Novel β-1,4-Mannanase Belonging to a New Glycoside Hydrolase Family in Aspergillus nidulans*

Received for publication, April 27, 2015, and in revised form, September 16, 2015 Published, JBC Papers in Press, September 18, 2015, DOI 10.1074/jbc.M115.661645

Motoyuki Shimizu1,†, Yuhei Kaneko3, Saaya Ishihara2, Mai Mochizuki4, Kiyota Sakai3, Miyuki Yamada3, Shunsuke Murata†, Eriko Itoh5, Tatsuya Yamamoto5, Yu Sugimura5, Tatsuya Hirano6, Naoki Takaya5, Tetsuo Kobayashi3,†, and Masashi Kato‡

From the 1Faculty of Agriculture, Meijo University, Nagoya, Aichi 468-8502, Japan, 2Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan, and 3Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan

Background: Filamentous fungi produce various mannanolytic enzymes including β-1,4-mannanases for β-mannan degradation.

Results: Fungal β-1,4-mannanase, Man134A, has an unusual sequence and substrate specificity that differs from Man5C belonging to the GH5 family.

Conclusion: Man134A is involved in β-mannan degradation in vivo.

Significance: An Aspergillus nidulans β-1,4-mannanase reveals a novel glycoside hydrolase family, GH134.

Many filamentous fungi produce β-mannan-degrading β-1,4-mannanases that belong to the glycoside hydrolase 5 (GH5) and GH26 families. Here we identified a novel β-1,4-mannanase (Man134A) that belongs to a new glycoside hydrolase (GH) family (GH134) in Aspergillus nidulans. Blast analysis of the amino acid sequence using the NCBI protein database revealed that this enzyme had no similarity to any sequences and no putative conserved domains. Protein homologs of the enzyme were distributed to limited fungal and bacterial species. Man134A released mannobiose (M2), mannotriose (M3), and mannotetraose (M4) but not mannopentaose (M5) or higher manno-oligosaccharides when galactose-free β-mannan was the substrate from the initial stage of the reaction, suggesting that Man134A preferentially reacts with β-mannan via a unique catalytic mode. Man134A had high catalytic efficiency (kcat/Km) toward mannohexaose (M6) compared with the endo-β-1,4-mannanase Man5C and notably converted M6 to M5, M3, and M2, with M3 being the predominant reaction product. The action of Man5C toward β-mannans was synergistic. The growth phenotype of a Man134A disruptant was poor when β-mannans were the sole carbon source, indicating that Man134A is involved in β-mannan degradation in vivo. These findings indicate a hitherto undiscovered mechanism of β-mannan degradation that is enhanced by the novel β-1,4-mannanase, Man134A, when combined with other mannanolytic enzymes including various endo-β-1,4-mannanases.

β-Mannan polysaccharides including glucomannan, galactomannan, and galactoglucomannan are widely distributed in nature. They constitute the main components of plant cell walls and serve as storage polysaccharides in some plants. Ivory nuts, konjac, coffee beans, and red algae contain abundant amounts of β-mannan (1–4). Glucomannan consists of a β-1,4-linked backbone containing mannose or a combination of glucose and mannose moieties, and it is acetylated at the O-2 and/or O-3 positions (5, 6). Galactomannan and galactoglucomannan have branched galactose side chains linked to backbone mannoses by an α-1,6-bond (7). Konjac glucomannan and guar gum galactomannan are storage polysaccharides that are useful to the food industry because of their gelification properties (8, 9).

Mannanolytic enzymes have recently become important natural resources for industrial biorefinery processes to produce second-generation biofuels from plant biomass (10). As β-mannans have a complex structure, a set of mannanolytic enzymes with different substrate specificities is necessary for complete degradation. Filamentous fungi produce various mannanolytic enzymes including β-1,4-mannanase (EC 3.2.1.78), α-galactosidase (EC 3.2.1.22), β-mannosidase (EC 3.2.1.25), acetylmannan esterase (EC 3.1.1.6), and β-glucosidase (EC 3.2.1.21), making these organisms excellent sources of these enzymes (7, 11, 12). Endo-β-1,4-Mannanases that randomly hydrolyze the internal β-1,4-linkage of the mannan backbone (7) are ubiquitous in viruses, bacteria, and eukaryotes (13). They are classified according to sequence similarity into the glycoside hydrolase (GH) families GH5, GH26, and GH113 in the Carbohydrate-Active enZYmes (CAZy) database, and filamentous fungi produce β-1,4-mannanases of the GH5 and GH26 families (14).

Aspergillus nidulans is a classical model eukaryote for genetic and biological studies, and it produces β-1,4-mannanases and other glycoside hydrolases (12, 14). Fifteen and three genes belonging to the GH5 and GH26 families, respec-
tively, have been identified in the *A. nidulans* genome based on the CAZy database. Among them, secreted proteins encoded by ANID_03297, ANID_03358, and ANID_06427 have known β-1,4-mannanase activity (15–18).

The production of mannanolytic enzymes is generally induced in the presence of β-mannans and regulated by the transcription factor ManR in *Aspergillus oryzae* (19, 20). Growth on konjac glucomannan is significantly decreased by disrupting manR. ManR-regulated genes not only include orthologs and homologs of extant glycoside hydrolase genes but also hypothetical protein genes. These findings imply that β-mannan-induced and/or ManR-regulated hypothetical genes are involved in β-mannan degradation.

Here, we investigated the enzymatic functions of a secreted hypothetical protein of which the production was induced by β-mannans. The protein shared no homology to extant β-mannanases but displayed hydrolytic activity toward β-mannan. Furthermore, the protein had no homology to any proteins with known functions, and thus we propose that the protein is a novel β-1,4-mannanase belonging to a new GH family. The β-1,4-mannanase reacted with β-mannan and manno-oligosaccharides with mannotriose recognition and had high catalytic efficiency (*k*_cat/*K*_m) toward mannohexaose (M6) compared with the endo-β-1,4-mannanase Man5C belonging to a GH5 family, indicating that the enzyme had a unique catalytic property. Moreover, the novel β-1,4-mannanase had a synergistic effect with Man5C toward glucomannan and galactomannan, suggesting that would be useful for diverse industrial applications including conversion technology of lignocellulosic biomass. We also show that the novel β-1,4-mannanase plays a critical role in β-mannan degradation in vivo.

**Experimental Procedures**

**Chemicals**—Mannobiose (M₃), mannotriose (M₄), mannolactose (M₅), mannopentaose (M₆), mannohexaose (M₇), glucomannan, galactomannan, and galactose-free β-mannan (prepared from carob galactomannan with removal of all α-galactosyl residues by α-galactosidase) were purchased from Megazyme International (Bray, Ireland). Microcrystalline cellulose (MCC; Funakoshi, Tokyo, Japan), carboxymethylcellulose (CMC; Hercules Inc., Wilmington, DE), xylan from beech wood (Sigma), and chitin (Wako Pure Chemical Industries, Osaka, Japan) served as the carbon sources in cultures and in enzymatic assays.

