Cloning and Expression in *Saccharomyces cerevisiae* of a *Trichoderma reesei* β -Mannanase Gene Containing a Cellulose Binding Domain

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β -Mannanase (endo-1,4- β -mannanase; mannan endo-1,4- β -mannosidase; EC 3.2.1.78) catalyzes endo-wise hydrolysis of the backbone of mannan and heteromannans, including hemicellulose polysaccharides, which are among the major components of plant cell walls. The gene *man1*, which encodes β -mannanase, of the filamentous fungus *Trichoderma reesei* was isolated from an expression library by using antiserum raised towards the earlier-purified β -mannanase protein. The deduced β -mannanase consists of 410 amino acids. On the basis of hydrophobic cluster analysis, the β -mannanase was assigned to family 5 of glycosyl hydrolases (cellulase family A). The C terminus of the β -mannanase has strong amino acid sequence similarity to the cellulose binding domains of fungal cellulases and is preceded by a serine-, threonine-, and proline-rich region. Consequently, the β -mannanase is probably organized similarly to the *T. reesei* cellulases, having a catalytic core domain separated from the substrate-binding domain by an O-glycosylated linker. Active β -mannanase was expressed and secreted by using the yeast *Saccharomyces cerevisiae* as the host. The results indicate that the *man1* gene encodes the two β -mannanases with different isoelectric points (pIs 4.6 and 5.4) purified earlier from *T. reesei*.

Fungal plant polysaccharide-hydrolyzing enzymes, cellulases and hemicellulases, are industrially important enzymes with a wide spectrum of well-established applications. Cellulolytic enzymes have been extensively studied at the biochemical, molecular, and structural levels for several years. Recently, growing interest has been focused on hemicellulases, not only for academic reasons but also because of their possible applicability in, for example, the food and feed and pulp and paper industries (45, 50). Attention in the pulp and paper industry has been drawn mostly towards xylanases because of the demonstration that loading of chlorine-containing chemicals during the bleaching of pulp can be reduced by addition of xylanases to the process (44). However, not only xylanase but also β -mannanase from the filamentous fungus *Trichoderma reesei* have been shown to facilitate bleaching (4, 43).

Hemicelluloses are heteropolysaccharides that vary in chemical composition and are abundant in plant cell walls. The major hemicelluloses in softwoods are acetylated galactoglucomannans. These consist of a backbone of β -1,4-linked mannose and glucose residues substituted with α -1,6-linked galactosyl side groups. Hardwoods also have, besides the major hemicelluloses which are substituted xylans, a minor glucomannan content (44). Other types of heteromannans can be found in plant seeds, e.g., locust bean gum, a galactomannan polymer with a main chain of β -1,4-mannan with α -1,6-bound galactosyl side groups.

β -Mannanase (endo-1,4- β -mannanase; mannan endo-1,4- β -mannosidase; EC 3.2.1.78) catalyzes random hydrolysis of β -1,4-mannosidic linkages in the main chain of mannan and

heteromannans. β -Mannanases are produced by plants, bacteria, and fungi. β -Mannanases from several fungi have been purified and characterized (45) in addition to the β -mannanase from *T. reesei* (3, 36). No report on the cloning of genes encoding fungal β -mannanases has been published, but three β -mannanase sequences from bacteria are available, those of *Bacillus* sp. strain AM-001 (1), *Caldocellum saccharolyticum* (10), and *Streptomyces lividans* 66 (2).

The filamentous fungus *T. reesei* is one of the most extensively studied organisms producing cellulases and hemicellulases and is an industrially important production organism. We have previously reported on the purification and biochemical characterization of β -mannanases from *T. reesei* RutC30. Five β -mannanase forms with pIs between 3.6 and 6.5 could be detected by isoelectric focusing (IEF) and the zymogram technique. Two of the β -mannanases (pIs 4.6 and 5.4) were purified and characterized (36), and these have been shown to enhance the bleachability of pulp (4, 43). This report describes the cloning of a β -mannanase gene from *T. reesei* which appears to encode both the pI 4.6 and pI 5.4 β -mannanase forms.

