

A Gene Encoding a Novel Multidomain β -1,4-Mannanase from *Caldibacillus cellulovorans* and Action of the Recombinant Enzyme on Kraft Pulp

ANWAR SUNNA,¹ MORELAND D. GIBBS,¹ CHARLES W. J. CHIN,² PETER J. NELSON,²
AND PETER L. BERGQUIST^{1,3*}

Department of Biological Sciences, Macquarie University, Sydney, New South Wales 2109,¹ and CSIRO Forestry and Forest Products, Clayton, Victoria 3168,² Australia, and Department of Molecular Medicine, University of Auckland Medical School, Auckland, New Zealand³

Received 31 August 1999/Accepted 9 November 1999

Genomic walking PCR was used to obtain a 4,567-bp nucleotide sequence from *Caldibacillus cellulovorans*. Analysis of this sequence revealed that there were three open reading frames, designated ORF1, ORF2, and ORF3. Incomplete ORF1 encoded a putative C-terminal cellulose-binding domain (CBD) homologous to members of CBD family IIIb, while putative ORF3 encoded a protein of unknown function. The putative ManA protein encoded by complete *manA* ORF2 was an enzyme with a novel multidomain structure and was composed of four domains in the following order: a putative N-terminal domain (D1) of unknown function, an internal CBD (D2), a β -mannanase catalytic domain (D3), and a C-terminal CBD (D4). All four domains were linked via proline-threonine-rich peptides. Both of the CBDs exhibited sequence similarity to family IIIb CBDs, while the mannanase catalytic domain exhibited homology to the family 5 glycosyl hydrolases. The purified recombinant enzyme ManAd3 expressed from the cloned catalytic domain (D3) exhibited optimum activity at 85°C and pH 6.0 and was extremely thermostable at 70°C. This enzyme exhibited high specificity with the substituted galactomannan locust bean gum, while more substituted galacto- and glucomannans were poorly hydrolyzed. Preliminary studies to determine the effect of the recombinant ManAd3 and a recombinant thermostable β -xylanase on oxygen-delignified *Pinus radiata* kraft pulp revealed that there was an increase in the brightness of the bleached pulp.

The use of hemicellulases in the manufacture of kraft pulp has been shown to promote pulp bleaching, and in most application studies the workers have focused on using xylanases (24, 47, 48). Using mannanases was not studied until recently, when it was shown that mannanases, acting in combination with xylanases, enhance enzyme-aided bleaching of pulps in modified kraft processes (10, 40).

1,4- β -Mannans, which are some of the major constituents of hemicellulose, are hydrolyzed to mannose by endo-acting β -mannanases and exo-acting β -mannosidases. However, due to the complexity of the polysaccharide and the presence of side chain sugars, additional enzymes, including α -galactosidase, β -glucosidase, and acetylmannan esterase, are required to completely hydrolyze mannans (34). Extremely thermophilic bacteria and hyperthermophilic archaea are known to produce several types of thermostable glycosyl hydrolases (38). Few thermostable β -mannanases from thermophilic bacteria have been characterized and sequenced (14, 17–19, 33), while no archaeal β -mannanase has been described so far. The mannanases that have been sequenced belong to either glycosyl hydrolase family 5 or glycosyl hydrolase family 26 (22).

Huang et al. (X. P. Huang, J. A. Hudson, F. A. Rainey, P. D. Nichols, and H. W. Morgan, submitted for publication) described a new thermophilic, spore-forming, aerobic bacterium related to the bacilli that was able to grow on crystalline cellulose, and they named this organism *Caldibacillus cellulovorans*. Their isolate was related to members of the genus

Alicyclobacillus, as determined by small-subunit ribosomal DNA sequence analysis, but it lacked alicyclic fatty acids in its cell membrane. It could also utilize xylan and wood pulp as substrates, which indicated that it was also hemicellulolytic. Using genomic walking PCR (GWPCR) techniques, we identified a multidomain β -mannanase gene, *manA*, in *C. cellulovorans* that codes for a thermostable enzyme, ManA, belonging to glycosyl hydrolase family 5. The ManA protein has an unusual structure; it consists of an N-terminal domain of unknown function and a catalytic domain flanked by two cellulose-binding domains (CBDs) homologous to the domains found in a variety of cellulases (45, 46). In this paper we describe cloning, sequencing, and expression of recombinant ManA, as well as biochemical properties of the purified enzyme and its action on kraft pulp.