**Strains, Cultures, and Media**—*A. nidulans* strain A26 (biA1) and ABPU1 (biA1, pyrG89, wA3, argB2, pyroA4) were obtained from the Fungal Genetic Stock Center (Kansas State University, Manhattan, KS) and cultured on MM agar medium (10 mM NaNO₃, 10 mM KH₂PO₄, 7 mM KCl, 2 mM MgSO₄, 2 ml/liter Hutton’s trace metals, 1.5% agar (pH 6.5)) containing 0.01–1.0% glucose, mannose, xylan, chitin, MCC, CMC, β-glucanomannan, or β-galactomannan as the sole carbon source at 37 °C. Conidia (1 × 10⁵) of *A. nidulans* and *A. oryzae* RIB40 obtained from NITE Biological Resource Center were transferred to 300-ml flasks containing 30 ml of MM medium with 1.0% glucomannan (w/v) and rotary shaken at 120 rpm at 37 °C. Arginine (0.2 mg liter⁻¹), pyridoxine (0.1 mg liter⁻¹), uracil and uridine (1.12 and 1.2 g liter⁻¹), and biotin (0.25 mg liter⁻¹) were added to the culture medium for auxotrophic mutants.

**Protein Identification**—Mycelia were cultured in MM media containing several carbon sources for 24 h at 37 °C. Extracellular proteins from culture filtrates were separated by SDS-PAGE and two-dimensional gel electrophoresis and then stained with Coomassie Brilliant Blue. Protein bands and spots were excised from the gels, digested with trypsin, and analyzed using MALDI-TOF/TOF-MS as described (21, 22). Peptide mass fingerprints and MS/MS spectra were analyzed using the Mascot search engine (Matrix Science Ltd., London, UK) as described (21, 22).

**Quantitative PCR**—Single-strand cDNA was synthesized from total RNA extracted from disrupted fungal cells, and then quantitative PCR proceeded as described (23). Table 1 shows the gene-specific primers. The expression of each gene was normalized against that of the actin gene (*actA*). Results are shown as relative expression.

**Gene Disruption of ANID_02710**—Table 1 lists the primers used for gene disruption. PrimeSTAR HS DNA polymerase (Takara Bio, Otsu, Japan) was used for PCR. The 5’- and 3’-untranslated regions (1 kb) of the ANID_02710 gene (*ANID* is the prefix of gene identifiers in the *Aspergillus* Comparative Database at the Broad Institute) were amplified by PCR using *A. nidulans* DNA and the respective primer pairs (Table 1). The *argB* gene was amplified using *A. nidulans* A26 genomic DNA and *argB*-f and *argB*-r primers. The three DNA fragments were fused by PCR using primers an2710–5-f and an2710–3-r to generate an ANID_02710 disruption cassette that was introduced into the ABPU1 strain as described (23, 24) to create the ANID_02710 deletion strain ANID_02710Δ. Targeted gene disruption was confirmed by Southern blotting fungal total DNA using a DIG DNA labeling and detection kit (Roche Diagnostics) according to the manufacturer’s instructions.

**Introducing ANID_02710 into Gene Disruptant**—A genomic DNA fragment encoding the ANID_02710 gene with additional NotI recognition sequences was amplified using primers shown in Table 1. The DNA fragments were digested with NotI and ligated with pBSpyrG (23) that had been spliced with the same restriction enzyme to generate pAN2710, which was then introduced into strain ANID_02710Δ.

**Preparation of Recombinant Proteins**—We prepared ANID_02710 cDNA by PCR using the *A. nidulans* cDNA and a set of oligonucleotide primers (Table 1). The PCR product was purified, digested by HindIII and XhoI, and then ligated to pET28a (Novagen, Darmstadt, Germany) that had been digested with pETAN2710 cDNA by PCR using the *A. nidulans* cDNA and a set of oligonucleotide primers (Table 1). The PCR product was purified, digested by HindIII and XhoI, and then ligated to pET28a (Novagen, Darmstadt, Germany) that had been digested with the same restriction enzymes to generate pETAN2710. The pETAN2710 was introduced into *Escherichia coli* BL21-CodonPlus(DE3) (Novagen) and cultured in LB containing 50 μg ml⁻¹ kanamycin sulfate for 16 h, and then a portion (2 ml) was agitated at 120 rpm in 200 ml of LB containing 50 μg ml⁻¹ kanamycin sulfate at 28 °C. After the optical density reached 1.0, isopropyl-thio-β-D-galactoside (0.2 mM) was added to the medium, and the flask were shaken at 120 rpm for 12 h at 28 °C. The *E. coli* cells were harvested, suspended in 50 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, and disrupted by sonication. Cell-free extract was obtained from the suspension after centrifugation at 6,000 × g for 15 min, and...
Fungal β-1,4-Mannanase Belonging to New GH134 Family

Table 1
Oligonucleotide primers used in this study

| Primers          | Gene    | Nucleotide sequence                          | Application |
|------------------|---------|----------------------------------------------|-------------|
| Real time PCR    | RTan2710-f | 5'-GGGACCCCTCTTCTATCTTATCCCTTTG-3'           |             |
|                  | RTan2710-r | 5'-AAGATCTCTTCTCCTGAACTGGATTTTTTGGTAT-3'    |             |
|                  | RTan6427-f | 5'-CAATTCGATTTAAAGTCAACGATGACTTAAATCTGAATTG-3' |             |
|                  | RTan6427-r | 5'-CTCGAGGACTTGAATGACTGCTTACAGCTTAT-3'      |             |
|                  | RTan9276-f | 5'-GAGGGAATGACTGGTCTGGA-3'                   |             |
|                  | RTan9276-r | 5'-TAACGGGTGACCATTTCCTT-3'                   |             |
|                  | RTan2709-f | 5'-ACAGTGAACCTGGATTTTTTGGTAT-3'             |             |
|                  | RTan2709-r | 5'-GCGGAAAAGCTCGGATGTCTA-3'                  |             |
|                  | RTao0945-f | 5'-ATGGAACCGAGATCTGTAAGTT-3'                 |             |
|                  | RTao0945-r | 5'-ATGGAACCGAGATCTGTAAGTT-3'                 |             |

Construction of gene disruption cassette

| an2710–5-f | an2710–5-r | an2710–3-f | an2710–3-r |

Plasmid for expressing ANID_02710 in A. nidulans

| an2710RE-f | an2710RE-r | RTan2710-r |

Plasmids for recombinant protein production

| Man5C-f | Man5C-r |
|---------|---------|

then soluble fractions were separated by further centrifugation at 100,000 × g for 30 min. These fractions were passed through a column (φ5 × 20 mm) containing nickel-nitrilotriacetic acid-agarose (QiAgen, Hilden, Germany). The column was washed with 10 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM imidazole, and proteins were eluted with the same buffer containing 300 mM imidazole. After dialysis with 20 mM Tris-HCl (pH 8.0), the purified recombinant proteins were eluted with 20 mM Tris-HCl containing 300 mM imidazole. The deglycosylated solution was concentrated protein solution was fractionated on a HiTrap Q column (GE Healthcare) using a linear gradient of NaCl (0–0.5 M) in buffer A (50 mM Tris-HCl, pH 8.0). Fractions containing Man5C or ANID_02710 product were applied to a Superose 6 10/300 GL column (GE Healthcare) equilibrated with buffer A containing 150 mM NaCl and dialyzed against 20 mM Tris-HCl (pH 8.0). All protein purification steps proceeded at 4 °C.