MATERIALS AND METHODS

Strains and vectors. *T. reesei* RutC30 (ATCC 56765), QM9414 (ATCC 26921), and VTT-D-79125 are descendants of the natural isolate *T. reesei* QM6a (ATCC 13631), and the pedigree is described in reference 20. The *T. reesei* RutC30 cDNA library was prepared in the Uni-ZAP XR vector (Lambda ZAP II vector digested with *Eco*RI and *Xho*I; Stratagene). Strains XL1 Blue, XL1 Blue MRF', and SOLR (Stratagene) were used as hosts in preparation and screening of the cDNA library. *Saccharomyces cerevisiae* DBY746 (α *his3-1 leu2-3 leu2-112 ura3-52 trp1-289* Cyt⁺ Cir⁺) (D. Botstein, Massachusetts Institute of Technology, Cambridge) was used as a yeast host. Plasmid pAJ401 (29), used as a yeast expression vector, contains the *URA3* marker gene and the 2 μ origin of replication. The cloning sites (*Eco*RI and *Xho*I) are situated between the constitutive yeast phosphoglycerate kinase (*PGK*) promoter and terminator.

Construction of the cDNA library. *T. reesei* RutC30 was grown in 250-ml shake flasks at 28°C and 200 rpm in 50 ml of medium containing the following (in grams

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per liter): Solka floc cellulose, 20; spent grain, 10; locust bean gum (Sigma 0753), 2; lactose, 1; acetyl glucuronoxylan (Birke 150), 1; arabinoxylan (Sigma 0376), 1; KH_2PO_4 , 15; $(\text{NH}_4)_2\text{SO}_4$, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6; and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.6. The following trace metal salt amounts (in grams per liter) were included: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.6; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 3.7. The pH was adjusted to 5.0 prior to inoculation with 10^7 spores per 50 ml of medium. After 48 h of growth, the mycelia were harvested and frozen at -70°C , and total RNA was isolated as described by Chirgwin et al. (5). Isolation of mRNA was done with a Quick prep mRNA purification kit (Pharmacia). cDNA was synthesized from the isolated mRNA, and a cDNA library was established in the Uni-ZAP XR vector by using the ZAP-cDNA Synthesis kit (Stratagene) in accordance with the manual. The size of the library was 1.2×10^6 separate clones, and the average length of the cDNA inserts was 1.2 kb.

Preparation of antibodies and screening of the cDNA library. Antiserum was prepared (31) against the purified β -mannanase (pI 5.4) of *T. reesei* RutC30. During the 10-week immunization period, 0.9 mg of protein emulsified with 0.5 ml of complete (first injection) or incomplete (the following injections) Freund's adjuvant was injected subcutaneously into rabbits at 3-week intervals. The collected sera were purified with protein A-Sepharose CL-4B columns (Pharmacia).

Clones (4.2×10^5) from the unamplified cDNA library were plated to a density of 7,000 PFU/87-mm-diameter plate and screened with β -mannanase antiserum by using the alkaline phosphatase detection system (Bio-Rad) in accordance with the manufacturer's instructions. Nine positive clones of a total of 29 detected were in vivo excised into phagemid pBluescriptSK(-) form, resulting in phagemids pHSM1, pHSM3, pHSM5, pHSM7, pHSM10, pHSM11, pHSM20, pHSM21, and pHSM24.

Isolation of the chromosomal gene. The *man1* genomic copy was isolated from a *T. reesei* QM6a genomic cosmid library (20) by using as a probe a digoxigenin-labeled internal PCR fragment derived from the *man1* cDNA (see Fig. 1) in accordance with the supplier's (Boehringer) instructions. Seven positive clones were obtained. The gene was subcloned as a 2.0-kb *Xba*I fragment into pBlue-scriptSK(-), resulting in plasmid pALK133.

Sequencing. Both strands of the cDNA and the chromosomal copies were sequenced by using ABI (Applied Biosystems) kits based on fluorescence-labeled T7 and T3 primers or, when sequence-specific primers were used, on fluorescence-labeled dideoxynucleotides, by the *Taq* cycle sequencing method in accordance with the supplier's instructions. Sequencing reactions were analyzed on an ABI 373A sequencer, and the sequences obtained were characterized by using the Genetics Computer Group Sequence Analysis Software Package, version 7.2.

