture conditions and purification procedures, the amount of recombinant β-glucosidases produced by each transformant was estimated to be about

50 to 200 μg/ml of recombinant β-glucosidases in the culture supernatant. These production levels are equivalent to those of recombinant cellulases produced by *A. oryzae* (*11, 31*). The apparent molecular weight of recombinant *H. grisea* BGL4 estimated by SDS-PAGE is about 57,000, which is slightly larger than the molecular mass of the deduced amino acid sequence (54,061 Da). The discrepancy between the molecular weight estimated by SDS-PAGE and the molecular mass from the deduced amino acid sequence may be due to minor glycosylation of the recombinant *H. grisea* BGL4 by *A. oryzae*. On the other hand, the apparent molecular weight of recombinant *T. reesei* BGL2 as estimated by SDS-PAGE is about 52,000, which corresponds to the molecular mass from the deduced amino acid sequence.

Enzymatic Properties of Recombinant β-Glucosidases—The optimal temperature, thermal stability, optimal pH, and pH stability of these recombinant enzymes were measured using PNPG as a substrate. Although *H. grisea* BGL4 and *T. reesei* BGL2 are homologous to each other, some differences were observed in their enzymatic properties. The optimal temperatures for the reaction of recombinant *H. grisea* BGL4 and recombinant *T. reesei* BGL2 were found to be 55 and 40°C, respectively. Recombinant *H. grisea* BGL4 and recombinant *T. reesei* BGL2 retain more than 80% of their relative activity following heating to 50 and 45°C for 10 min, respectively. These differences may be related to the fact that *H. grisea* is a thermophilic fungus and produces thermostable cellulases (*6, 7*).

The pH optima of these enzymes was found to be pH 6.0, and they retain more than 80% of their relative activity within the range of pH 6.0–11.0 after 20 h at 4°C.

The optimal temperature, thermal stability, optimal pH, and pH stability of recombinant *H. grisea* BGL4 are similar to those of native *H. grisea* BGL4 (*6*).

The purified recombinant β-glucosidases were tested for substrate specificity (Table I). Both enzymes show relatively high activity toward PNPG, while they show very weak or no activity toward *p*-nitrophenyl-β-D-cellobioside (PNPC). Native *H. grisea* BGL4 has β-galactosidase activity (*6*), so we examined the β-galactosidase activity of the recombinant enzymes using *p*-nitrophenyl-β-D-galactoside (PNPGal) as a substrate. Recombinant *H. grisea* BGL4 shows significant activity toward PNPGal, while recombinant *T. reesei* BGL2 shows weak activity. The β-galactosidase activity of recombinant *H. grisea* BGL4 is about 6.5 times higher than that of recombinant *T. reesei* BGL2.

The kinetic parameters for the hydrolysis of PNPG and PNPGal were calculated by Lineweaver-Burk plots to evaluate the efficiency of substrate use. As shown in Table II, the V_{max}/K_m values of recombinant *H. grisea* BGL4 toward PNPG and PNPGal were 4.3 times and 11.1 times higher than those of recombinant *T. reesei* BGL2, respectively. In addition, *H. grisea* BGL4 is a thermostable enzyme compared with *T. reesei* BGL2, suggesting that *H. grisea* BGL4 is a stronger enzyme than *T. reesei* BGL2. These results may be related to the fact that the β-glucosidase activity of the *H. grisea* cellulase system is higher than that of the *T. reesei* cellulase system (*8*). It is interesting that *H. grisea* BGL4 contains a unique amino acid stretch between His 183 and Gly 194 when compared with *T. reesei* BGL2 (Fig. 3). This stretch may be related to the