A cDNA fragment encoding the ManSC gene (manC; ANID_06427) was digested with KpnI and NotI and ligated into pPICZα-A (Invitrogen) that had been digested with the same restriction enzymes to generate pMan5C. The plasmid was introduced into Pichia pastoris KM71H (Invitrogen), and the resulting strain was cultured to produce recombinant Man5C as described (17, 18). The culture supernatant was concentrated using an Amicon Ultra filter unit (Merck-Millipore). The concentrated protein solution was fractionated on a HiTrap Q column (GE Healthcare) using a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl (pH 8.0). The sample was then digested with endoglucosidase H (New England Biolabs, Ipswich, MA) according to the manufacturer’s instructions to remove N-linked glycans. The deglycosylated solution was applied to a Superose 6 10/300 GL column (GE Healthcare), and recombinant proteins were eluted with 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and dialyzed against 20 mM Tris-HCl (pH 8.0). All protein purification steps proceeded at 4 °C. Protein concentrations were assayed using Bradford Protein Assays (Bio-Rad) with bovine serum albumin as the standard.

Purification of Proteins from Culture Filtrate—A. nidulans

was cultured in 2 liters of MM medium with 1.0% glucose as the sole carbon source for 36 h. The culture filtrate was applied to a column containing DEAE-cellulose (GE Healthcare) and equilibrated with buffer A (50 mM Tris-HCl, pH 8.0). Proteins were eluted from the column using a linear gradient of NaCl (0–0.5 M) in buffer A. Fractions containing Man5C or ANID_02710 were pooled, dialyzed against buffer A, and then eluted with a linear gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 1.0 ml min⁻¹. Fractions containing Man5C or ANID_02710 product were applied to a Superoxide 6 10/300 GL column (GE Healthcare) equilibrated with buffer A containing 150 mM NaCl and eluted at a flow rate of 0.5 ml min⁻¹. All of these steps proceeded at 4 °C. Fractions containing Man5C or ANID_02710 product served as purified preparations.

Enzyme Assays—β-1,4-Mannanase activity was assayed in 0.5-ml reaction mixtures containing 50 mM sodium phosphate (pH 6.0), 0.2–5% (w/v) substrates, and purified proteins or culture supernatants. Reactions were incubated at 37 °C and then stopped by boiling at 100 °C for 10 min. The reducing sugars produced by β-1,4-mannanase were measured using tetrazonium blue as described (25, 26). Standard curves were prepared based on solutions containing different concentrations of mannose. One unit of β-1,4-mannanase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar (mannose equivalents) per min. The effect of temperature on the activity was determined using 1.0%
Glucomannan as a substrate in 50 mM sodium phosphate (pH 6.0). The optimum temperature was determined by measuring the activity over the range of 30–70 °C for 15 min. The optimum pH was determined using 50 mM sodium acetate (pH 3.0–6.0), 50 mM sodium phosphate (pH 5.0–7.0), and 50 mM Tris-HCl (pH 7.0–10.0) and assayed over a range of 3.0–10.0 at 37 °C for 15 min.

The reaction products released from glucomannan, galactose-free β-mannan and manno-oligosaccharides were separated on TLC Silica gel 60 plates (Merck-Millipore) using n-butanol:ethanol:water (10:8:7), visualized by staining with 0.82% (v/v) N-(1-naphthyl)ethylenediamine dihydrochloride and 8.6% (v/v) sulfinic acid in ethanol, and baked at 105 °C for 5 min. The soluble products released from galactose-free β-mannan and manno-oligosaccharides were determined by monitoring post-column derivatized reducing sugars that were separated using a Prominence reducing-sugar HPLC analytical system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector. The supernatant was separated on a Shim-pack ISA-07/S2504 column (4.0 × 250 mm, Shimadzu, Kyoto, Japan) with a linear gradient of 0.1 M potassium borate buffer (pH 8.0) and 0.4 M potassium borate buffer (pH 9.0) for 90 min at a flow rate of 0.6 ml min⁻¹.

Table 2

Proteins identified in the secretome of A. nidulans cultured with glucomannan as sole carbon source

| No. | Annotation name | Gene ID | Score | MMr | Cov. | Identified peptide sequence |
|-----|-----------------|---------|-------|-----|------|-----------------------------|
| 1   | Putative chitinase (GH18) | ANID_04871 | 172   | 44.2| 44   | ILGMP1YGR                   |
| 2   | Endo-β-1,4-Mannanase (GH5) | ANID_06427 | 164   | 45.3| 34   | LEAVGGWQSISLR, WIVDHAR, LEAVGGWQSISLR |
| 3   | Putative endo-β-1,4-mannanase (GH5) | ANID_07639 | 71    | 44.2| 21   | YVDSPAIFAWELANEPR          |
| 4   | Putative endo-β-1,4-mannanase (GH5) | ANID_09276 | 80    | 41.7| 22   | 127                        |
| 5   | Putative trypsin-like protease | ANID_02366 | 84    | 25.4| 22   | AGYGGVYSSPARYR              |
| 6   | Putative dipeptidyl-peptidase | ANID_02572 | 63    | 79.4| 13   | FVAYAQSRY                   |
| 7   | Hypothetical protein | ANID_02710 | 136   | 20.9| 54   | 88                         |

*Protein names and accession numbers are according to Aspergillus nidulans Genome Database.

The amount of each product was quantified using manno-oligosaccharides with degree-of-polymerization (DP) values of 1 to 6 as standards. Initial rates of hydrolysis of manno-
oligosaccharides by \( \beta-1,4 \)-mannanases were determined by HPLC as decreases in substrates. The kinetic parameters \( K_m \) and \( k_{cat} \) were calculated by fitting the Michaelis-Menten equation to initial rates using Origin Version 6.0 software (OriginLab, Northampton, MA).

**Other Methods**—The hydrolysis products of galactose-free \( \beta \)-mannan were analyzed using MALDI-TOF-MS as described (18). Amino acid sequences were aligned using ClustalW (27). Dry mycelial weight was determined as described (28). Phylogenetic analyses of full-length amino
acid sequences proceeded by adapting the neighbor-joining method using MEGA 6 software (29).

Results

Identification of Extracellular Proteins Induced by β-Mannans—The extracellular proteins of A. nidulans produced using various sole carbon sources were analyzed by SDS-PAGE (Fig. 1A). Whereas proteins were undetectable with glucose and mannose, three major bands (Fig. 1A, arrows 2, 5, and 7) comprising several proteins were detected with galactomannan and glucomannan, and seven of them were identified by peptide mass fingerprinting and MS/MS spectrum analysis using MALDI-TOF/TOF-MS (Fig. 1A and Table 2). The three major proteins were endo-β-1,4-mannanase belonging to the GH5 family (no. 2; Man5C, ANID_06427), a protease (no. 5; ANID_02366), and a hypothetical protein (no. 7; ANID_02710) (see Table 2). The others included two more GH5 family endo-β-1,4-mannanases (nos. 3 and 4; ANID_07639 and ANID_09276). All of them possessed signal peptides based on prediction by the downloadable SignalP server 4.0 (Technical University of Denmark, Lyngby, Denmark). The extracellular proteins of A. nidulans grown in MM medium containing glucomannan as the sole carbon source were also resolved by two-dimensional gel electrophoresis (Fig. 1B), which also identified the same proteins as those found by SDS-PAGE. These results indicated that A. nidulans grown with glucomannan predominantly secreted endo-β-1,4-mannanase (no. 2; ANID_06427) and the hypothetical protein (no. 7; ANID_02710).