Southern hybridizations. Genomic DNAs from *T. reesei* RutC30 and QM9414 were digested with *Sac*I or *Pvu*II, separated on an agarose gel, and blotted onto a nylon membrane. The membrane was probed with ^{32}P -labeled (5×10^8 cpm μg^{-1}) *man1* cDNA (1.5-kb insert from pHSM1). The membrane was prehybridized ($6 \times \text{SSC}$ [$1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate], $5 \times$ Denhardt's reagent, 0.5% sodium dodecyl sulfate [SDS], 50% formamide, 100 μg of denatured herring sperm DNA ml^{-1}) for 2 h at 42°C and hybridized with 10^6 cpm of the probe ml^{-1} for 16 h at 42°C . The membrane was washed with $2 \times \text{SSC}$ –0.1% SDS at room temperature and then with 0.1% SSC –0.1% SDS for 1 h at 68°C . Genomic DNA from *T. reesei* VTT-D-79125 was cut with either *Bam*HI, *Bgl*II, or *Pvu*II and treated as described above, except that the probe was digoxigenin labeled (Boehringer) and the hybridization solution was prepared in accordance with the supplier's instructions. The internal PCR fragment derived from cDNA clone pHSM1 (see Fig. 1) was used as a probe when *Pvu*II was used to cut the DNA.

DNAs from the seven cosmid clones recovered from the genomic library were digested with restriction enzymes *Bgl*II, *Pvu*II, *Pst*I, *Eco*RI, *Xba*I, *Hind*III, *Cla*I, and *Bam*HI and separated on an agarose gel. The clones possessed several common bands. The DNA was transferred onto a nylon membrane and hybridized with the *man1* internal PCR fragment as described above. All clones gave the same hybridization pattern.

Hydrophobic cluster analysis. Bidimensional helical net representations of the amino acid sequences were done with the HCA-PLOT program from Doriane S.A. Clusters of hydrophobic residues (VILMFYW) were automatically drawn by the program. Analysis of the plots was conducted by following published guidelines (9, 18).

Production of β -mannanase in *S. cerevisiae*. The 1.5-kb *man1* cDNA was released from pHSM1 with *Eco*RI and *Xho*I and ligated to *Eco*RI- and *Xho*I-cut plasmid pAJ401, resulting in plasmid pHSM30. pHSM30 was transformed into *S. cerevisiae* DBY746 by using the Li acetate method of Ito et al. (16), resulting in yeast strain VTT-C-94193.

Yeast cells producing extracellular β -mannanase were detected by plate assay on uracil-deficient synthetic complete medium (34) plates supplemented with 0.1% locust bean gum (Sigma). Yeasts were grown for 4 days at 30°C , and the plates were stained with Congo red as described by Penttilä et al. (25) to reveal hydrolysis halos. For analysis of the β -mannanase in the yeast culture supernatant, the strains were cultivated in uracil-deficient synthetic complete medium at 30°C in 100-ml shake flasks at 200 rpm. The culture fluids were separated from the cells at the stationary phase (after 48 h of growth) by centrifugation and concentrated with an Omegacell unit with a molecular weight cutoff of 5,000 (Filtron).

FIG. 1. Analysis of *man1* cDNA clones and chromosomal copies. The diagram at the top shows the chromosomal cosmid copy which was studied in detail in this work (2.0-kb fragment in pALK133) together with nucleotide numbering and recognition sites for the restriction enzymes used (i) to analyze the seven chromosomal cosmid copies isolated (one insert in pALK133 originates from one of these) (ii) or for Southern analysis of total genomic DNA. *Eco*RI, *Hind*III, *Sac*I, *Bam*HI, and *Bgl*II do not cut in the pALK133 insert. The protein encoding region is black and is interrupted by two introns. Below, drawn to scale and aligned with the corresponding regions in pALK133, are the eight cDNA copies (inserts of pHSM clones) isolated from the cDNA library. The thick lines illustrate the regions sequenced and found to be identical in all of the cDNA clones and pALK133. The thin lines mark the positions of introns present in the chromosomal copy. The broken lines represent unsequenced regions in the cDNA clones. The filled circles mark the positions of the primers used in PCR amplification (see text).

Biochemical methods. The β -mannanase activity assay used has already been described (36). The amino-terminal protein sequences were determined by Edman degradation at the Biomolecular Resource Facility at Lund University. IEF and subsequent mannanase activity detection (zymogram) with a locust bean gum overlay gel and staining with Congo red have been described previously (36). SDS-polyacrylamide gel electrophoresis (PAGE) was done with 12.5% polyacrylamide gels in the Pharmacia PhastSystem in accordance with the manufacturer's instructions. Samples were dialyzed prior to electrophoresis. Proteins were blotted onto nitrocellulose membranes by using the Phast Transfer Semidry Transfer System (Pharmacia) and treated with β -mannanase antiserum. *N*-Glycosidase F treatment was carried out for 15 h at 20 or 37°C after denaturation of the proteins with SDS in accordance with the supplier's (New England Biolabs, Inc.) recommendation.

