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A mannanase, ManA, of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2 has carbohydrate binding and docking modules

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Abstract

The anaerobic fungus *Orpinomyces* sp. strain PC-2 produces a broad spectrum of glycoside hydrolases, most of which are components of a high molecular mass cellulosomal complex. Here we report about a cDNA (*manA*) having 1924 bp isolated from the fungus and found to encode a polypeptide of 579 amino acid residues. Analysis of the deduced sequence revealed that it had a mannanase catalytic module, a family 1 carbohydrate-binding module, and a noncatalytic docking module. The catalytic module was homologous to aerobic fungal mannanases belonging to family 5 glycoside hydrolases, but unrelated to the previously isolated mannanases (family 26) of the

²Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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anaerobic fungus *Piromyces*. No mannanase activity could be detected in *Escherichia coli* harboring a *manA*-containing plasmid. The *manA* was expressed in *Saccharomyces cerevisiae* and ManA was secreted into the culture medium in multiple forms. The purified extracellular heterologous mannanase hydrolyzed several types of mannan but lacked activity against cellulose, chitin, or β -glucan. The enzyme had high specific activity toward locust bean mannan and an extremely broad pH profile. It was stable for several hours at 50 °C, but was rapidly inactivated at 60 °C. The carbohydrate-binding module of the Man A produced separately in *E. coli* bound preferably to insoluble lignocellulosic substrates, suggesting that it might play an important role in the complex enzyme system of the fungus for lignocellulose degradation.

Résumé:

Le champignon anaérobie *Orpinomyces* sp. souche PC-2 produit un large spectre d'hydrolases glycosidiques, la plupart faisant partie d'un complexe cellulosomal de masse moléculaire élevée. Nous faisons ici la description d'un ADNc (*manA*) de 1924 pb isolé du champignon et codant un polypeptide de 579 acides aminés. L'analyse de la séquence déduite a révélé qu'il renfermait un module catalytique de mannanase, un module de liaison aux glucides de la famille 1 et un module d'arrimage non catalytique. Le module catalytique était homologue à des mannanases de champignons aérobies appartenant aux hydrolases glycosidiques que la famille 5 mais n'était pas apparenté à des mannanases isolées précédemment (famille 26) du champignon anaérobie *Piromyces*. Aucune activité mannanases n'a pu être détectée chez *Escherichia coli* renfermant un plasmide contenant *manA*. Le *manA* fut exprimé chez *Saccharomyces cerevisiae* et ManA fut secrète dans le milieu culture sous des formes multiples. La mannanase hétérologue extracellulaire purifiée a hydrolysé plusieurs types de mannane mais n'a démontré aucune activité envers le cellulose, la chitine ou le β -glucane. L'enzyme a démontré une activité spécifique élevée envers le mannane de caroube et un profil de pH extrêmement large. Elle était stable pendant plusieurs heures à 50 °C mais fut rapidement inactivée à 60 °C. Le module de liaison aux glucides de la mannanase A produite séparément chez *E. coli* s'est lié de préférence à des substrats lignocellulosiques insolubles, indiquant qu'il pourrait jouer un rôle important dans la dégradation du lignocellulose par le système enzymatique complexe du champignon.

Keywords

Orpinomyces; anaerobic fungi; mannanase; cellulose-binding module; cellulosome

Mots-clés

Orpinomyces; champignons anaérobies; mannanase; module de liaison au cellulose; cellulosome

Introduction

Plant cell wall, an abundant organic matter on earth, is composed of 3 major constituents: cellulose, hemicelluloses, and lignin. The ratio of these components varies depending on the source of cell walls. Hemicelluloses refer to a number of heterologous structures, such as arabinoxylan, mannan, and xyloglucan. These chemically diverse polymers are linked together through physical attachment and chemical bonds. The cross-linking and partially

crystalline nature of the matrix offer great resistance to enzymatic degradation. To date, hydrolytic enzymes for the cleavage of almost all chemical bonds found in plant structures have been identified in microbial sources. These enzymes often are modular and, in addition to catalytic domains, they have modules for carbohydrate binding (CBM) and cellulose surface modification and disruption (Bayer et al. 1998; Kataeva et al. 2002; Saloheimo et al. 2000). In addition, especially with anaerobic bacteria and fungi, in which glycoside hydrolases are found in extracellular protein complexes called cellulosomes, the enzymatically active components contain noncatalytic dockerin modules (NCDM) that bind to cohesins of scaffolding proteins (Bayer et al. 1998; Fannuti et al. 1995; Ljungdahl et al. 1998).

Mannanase (endo-1,4- β -mannanase; mannan endo-1,4- β -mannosidase; EC 3.2.1.78) randomly hydrolyzes the 1,4- β -mannosidic bonds of the main chain of glucomannan and galactomannan. On the basis of sequence similarity, microbial β -mannanases are classified into the glycoside hydrolase families 5 and 26 (<http://afmb.cnrs-mrs.fr/CAZY/>). Among the family 5 members are the mannanases from *Trichoderma reesei*, *Aspergillus aculeatus*, and *Agaricus bisporus*. Although their catalytic modules (CMs) are similar, the presence and location of CBMs vary among them. A mannanase, ManA from *Clostridium cellulovorans*, belonging to family 5, has a dockerin and is a cellulosomal component. It does not have a CBM (Tamaru and Doi 2000). *Clostridium thermocellum* produces a mannanase belonging to family 26, which has a dockerin and possibly also a CBM (Halstead et al. 1999). The family 5 mannanase from *Caldobacillus cellulovorans* has 2 CBMs, one on each side of the CM, and an unknown module (Sunna et al. 2000). There is no conclusive evidence of a fungal mannanase with both a dockerin and a CBM (Bayer et al. 1998; Lynd et al. 2002).

Anaerobic fungi were first described by Orpin (Orpin 1975). At least 17 different anaerobic fungi have been described and classified into 5 genera. The classification was done according to ultrastructural characteristics, type of growth (monocentric or polycentric), and uni-, bi-, or poly-flagellated zoospores. The anaerobic fungi differ from aerobic fungi in that they are very sensitive to oxygen and that instead of mitochondria, they have hydrogenosomes, which are involved in energy metabolism (Ljungdahl et al. 1998; van der Giezen et al. 1997). Many anaerobic fungi are able to completely degrade recalcitrant cell wall materials because of their ability to secrete a broad spectrum of hydrolytic enzymes (Borneman et al. 1989). Some of these enzymes are found free, but most of them are bound to cellulosomal complexes (Fannuti et al. 1995; Steenbakkens et al. 2001). The enzymes of the complexes contain noncatalytic docking modules (NCDM), equivalent to the dockerins of clostridial cellulosomes. But the fungal and bacterial docking modules do not share sequence similarities (Steenbakkens et al. 2001). Evidence for scaffolding polypeptides for the fungal cellulosomes has been presented (Fannuti et al. 1995; Steenbakkens et al. 2001), but genes coding for the scaffolding polypeptides have not been reported.