Regulation of the Genes Encoding the Proteins Produced in the Glucomannan and Galactomannan Media—Gene transcripts were quantified using real-time PCR. A. nidulans generated 19-, 18-, and 29-fold more endo-β-1,4-mannanase (Man5C; ANID_06427), putative β-mannanase (ANID_09276), and hypothetical protein (ANID_02710) transcripts, respectively, on glucomannan compared with glucose (Fig. 2). These genes were not induced in A. nidulans grown with mannose, xylan, and chitin as the sole carbon sources (Fig. 2). Galactomannan was more effective than glucose for expression of the genes reaching 41-, 72-, and 100-fold, respectively (Fig. 2). Both MCC and CMC slightly induced the expression of these genes (Fig. 2).

In addition to the β-1,4-mannanases, ANID_02710 protein with a molecular mass of 18 kDa was produced in the presence of β-mannans (Fig. 1). These results indicated that β-mannans-induced expression of the genes encoding ANID_02710 and endo-β-1,4-mannanases belonging to the GH5 family. The hypothetical protein XP_001825366 encoded by the AO090038000445 gene in A. oryzae was orthologous to the ANID_02710 product with 70% amino acid sequence identity (19). Expression levels of the A. oryzae gene were 62- and 37-fold higher on galactomannan and glucomannan as compared with glucose. The β-mannan induced expression of ANID_02710 and its ortholog AO090038000445, suggesting that these proteins are involved in β-mannan degradation. Amino acid sequence analysis using the NCBI protein database did not reveal any putative conserved domains in the hypothetical protein.

The ANID_02709 gene encoding a putative β-1,4-mannanase adjoins ANID_02710, but a transcript encoding ANID_02709 was undetectable (data not shown), implying that ANID_02709 is not involved in β-mannan degradation. These findings also indicated that ANID_02709 and ANID_02710 gene expression is differently regulated in the presence of β-mannans.

ANID_02710 Is Involved in β-Mannan Utilization—We constructed a disruptant of the gene that expresses ANID_02710. We prepared an ANID_02710 gene disruption cassette that is designed to double-crossover with the fungal chromosome at the 5′- and 3′-regions of ANID_02710 and then introduced it into A. nidulans. Southern blotting and PCR analysis of total DNA from the transformant ANID_02710Δ revealed that ANID_02710 was deleted from the strain (Fig. 3A). Compared with the wild type strain (WT), the phenotype of ANID_02710Δ growth was poor on agar containing either gluco-
Fungal β-1,4-Mannanase Belonging to New GH134 Family

nan or galactomannan as the sole carbon source (Fig. 3, B and C). The hyphal growth of the ANID_02710Δ and WT strain on agar containing any of glucose, mannos, xylose, chitin, MCC, or CMC as the sole carbon source did not significantly differ (Fig. 3, D–I). Introducing the ANID_02710 gene into ANID_02710Δ restored growth in glucosmanan and galactomannan medium, suggesting that that ANID_02710 protein plays a specific role in fungal β-mannan degradation.

**ANID_02710 Product Has Mannanolytic Activity**—A purified recombinant ANID_02710 product was generated as an

---

**FIGURE 5. Hydrolysis of galactose-free β-mannan by purified ANID_02710 product.** A, MALDI-TOF-MS analysis of hydrolytic products from galactose-free β-mannan generated by ANID_02710 product. Recombinant ANID_02710 product (1.0 μM) was incubated with 0.5% galactose-free β-mannan in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C for 60 min, and then reaction products were detected as corresponding sodium adducts (molecular weight + 23). B, hydrolysis products of galactose-free β-mannan incubated without (blank) or with recombinant ANID_02710 product for 10–60 min monitored using HPLC. C, TLC analysis of soluble products in reaction mixtures of ANID_02710 product with galactose-free β-mannan. Recombinant ANID_02710 product (1.0 μM) was incubated with 0.5% substrate in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C for 10 to 60 min. M₁, mannos; M₂, mannobiose; M₃, mannotriose; M₄, mannotetraose; M₅, mannopentaose; M₆, mannohexaose. Std, manno-oligosaccharides standard (0.5 mM each).
N-terminal His<sub>6</sub>-tagged protein in the E. coli expression system as described under “Experimental Procedures.” The recombinant ANID_02710 product migrated as a single band on SDS-PAGE at a molecular mass of 18 kDa after removal of the His<sub>6</sub> tag by thrombin digestion followed by purification (Fig. 4A). The hydrolase activity of the recombinant ANID_02710 product was significant toward /H<sub>9252</sub>-mannans but undetectable toward xylan, chitin, MCC, or CMC (Fig. 4B). We also purified native ANID_02710 product from culture supernatants with 40% recovery using three chromatographic separations, each of which resulted in a single band on SDS-PAGE (Fig. 4C). The native ANID_02710 product was essentially similar to the recombinant ANID_02710 product generated in the E. coli expression system (data not shown).

**ANID_02710 Product Is a Novel Glycoside Hydrolase**—The reaction products of galactose-free β-mannan generated by recombinant ANID_02710 product were analyzed using MALDI-TOF-MS (Fig. 5A). Considering that polysaccharides are degraded by hydrolases, lyases, and lytic polysaccharide monooxygenases, the reaction products were analyzed using MS to determine the type of reaction that the ANID_02710 product catalyzes. The molecular weights of the three products detected by MALDI-TOF-MS were identical to those of the Na<sup>+</sup> adducts of mannobiose (M<sub>2</sub>), mannnotriose (M<sub>3</sub>), and mannnotetraose (M<sub>4</sub>) (Fig. 5A). The soluble products from galactose-free /H<sub>9252</sub>-mannan produced by recombinant ANID_02710 product were also analyzed using HPLC and TLC (Fig. 5B and C). Adding recombinant ANID_02710 product to the reaction mix resulted in the generation of M<sub>2</sub>, M<sub>3</sub>, and M<sub>4</sub>, whereas mannose (M<sub>1</sub>), mannopentaose (M<sub>5</sub>), and mannohexaose (M<sub>6</sub>) were undetectable (Fig. 5B and C). These results indicated that the ANID_02710 product is a novel /H<sub>9252</sub>-1,4-mannanase that belongs to a new family of glycoside hydrolases (family GH134 in the CAZy classification). Thus, we designated the recombinant ANID_02710 product as a /H<sub>9252</sub>-1,4-mannanase belonging to the glycoside hydrolase 134 family (GH134). Homologs of Man134A are distributed in some Proteobacteria, Actinobacteria, Zygomycota, Basidiomycota, and Ascomycota including aspergilli except for Aspergillus niger (Fig. 6 and Table 3). Fig. 7