Nucleotide sequence accession number. The GenBank accession number of the *man1* cDNA sequence is L25310.

RESULTS

Isolation of the *man1* gene. The two β -mannanase forms (pIs 5.4 and 4.6) of *T. reesei* RutC30 purified earlier have very similar features (36). To clone the gene coding for a β -mannanase, we prepared antibodies to the pI 5.4 β -mannanase which were found to react to the same extent with both the pI 5.4 and pI 4.6 mannanases (data not shown). The N-terminal amino acid sequences of both mannanase forms were determined and seemed to be identical (see Fig. 2 legend).

A *T. reesei* RutC30 cDNA library was prepared in the Uni-ZAP XR vector and screened with anti-mannanase antibodies. Nine positive clones were in vivo excised into the pBlue-scriptSK(-) phagemid form. Restriction enzyme analysis and partial sequencing (see Fig. 1) revealed that all were copies of the same cDNA, except one (pHSM24) which represented a previously characterized gene (*egl5*) encoding endoglucanase V (EGV) (29). Phagemid pHSM1 carried a 1.5-kb insert (Fig.

FIG. 2. Nucleotide sequence of the isolated *man1* gene and deduced amino acid sequence of the β -mannanase protein. Intron sequences are in lowercase. The standard one-letter amino acid code is used. The first N-terminal amino acid in the mature protein is numbered 1, and the presumed signal sequence and propeptide cleavage sites are indicated by arrows. The N-terminal amino acid sequences determined for the *T. reesei* β -mannanase pI 5.4 (XXXFXITISGTQFXID) and pI 4.6 (XXXFVTISGTQFNID) forms are underlined. Putative N-glycosylation sites are indicated by stars, and putative catalytic amino acid residues (predicted with HCA) are indicated by dots.

1) with an open reading frame corresponding to the N-terminal amino acid sequences of the pI 5.4 and 4.6 β -mannanases (Fig. 2). The gene was named *man1*. The corresponding genomic copy was isolated from a *T. reesei* QM6a genomic cosmid library by using a PCR fragment of the *man1* cDNA clone as a probe. The seven positive clones picked appeared to originate from the same region of the genome and seemed to carry the same *man1* gene as studied by eight different restriction

enzymes and Southern hybridization (Fig. 1; see Materials and Methods). Furthermore, PCR amplification covering a region containing the two introns (Fig. 1) gave products with the same apparent size (430 bp) with all seven clones, indicating that there are no major differences in this part of the clones (data not shown).

Results of Southern hybridization of restriction enzyme-digested genomic DNAs of three different *T. reesei* strains

(QM9414, RutC30, and VTT-D-79125) carried out with the *man1* cDNA, or a fragment of it, as a probe were consistent between the strains and gave no indication of more than one copy of the *man1* gene in the *T. reesei* genome. Cleavage with *SacI* visualized one band of 8.2 kb, cleavage with *BamHI* revealed one band of about 20 kb, cleavage with *PvuII* showed one band of 3.4 kb, and cleavage with *BglII* revealed two bands of about 9 and 7.5 kb.

The *man1* gene and the deduced β -mannanase protein. The genomic sequence of the *man1* gene and the deduced amino acid sequence of the gene product are shown in Fig. 2. There is an open reading frame of 1,440 bp starting from the first start codon in the cDNA. Two introns interrupt the coding sequence. The nucleotide sequences of the *man1* cDNA derived from *T. reesei* RutC30 and the coding region of the chromosomal copy of *T. reesei* QM6a were identical, demonstrating that these two strains express identical *man1* gene products.

A putative signal peptide cleavage site (46, 49) is situated 19 amino acids after the initiation codon (Fig. 2). This cleavage site leaves an eight-amino-acid putative propeptide preceding the N-terminal amino acid sequence of the mature protein, which may be cleaved off by a proline-directed arginyl protease (33). Two-step proteolytic processing has also been suggested for many other secreted fungal proteins, such as cellobiohydrolase II (CBHII) (38) and endoxylanase II (27) of *T. reesei* and glucoamylase of *Aspergillus niger* (15).