MATERIALS AND METHODS

Bacterial strains and genomic DNA. *Escherichia coli* JM101 [Δ (*lac-proAB*) *thi-1 supE44 F'* (*traD36 proAB⁺ lacZAM15*)] was used as the bacterial host in all DNA cloning and expression studies. *C. cellulovorans* cells were kindly provided by Hugh W. Morgan, University of Waikato, Hamilton, New Zealand. Genomic DNA was prepared as described previously (33). The media and other reagents used have been described by Croft et al. (11).

GWPCR. Linker assembly, linker library construction, and GWPCR were performed as described by Morris et al. (31, 33). The forward and reverse genomic walking primers used in this study are shown in Table 1.

DNA sequencing. DNA sequencing was performed with an Applied Biosystems model 377 DNA sequencer by using Big Dye terminator chemistry. A computer analysis of sequence data was carried out by using the Genetics Computer Group software package (12).

Construction of a recombinant pJLA602 plasmid containing *manA*. The catalytic or binding domains of ManA were numbered from the N terminus, and the linkers were ignored in this nomenclature (Fig. 1). The *manA*-specific primers dom1F and newcbdt1R (Table 1) were synthesized in order to PCR amplify DNA coding for the full-length *manA* gene from *C. cellulovorans* and were designed to include *Nco*I and *Eco*RI restriction enzyme sites, respectively, which

* Corresponding author. Mailing address: Research Office, Macquarie University, North Ryde, Sydney, New South Wales 2109, Australia. Phone: 61 2 9850 8614. Fax: 61 2 9850 8799. E-mail: peter.bergquist@mq.edu.au.

TABLE 1. Oligonucleotides used in this study

Primer	Sequence
Genomic walking primers	
GW3F	5' CGACGCAGGCCGAACAGTACTTCTG 3'
GW3R	5' CGTCGGATCATAACGAGTAGTCGTTG 3'
GW4F	5' GCAACTTGGTTCGTACAGTACCGCG 3'
GW4R	5' CAACCGTTGCTCAGCACGATCCGC 3'
GW5F	5' CCGGAGACCGCCGACATTCGAAGCACCGCG 3'
uorf1F	5' GACGATAAACGGCGAATCCGGCGCGAAAACATCG 3'
<i>manA</i>-specific primers^a	
dom1F	5' TAGAGGATTTCCATGGTTCGACGGCTTATCGC 3'
newcbdt1R	5' GTCGGCGGAATTCGGTTACGGCTCGACGCC 3'
mannF	5' GCAACGCCACCATGGGCGGTGCCAGCCACTGAG 3'
mannR	5' GGGTGCGGAATTCACGAGGTCCCGATCGCTTCG 3'

^a Engineered restrictions sites are underlined.

allowed directional in-frame ligation of the *manA* PCR fragment into expression plasmid pJLA602 (36); this resulted in recombinant plasmid pSUN22. Mannanase-positive transformants were identified on plates containing galactomannan by using Congo red stain (43). Both strands of the recombinant plasmid encoding ManA were sequenced in order to confirm that there were no PCR-derived base changes in the DNA except those introduced in the engineered restrictions sites of the PCR primers (Asn→Val change in the second amino acid at the N terminus of the ManA peptide).

Construction of *manAd3* recombinant pJLA602 plasmid. The *manAd3* PCR product coding for the mannanase domain of *manA* was amplified from recombinant plasmid pSUN22 by using the *manA*-specific primers mannF and mannR (Table 1). These primers were designed to include the *Nco*I and *Eco*RI restriction sites, respectively, which allowed directional in-frame ligation of the *manAd3* PCR fragment into pJLA602; this resulted in recombinant plasmid pSUN23. Both strands of the recombinant plasmid encoding the ManAd3 mannanase were sequenced in order to confirm that there were no PCR-derived base changes in the DNA except those introduced in the engineered restriction sites of the PCR primers (Pro→Met and Ser→Gly changes at the beginning of the D3 peptide and introduction of a stop codon at the end of the D3 peptide).