**TABLE 3**

| Species                     | Number of Man134A homologs<sup>a</sup> |
|-----------------------------|----------------------------------------|
| Aspergillus clavatus        | 2                                      |
| Aspergillus fumigatus       | 1                                      |
| A. nidulans                 | 4                                      |
| A. oryzae                   | 2                                      |
| A. terreus                  | 1                                      |
| Blastomyces dermatitidis    |                                        |
| Botrytis cinerea            |                                        |
| Candida albicans            |                                        |
| Candida guilliermondii      |                                        |
| Claviceps fusiformis        |                                        |
| Coccidiotheca immitis       |                                        |
| Fusarium fujikuroi          | 1                                      |
| Fusarium graminearum        | 1                                      |
| F. oxysporum sp.            | 1                                      |
| F. verticilloides           | 1                                      |
| Histoplasma capsulatum      |                                        |
| Lodderomyces elongisporus   |                                        |
| Neurospora crassa           |                                        |
| Microsporus canis           |                                        |
| Microsporus gyipseum        | 5                                      |
| Penicillium expansum        |                                        |
| Pheosphaeria nodorum        |                                        |
| Pyrenophora tritici-repentis|                                        |
| Schizosaccharomyces cryophilus|                                       |
| Schizosaccharomyces japonicus|                                       |
| Schizosaccharomyces octopus |                                        |
| Schizosaccharomyces pombe   | 4                                      |
| Sclerotinia sclerotiorum    |                                        |
| Stachybotrys chartarum      |                                        |
| Trichoderma reesei          |                                        |
| Trichophyton equinum        |                                        |
| Trichophyton rubrum         |                                        |
| Trichophyton tonsurans      |                                        |
| Uncinocarpus reesi          |                                        |
| Verticillium dahliae        | 1                                      |
| Zygomycota                  |                                        |
| Mucor ambigius              | 3                                      |
| Mucor circinelloides        | 1                                      |
| R. delemar                  | 9                                      |
| Rhizopus microsporus        | 5                                      |
| Basidiomycota               |                                        |
| Botryobasidium botryosum    | 1                                      |
| Coprinopsis cinerea         |                                        |
| Cryptococcus neoformans     |                                        |
| Galerina marginata          | 1                                      |
| Phanerochaete chrysosporium |                                        |
| Puccinia graminis f. sp.    |                                        |
| Puccinia tricitina          |                                        |
| Serendipita vermifera       | 2                                      |
| Sphaerobolus stellatus      | 1                                      |
| Ustilago maydis             |                                        |

<sup>a</sup> Man134A homologs include amino acid sequences having at least 50% identity to Man134A (ANID_02710).
shows the sequence alignment of the Man134A orthologs. The LAIXMLE and WFXGHRNG motifs in the N-terminal and C-terminal regions, respectively, were highly conserved, suggesting that the motifs play important roles for the functions of GH134 family proteins (Fig. 7).

Comparison of Biochemical Properties between Man134A and Man5C—We compared the biochemical properties of the major mannanolytic enzymes Man134A and Man5C that were produced when glucomannan was the sole carbon source to understand how they each contribute to /H9252-mannan hydrolysis.

We prepared recombinant Man5C (rMan5C) in the P. pastoris expression system as described (17, 18). The optimal temperature and pH for the activities of the recombinant Man134A (rMan134A) and rMan5C were determined using glucomannan as a substrate (Fig. 8). The optimal reaction conditions for rMan134A and rMan5C were 30 °C and 50 °C, respectively (Fig. 8, A and B). The optimal pH for rMan134A activity was 6.0, and it preferred a neutral pH environment, whereas that for rMan5C activity was 4.0 (Fig. 8, C and D). These results were similar to those of native Man134A and Man5C purified from A. nidulans (data not shown). We investigated the kinetic parameters of Man134A and Man5C to determine the profiles of glucomannan and galactomannan hydrolysis (Table 4). The catalytic efficiency (kcat/Km) of rMan134A was 6.5-fold higher toward glucomannan (kcat/Km = 330 ml s⁻¹ mg⁻¹) than galactomannan (kcat/Km = 51 ml s⁻¹ mg⁻¹), both of which were slightly lower than those of rMan5C. Together with the activity-pH profiles in Fig. 8, these results imply that Man134A mainly contributes to /H9252-mannan hydrolysis within a near-neutral range of pH.

We analyzed the reaction products of linear manno-oligosaccharides generated by rMan134A and rMan5C. Recombinant Man5C hydrolyzed manno-oligosaccharides with a DP /H11022 4 (Fig. 9 A) and exhibited transglycosylation activity, which confirmed previous findings of recombinant Man5C expression in P. pastoris (17, 18). Although rMan134A did not hydrolyze M2, M3, and M4 (Fig. 9 B), it produced M2 and M3 from M5 (Figs. 9 B and 10 A) and notably converted M6 to M2, M3, and M4 with M3 being the predominant reaction product (Figs. 9 B and 10 B). These findings suggested that the action of Man134A against M6 differs from that of other /H9252-1,4-mannanases (26, 30–33).

We also determined the transglycosylation activity of Man134A toward M6 in the range of 10, 20, and 30 mM together with 25, 50, and 100 mM M4 as a non-cleaved acceptor. However, transglycosylation activity was not detected (data not shown). These results indicated that Man134A has a unique
Catalytic property that hydrolyzes manno-oligosaccharides with a degree of polymerization \( \leq 5 \), which was consistent with the results of the galactose-free \( \beta \)-mannan hydrolysis (Fig. 5).

The substrate specificity of rMan134A toward manno-oligosaccharides was analyzed (Table 5). The catalytic efficiency \( (k_{cat}/K_m) \) of rMan134A was higher toward M6 than M5, suggesting that manno-oligosaccharides with a higher DP are preferable substrates for Man134A. The \( k_{cat}/K_m \) value of rMan134A toward M6 was 20-fold higher than that of rMan5C, whereas the \( k_{cat}/K_m \) values of both enzymes were similar toward M5 (Table 5).

Synergistic Action of Man134A and Man5C upon Hydrolysis of \( \beta \)-Mannans—rMan134A, rMan5C, and mixtures of the enzymes at various ratios were used for \( \beta \)-mannan hydrolysis to determine whether or not the enzymes have synergistic action. Fig. 11A shows significant increases in the production of reducing sugars from glucomannan (solid lines) with the enzymes at any ratio compared with the calculated sums of specific activities and amounts of enzymes (dashed lines). After a 120-min reaction, the production of reducing sugars reached 2.4- and 2.3-fold of the calculated values at Man134A:Man5C ratios of 10:90 and 25:75, respectively (Fig. 11A). Synergistic hydrolysis

---

**FIGURE 8. Temperature and pH optima of rMan134A and rMan5C.** A and B, optimal temperature of rMan134A (A) and rMan5C (B). Enzyme reactions proceeded at temperatures ranging from 30 °C to 70 °C for 15 min. C and D, optimal pH of rMan134A (C) and rMan5C (D). Enzyme reactions proceeded over pH range 3.0–10.0. Buffers were 50 mM sodium acetate (pH 3.0–5.0; ●), 50 mM sodium phosphate (pH 5.0–7.0; ■), and 50 mM glycine-NaOH (pH 7.0–10.0; ▲). Error bars are shown as the means ± S.E. of three independent experiments.

**FIGURE 9. TLC profiles of hydrolytic products generated by rMan5C and rMan134A from linear manno-oligosaccharides.** A and B, various manno-oligosaccharides (3 mM) were digested with rMan5C (A) and rMan134A (B) at 37 °C for 60 min, and reaction products were compared. Plus (+) and minus (−) indicate the presence and absence of 2.0 mM enzymes. M1, mannose; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M5, mannopentaose; M6, mannohexaose; Std, manno-oligosaccharides standard (0.5 mM each).

**TABLE 4**

| Enzyme | Substrate       | \( K_m \) \( \text{mg/ml} \) | \( k_{cat} \) \( \text{s}^{-1} \) | \( k_{cat}/K_m \) \( \text{ml s}^{-1} \text{mg}^{-1} \) |
|--------|----------------|--------------------------|------------------------|---------------------|
| rMan134A | Glucomannan    | 1.2 ± 0.1                | 390 ± 30               | 330                 |
|         | Galactomannan  | 4.7 ± 0.2                | 240 ± 20               | 51                  |
| rMan5C  | Glucomannan    | 0.81 ± 0.1               | 540 ± 40               | 620                 |
|         | Galactomannan  | 2.7 ± 0.2                | 200 ± 20               | 74                  |
by the two enzymes was also evident with galactomannan as a substrate (Fig. 11B).