The deduced mature protein has a length of 410 amino acids and a calculated molecular mass of 44.3 kDa. This is in rough agreement with the molecular mass estimations for the *T. reesei* β -mannanase pI 5.4 and 4.6 forms, which are 53 and 51 kDa, respectively (36). This difference may be explained by glycosylation. Arisan-Atac et al. (3) reported on a *T. reesei* β -mannanase with a pI of 5.2 and a molecular mass of 46 kDa, which is probably identical to the pI 5.4 β -mannanase purified by us (36). This protein was found to be glycosylated.

Domain structure of the *T. reesei* β -mannanase. Three previously characterized β -mannanase sequences were available in the GenBank and Swiss-Prot data banks. Two of these, those of *S. lividans* and *C. saccharolyticum*, have been shown to belong to glycosyl hydrolase family 5 (also named family A in the classification of cellulases) (12). The third mannanase, that of *Bacillus* sp., has been assigned to family 26 (family I in the cellulase classification) (12). This classification has been done by hydrophobic cluster analysis (HCA) (9, 18), which predicts structural similarities in proteins and has been particularly adapted and useful for the classification of cellulases (13). HCA is a very sensitive method, allowing confident sequence similarity detection and alignment at amino acid sequence identity levels as low as 15%.

Computer searches revealed extremely weak similarities between the *T. reesei* β -mannanase and the two mannanases belonging to family 5. To assess the validity of the similarities, HCA was performed for the *T. reesei* β -mannanase. Family 5 contains enzymes that are widely diverged, and there are only a very limited number of semi-invariant and invariant residues (19, 47). The presence of these residues was searched for along with similarities in shape, distribution, and size between hydrophobic clusters on the HCA plots of the *T. reesei* β -mannanase and the other members of the family (Fig. 3). All invariant and semi-invariant residues typical of the family could be identified in the *T. reesei* β -mannanase (Fig. 3), including the two glutamic acid residues which have been shown to be catalytic in EGII (formerly named EGIII) of *T. reesei* (19) and CelC of *Clostridium thermocellum* (47). These residues are Glu-169 and Glu-276 in the *T. reesei* β -mannanase. The *T. reesei* β -mannanase can therefore be assigned to glycosyl hy-

drolase family 5 (cellulase family A). It is interesting that the β -mannanases of *S. lividans* and *C. saccharolyticum* appear to be more closely related to each other than to the *T. reesei* enzyme, thereby suggesting possible differences in activity between the bacterial and *T. reesei* enzymes.

Interestingly, a strong amino acid similarity with the cellulose binding domains (CBDs) of fungal cellulases was detected at the C terminus of the deduced β -mannanase protein. This region is preceded by a serine-, threonine-, and proline-rich region in the β -mannanase, similar to the O-glycosylated linker regions separating the CBDs from the catalytic cores in fungal cellulases. A comparison of the C-terminal region of the β -mannanase with the five *T. reesei* cellulase CBDs is shown in Fig. 4. The tryptophan and tyrosine residues forming a flat surface presumably interacting with the substrate (6, 26, 29), cysteines forming stabilizing disulfide bridges, and other conserved amino acids are present in the β -mannanase. The strong similarity is further emphasized by the fact that only a single amino acid gap compared with the cellulase CBDs is present, and this is situated in a region where variation between the cellulase CBDs also occurs. On the basis of the nuclear magnetic resonance-determined structure of the CBHI CBD (17) and the modelled structures of the other four *Trichoderma* cellulase CBDs (14, 29), this region forms a loop structure. Variations or even deletions in this region are unlikely to cause big changes to the overall CBD structure. There are two extra cysteine residues, Cys-374 and Cys-391, in the β -mannanase CBD compared with the CBD of CBHI, EGII, and EGV. Corresponding cysteine residues are also found in the CBD of CBHII and EGI, in which they have been predicted to form an extra disulfide bridge (14). To conclude, the putative CBD of the *T. reesei* β -mannanase does not appear to differ from the cellulase CBDs more than these differ from each other.

Expression of the *man1* gene in *S. cerevisiae*. The *man1* cDNA was cloned into yeast multicopy expression vector pAJ401 under control of the constitutive *PGK* promoter, and the resulting plasmid, pHSM30, was transformed into *S. cerevisiae* DBY746. The recombinant obtained, *S. cerevisiae* VTT-C-94193, was analyzed for production of β -mannanase activity on locust bean gum plates (Fig. 5). The strain produced clear halos, indicating hydrolysis of the substrate, and thus the *Trichoderma* enzyme was secreted in active form by the yeast. The control strain, *S. cerevisiae* DBY746, transformed with the vector pAJ401, showed no β -mannanase activity in the plate assay.