Production and purification of ManAd3. ManAd3 was produced as described previously (31). After host proteins were denatured at 70°C for 30 min, ManAd3 was purified further by anion-exchange chromatography by using a procedure similar to that used for XynB of *Dicystoglossus thermophilum* Rt46B.1 (31); a HiTrapQ column (Amersham Pharmacia Biotech) and an NaCl step gradient were used. The ManAd3 protein eluted at 75 mM NaCl.

Enzyme activity and protein determination. β-Mannanase activity was studied by using the dinitrosalicylic acid method of Bernfeld (6). The standard assay reaction mixture contained 0.5% (wt/vol) locust bean gum (LBG) supplemented

with 120 mM universal buffer (8) (pH 6.0) and enzyme, and the final volume was 0.1 ml. The reaction mixture was incubated at 85°C for 15 min. All assay reactions were linear (time versus enzyme concentration). One mannanase unit was defined as the amount of enzyme required to liberate 1 μmol of mannose per min at the assay temperature. Protein concentrations were determined by using a BCA protein quantification kit (Pierce, Rockford, Ill.).

Effects of temperature, pH, and thermostability on mannanase activity. The effect of temperature on the reaction rate was determined by incubating purified enzyme with the substrate at temperatures ranging from 40 to 95°C under standard assay conditions. The enzyme activity of purified ManAd3 was also assayed at pH values ranging from 3.0 to 7.5 (120 mM universal buffer) and from 6.5 to 8.5 {120 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane buffer} at the optimal temperature for activity. All pH values were adjusted at 85°C.

The purified enzyme was incubated at 70 and 80°C in the absence of substrate in the thermostability experiments. Samples were removed at different times, and the residual mannanase activity was measured under standard conditions by using an assay time of 5 min.

Electrophoresis and molecular weight estimation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using the method of Laemmli (26) and 10% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.). Broad-range molecular weight standards obtained from Bio-Rad (Richmond, Calif.) were used to determine the molecular mass of the purified protein.

Substrate specificity and mode of action of ManAd3. The substrate specificity of ManAd3 was determined by incubating the purified enzyme with different polymeric substrates under standard assay conditions. The hydrolysis products arising after the action of ManAd3 on LBG (at 70°C) were analyzed as previously described (18). In addition, manno oligosaccharide and galactosyl-manno oligo-

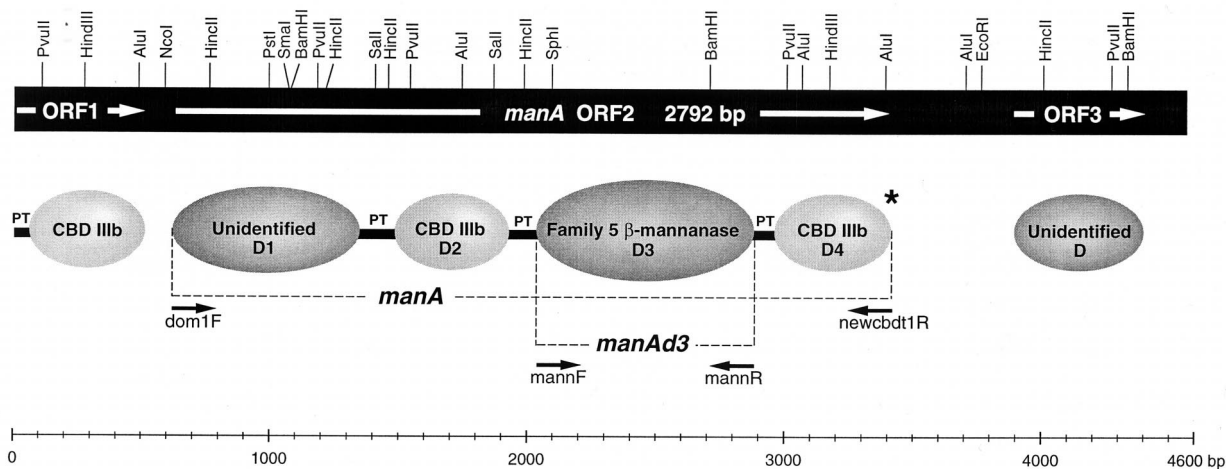


FIG. 1. Diagrammatic representation and partial restriction map of ORF1, ORF3, and *manA* and the domains encoded by the genes. The positions of PCR primers dom1F and newcbdt1R used to amplify the *manA* fragment from the genomic DNA of *C. cellulovorans* for construction of pSUN22 are shown. The positions of PCR primers mannF and mannR used to amplify the *manAd3* fragment from pSUN22 for construction of expression vector pSUN23 are also shown. The asterisk indicates the stop codon. D, domain; PT, proline-threonine linker peptide.