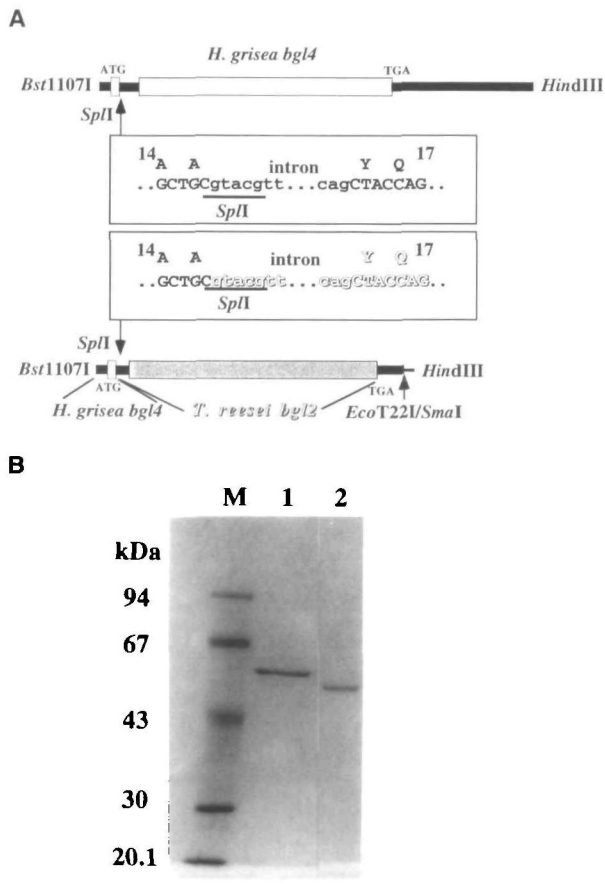

Fig. 4. **Construction of the fusion gene (A) and SDS-PAGE of the purified recombinant β-glucosidases produced by *A. oryzae* (B).** Lane M, molecular mass standard; lane 1, recombinant *H. grisea* BGL4; lane 2, recombinant *T. reesei* BGL2.

TABLE I. **Substrate specificity of purified β-glucosidases.**

Enzyme	Specific activity toward (U·mg^{-1})		
	PNPG	PNPC	PNPGal
Recombinant *H. grisea* BGL4	26.1	nd	11.2
Native *H. grisea* BGL4[a]	8.38	1.80	2.70
Recombinant *T. reesei* BGL2	23.9	0.89	1.72

nd, not detected. [a]Data from Ref. *6*.

TABLE II. **Kinetic parameters of purified β-glucosidases in the hydrolysis of PNPG and PNPGal.**

Enzyme	PNPG			PNPGal		
	K_m (mM)	V_{max} (μmol PNP·min^{-1}·mg^{-1})	V_{max}/K_m	K_m (mM)	V_{max} (μmol PNP·min^{-1}·mg^{-1})	V_{max}/K_m
Recombinant *H. grisea* BGL4	0.32	25.0	78.1	1.82	21.1	11.6
Native *H. grisea* BGL4[a]	0.34	8.70	25.6	—	—	—
Recombinant *T. reesei* BGL2	2.22	40.0	18.0	20.0	20.8	1.04

[a]Data from Ref. *6*.

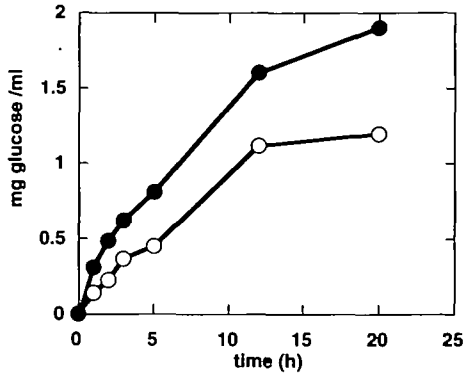

Fig. 5. **Improvement of saccharification of cellulose by *Trichoderma* cellulases in the presence of recombinant *H. grisea* BGL4.** Saccharification of cellulose by *Trichoderma* cellulases in the presence (closed circles) and absence (open circles) of recombinant *H. grisea* BGL4 was measured by the mutarotase-glucose oxidase method.

thermostability and enzymatic activity of *H. grisea* BGL4.

The activities of recombinant *H. grisea* BGL4 toward PNPG and PNPGal are about 3-4 times higher than those of the native enzyme (Table I). We think this is mainly due to differences in the assay conditions used, that is, buffer system [50 mM sodium phosphate buffer (pH 6.0) *vs.* 50 mM sodium acetate buffer (pH 5.0)], incubation time (10 min *vs.* 30 min), incubation temperature (55°C *vs.* 50°C), and so on. In some cases, recombinant enzymes produced by *A. oryzae* are glycosylated differently compared with the native enzymes (*31*), and this may also affect some enzymatic properties. The V_{max} value of recombinant *H. grisea* BGL4 is about 3 times higher than that of the native enzyme (Table II), possibly for the same reasons described above.