Orpinomyces sp. strain PC-2, a polycentric fungus isolated from bovine rumen (Borneman et al. 1989), secretes cellulase, β -glucanase, xylanase, feruloyl and *p*-coumaroyl esterase, acetylxytan esterase, β -glucosidase, and mannanase activities. Many of these enzymes are cellulosomal components (Li et al. 1997a, 1997b; Steenbakkens et al. 2001). Evidence that

genes coding for enzymes of anaerobic fungi are of either bacterial or fungal origins has been provided (Ljungdahl et al. 1998).

We report here the isolation of a cDNA coding for ManA of *Orpinomyces* sp. strain PC-2. Sequence analysis revealed that the enzyme contains 3 modules: a CM, a CBM, and a fungal-type dockerin. The CM with and without the CBM and CBM alone were heterologously expressed and characterized. It is suggested that the complete gene evolved by combination of DNA sequences coding for the various modules.

Materials and methods

Strains and vectors

Escherichia coli INV α F' and BL21(DE3) were products of Invitrogen (Carlsbad, Calif.) and Novagen (Madison, Wis.), respectively. *Saccharomyces cerevisiae* INVSc1 (MAT α his 3-D 1 *El2 trp1-289 ura3-52*) and plasmid pYES2 were purchased from Invitrogen Corp. pYES2 possesses ampicillin and tetracycline resistance genes for selection in *E. coli*, a *URA3* gene for high-copy-number maintenance and selection in *S. cerevisiae* INVSc1, and a GAL1 promoter sequence. Plasmid pET24 (b) was purchased from Novagen. A cDNA library of *Orpinomyces* sp. strain PC-2 was the same as described before (Chen et al. 1995).

Generation of DNA hybridization probes

To assess the diversity of docking module-containing components of *Orpinomyces* PC-2 cellulosomes, a PCR-based approach was developed (Steenbakketers et al. 2001) to amplify possible sequences coding for the partial docking modules. A number of distinct DNA sequences were amplified from an *Orpinomyces* cDNA library (Chen et al. 1995), and 9 were found to be different from those already isolated by activity screening. Degenerate primer designs (Table 1), PCR conditions, cloning and sequencing of PCR products, and analysis of sequence data were done as described by Steenbakketers et al. (2001). One of the sequences, RP2, was reamplified and labeled with digoxigenin (Roche Molecular Biochemicals, Indianapolis, Ind.) by PCR using the same degenerate primers and the RP2-specific PCR clone as template. The incorporation of digoxigenin into PCR products was detected by the increase of sizes on a 4.0% agarose gel.

DNA hybridization screening

The digoxigenin labeled RP2 probe was used to screen about 10^7 plaque-forming units (PFUs) of the *Orpinomyces* cDNA library. Twenty primary positive plaques were identified and purified into pure clones by a second round. Conditions for hybridization using DIG Easy Hyb buffer (Roche), washing ($0.5 \times$ SSC (1×0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 0.1% SDS (sodium dodecyl sulfate), 60 °C), and color development were as described by Roche. The recombinant pBluecript phagemids were excised from positive λ ZAP clones using a kit provided by Stratagene (La Jolla, Calif.), and the DNA inserts were sequenced as described by Li et al. (1997a). A 5' region of the clone with the longest insert was amplified by PCR using ManAF and ManAR (Table 1) as primers and labeled with digoxigenin. The new probe was used to isolate the full length cDNA clone (pManA).

Construction of plasmid cassette

Plasmid pYES2 was digested with *SacI* and *XbaI* overnight. The digested plasmid was purified using the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.). Primers PFMan and PRMan were synthesized based on the nucleotide sequence of the cloned gene (Table 1). PFMan codes for the first 7 amino acids of the open reading frame (ORF) and had a *SacI* site attached. PRMan is complementary to the sequence coding for amino acids 461–468, CTA nucleotides (complementary to the UAG stop codon), and a *XbaI* site attached. The encoded polypeptide should yield ManA without the NCDM. Using PFMan and PRMan as primers and the plasmid isolated from *E. coli* containing the complete *Orpinomyces* mannanase cDNA sequence (pManA) as template, the DNA region was amplified by PCR with 30 cycles of denaturation (1 min at 94 °C), annealing (1.0 min at 55 °C), and extension (1.5 min at 72 °C) on a 480 thermocycler (Perkin-Elmer Co., Norwalk, Conn.). PFU polymerase (Stratagene) was used for the PCR reactions. PCR products were purified using the GeneClean kit and digested with *SacI* and *XbaI*. Digested DNA fragments were purified and concentrated before they were ligated with the *SacI*- and *XbaI*-digested pYES2 using T4 DNA ligase. The plasmid pYES2 containing the insert was named pTmanA.

Transformation of *E. coli* and propagation of the plasmids

Ligation reactions were performed using a rapid ligation kit (Roche). Transformation was done following instructions of the Original TA Cloning Kit (Invitrogen, Carlsbad, Calif.). *Escherichia coli* INV α F' transformants were plated out on Luria–Bertani (LB) plates containing ampicillin (100 μ g/mL) and X-Gal (40 μ g/mL). White colonies were picked and grown overnight in LB liquid medium containing ampicillin. Plasmids were purified with the spin column kit from Qiagen (Valencia, Calif.). Restriction digestion and PCR amplification were performed to verify the presence of the insert.

Transformation of *S. cerevisiae*

A single colony of the yeast strain INVSc1 was grown to an OD₆₀₀ of 1.3 in yeast extract – Bacto-peptone – dextrose (YPD) medium, pH 6.5, containing 1% (w/v) yeast extract, 1% (w/v) bacto-peptone, and 1% (w/v) dextrose (Difco). Cells were harvested by centrifugation at 4000g for 5 min at 4 °C and washed twice with ice-cold sterile H₂O and twice with ice-cold 1 mol/L sterile sorbitol. The cells were then resuspended in 0.5 mL of 1 mol/L sorbitol. Approximately 5 μ g of pYES2 or pTmanA was added to transform 40 μ L of the yeast cells using an electroporator (Bio-Rad Laboratories, Hercules, Calif.). Transformants were grown in a drop-out base (DOB) medium containing 0.17% (w/v) yeast nitrogen base, 2.0% (w/v) dextrose, 0.08% (w/v) drop out supplements lacking uracil (Bio101, Inc.), as well as 2% (w/v) agarose and 1 mol/L sorbitol. The plates were incubated at 30 °C for 3–5 d.