**Man134A Is Involved in β-Mannan Degradation under Neutral pH**—We investigated the physiological importance of Man134A in β-mannan utilization in A. nidulans. Disrupting the Man134A gene affected fungal growth rates in liquid MM medium containing glucomannan as the sole carbon source (liquid GM medium). The growth of Man134AΔ (ANID_02710Δ was changed to Man134AΔ), and the WT strains were similar in liquid GM medium at acidic pH, whereas that of the Man134A strain was defective at neutral pH (Fig. 12A). By contrast, glucose did not affect the hyphal growth of Man134AΔ at either acidic or neutral pH (Fig. 12B). We investigated the time course of the growth of Man134AΔ and the WT strains and β-1,4-mannanase activities toward glucomannan (Fig. 12C, D). After incubation for 1 day, the dry weight of mycelia from the Man134AΔ strain was 38% that of the WT in liquid GM medium at pH 6.5 (Fig. 12C), and the β-1,4-mannanase activity of Man134AΔ toward glucomannan was 21% that of the WT (Fig. 12D). The expression of extracellular proteins other than Man134A remained essentially unchanged between WT and Man134AΔ during culture (Fig. 12E), indicating that Man134A is the predominant β-1,4-mannanase involved in β-mannan degradation under conditions of neutral pH in A. nidulans.

**Discussion**

Here we identified a novel β-1,4-mannanase that shared no amino acid sequence homology with any extant β-1,4-mannanases or to any proteins with a known function and apparently lacked a conserved motif sequence. The glycoside hydrolase family GH134 was created for the enzyme in the CAZy database based on the present findings.

Filamentous fungi secrete various mannanolytic enzymes including endo-β-1,4-mannanases, β-mannosidases, and accessory enzymes such as α-galactosidas and acetylmann esteras that all act in concert for efficient β-mannan hydrolysis. Expression of the mannanolytic and cellulosytic enzyme genes is controlled by the transcriptional activator ManR in response to manno-oligosaccharides as well as cellulose in A. oryzae (19, 20). The genes encoding the hypothetical protein XP_001825366 and β-1,4-mannanase ManG, which are orthologs of Man134A and Man5C, are also regulated by ManR in A. oryzae. The ortholog of ManR in A. nidulans is ClrB, which regulates genes encoding cellulosytic and hemicellulosytic enzymes in response to cellulose (34, 35). The ClrB target genes comprise various mannanolytic enzyme genes including the Man5C gene (*manC*). However, the Man134A gene is not included among the ClrB targets (35), suggesting that another transcription factor(s) is responsible for inducing expression of the gene. In fact, A. oryzae and A. nidulans differ in terms of regulation of the genes encoding mannanolytic enzymes.

**TABLE 5**

| Enzyme         | Substrate          | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) |
|----------------|--------------------|------------|---------------------|-------------------------------------|
| rMan134A       | Mannotetraose (M₄) | 1.6 ± 0.1  | 76 ± 0.1            | 48                                  |
|                | Mannopentaose (M₅) | 0.083 ± 0.01 | 360 ± 10           | 4600                                |
| rMan5C         | Mannotetraose (M₄) | 3.0 ± 0.2  | 19 ± 2              | 63                                  |
|                | Mannopentaose (M₅) | 2.1 ± 0.1  | 120 ± 10            | 57                                  |
|                | Mannohexaose (M₆)  | 0.53 ± 0.1 | 120 ± 10            | 230                                 |

**FIGURE 10.** Hydrolysis of mannopentaose and mannohexaose by rMan134A. Reaction products of mannopentaose (A) and mannohexaose (B) catalyzed by rMan134A were sequentially monitored using HPLC. $M_i$: mannose; $M_2$: mannobiose; $M_3$: mannotriose; $M_4$: mannopentaose; $M_5$: mannopentaose; $M_6$: mannohexaose. **FIGURE 11.** Effect of glucose on the growth of Man134A. A. oryzae strains harboring the Man5C and Man134A genes were incubated with linear manno-oligosaccharides in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C. Recombinant Man5C (1.0 μM) was incubated with manno-oligosaccharides in 50 mM sodium acetate buffer (pH 4.0) at 50 °C.

**FIGURE 12.** Expression patterns of the Man5C and Man134A genes during culture. **A** and **B**. The expression of extracellular proteins other than Man134A remained essentially unchanged between WT and Man134AΔ during culture (Fig. 12E), indicating that Man134A is the predominant β-1,4-mannanase involved in β-mannan degradation under conditions of neutral pH in A. nidulans. **C**. Disrupting the Man134A gene affected fungal growth rates in liquid MM medium containing glucomannan as the sole carbon source (liquid GM medium). The growth of Man134AΔ (ANID_02710Δ was changed to Man134AΔ), and the WT strains were similar in liquid GM medium at acidic pH, whereas that of the Man134A strain was defective at neutral pH (Fig. 12A). By contrast, glucose did not affect the hyphal growth of Man134AΔ at either acidic or neutral pH (Fig. 12B). We investigated the time course of the growth of Man134AΔ and the WT strains and β-1,4-mannanase activities toward glucomannan (Fig. 12C, D). After incubation for 1 day, the dry weight of mycelia from the Man134AΔ strain was 38% that of the WT in liquid GM medium at pH 6.5 (Fig. 12C), and the β-1,4-mannanase activity of Man134AΔ toward glucomannan was 21% that of the WT (Fig. 12D). The expression of extracellular proteins other than Man134A remained essentially unchanged between WT and Man134AΔ during culture (Fig. 12E), indicating that Man134A is the predominant β-1,4-mannanase involved in β-mannan degradation under conditions of neutral pH in A. nidulans.
FIGURE 11. Synergistic action of Man134A and Man5C for hydrolysis of β-mannans. A and B, synergy plots of reducing sugar production from glucomannan (A) and galactomannan (B) by Man134A and Man5C (ratios of Man134A:Man5C of 100:0, 90:10, 50:50, 25:75, 10:90, 0:100) after incubation for 15 min (circles), 30 min (triangles), 60 min (squares), and 120 min (diamonds). Man134A and Man5C (0.4 μM as a total) were incubated with 1.0% substrate in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C. Dashed lines indicates the theoretical sums of reducing sugar production by the enzymes when there is no synergy.

Although ManR is crucial for the β-mannan- and cellulose-induced expression of both mannanolytic and cellulolytic enzyme genes in A. oryzae, expression of the mannanolytic enzyme genes in A. nidulans is controlled by ClrB and its paralog ManS, which is not found in A. oryzae (36). Expression of the Man134A gene is regulated by the ClrB paralog.3

Our results indicated that Man134A plays an important role for growth on β-mannans at neutral pH. Some fungal β-mannanases including Man5C in this study have optimal pH for activity within the acidic range (13), whereas Man134A activity has an optimal pH near neutral. Because of this property, Man134A supports the growth of A. nidulans on β-mannans at neutral pH; however, no information is available about Man134A homologs in other organisms. Other features of Man134A were determined in addition to the optimal pH. One is that it released M2, M3, and M4 from the initial stage of reaction, with M3 being the predominant reaction product, when galactomannan digested with α-galactosidase was the substrate. Mannose (M1), M2, and M6 were not generated. This suggests exolytic hydrolysis with a preference for M2 units or, alternatively, endolytic initial attack followed by processive hydrolysis that releases M3. Transglycosylation products were not detectable in the reaction catalyzed by Man134A. Fungal β-mannanases characterized to date belong to the GH5 and 26 families. Members of these families catalyze hydrolysis via a retaining reaction mechanism (37). The crystal structures of the β-1,4-mannanases in both GH families from a wide range of bacteria and fungi (26, 31, 33, 38, 40–42) have revealed an open active-site cleft with at least four subsites and two stiffly conserved catalytic glutamates (nucleophiles and acid/base) in β-strands 4 and 7, respectively. The present study found that Man134A is the smallest protein so far identified with β-1,4-mannanase activity. Because of the absence of homology with any described functional proteins and of any putative conserved domains, the structure of Man134A and which of its amino acid residues are important for catalysis are impossible to determine without mutational and/or structural studies. The Phyre server was unable to predict the three-dimensional structure of Man134A (43). All of these imply that Man134A has a novel structure and provides insight into a novel mechanism of β-mannan hydrolysis.