saccharide (Megazyme International, Bray, Ireland) hydrolysis was investigated by using high-performance thin-layer chromatography (HPTLC). Each hydrolysis reaction mixture contained 4 μ l of 10 mM oligosaccharide substrate, 4 μ l of sterile distilled water, 1 μ l of 120 mM universal buffer (pH 6.0), and 1 μ l of purified mannanase. The reaction mixtures were incubated at 70°C for 3 h, and then 3 μ l of each reaction mixture was applied to a Silica Gel 60 HPTLC plate (Merck, Darmstadt, Germany) and separated with an *n*-propanol-ethanol-water (70:10:20, vol/vol/vol) solvent system. Sugars were detected after the plate was dried and dipped in a 5% sulfuric acid solution in ethanol and then incubated at 110°C for 10 min.

Enzyme treatment of oxygen-delignified kraft pulp from *Pinus radiata*. Oxygen-delignified kraft pulp prepared from *Pinus radiata* wood was enzymatically treated with recombinant β -mannanase ManAd3 from *C. cellulovorans* and with recombinant xylanase XynB3 from *D. thermophilum* Rt46B.1 as previously described (31) and then subjected to elemental-chlorine-free (ECF) bleaching.

Nucleotide sequence accession number. The DNA sequence described here has been deposited in the GenBank database under accession no. AF163837.

RESULTS

GWPCR and analysis of the 4,567-bp nucleotide sequence.

As part of our effort to identify a *C. cellulovorans* xylanase gene by using GWPCR, we obtained a product that exhibited sequence homology to β -mannanases belonging to glycosyl hydrolases family 5. This product was generated by using genomic walking primers (GW3F and GW3R [Table 1]) which bind in the sequence coding for family IIIb CBDs. Preliminary observations revealed that highly homologous CBD type IIIb domains were present in other glycosyl hydrolase structures found in *C. cellulovorans* (unpublished results). The nucleotide sequence data obtained for regions upstream and downstream from the GWPCR fragments were combined to generate a 4,567-bp sequence.

Analysis of the nucleotide sequence revealed that three open reading frames (ORFs), ORF1, *manA*, and ORF3, were present. Part of ORF1 consisted of a sequence that coded for a putative C-terminal type IIIb CBD (61 to 513 bp). Downstream of the ORF1 terminator codon, TGA, there was a 105-bp intergenic region (514 to 619 bp), and this region contained a 15-bp palindromic repeat sequence which may have acted as a transcription terminator signal.

The second ORF, *manA* (620 to 3,412 bp), consisted of 2,792 nucleotides that encoded a multidomain β -mannanase, ManA, whose putative size was 930 amino acids. The initiation codon, ATG, was preceded at a spacing of 5 bp by a potential ribosome-binding sequence (GAGGA). Downstream of the termination codon, TAA, there was a 481-bp intergenic region (3,413 to 3,893 bp) that contained a 19-bp palindromic repeat sequence, which also may have acted as a transcriptional terminator signal. Further downstream at position 3,894 was the putative ATG initiation codon of ORF3 (3,894 to 4,400 bp).

Amino acid sequences of the ORFs and the multidomain structure of ManA. Figure 1 is a diagrammatic representation of the elements encoded by the three ORFs of the 4,567-bp sequence. The translated amino acid sequence of ORF3 exhibited no sequence similarity to any of the entries in the GenBank and SwissProt databases. The incomplete nucleotide sequence of ORF1 encodes a proline-threonine (PT) linker peptide followed by a C-terminal CBD homologous to family IIIb CBDs, as classified by Tormo et al. (46). The family IIIb CBD ORF1 exhibits only moderate sequence similarity to the partial ORF encoding a CBD directly upstream of *Bacillus lautus* CelA (level of sequence identity, 59%) (21), the internal CBD of *Caldicellulosiruptor* sp. strain Rt69B.1 multidomain XynC (level of sequence identity, 56%) (32), and the C-terminal CBD of *Clostridium stercoarium* CelY (level of sequence identity, 53%) (9).