Induction and expression of *manA*

Putative transformants were chosen for induction experiments. A single colony of a transformed yeast was transferred to 5 mL of DOB medium containing 0.17% (w/v) yeast nitrogen base, 0.08% (w/v) drop out supplement lacking uracil, and 4% (w/v) raffinose. After growth reached an OD₆₀₀ of about 1.0, sterile galactose (50%, w/v) was added to a concentration of 2.0% (w/v). During continuous culturing for an additional 40 h at 30 °C,

aliquot samples were drawn from the culture for OD₆₀₀ and enzymatic activity measurements. Cells were harvested by centrifugation (5000g, 20 min) at 4 °C. The supernatant obtained was stored at -20 °C until analyzed. The supernatant from five 500-mL cultures was used for the purification of the mannanase.

CBM production by *E. coli*

To produce the CBM, 2 oligonucleotides, PFMC and PRMC, containing *EcoRI* and *NoI* restriction sites, respectively, were synthesized (Table 1). With these as primers and pManA as template, PCR produced a DNA fragment coding for a polypeptide of 69 amino acid residues (401–469) of ManA. The fragment was cloned into pET24 (b) digested with *EcoRI* and *NoI*. The polypeptide was 6× His tagged at the C-terminus to facilitate purification. Sequencing of the nucleotide confirmed the lack of unwanted mutation. A plasmid with the CBM coding sequence inserted was named pCBM and was used for the transformation of *E. coli* BL21 (DE3). Transformed cells were grown on LB medium containing 50 µg/mL kanamycin. The presence of recombinant CBM production was verified by comparing protein binding patterns among cells transformed with pET24 (b) with or without the insert.

Mannanase and CBM purification

Saccharomyces cerevisiae cells transformed with pTmanA were grown in raffinose YPD medium (5 × 500 mL) to an OD₆₀₀ of 1.0. Galactose was added to 2.0% (w/v), and the cultures were shaken for an additional 30 h. Cultures were harvested and cell debris removed by centrifugation at 5000g for 20 min. The supernatant (2200 mL) was concentrated to 30 mL using an ultrafiltration cell equipped with a 10-kDa cutoff membrane (PM10; Millipore, Bedford, Mass.) and dialyzed against 20 mmol/L Tris-HCl, pH 7.0 (starting buffer). A fast protein liquid chromatography (FPLC) system was used for all of the column chromatographic steps. The concentrated sample was applied to a HiPrep 16/10 Q XL column equilibrated with the starting buffer. The column was washed with 500 mL of the same buffer. The mannanase was then eluted with a 400-mL linear gradient of NaCl from 0 to 1 mol/L. Fractions containing about 80% of the mannanase activity were combined, concentrated, dialyzed against the starting buffer, and then applied to an equilibrated gel filtration column packed with Superdex 75 (Pharmacia). The fractions displaying activity after being applied to both columns were analyzed by SDS – polyacrylamide gel electrophoresis (SDS-PAGE) to assess the purity of the mannanase.

To purify the CBM fusion protein, an *E. coli* culture (2.0 L) was grown to an OD₆₀₀ of 1.0, induced with 1 mmol/L of isopropyl-β-D-thiogalactopyranoside, and then grown for 5 h. The cells recovered by centrifugation (5000g, 20 min) were washed with 20 mmol/L sodium phosphate buffer, pH 7.0, containing 0.5 mol/L NaCl (starting buffer), and then disrupted using a french press. Cell debris was removed by centrifugation. All purification steps were done at 4 °C, except for the FPLC column chromatography, which was performed at room temperature. Clear supernatant was applied onto a Ni – nitrilotriacetic acid (NTA) agarose (Qiagen Inc., Valencia, Calif.) column equilibrated with the starting buffer. The column was washed with 20 mmol/L sodium phosphate (0.5 mol/L NaCl, pH 6.0). Proteins were eluted by a gradient of 0.5 mol/L imidazole, pH 6.0. Fractions containing the CBM were combined, concentrated by precipitation with ammonium sulfate (60% saturation), and the

precipitated protein was dialyzed against 20 mmol/L Tris–HCl buffer, pH 7.5, containing 0.1 mol/L NaCl. Dialyzed CBM was further purified by gel-exclusion chromatography on a Superdex-200 prepacked column (Pharmacia Biotech, Piscataway, N.J.). Eluted CBM was concentrated using Centricon 3 concentrator (Amicon, Inc., Beverly, Mass.) and stored at 4 °C. Purity of the CBM during purification was monitored by gradient (8%–16%) SDS–PAGE.

Enzyme activity and protein determination

Mannanase activity was routinely measured using 1% (*w/v*) locust bean mannan (Sigma) dissolved in 50 mmol/L sodium citrate buffer, pH 6.0, as substrate. The assay mixture contained 50 µL of an appropriately diluted enzyme solution and 200 µL of the substrate. Reaction mixtures were incubated for 15 min at 50 °C. Reducing sugars were measured by the dinitrosalicylic acid procedure of Miller (Miller 1959). Xylanase, CMCase, and filter paper hydrolase activities were measured in the presence of 1% xylan, 1% C, and 20 mg of filter paper in 200 µL of 50 mmol/L sodium citrate buffer, pH 6.0. One unit of enzyme is defined as the amount required for the generation of 1 µmol/min of reducing sugars under the assay conditions.

Insoluble polysaccharide binding assay

Binding of the CBM to insoluble polysaccharides (10 g/L) was done at room temperature in 1.5-mL microcentrifuge tubes. The CBM (18 nmol) was mixed with carbohydrates in 50 mmol/L sodium citrate buffer, pH 6.0, in a final volume of 0.5 mL. Tube contents were continuously mixed by rotation. After equilibration for 2 h, the carbohydrates were removed by centrifugation at 10 000*g* for 10 min. Centrifugation was repeated twice to ensure the removal of all of the carbohydrates. The amount of unbound protein was determined. The amount of bound protein was calculated as the difference between the initial amount of protein and unbound protein. Each data point is the mean of 5 replicates.

Affinity electrophoresis

Affinity electrophoresis using polyacrylamide gels with soluble polysaccharide ligands was performed as described earlier (Kataeva et al. 2001; Tomme et al. 2000). The polysaccharides were incorporated into the gel at a concentration of 0.2% prior to polymerization. A control gel without polysaccharides was prepared and run simultaneously. Electrophoresis was at 4 °C and 150 V for 3 h.

Analytical methods

Gel (10%) electrophoresis (SDS–PAGE) was done according to Laemmli (1970). Molecular-weight protein standards (Bio-Rad) were used as markers. Electrophoresis was performed in a Mini-Protein II cell, and gels were stained with Coomassie Brilliant Blue R-250 (Fairbanks et al. 1971). Mannanase active bands were visualized in a zymogram gel containing 0.5% mannan (Locust bean gum) as substrate after staining with 1.0% Congo Red as done before (Chen et al. 1997).