S. cerevisiae VTT-C-94193 and the control yeast were cultivated in shake flasks in uracil-deficient synthetic complete medium for 48 h, and the concentrated culture supernatants were analyzed by SDS-PAGE and immunoblotting (Fig. 6). The apparent molecular mass of the extracellular β -mannanase produced by the yeast was approximately 75 kDa. Additional high-molecular-weight immunoreactive material was visible on the gel. Treatment of the culture fluid with N-glycosidase F altered the apparent molecular weight position of the β -mannanase to a faint single band of about 53 kDa. This indicates that the extracellular β -mannanase produced in *S. cerevisiae* is more heavily N glycosylated than the native enzyme, as has been shown for many other extracellular heterologous proteins expressed in *S. cerevisiae*, such as the *T. reesei* cellulases CBHI, CBHII, and EGI (24, 25). The apparent molecular mass of the purified pI 5.4 β -mannanase (53 kDa) produced by the native organism *T. reesei* did not appear to be significantly altered by N-glycosidase F treatment under the conditions used (Fig. 6), nor did the molecular mass of the pI 4.6 form (data not shown).

The concentrated VTT-C-94193 culture filtrate was also analyzed by analytical IEF combined with the zymogram tech-

FIG. 3. HCA of some family 5 glycosyl hydrolases. Comparison of the β -mannanase of *T. reesei* (A) with those of *S. lividans* (B) and *C. saccharohydricum* (C) and with *T. reesei* cellulase EGH (D). The standard one-letter code is used for all amino acids except proline ($\star$), glycine ($\diamond$), serine ($\square$), and threonine ($\square$). Hydrophobic clusters are boxed. Correspondences between the sequences are indicated by vertical lines. The invariant and semi-invariant residues typical of family 5 are in yellow, and the two putative catalytic Glu residues (also invariant) are in red.

MANI	373	S	C	S	P	L	Y	G	Q	C	G	G	S	G	Y	T	G	P	T	C	C	-	A	Q	G	T	-	C	I	Y	S	N	Y	W	Y	S	Q	C	L	N	T	410
CBHI	461	P	T	Q	S	H	Y	G	Q	C	G	G	I	G	Y	S	G	P	T	V	C	-	A	S	G	T	T	C	Q	V	L	N	P	Y	Y	S	Q	C	L	497		
CBHII	2	A	C	S	S	V	W	G	Q	C	G	G	Q	N	W	S	G	P	T	C	C	-	A	S	G	S	T	C	V	Y	S	N	D	Y	Y	S	Q	C	L	...	38	
EGI	401	C	T	Q	T	H	W	G	Q	C	G	G	I	G	Y	S	G	C	K	T	C	-	T	S	G	T	T	C	Q	Y	S	N	D	Y	Y	S	Q	C	L	437		
EGII	1	Q	Q	T	V	W	G	Q	C	G	G	I	G	W	S	G	P	T	N	C	-	A	P	G	S	A	C	S	T	L	N	P	Y	Y	A	Q	C	I	...	36		
EGV	205	G	Q	Q	T	L	Y	G	Q	C	G	G	A	G	W	T	G	P	T	T	C	-	Q	A	P	G	T	-	C	K	V	Q	N	Q	W	Y	S	Q	C	L	P	242

FIG. 4. Alignment of the β -mannanase CBD (MANI) with those of *T. reesei* cellulases CBHI (35), CBHII (38), EGI (26), EGII (30), and EGV (29). The standard one-letter amino acid code is used. Identical amino acid residues are shaded light grey, and tyrosine and tryptophan residues that presumably interact with the substrate are shaded dark grey. Cysteine residues are circled. Numbering refers to the first and last amino acid residues of the CBD. Hyphens indicate gaps, and dots indicate continuation of the sequence.

nique, which assays for β -mannanase activity (Fig. 7). β -Mannanase activity was detected as three distinct bands with approximate pIs of 4.7, 5.5, and 6.7. At least two additional, poorly separated β -mannanase bands in the pI 3.6-to-4.2 region were also visible. Comparison with two different culture supernatants of *T. reesei* shows that the patterns produced by the recombinant yeast and *T. reesei* are very similar. The approximate pIs detected in a similar *T. reesei* culture have previously been determined to be 3.6, 4.2, 4.8, 5.5, and 6.5 (36).