Analysis of the sequence of ManA revealed a complex and unique multidomain structure. The N-terminal sequence con-

tained a putative signal peptide sequence with a predicted cleavage site between position 33 (Ala) and position 34 (His). Removal of the signal peptide yielded a mature protein that had a predicted M_r of 98.1×10^3 and contained a domain with an unidentified function at its N terminus, ManAd1 (620 to 1,348 bp) (Fig. 1), followed by a 45-amino-acid PT linker peptide. ManAd1 was compared to translated DNA sequences in the GenBank database and was found to be most closely related to the N-terminal domain of the cellulose binding protein (p40) of *Streptomyces halstedii* (level of identity, 45%) (16). The *S. halstedii* p40 N-terminal domain was associated via a PT linker with a C-terminal type IIa CBD and was shown to be able to bind to Avicel. The p40 protein exhibited only very weak catalytic activity when it was incubated for 12 h with methylumbelliferyl- β -D-glucoside. Preliminary hydrolysis experiments performed with different substrates did not reveal any ManAd1 catalytic activity (unpublished results).

ManAd1 also exhibited some sequence similarity with a number of proteins, some of which have been shown experimentally to bind to chitin (25, 35, 37, 41, 44), and in one instance a chitinase (15). An alignment of the ManAd1 sequence with the sequences of related catalytic domains is shown in Fig. 2.

ManAd1 was followed by a second domain, ManAd2 (1,484 to 1,927 bp) (Fig. 1), which was identified as a family IIIb CBD. This CBD exhibited the highest levels of sequence similarity with the internal CBD of *Caldicellulosiruptor* sp. strain Rt69B.1 XynC (level of sequence identity, 45%) (32) and the C-terminal CBD of *Bacillus subtilis* endoglucanase EglS (level of sequence identity, 44%) (29). The internal CBD was linked through a 35-amino-acid PT linker to an internal domain, ManAd3 (2,033 to 2,884 bp) (Fig. 1), which was identified as a β -1,4-mannanase belonging to glycosyl hydrolase family 5. The three conserved active site residues (Asn-121, Glu-122, and Glu-223) and the strictly conserved residues Arg-43, His-80, His-188, Tyr-190, and Trp-248 that are characteristic of all family 5 enzymes (2, 4, 20, 28, 49) were also found in ManAd3. The mannanase domain of ManA exhibited 71% sequence identity with the mannanase catalytic domains of ManA and CelC of *Caldicellulosiruptor saccharolyticus* (19, 33) and 56% sequence identity with the β -mannanase ManA of *Streptomyces lividans* (1).

The ManAd3 catalytic domain was linked to a C-terminal family IIIb CBD, ManAd4 (2,960 to 3,412 bp) (Fig. 1), through a 25-amino-acid PT linker. This CBD was almost identical (level of sequence identity, 97%) to the C-terminal CBD of ORF1 but exhibited only 54% sequence identity to the internal ManAd2 CBD. Furthermore, ManAd4 exhibited 59, 57, and 53% sequence identity to the CBDs of ORF, XynC, and CelY of *Bacillus lautus* (21), *Caldicellulosiruptor* sp. strain Rt69B.1 (32), and *Clostridium stercoarium* (9), respectively.

Purification and biochemical properties of the recombinant ManAd3 enzyme. Recombinant ManAd3 mannanase was purified from lysed *E. coli* cells harboring pSUN23 as described above to electrophoretic homogeneity, and the final specific activity was 1,949 mannanase units/mg. The apparent M_r of the purified enzyme was estimated to be 30.7×10^3 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), which is consistent with the M_r of 31.3×10^3 deduced from the translated amino acid sequence of ManAd3.

Recombinant ManAd3 enzyme was active at temperatures between 40 and 95°C, and the optimal temperature for activity was 85°C (data not shown), which is 20°C above the optimum growth temperature for *C. cellulovorans* (Huang et al., submitted). More than 30% of the initial activity was still detected at 95°C. Thermostability experiments revealed that in the ab-