Protein was determined with the Coomassie Protein Assay Reagent (Pierce, Rockford, Ill.) using bovine serum albumin as standard. In CBM binding experiments, protein

concentrations were determined on the basis of A_{278} values. Molar absorption coefficient of the CBM (calculated from tryptophan and tyrosine content) was $9140 \text{ M}^{-1}\text{cm}^{-1}$.

The metal content of the CBM was determined by plasma emission spectrophotometry (Jarrell-Ash 965 ICP). Each data point was a mean of 3 replicates.

The pH optimum of ManA was determined by performing assays with 1% mannan as substrate at 50 °C in 0.1 mol/L sodium acetate (pH 3.8–5.6) and 0.1 mol/L sodium phosphate (pH 5.8–8.2). Enzyme stability at different pH values was determined by measuring the residual activity after incubating the enzyme for 24 h at 4 °C with the buffers above. The effect of the temperature on mannanase activity was determined by assaying the enzyme at temperatures from 30 to 65 °C. The stability of the ManA activity at various temperatures was determined by incubating the enzyme in 100 mmol/L sodium phosphate buffer, pH 6.0, from 30 min to 8 h at 50 and 55 °C. During the time course, samples were withdrawn and tested for remaining activity.

Nucleotide sequence accession No.

The nucleotide sequence reported in this paper was submitted to Genbank nucleotide sequence databases under access No. [AF177206](#).

Results

Isolation of *Orpinomyces mana*

In addition to the corresponding regions coding for *celA*, *celB*, *celC*, *celD*, *celE*, and *xynA*, 11 novel docking module specific sequences of *Orpinomyces* PC-2 were obtained by PCR amplification and cloning (Steenbakkers et al. 2001). Among the 11 novel sequences, the insert in pRP2 was reamplified, labeled with digoxigenin, and used as hybridization probe for screening of the cDNA library. Restriction analysis by *EcoRI* digestion of the excised positive plasmids revealed that the longest insert was 1.8 kb. It did not contain a full-length ORF. Therefore, a 300-bp region at the 5' region of the longest clone was amplified using primers ManAF and ManAR (Table 1) and labeled with digoxigenin. Using the new amplified sequence as a probe, 9 positive λ plaques were isolated from the library and were used to recover the full length cDNA (pManA).

Nucleotide and deduced amino acid sequences of *mana*

The DNA fragment obtained consisted of a total of 1924 nucleotides. It contained an open reading frame of 1740 bp coding for a polypeptide of 579 amino acid residues. The calculated mass of the polypeptide was 64 425 Da. A putative translation start codon (ATG) was assigned based on the fact that the preceding region was highly A+T rich, contained no ATG codon, and the amino-terminal region of the deduced polypeptide had the characteristics of a signal peptide (von Heijne 1986). The G+C content of the entire cDNA sequence and the ORF were 31.8% and 34.4%, respectively, while that of the 5' and 3' noncoding region was 17.6%, in agreement with other genes of the fungus (Chen et al. 1995; Li et al. 1997a). No long poly(A) tail at the 3' end was found. The nucleotide sequence between NDDCF and NDDCR in pRP2 matched perfectly with nucleotides 1709–1819

(amino acid residues 525–561) of the *manA* cDNA and the corresponding regions of all the partial cDNA clones. It suggests that there is no other gene highly homologous to *manA* in the fungal genome.

Modular structure and similarity analysis of ManA

Similarity search with the full length and various regions of the deduced ManA amino acid sequence using FASTA and BLAST revealed that ManA has a modular structure, as illustrated in Fig. 1. Starting with the N-terminus, there is a putative signal peptide of about 20 amino acid residues rich in nonpolar side chains. It is followed by about a 350-amino-acid residue, CM, a CBM, a linker, and 2 NCDMs. The CM of ManA belongs to glycoside hydrolase family 5, which includes hydrolases of bacteria, fungi, and plants. ManA has about 30% sequence identity with the CMs of mannanases from *T. reesei* (Stålbrand et al. 1995), *Aspergillus aculeatus* (Christgau et al. 1994), and *Agaricus bisporus* (Yagüe et al. 1997) (Fig. 2). It is also homologous to endoglucanases, xylanases, and other mannanases of family 5 glycoside hydrolases. Residues corresponding to Glu-192 and Glu-313 of ManA are highly conserved among family 5 glycoside hydrolases and shown to be involved in catalysis (Maccarón et al. 1993; Stålbrand et al. 1995; Fig. 2). However, the CM of ManA has several short insertions and is longer than those of the enzymes from the aerobic fungi. The functional significances of these insertions in ManA or deletions in the mannanases of aerobic fungi are not known.

The amino acid sequence spanning residues 406–463 showed homology to CBMs of fungal hydrolases belonging to CBM family 1 (Tomme et al. 1995; Fig. 3). CBMs of family 1, which are composed of about 40 amino acid residues, bind to insoluble substrates with varying reversibility, and form flat binding surfaces accessible to crystalline substrates (Carrard and Linder 1999; Linder and Teeri 1996). Three conserved aromatic residues (corresponding to Tyr-5, Tyr-31, and Tyr-32 of *T. reesei* CBHI CBM; Linder and Teeri 1996) form the binding surface. A glutamine (Gln-34 of *T. reesei* CBHI CBM) stabilizes Tyr-32 (Kraulis et al. 1989; Mattinen et al. 1998). In the CBM of ManA, 2 of the 3 corresponding aromatic residues (Tyr-458 and Tyr-459) and the glutamine (Gln-461) are conserved (Fig. 3).

Following the CBM of ManA is a Thr- and Ser-rich linker, which connects to a typical NCDM at the C-terminus. This module was used for the cloning of the *manA* cDNA and its partial sequence was reported as NCDM-PCR2 (Steenbakketers et al. 2001). NCDM sequences of anaerobic fungi have been placed into 3 groups according to their number and conservation of Cys residues. The ManA NCDM belongs to Group B.

Expression of *manA* in, and secretion of, the ManA from *S. cerevisiae*

In contrast with *xynA*, *celA*, *celB*, *celC*, and *axeA* cDNAs (Blum et al. 1999; Li et al. 1997a, 1997b), active mannanase was not expressed in *E. coli* harboring the plasmid containing the full-length *manA* cDNA. To confirm that the cDNA codes for a mannanase and to study its enzymatic properties, *S. cerevisiae* was used as an expression host. The region (Fig. 1) coding for the putative signal peptide, CM, and CBM (amino acid residues 1–500) was inserted into pYES2. Yeast transformed with the plasmid produced active mannanase in both cell-associated and extracellular fractions. The yeast cells transformed with the plasmid with

and without the *manA* insertion grew similarly in shake flask cultures (Fig. 4), indicating that the ManA insert had no negative effect on the yeast cells. The production level of the mannanase by the yeast cells was about 1.15 U/mL after 30 h induction by galactose. The amount of ManA was about 6 µg/mL, considering that the specific activity of the enzyme is 179 U/mg (see below). A much higher level (150 µg/mL) was obtained using similar conditions when a *T. reesei* mannanase gene was expressed in *S. cerevisiae* (Stålbrand et al. 1995).