The classification of glycoside hydrolases based on amino acid sequence similarity has led to >130 defined families being listed in the CAZy database. Families with similar three-dimensional structures have been classified into clans, and the GH5 and GH26 families that include fungal β-1,4-mannanases belong to the largest glycoside hydrolase family, GH-A. The GH-A enzymes share the triose phosphate isomerase (β/α)s barrel-fold and a retaining reaction mechanism (37). The crystal structures of the β-1,4-mannanases in both GH families from a wide range of bacteria and fungi (26, 31, 33, 38, 40–42) have revealed an open active-site cleft with at least four subsites and two stiffly conserved catalytic glutamates (nucleophiles and acid/base) in β-strands 4 and 7, respectively. The present study found that Man134A is the smallest protein so far identified with β-1,4-mannanase activity. Because of the absence of homology with any described functional proteins and of any putative conserved domains, the structure of Man134A and which of its amino acid residues are important for catalysis are impossible to determine without mutational and/or structural studies. The Phyre server was unable to predict the three-dimensional structure of Man134A (43). All of these imply that Man134A has a novel structure and provides insight into a novel mechanism of β-mannan hydrolysis.

Although Man134A homologs are distributed among Proteobacteria, Actinobacteria, Zygomycota, Basidiomycota, and Ascomycota (Fig. 6), only a few genera/species actually have them (Table 3). Among the bacterial genomes in the NCBI database only five encode Man134A-like proteins. Around 50 fungal genome sequences are available from the Broad Institute database, and only A. nidulans, Rhizopus delemar RA 99–880, Fusarium oxysporum 4287 (FO2), Fusarium verticilloides 7600 (FV3), Aspergillus terreus, and Verticillium alfa aureum VaMs.102 encode Man134A-like proteins. Homologs have not been detected in plants and animals. The distribution of homologs suggests horizontal gene transfer including that between bacteria and fungi. Some pathogenic bacteria and fungi possess Man134A homologs. Although the physiological functions of the homologs remain obscure, they might be involved in the degradation of cell wall polysaccharides to infect plants.

3 N. Li, E. Kunitake, A. Watanabe, K. Kanamaru, M. Kimura, and T. Kobayashi, unpublished results.
Fungal β-1,4-Mannanase Belonging to New GH134 Family

The diverse industrial applications of β-mannanases include deinking paper waste, clarifying fruit juices, and pulp industries and to studies of the conversion technology of lignocellulosic biomass. The novel β-mannanase described herein should be of interest to the food and pulp industries and to studies of the conversion technology of lignocellulosic biomass.

**References**

1. Puls, J., and Schusele, J. (1993) Chemistry of hemicellulose: relationship between hemicellulose structure and enzyme required for hydrolysis. In *Hemicellulose and Hemicellulases*, pp. 1–27, London, Portland Press
2. Schröder, R., Atkinson, R. G., and Redgwell, R. J. (2009) Re-interpreting the role of endo-β-mannanases as mannan endotransglycosylases/hydrolases in the plant cell wall. *Ann. Bot.* 104, 197–204
3. Nunes, F. M., Reis, A., Domingues, M. R., and Coimbra, M. A. (2006) Characterization of galactomannan derivatives in roasted coffee beverages. *J. Agric. Food Chem.* 54, 3428–3439
4. Pérez Recalde, M., Carlucci, M. J., Noseda, M. D., and Matulewicz, M. C. (2012) Chemical modifications of algal mannans and xylanoxans: effects on antiviral activity. *Phytochemistry* 73, 57–64
5. Pawar, P. M., Koutaniami, S., Tenkenan, M., and Mellerowicz, E. J. (2013) Acetylation of woody lignocellulose: significance and regulation. *Front. Plant Sci.* 4, 118
6. Teleman, A., Nordström, M., Tenkenan, M., Jacobs, A., and Dahlman, O. (2003) Isolation and characterization of O-acetylated glucomannans from aspen and birch wood. *Carbohydr. Res.* 338, 525–534
7. Moreira, L. R., and Filho, E. X. (2008) An overview of mannan structure and mannan-degrading enzyme systems. *Appl. Microbiol. Biotechnol.* 79, 165–178
8. Barak, S., and Mudgil, D. (2014) Locust bean gum: processing, properties and food applications—a review. *Int. J. Biol. Macromol.* 66, 74–80
9. Simkovics, I. (2013) Unexplored possibilities of all-polysaccharide composites. *Carbohydr. Polym.* 95, 697–715
10. Sims, R. E., Mabee, W., Saddler, J. N., and Taylor, M. (2010) An overview of second generation biofuel technologies. *Bioresour. Technol.* 101, 1570–1580
11. Dhawan, S., and Kaur, J. (2007) Microbial mannanases: an overview of production and applications. *Crit. Rev. Biotechnol.* 27, 197–216
12. de Vries, R. P., and Visser, J. (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev.* 65, 497–522
13. Chauhan, P. S., Puri, N., Sharma, P., and Gupta, N. (2012) Mannanases: microbial sources, production, properties and potential biotechnological applications. *Appl. Microbiol. Biotechnol.* 93, 1817–1830
14. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The carbohydrate-active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238
15. Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11417–11422
16. Lu, H., Luo, H., Shi, P., Huang, H., Meng, K., Yang, P., and Yao, B. (2014) A novel thermophilic endo-β-1,4-mannanase from *Aspergillus nidulans* XZ3: functional roles of carbohydrate-binding module and Thr/Ser-rich linker region. *Appl. Microbiol. Biotechnol.* 98, 2155–2163
17. Dilkopimol, A., Nakai, H., Gottfredsen, C. H., Baumann, M. J., Nakai, N., Abou Hachem, M., and Svensson, B. (2011) Recombinant production and characterisation of two related GH5 endo-β-1,4-mannanases from *Aspergillus nidulans* FGSC A4 showing distinctly different transglycosylation capacity. *Biochim. Biophys. Acta* 1814, 1720–1729

We thank Norma Foster for critical reading of the manuscript.
18. Rosengren, A., Reddy, S. K., Sjöberg, J. S., Aurelius, O., Logan, D. T., Kolenov, K., and Stålbbrand, H. (2014) An _Aspergillus nidulans_ β-mannanase with high transglycosylation capacity revealed through comparative studies within glycosidase family 5. _Appl. Microbiol. Biotechnol._ **98**, 10091–10104

19. Ogawa, M., Kobayashi, T., and Koyama, Y. (2012) ManR, a novel Zn(II)Cys6 transcriptional activator, controls the β-mannan utilization system in _Aspergillus oryzae_. _Fungal Genet. Biol._ **49**, 987–995