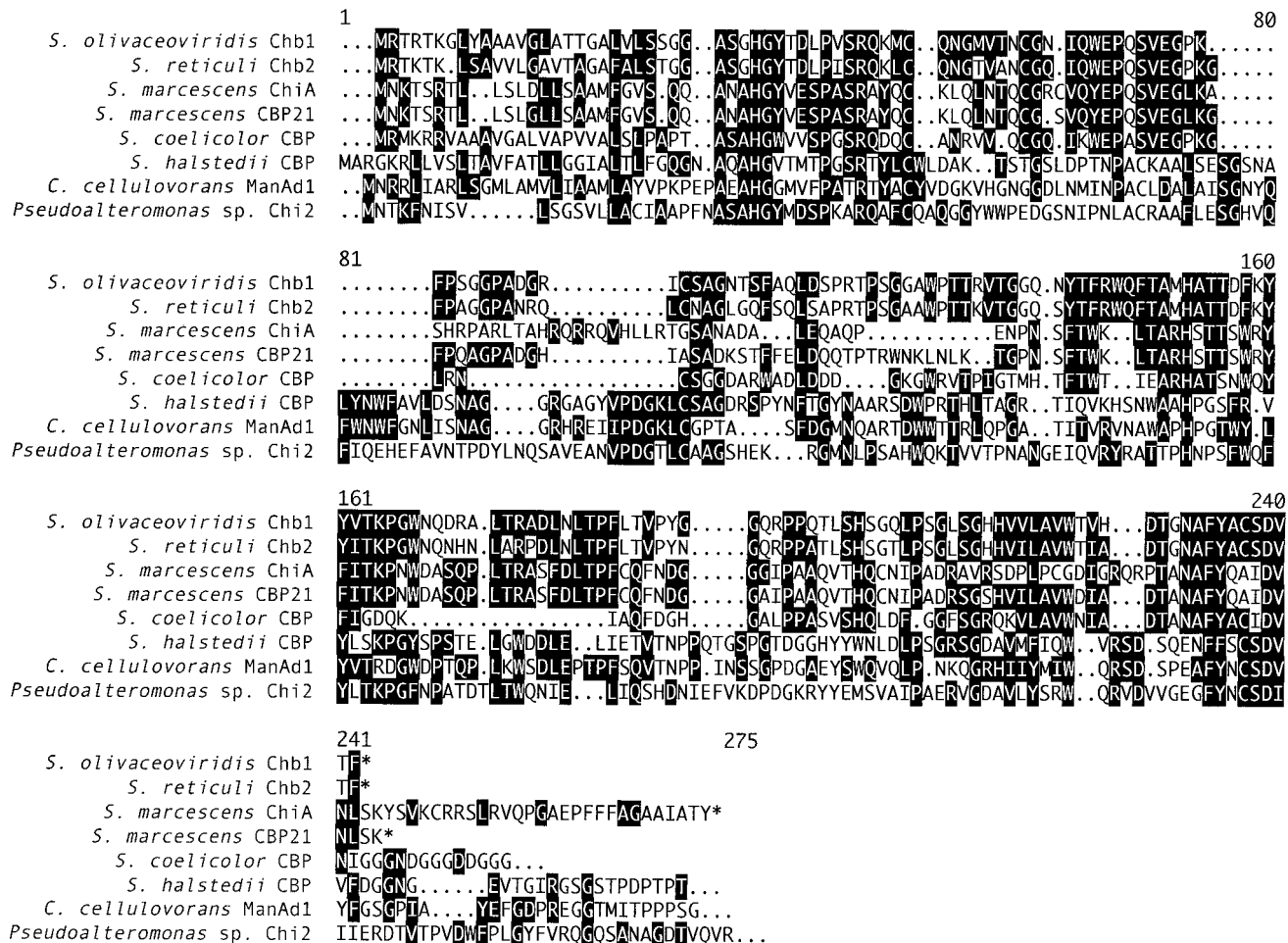


FIG. 2. Alignment of sequences homologous to the *C. cellulovorans* ManAd1 sequence. Conserved residues are highlighted. The sequences used were sequences of *Streptomyces olivaceoviridis* Chb1 (GenBank accession no. X78535) (37), *Streptomyces reticuli* Chb2 (GenBank accession no. Y14315) (25), *Serratia marcescens* ChiA (GenBank accession no. L38384) (15), *Serratia marcescens* CBP21 (GenBank accession no. AB015998) (41), *Streptomyces coelicolor* CBP (GenBank accession no. AL031155) (35), *Streptomyces halstedii* CBP (GenBank accession no. U51222) (16), *Caldibacillus cellulovorans* ManAd1 (GenBank accession no. AF163837) (this study), and *Pseudoalteromonas* sp. Chi2 (GenBank accession no. AF007895) (44).

sence of the substrate (LBG), the enzyme had a half-life at 80°C of 15 min, while at 70°C the enzyme retained 95% of its initial activity after 24 h of incubation (data not shown). When assays were performed at 85°C, the purified *Caldibacillus* mannanase was active at pH values between 5.0 and 8.5 (data not shown). The optimal pH for activity was 6.0, and 60 and 37% of the initial mannanase activity remained at pH 8.0 and 8.5, respectively.