Purification and analysis of the ManA

A crude mannanase preparation from the culture medium after 30 h of induction by galactose was concentrated and analyzed by zymogram after separation on SDS–PAGE. Although a major activity band (52 kDa) was revealed, other minor bands from 50 to 70 kDa (data not shown) of activity were also visualized on the gel, suggesting that the secreted mannanase might be glycosylated at various degrees, and (or) various truncated forms of the mannanase were secreted. A total of 3 *N*-glycosylation sites were found within the whole sequence, and 2 of them are located within the CM region. A total of 3 *N*-glycosylation sites were found within the whole sequence and 2 of those are within the CM region. Purification of the mannanase using ion exchange and gel filtration chromatography from 2.0 L of culture medium yielded 2.3 mg of purified mannanase that appeared as a single band of 55 kDa on SDS–PAGE gel (Fig. 5A).

Properties of the ManA

Among soluble substrates, the enzyme had the highest activity toward locust bean mannan (138.1 U/mg), followed by ivory nut mannan (46.7 U/mg), and oat spelt xylan (23.5 U/mg) (Table 2). It did not hydrolyze barley β-glucan or carboxymethylcellulose. Using various concentrations of locust bean mannan, a Lineweaver–Burk plot of activity levels over substrate concentrations revealed that the K_m and V_{max} of the enzyme were 3.4 mg/mL and 179.1 U/mg, respectively. With locust bean mannan, the enzyme was active from pH 3.8 to 8.0 at 50 °C. The optimum pH was from 4.6. to 7.2 (Fig. 6). The enzyme was stable at room temperature from pH 3.8 to 9.5 for 24 h. At pH 6.0, the highest activity was around 50 °C when the enzyme was assayed between 20 and 65 °C. The enzyme maintained about 70% of its activity for 8 h when stored at pH 6.0 and 50 °C, but after 1 h at 55 °C, only 50% of the activity remained. At 60 °C, the mannanase activity was lost within 2 min.

The ManA CBM binds to insoluble substrates

The ManA CBM (Fig. 1) was over-expressed in *E. coli* and purified to homogeneity (Fig. 5B). No metal ions were detected when the purified CBM was analyzed by plasma emission spectroscopy. Among the many polysaccharides used for binding assay, the CBM bound strongly to acid-swollen cellulose, Avicel PH-105, bacterial crystalline cellulose, filter paper, dewaxed cotton, chitin, and insoluble oat spelt xylan, but failed to bind to soluble oat spelt xylan, carboxymethylcellulose, arabinan, pectic galactan, laminarin, or lichenan (Table 3).

Discussion

To isolate genes coding for cellulosomal components of anaerobic fungi, several approaches have been employed. The most straightforward has been screening fungal cDNA expression libraries made in *E. coli* for various enzymatic activities. This has achieved the isolation of many genes coding for hydrolases of the anaerobic fungi. Many of the enzymes contain NCDMs and they are associated with the cellulosomal complexes produced by the anaerobic fungi (Fannuti et al. 1995; Ljungdahl et al. 1998), whereas some are devoid of the NCDM and therefore act individually. Examples of the former are *Neocallimastix patriciarum* XylA (Gilbert et al. 1992) and *Orpinomyces* CelA, CelB, CelC, CelE, and XynA (Li et al. 1997a, 1997b; Chen et al. 1998) and those of the later are *Orpinomyces* CelF (Chen et al. 2003), LicA (Chen et al. 1997), AxeA (Blum et al. 1999), and BglA (Li et al. 2004). The use of polyclonal antibodies against the *Piromyces* cellulosomes have been proven useful for the isolation of genes encoding a noncatalytic cellulose-somal component (Freelove et al. 2001). In addition, a PCR-based approach has been developed for the isolation of genes coding for NCDM-containing components (Steenbakkers et al. 2001). With this method, 16 and 19 distinct NCDM sequences were found in *Orpinomyces* strain PC-2 and *Piromyces* strain E2, respectively. Of these, 1 from *Piromyces* and 7 from *Orpinomyces* were identified with enzymes previously isolated from cDNA expression libraries. The other sequences are presumably part of genes coding for novel cellulosomal components. On the basis of these sequences, we have successfully isolated some full-length cDNA sequences from the *Orpinomyces* library. They code for 3 new cellulases (unpublished) and the ManA that is reported here.

The CM of ManA from *Orpinomyces* belongs to family 5 glycoside hydrolases. This family contains diverse enzymes hydrolyzing β -glycosidic bonds of cellulose, mannan, and xylan. The *Orpinomyces* ManA has the same modular structure as that of *T. reesei*, except that it has 2 NCDMs after the CBM separated by a linker (Fig. 1). In contrast, the 3 mannanases of the anaerobic fungus *Piromyces* (Ali et al. 1995; Fannuti et al. 1995; Millward-Sadler et al. 1996) contain 2 NCDMs and a CM of family 26 glycoside hydrolases. The family 26 mannanases had previously been found only in bacteria. One may propose that the *Orpinomyces* ManA has a fungal origin, whereas the *Piromyces* mannanase genes are of bacterial origin. Though there are no more debates regarding being placed as eukaryotes, anaerobic fungi have acquired genes from bacterial sources. Lateral exchanges of genetic resources between fungi and bacteria seem common and are probably due to their close interaction within the rumen and intestinal ecosystems (Ljungdahl et al. 1998). Typical examples of genes common with bacterial sources are those coding for the *Orpinomyces* LicA (Chen et al. 1997) and the 3 *Piromyces* mannanases (Fannuti et al. 1995; Millward-Sadler et al. 1996). Genes coding for hydrolases commonly found in fungi are *Neocallimastix celA* (Denman et al. 1996) and *Orpinomyces celF* (Chen et al. 2003), as well as *manA* reported here. The presence of these genes reflects the diversity of the genetic background of hydrolytic enzymes in anaerobic fungi for plant cell wall degradation.