The *S. cerevisiae* VTT-C-94193 culture filtrate was assayed for β -mannanase activity by using 24 h of incubation with a locust bean gum substrate. The activity was 0.22 nkat/ml, whereas the activity of the control yeast was negligible (<0.02 nkat/ml). Assuming the same specific activity for the yeast-produced enzyme as estimated for the purified *T. reesei* pI 5.4 mannanase (1,430 nkat/mg) (36), the expression level in this cultivation would be roughly 150 ng/ml. The value judged from immunoblotting is comparable, 80 ng/ml (data not shown).

DISCUSSION

The *T. reesei* *man1* gene reported here is the first fungal β -mannanase gene characterized (see Addendum in Proof, below). The *man1* gene codes for β -mannanase, which on the basis of HCA, belongs to glycosyl hydrolase family 5 and exhibits similarity at the amino acid level to the mannanases of the bacteria *S. lividans* and *C. saccharolyticum*. This family is

highly divergent at the amino acid level, as well as in enzymatic activity, including mannanases, cellulases, and β -1,3-glucanases. *Trichoderma* cellulase EGII also belongs to this family.

It is common that an organism secretes several plant polysaccharide-hydrolyzing enzymes with similar catalytic properties encoded by separate genes. For instance, two xylanase genes (27, 42), two different CBH genes, and four EG genes have been isolated from *T. reesei*, the two isolated most recently encoding EGIII (48) and EGV (29). Thus, it would be expected that the five different pI forms of β -mannanase present in the *T. reesei* culture medium (36) would be products of more than one gene. However, there are several facts which strongly suggest that at least the two major pI forms, 5.4 and 4.6, occurring in roughly equal amounts in the fungal culture medium (36), are products of the *man1* gene isolated in this work. The two pI forms purified from *T. reesei* seem to have identical N termini. Antibodies produced against the pI 5.4 form react similarly with the pI 4.6 form. By using these antibodies, cDNA clones were isolated. The eight cDNA clones studied had identical nucleotide sequences in the regions analyzed, including the complete 3' noncoding regions, where nucleotide changes are likely to occur, if more than one gene is in question. The only different clone picked represented an already known gene, cellulase gene *egl5*. By using the *man1* cDNA as a probe, genomic copies originating from only one chromosomal region were obtained and the gene was shown to be a single-copy gene by Southern analysis. Also, in the karyotyping of *T. reesei* QM6a, QM9414, VTT-79125, and RutC30 by contour-clamped homogeneous-field electrophoresis, the

FIG. 5. Hydrolysis halos produced on a locust bean gum plate by three streaks of *S. cerevisiae* VTT-C-94193 expressing the β -mannanase gene of *T. reesei* (A). Three streaks of the control strain containing the pAJ401 vector only were made on the other half of the plate (B).

FIG. 6. SDS-PAGE and immunostaining of extracellular β -mannanases produced by *S. cerevisiae*. Culture supernatants of *S. cerevisiae* VTT-C-94193 concentrated 100-fold and treated with endoglycosidase F at 20°C (A) and untreated (B and F), purified *T. reesei* pI 5.4 β -mannanase (0.3 ng) treated with endoglycosidase F at 37°C (C) and untreated (D), and culture supernatant of the control yeast strain carrying the pAJ401 vector concentrated 80-fold (E) are shown. Samples of 4 μ l were applied.

FIG. 7. IEF followed by β -mannanase activity detection of culture supernatants of *S. cerevisiae* and *T. reesei*. Yeast culture supernatants (concentrated 25-fold) of the control strain carrying the pAJ401 vector (A and B) and *S. cerevisiae* VTT-C-94193 (C and D) and culture supernatants of *T. reesei* RutC30 cultivated as described in Materials and Methods (diluted 1:5) (E) and grown on medium containing cellulose and spent grain (36) (diluted 1:10) (F) are shown. Samples of 4 μ l were applied. The values on the right are pIs.

man1 cDNA probe hybridized with only one chromosomal band (19a). Furthermore, yeast expression resulted in both the pI 4.6 and 5.4 forms. That the two pI forms would be encoded by one gene is also in accordance with their similar biochemical properties (36). The slight difference in molecular mass between the two forms produced by *T. reesei* (36) could be explained, for instance, by differences in glycosylation.