Improvement of Saccharification of Cellulose by Trichoderma Cellulases with Recombinant H. grisea BGL4— Some *Trichoderma* species such as *T. reesei*, *T. viride*, and so on have strong cellulose degrading activities, and their cellulases are commercially available. However, these species produce only small amounts of β-glucosidases. So, in the process of saccharification of cellulose by *Trichoderma* cellulases, large amounts of cellobiose should remain. By using the strong β-glucosidases of *H. grisea*, it might be possible to improve the saccharification of cellulose by *Trichoderma* cellulases. To examine this, we compared the level of cellulose saccharification by *Trichoderma* cellulases with and without recombinant *H. grisea* BGL4. *H. grisea* BGL4 shows strong activity toward cellobiose (13.1 U mg^{-1}, *6*), and the recombinant enzyme also has this activity (9 U mg^{-1}). As shown in Fig. 5, the level of saccharification in the presence of recombinant *H. grisea* BGL4 was 1.4-2.2 times higher than in its absence, indicating that the level of cellulose saccharification by *Trichoderma* cellulases can be improved by adding recombinant *H. grisea* BGL4. In these assays, we used purified recombinant *H. grisea* BGL4, but the production level of recombinant *H. grisea* BGL4 by *A. oryzae* transformants is high enough that the culture supernatant could be used instead of the purified enzyme, although there is the possibility of contamination by *A. oryzae* proteases. It is also possible that the overexpression of the *H. grisea bgl4* gene in *T. reesei* or *T. viride* improves the saccharification of cellulose by cellulases from these transformants. To utilize the cellulose resources efficiently, it is necessary to establish an efficient system for cellulose saccharification. In this study, we show that *H. grisea* β-glucosidase can replace the β-glucosidase activity of *Trichoderma* cellulases. A combination of *Trichoderma* and *Humicola* cellulases may provide a strong cellulose saccharification system.

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REFERENCES

1. Henrissat, B., Driguez, H., Viet, C., and Schülein, M. (1985) Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Bio/technology* **3**, 722-726
2. Uusitalo, J.M., Helena Nevalainen, K.M., Harkki, A.M., Knowles, J.K.C., and Penttilä, M.E. (1991) Enzyme production by recombinant *Trichoderma reesei* strains. *J. Biotechnol.* **17**, 35-50
3. Sternberg, D., Vijayakumar, P., and Reese, E.T. (1977) β-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. *Can. J. Microbiol.* **23**, 139-147
4. Kawamori, M., Ado, Y., and Takasawa, S. (1986) Preparation and application of *Trichoderma reesei* mutants with enhanced β-glucosidase. *Agric. Biol. Chem.* **50**, 2477-2482
5. Barnett, C.C., Berka, R.M., and Fowler, T. (1991) Cloning and amplification of the gene encoding an extracellular β-glucosidase from *Trichoderma reesei*: evidence for improved rates of saccharification of cellulosic substrates. *Bio/technology* **9**, 562-567
6. Takashima, S., Nakamura, A., Masaki, H., and Uozumi, T. (1996) Purification and characterization of cellulases from *Humicola grisea*. *Biosci. Biotechnol. Biochem.* **60**, 77-82
7. Hayashida, S. and Yoshioka, H. (1980) Production and purification of thermostable cellulases from *Humicola insolens* YH-8. *Agric. Biol. Chem.* **44**, 1721-1728
8. Araujo, E.F., Barros, E.G., Caldas, R.A., and Silvia, D.O. (1983) Beta-glucosidase activity of a thermophilic cellulolytic fungus, *Humicola* sp. *Biotechnol. Lett.* **11**, 781-784
9. Peralta, R.M., Terenzi, H.F., and Jorge, J.A. (1990) β-D-Glycosidase activities of *Humicola grisea*: biochemical and kinetic characterization of a multifunctional enzyme. *Biochim. Biophys. Acta* **1033**, 243-249
10. Dalbøge, H. and Heldt-Hansen, H.T. (1994) A novel method for efficient expression cloning of fungal enzyme genes. *Mol. Gen. Genet.* **243**, 253-260
11. Takashima, S., Nakamura, A., Hidaka, M., Masaki, H., and Uozumi, T. (1996) Cloning, sequencing, and expression of the cellulase genes of *Humicola grisea* var. *thermoidea*. *J. Biotechnol.* **50**, 137-147
12. Takashima, S., Nakamura, A., Masaki, H., and Uozumi, T. (1997) Cloning, sequencing, and expression of a thermostable cellulase gene of *Humicola grisea*. *Biosci. Biotechnol. Biochem.* **61**, 245-250
13. Azevedo, M.O., Felipe, M.S.S., Astolfi-Filho, S., and Radford, A. (1990) Cloning, sequencing and homologies of *cbh-1* (exoglucanase) gene of *Humicola grisea* var. *thermoidea*. *J. Gen. Microbiol.* **136**, 2569-2576
14. Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Innis, M. (1983) Molecular cloning of exocellobiohydrolase I derived from *Trichoderma reesei* strain L27. *Bio/technology* **1**, 691-696
15. Chen, C.M., Gritzali, M., and Stafford, D.W. (1987) Nucleotide sequence and deduced primary structure of cellobiohydrolase II from *Trichoderma reesei*. *Bio/technology* **5**, 274-278
16. Penttilä, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R., and Knowles, J. (1986) Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **45**, 253-263