The CBM of *Orpinomyces* ManA belongs to family 1 (Tomme et al. 1995, <http://afmb.cnrs-mrs.fr/~cazy/CAZY/>). The solution structures of 2 family 1 CBMs (formerly cellulose-binding domains or CBDs) of *T. reesei* cellobio-hydrolase I (Kraulis et al. 1989) and

endoglucanase I (Mattinen et al. 1998) form flat binding faces with 1 glutamine and 3 aromatic residues for binding to the crystalline cellulose surface. In ManA CBM at the C-terminal end, glutamine and tyrosine residues are aligned, whereas a tyrosine located at the N-terminal end is not aligned with those of the *Trichoderma* CBMs (Fig. 3). The binding of CBHI CBM is reversible, whereas that of CBHII CBM is irreversible (Carrard and Linder 1995; Linder and Teeri 1996). However, the binding preferences of the ManA CBM reported here are similar to those of both CBHI and CBHII CBMs. Thus, the ManA CBM binds preferably to insoluble polysaccharides that include both cellulose and hemicellulose structures. In fact, the module displays a higher affinity to crystalline cellulose and insoluble xylan than to mannan. This implies that the module within the complex plays a larger role than just helping the hydrolysis of mannan within the wall structures. Rather, it may facilitate the anchorage of the whole complex to the network surface of lignocellulosic structures. It can be noted that the *Piromyces* noncatalytic protein NCP1 (Freelove et al. 2001) contains 2 novel CBMs belonging to CBM family 29 and a docking module but is devoid of a CM. The CBMs of the NCP1 bind preferably to soluble polysaccharides. Cella of *N. patriciarum* and CelF of *Orpinomyces* have family I CBMs. However, they lack docking modules, are not cellulosomal, and act individually in the hydrolysis of plant cell wall constituents (Chen et al. 2003; Denman et al. 1996). Many bacterial cellulosomal catalytic components have CBMs in addition to dockerins (Bayer et al. 1998), but none of them has a family 1 CBM. In terms of fungal cellulosomes, a *N. patriciarum* xylanase was found to have a CBM and a NCDM (Liu et al. 1999), but the order of the CBM and the NCDM is reversed in comparison with that in ManA reported here.

The *Orpinomyces* ManA produced by *S. cerevisiae* hydrolyzes the β -1,4-mannosyl chain of locust bean and ivory nut mannan. It lacks activity against other structurally related polysaccharides, such as cellulose, β -glucan, or xylan, indicating that it is an endo-type mannanase. In contrast, many cellulases display substantial levels of activity against mannan substrates (Biely and Tenkanen 1998). The V_{\max} of the enzyme on locust bean mannan, 179 U/mg, is somewhat higher than that (113 and 103 U/mg) of 2 mannanases purified from *T. reesei* (Harjunt et al. 1995). A unique feature of the ManA is that it has an extremely broad pH optimum (Fig. 6), and this may prove to be attractive for several industrial applications.

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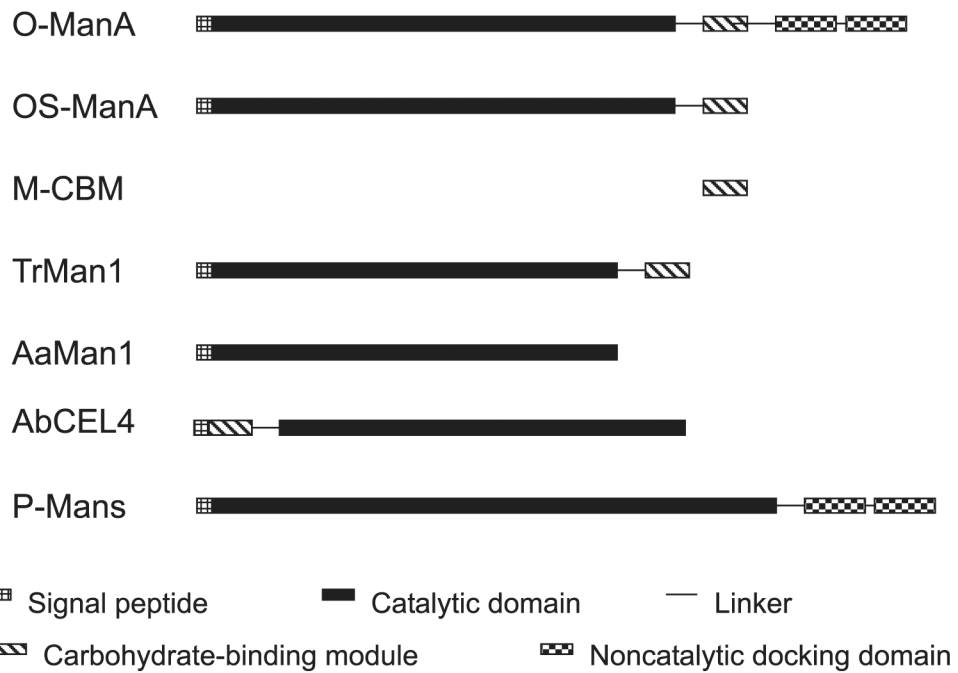


Fig. 1. Illustration of module organizations of some mannanases from various microbial sources. O-ManA, *Orpinomyces* mannanase A; OS-ManA, *Orpinomyces* mannanase A expressed in *S. cerevisiae* with NCDM truncated; M-CBM, *Orpinomyces* mannanase A CBM expressed in *E. coli*; TrMan1, *T. reesei* mannanase 1 (Stålbrand et al. 1995); AaMan1, *Aspergillus aculeatus* mannanase 1 (Christgau et al. 1994); AbCEL4, *Agaricus bisporus* CEL4 (Tang et al. 2001; Yagüe et al. 1997); P-Mans, mannanases of the anaerobic fungus *Piromyces equi* (Fannuti et al. 1995; Millward-Sadler et al. 1996).


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25 -----FVQIDGNEVVDGCKRYFSGSNITYYLMVS--NHERVDLALFTYARHNLNVVRAWAFCDCECE----DATRLVDFE O-ManA
26 -BRASSFWTISGTCQENIDGRVGYFAGTNCYWCSEFL--TNHADVDSTFESHSSGLKVVRVWGFENDVNTQPSPCQIWECKLS TrMan1
27 -AATTAHPSTISGLHFTIDGKTCYFAGTNSYWIIGFL--TNNDVDLIVMSQLAASDLKILRVWGFENDVNTKPTDCTWVYOLHA AaMan1
81 HEVSTICFVKASGIRRETLNGQRYTVVCGNSYVWGLTGLSTSAMNQAFSDTANACGTTVRRTWGFENEVTS---PNGNYICSWS AbCEL4

93 GPEVTLNG--ENMEKVVDYIATAAAQRNIRVVLTIINNWTDYGGMDVWVKQFGGKYHDE--FYTNKDIKGYKQYIKAMIN O-manA
104 ATGSTINTGADGLQILDYVVOQSAEQHNKLIIPFVNNWSDYGGINAYVNAFEGNATT---WYTNTAACTOYRKYVCAVVS TrMan1
105 NGTSTINTGADGLQILDYVVOQSAEQHNKLIIPFVNNWSDYGGINAYVNAFEGNATT---WYTNTAACTOYRKYVCAVVS AaMan1
158 GARPTINTGASGLLNFDNVIATAAKANGIRLITVALTNNWADYGGMDVYVNVQMVGNCPHDLFYTNNTAIKDAFKSYVRTFVS AbCEL4