It is interesting that all of the β -mannanase forms detected in *S. cerevisiae* and *T. reesei* cultures by the IEF-zymogram technique show a striking pI pattern similarity. Thus, it is tempting to speculate that *man1* also encodes the other *T. reesei* mannanase pI forms besides the pI 5.4 and 4.6 forms. This is further supported by the preliminary observation that all of the forms detected in a *T. reesei* culture with the IEF-zymogram technique seem to react with the antibodies produced against the pI 5.4 form (35a). The reason why many fungal extracellular enzymes encoded by one gene occur in several pI forms is still unclear and needs to be investigated further. However, the possibility cannot be excluded that *T. reesei* has β -mannanase-encoding genes other than that described here which do not hybridize with the *man1* probe and whose protein products do not cross-react with the β -mannanase pI 5.4 antibodies and are not detectable with the zymogram technique used.

An interesting feature of the *T. reesei* β -mannanase is that it seems to consist of two functional domains. In addition to the catalytic core, it carries a putative substrate-binding domain separated from the core by a linker region. This organization is well known for several bacterial and fungal cellulases (11), the best-studied fungal ones being those of *T. reesei* (39) and *Humicola insolens* (32). The fungal CBDs so far characterized share a highly conserved structure (14). They are situated at either the N or C termini of the proteins but are not found in all cellulases (32, 48). Furthermore, EGI of *T. reesei* has a CBD whereas EGI of *H. insolens* does not, although the proteins have otherwise similar amino acid sequences and belong to the same HCA family. In cases studied so far, the CBDs are located at the same terminus in all fungal enzymes belonging to the same HCA family. The *T. reesei* β -mannanase, however, carries its CBD at the C terminus, whereas the fungal cellulases belonging to the same HCA family have N-terminal

CBDs (32). These observations indicate the possible occurrence of domain shuffling in the evolution of glycan-interacting polypeptides, as has also been discussed before, e.g., by Meinke et al. (21).

The bacterial CBDs characterized so far differ clearly from the fungal CBDs (7). It is interesting, however, that the β -mannanase from *C. saccharolyticum* also seems to carry a substrate-binding domain which shows amino acid similarity to cellulase CBDs of the same organism and of *Bacillus subtilis* (10). Furthermore, other hemicellulases also have CBDs; e.g., the bacterial xylanases encoded by the *xynA* gene from *Clostridium stercorarium* (28) and by the *xynD* gene of *Cellulomonas fimi* (22) have both been reported to have a bacterial type of CBD on the basis of sequence analysis. The *T. reesei* xylanases, on the other hand, do not carry CBDs (27, 42). It seems to be a general feature that enzymes catalyzing the hydrolysis of different plant polysaccharides can carry common substrate-binding motifs, not only in bacteria but also in fungi, as demonstrated in this report.

The strong amino acid similarity between the putative CBD of the *T. reesei* β -mannanase and the fungal cellulase CBDs strongly suggests that the β -mannanase CBD has substrate-binding properties similar to those of the cellulase CBDs. Initial binding studies indicate that the β -mannanase binds to cellulose and to unsubstituted mannan (ivory nut mannan). In these experiments, the binding behavior of the β -mannanase was similar to that of *T. reesei* cellulases CBHI and EGI (40).

The organization of hemicellulases having a catalytic domain and a CBD might be reflected by the fact that hemicelluloses are closely associated with cellulose in plant tissues (41). The binding domains may not only serve as an aid for binding close to the target to be hydrolyzed but may also act as disruptors of the crystalline substrate structures (8). The affinity of the β -mannanase and other enzymes carrying CBDs for cellulose and other carbohydrate compounds and the role of CBD in targeting the enzymes to the substrate in complex lignocellulosic materials remain to be studied. Because of already existing structure-function data (17, 26) on fungal CBDs, the fungal enzymes provide an excellent tool for this kind of analysis.

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ADDENDUM IN PROOF

A recent report (5a) demonstrated the cloning of a β -mannanase gene from the filamentous fungus *Aspergillus aculeatus* which has a high amino acid similarity to the *T. reesei* β -mannanase but lacks a CBD. Dalbøge and Heldt-Hansen (7a) described two *Humicola insolens* xylanases carrying CBDs.

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