17. Tsuchiya, K., Nagashima, T., Yamamoto, Y., Gomi, K., Kitamoto, K., Kumagai, C., and Tamura, G. (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **58**, 895-899
18. Gomi, K., Iimura, Y., and Hara, S. (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. *Agric. Biol. Chem.* **51**, 2549-2555
19. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Labolatory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
22. Béguin, P. (1990) Molecular biology of cellulose degradation. *Annu. Rev. Microbiol.* **44**, 219-248
23. Gräbnitz, F., Seiss, M., Rücknagel, K.P., and Staudenbauer, W.L. (1991) Structure of the β-glucosidase gene *bglA* of *Clostridium thermocellum*. *Eur. J. Biochem.* **200**, 301-309
24. Inoue, K., Shibuya, M., Yamamoto, K., and Ebisu, Y. (1996) Molecular cloning and bacterial expression of a cDNA encoding furostanol glycoside 26-*O*-β-glucosidase of *Costus speciosus*. *FEBS Lett.* **389**, 273-277
25. Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L., and Mornon, J.-P. (1989) Cellulase families revealed by hydrophobic clustar analysis. *Gene* **81**, 83-95
26. Cicek, M. and Esen, A. (1995) Cloning and sequencing of a cDNA coding for beta-glucosidase (Dhurrinase) from *Sorghum bicolor* (L). *Plant Physiol.* **109**, 1497-1499
27. Oxtoby, E., Dunn, M.A., Pancoro, A., and Hughes, M.A. (1991) Nucleotide and derived amino acid sequence of the cyanogenic β-glucosidase (linamarase) from white clover (*Trifolium repens* L.). *Plant Mol. Biol.* **17**, 209-219
28. Withers, S.G., Warren, R.A.J., Street, I.P., Rupitz, K., Kempton, J.B., and Aebersold, R. (1990) Unequivocal demonstration of the involvement of a glutamate residue as a nucleophile in the mechanism of a "retaining" glycosidase. *J. Am. Chem. Soc.* **112**, 5887-5889
29. Jenkins, J., Leggio, L.L., Harris, G., and Pickersgill, R. (1995) β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases from a superfamily of enzymes with 8-fold β/α architecture and with two conserved glutamates near the carboxy-terminal ends of β-strands four and seven. *FEBS Lett.* **362**, 281-285
30. Keresztessy, Z., Kiss, L., and Hughes, M.A. (1994) Investigation of the active site of the cyanogenic β-D-glucosidase (Linamarase) from *Manihot esculenta* Crantz (Cassava). II. Identification of Glu-198 as an active site carboxylate group with acid catalytic function. *Arch. Biochem. Biophys.* **315**, 323-330
31. Takashima, S., Iikura, H., Nakamura, A., Hidaka, M., Masaki, H., and Uozumi, T. (1998) Overproduction of recombinant *Trichoderma reesei* cellulases by *Aspergillus oryzae* and their enzymatic properties. *J. Biotechnol.* **65**, 163-171