169 RVNTYTQOLYKDDFTIFSWOLANEARCNGPHGLPVKNQNTDITIKWMDEIATFTHOEDNHLVSSCIIEGIGLIEPAGVD O-ManA
181 R-----YANSTAFWELGNEPRCN-G-----CSTDVTVQWATSVSQYVKSLSNHLVITGDEGIGLSTC---- TrMan1
183 R-----YSSSAATFAWELANEPRCQ-G-----CDTSVLNWIISDTSKYIKSLDSKHLVITGDEGFGLDVDS--- AaMan1
238 R-----YANETVMWELANEPROK-GSTGTTSGTCTTTTIVNWKEMSAFLKTTIDSNHLVAIGDEGEYNOQC---- AbCEL4

249 KNTYVYTYTEGTDYEAISALDSDYNTVHMYPVGWGLK--DYAKDGVTWIKAHADVDKFKFKPTVVEEWGLSISADNVPIE O-ManA
240 DGAYPYTYGEGTDEAKNVQIKSLDFGTFHLYPDSWGT---NYTWGNGWIOTHAACLAAGKPCVFEYGAOQNP----- TrMan1
243 DGSYPYTYGEGLENETKNIQISLIDFGTIFHLYPDSWGT---SYDWGNGWITAHAAACKAVGKPCILEEYGVTSNHC----- AaMan1
305 APTYPYQSGEGVDFEANLAISSVDEATEHSYEPFPGQGAIDAKAWCTQWITDHAASMKRVNKPVILEEFGVTTNQPF----- AbCEL4

328 QRDPIYTQWMNEVLIANDNIGMNFVWVCGEDYYCTDGYLLEEDETHAVIDPFTKKLIYANQTCENLDTISIV O-ManA
312 ---TNEAPWQTTISITTRGMGGDMFWQWGDTRFANGAQ---SNSDPYTVWYNSSNWQCLVKNHVDATNGGTTT TrMan1
315 ---AVESPWQOTAGNATGTSGLDYWQYGITTFSWG-O---SPNDGNTHYNTSDFTCIVTDHVAATNAQSK AaMan1
380 ---DTYAEWFN-EVESSCLTGLIWOACSHLSTC-D---THNDGYAVVDPGVPYP-LMKSHASAMKNRA AbCEL4

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Fig. 2.

Amino acid sequence alignment of the catalytic domains of fungal mannanases belonging to family 5 glycoside hydrolases of *Orpinomyces* PC-2 (O-ManA), *T. reesei* (TrMan1; Stålbrand et al. 1995), *Aspergillus aculeatus* (AaMan1; Christgau et al. 1994), and *Agaricus bisporus* (AbCEL4; Yagüe et al. 1997).

O-ManA	406	YYEVE- GCQPKYGTCTGGKC-C-AHGTRCEGS-EYYGQCR	463
T-Man1	373	SCSPLY G-QCG-GSGYTGPTCC-AQGT-CIYSNYWYSQCL	408
T-CbhI	461	PTQSHY G-QCG-GIGYSGPTVC-ASGTTQVLNPYYSQCL	497
T-CbhII	2	ACSSVW G-QCG-GQNWSGPTCC-ASGSTCVYSNDYYSQCL	38
T-EngI	401	CTQTHW G-QCG-GIGYSGCKTC-TSGTTQYSNDYYSQCL	437
T-EngII	1	QQT VW-G-QCG-GIGWSGPTNC-APGSACSTLNPYYAQCI	36
T-EngV	205	GQQTLY G-QCG-GAGWTGPTTCQAPGT-CKVQNQWYSQCL	241

Fig. 3.

Alignment of the *Orpinomyces* mannanase CBM (O-ManA) with those from *T. reesei* mannanase (T-Man1; Stålbrand et al. 1995), cellobiohydrolase I (T-CbhI; Shoemaker et al. 1983), cellobiohydrolase II (T-CbhII; Teeri et al. 1987), endoglucanase I (T-EngI; Penttilä et al. 1986), endoglucanase II (T-EngII; Saloheimo et al. 1988), and endoglucanase V (T-EngV; Saloheimo et al. 1994). Identical amino acids are in bold.

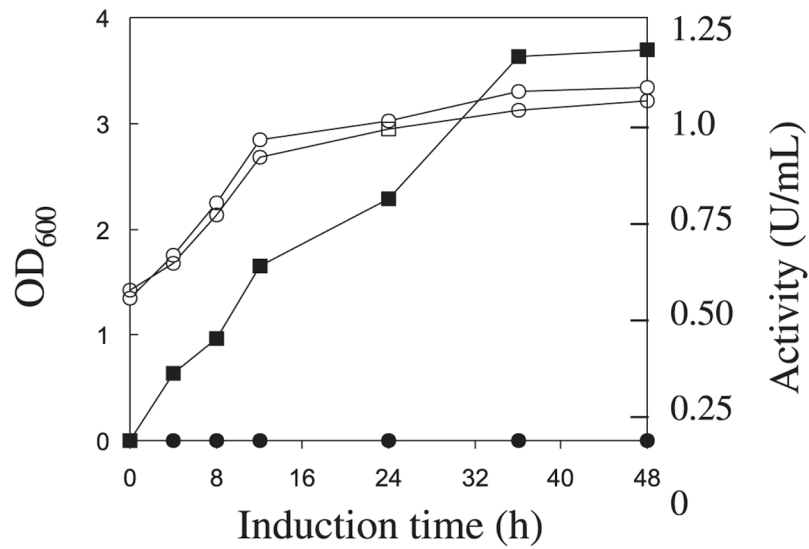


Fig. 4. Mannanase production by *S. cerevisiae* transformed with pManA after galactose induction. An aliquot of an overnight culture grown in DOB medium was used to inoculate raffinose – YPD medium. After growth to an OD₆₀₀ of 1.0, sterile galactose was added. Samples were withdrawn at time points shown in the figure. Levels of OD₆₀₀ (open symbols) and extracellular mannanase activity (filled symbols) are shown for the transformants containing pManA and pYES2.