20. Ogawa, M., Kobayashi, T., and Koyama, Y. (2013) ManR, a transcriptional regulator of the β-mannan utilization system, controls the cellulose utilization system in _Aspergillus oryzae_. _Biosci. Biotechnol. Biochem._ **77**, 426–429

21. Shimizu, M., Fujii, T., Masuo, S., Fujita, K., and Takaya, N. (2009) Proteomic analysis of _Aspergillus nidulans_ cultured under hypoxic conditions. _Proteomics_ **9**, 7–19

22. Shimizu, M., Fujii, T., Masuo, S., and Takaya, N. (2010) Mechanism of _de novo_ branched-chain amino acid synthesis as an alternative electron sink in hypoxic _Aspergillus nidulans_ cells. _Appl. Environ. Microbiol._ **76**, 1507–1515

23. Shimizu, M., Masuo, S., Fujita, T., Doi, Y., Kamimura, Y., and Takaya, N. (2012) Hydrolase controls cellular NAD, sirtuin, and secondary metabolites. _Mol. Cell. Biol._ **32**, 3743–3755

24. Shimizu, M., and Takaya, N. (2013) Nudix hydrolase controls nucleotides and glycolytic mechanisms in hypoxic _Aspergillus nidulans_. _Biosci. Biotechnol. Biochem._ **77**, 1888–1893

25. Jue, C. K., and Lipke, P. N. (1985) Determination of reducing sugars in the nanomole range with tetrazolium blue. _J. Biochem. Biophys. Methods_ **11**, 109–115

26. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. _Nucleic Acids Res._ **22**, 4673–4680

27. Takasaki, K., Shoun, H., Yamaguchi, M., Takeo, K., Nakamura, A., Hoshino, T., and Takaya, N. (2004) Fungal ammonia fermentation, a novel metabolic mechanism that couples the dissimilatory and assimilatory pathways of both nitrate and ethanol: role of acetyl CoA synthetase in anaerobic ATP synthesis. _J. Biol. Chem._ **279**, 12414–12420

28. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. _Mol. Biol. Evol._ **24**, 1596–1599

29. Couturier, M., Roussel, A., Rosengren, A., Leone, P., Stålbbrand, H., and Berrin, J. G. (2013) Structural and biochemical analyses of glycoside hydrolase families 5 and 26 β-(1,4)-mannanases from _Podospora anserina_ reveal differences upon manno-oligosaccharide catalysis. _J. Biol. Chem._ **288**, 14624–14635

30. Cartmell, A., Topakas, E., Ducros, V. M., Suits, M. D., Davies, G. J., and Gilbert, H. J. (2008) The _Cellvibrio japonicus_ mannanase CjMan26C displays a unique exo-mode of action that is conferred by subtle changes to the distal region of the active site. _J. Biol. Chem._ **283**, 34403–34413

31. Chen, Z., Friedland, G. D., Pereira, J. H., Revesco, S. A., Chan, R., Park, J. I., Thelen, M. P., Adams, P. D., Arkin, A. P., Keasling, J. D., Blanch, H. W., Simmons, B. A., Sale, K. L., Chivian, D., and Chhabra, S. R. (2012) Tracing determinants of dual substrate specificity in glycoside hydrolase family 5. _J. Biol. Chem._ **287**, 25335–25343

32. Tailford, L. E., Ducros, V. M., Flint, J. E., Roberts, S. M., Morland, C., Zechel, D. L., Smith, N., Björnud, M. E., Borchert, T. V., Wilson, K. S., Davies, G. J., and Gilbert, H. J. (2009) Understanding how diverse β-mannanases recognize heterogeneous substrates. _Biochemistry_ **48**, 7009–7018

33. Coradetti, S. T., Xiong, Y., and Glass, N. L. (2013) Analysis of a conserved cellulase transcriptional regulator reveals inducer-independent production of cellulolytic enzymes in _Neurospora crassa_. _Microbiologopen_ **2**, 595–609

34. Coradetti, S. T., Craig, J. P., Xiong, Y., Shock, T., Tian, C., and Glass, N. L. (2012) Conserved and essential transcription factors for cellulase gene expression in _Ascomycte fungi_. _Proc. Natl. Acad. Sci. U.S.A._ **109**, 7397–7402

35. Li, N., Kunitake, E., Watanabe, A., Kanamaru, K., Kimura, M., and Kobayashi, T. (2014) Regulation of cellulolytic and hemicellulolytic genes by McmA and ManR/ClrB in _Aspergillus nidulans_. In _Proceedings of the 12th European Conference on Fungal Genetics_, Seville, Spain, March 23–27, 2014, p. 219

36. Gilbert, H. J. (2010) The biochemistry and structural biology of plant cell wall deconstruction. _Plant Physiol._ **153**, 444–455

37. Dugg, J., Yuan, D., Do, C., and Glass, N. L. (2014) Application of endo-β-1,4,α-mannanase and cellulase for the release of manno-oligosaccharides from steam-pretreated spent coffee ground. _Appl. Biochem. Biotechnol._ **172**, 3538–3557

38. Le Nours, L., Rousset, A., Rosengren, A., Leone, P., Stålbbrand, H., and Lo Leggio, L. (2005) The structure and characterization of a modular endo-β-1,4-mannanase from _Cellulomonas fimii_. _Biochemistry_ **44**, 12700–12708

39. Sengsiriithigul, C., Labboobnrueng, S., Roytrakul, S., Haltrich, D., and Górgens, J. F. (2014) Crystal structure of mannanase 26 A from _Pseudomonas cellulosa_ and analysis of residues involved in substrate binding. _J. Biol. Chem._ **276**, 31186–31192

40. Chianzu, I., Breinzo, M., García-Aparicio, M. P., and Górgens, J. F. (2014) Structure and characterization of barley β-1,4-mannanase from _Cellulomonas fimii_. _Biochemistry_ **43**, 12700–12708

41. Sengsiriithigul, C., Labboobnrueng, S., Roytrakul, S., Haltrich, D., and Yamabhai, M. (2011) Crystallization and preliminary crystallographic analysis of _Cellvibrio japonicus_ mannanase CjMan26C dissection of dual substrate specificity in glycoside hydrolase family 5. _Appl. Environ. Microbiol._ **77**, 7397–7402

42. Jue, C. K., and Lipke, P. N. (1985) Determination of reducing sugars in the nanomole range with tetrazolium blue. _J. Biochem. Biophys. Methods_ **11**, 109–115

43. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. _Nucleic Acids Res._ **22**, 4673–4680

44. Takasaki, K., Shoun, H., Yamaguchi, M., Takeo, K., Nakamura, A., Hoshino, T., and Takaya, N. (2004) Fungal ammonia fermentation, a novel metabolic mechanism that couples the dissimilatory and assimilatory pathways of both nitrate and ethanol: role of acetyl CoA synthetase in anaerobic ATP synthesis. _J. Biol. Chem._ **279**, 12414–12420

45. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. _Mol. Biol. Evol._ **24**, 1596–1599

46. Couturier, M., Roussel, A., Rosengren, A., Leone, P., Stålbbrand, H., and Berrin, J. G. (2013) Structural and biochemical analyses of glycoside hydrolase families 5 and 26 β-(1,4)-mannanases from _Podospora anserina_ reveal differences upon manno-oligosaccharide catalysis. _J. Biol. Chem._ **288**, 14624–14635