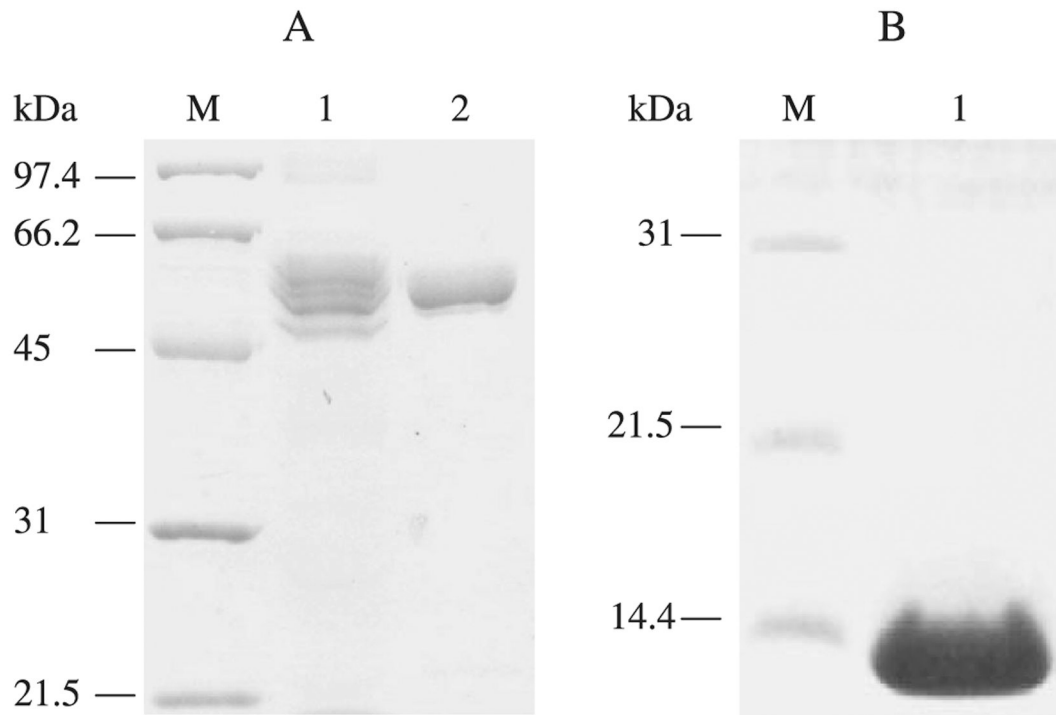


Fig. 5. SDS-PAGE analysis of the *Orpinomyces* ManA produced in *S. cerevisiae* and ManA CBM produced in *E. coli*. Gel was stained with Coomassie Brilliant Blue R-250 and destained before photographs were taken. Lane M, low molecular weight standards (Bio-Rad); Lane 1 of Panel A, 40-μg proteins of yeast culture supernatant; Lane 2 of Panel A, 10 μg ManA purified from yeast culture supernatant; Lane 1 of Panel B; 15 μg ManA CBM purified from *E. coli* cell lysate.

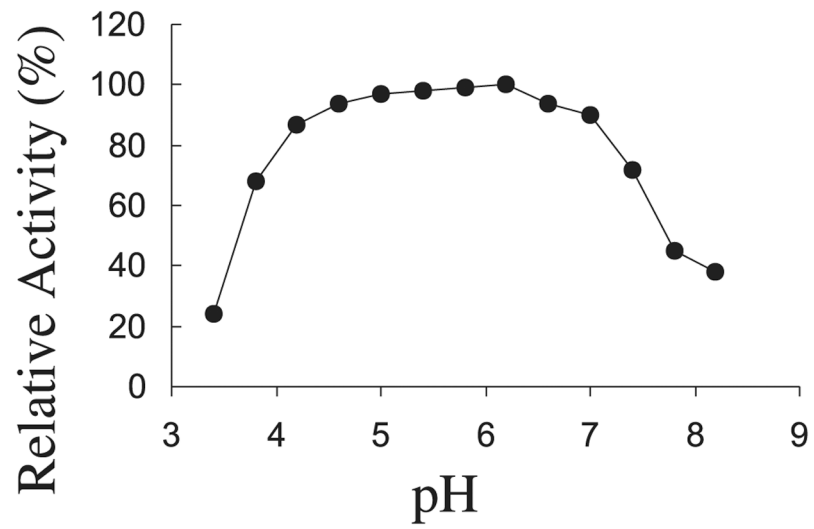


Fig. 6. Effect of pH on the activity of ManA assayed at 50 °C. For details see “Materials and methods” section.

Table 1.

Oligonucleotides used as PCR primers.

Primer	Sequence ^a	Orientation	Amino acid position ^b	Restriction site ^c
NDDCF	5'-GGIAAIIITGGGGIGTIGARAA-3'	Forward	518-524	
NDDCR	5'-TTYTClAClCCCAITlTlCC-3'	Reverse	562-568	
ManAF	5'-TGTTCAAACATGGAACCAA-3'	Forward	26-32	
ManAR	5'-CCAAACATCCATACCACCA-3'	Reverse	132-137	
PFMan	5'-ACCGAGCTC <u>GATGCATTTCAATAAAGTTAGTG</u> -3'	Forward	1-7	<i>SacI</i>
PRMan	5'-GCTCTAGAGTGGAAAGTACTTTTTGCACCTG-3'	Reverse	461-468	<i>XbaI</i>
PFMC	5'-GGAAITTCGGATTAGTTGTGTTTACTA-3'	Forward	401-405	<i>EcoRI</i>
PRMC	5'-GCTAGTGGCGCCCTTTGGAAAGTACTTTTTTGCA-3'	Reverse	462-469	<i>NotI</i>

^aR represents A and G; Y represents C and T; I represents inosine.

^bNumbering corresponds to that of Genbank acc. No. AF 177206.

^cRestriction sites are underlined.

Table 2.

Substrate specificity of the recombinant ManA.

Substrate	Activity ^a ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
Mannan (Locust bean)	138.1
Mannan (Ivory nut)	46.7
Xylan (Oat spelt)	23.5
β -Glucan (Barley)	ND ^b
Carboxymethylcellulose	ND

^a Activity was measured at 50 °C in pH 6.0 sodium citrate buffer (50 mmol/L) with a substrate concentration of 1.0% (w/v).

^b ND, activity level less than 1.0% of that obtained using locust bean mannan.

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Table 3.

Binding of the ManA CBM to polysaccharides.

Polysaccharide	Liquid state	Relative affinity
Acid swollen cellulose	Insoluble	+++
Avicel PH-105	Insoluble	+
Bacterial crystalline cellulose	Insoluble	++
Insoluble oat spelt xylan ^a	Insoluble	+++
Soluble oat spelt xylan ^a	Soluble	NB
Chitin	Insoluble	+++
Filter paper	Insoluble	+
Dewaxed cotton	Insoluble	++
Carboxymethyl cellulose	Soluble	NB
Soluble birchwood xylan ^a	Soluble	NB
Arabinan	Soluble	NB
Pectic galactan	Soluble	NB
Laminarin	Soluble	NB
Lichenan	Soluble	NB

Note: Reaction mixtures contained 10 mg polysaccharide and 18.3 nmol CBM in 0.5 mL sodium citrate buffer (50 mmol/L), pH 6.0. Adsorption scale:

+++ more than 50% adsorbed;

++ 20%–49% adsorbed;

+ 5%–19% adsorbed; NB, less than 5% adsorbed.

^aSoluble and insoluble xylan preparations were obtained by stirring 2.0% (w/v) xylan suspension for 1 h and separating the 2 fractions by centrifugation at 5000g for 15 min.