Substrate specificity and mode of action. No detectable reducing sugars were released from oat spelts, birchwood, beechwood, larchwood, and purified 4-*O*-methyl- β -glucuronid-xylans, β -glucan, hydroxyethyl or carboxymethyl cellulose, lichenan, laminarin, soluble starch, pullulan, and dextran. The largest amount (100%) of reducing sugars was liberated when the galactomannan LBG (mannose/galactose ratio, 4:1) was used as the substrate. Pure ivory nut mannan (99% mannose) was poorly hydrolyzed (0.7% compared to LBG), indicating that the enzyme was not able to hydrolyze unsubstituted mannan. In the presence of konjac gum glucomannan (mannose/glucose ratio, 1.5:1), only 15% of the maximum activity was observed, while replacement of LBG by the galactomannan guar gum (mannose/galactose ratio, 2:1) resulted in a reduc-

tion in the mannanase activity of more than 90%. Furthermore, no detectable reducing sugars were released during incubation with *Saccharomyces cerevisiae* mannan (with α -1,6, α -1,2, and α -1,3 mannosidic linkages).

The products released by the action of ManAd3 mannanase on LBG galactomannan were analyzed by anion-exchange chromatography (data not shown), and the results revealed that mannose, mannobiose, and mannotriose were the major end products of hydrolysis, which confirmed the endo-acting characteristic of the enzyme. In addition, larger oligosaccharides and trace amounts of galactose were also detected, as were unidentified products that probably represented galactosyl manno oligosaccharides. The ability of ManAd3 to hydrolyze manno oligosaccharides with different degrees of polymerization was investigated by using HPTLC (data not shown). After 3 h of incubation at 70°C, the purified recombinant β -mannanase completely hydrolyzed mannohexaose, manno-pentaose, and mannotetraose, yielding mannotriose, mannobiose, and mannose. Mannotriose was only partially hydrolyzed, which produced mannobiose and traces of mannose, while mannobiose was not hydrolyzed. This result indicates that the enzyme contains an active site capable of accommo-

TABLE 2. Effects of treatment with recombinant XynB3 and ManAd3 on D(EO)DD bleaching of *P. radiata* kraft pulp

Prepn	Enzyme stage yield (%)	D(EO) stage			D1 stage brightness (% ISO)	D2 stage brightness (% ISO)	Total bleached pulp yield (%)
		Kappa no.	Brightness (% ISO)	Yield (%)			
Control	99.2	4.6	53.9	96.5	71.3	81.8	95.3
XynB3	98.8	3.4	60.0	95.8	77.4	85.3	94.7
ManAd3	99.9	4.0	55.6	95.9	72.9	82.8	95.5
XynB3 + ManAd3	98.3	3.1	60.7	95.3	79.0	86.1	93.4

dating at least six mannose residues. Furthermore, no transferase activity was observed after 3 h of incubation with mannose. Purified ManAd3 was also incubated with the galactosyl manno-oligosaccharides 6¹- α -D-galactosyl-mannotriose and 6^{3,6}-di- α -D-galactosyl-mannopentaose. Only minimal hydrolysis of 6¹- α -D-galactosyl-mannotriose was observed after 3 h of incubation, and trace amounts of mannose and a second product, probably α -D-galactosyl-mannobiose, were released. 6^{3,6}-di- α -D-galactosyl-mannopentaose was not hydrolyzed by ManAd3.

Enzyme treatment of oxygen-delignified kraft pulp. Oxygen-delignified kraft pulp was treated with two thermostable recombinant enzymes, XynB3 of *D. thermophilum* Rt46B.1 (31) and ManAd3 of *C. cellulovorans*. The results of this experiment are summarized in Table 2. The individual xylanase and mannanase treatments resulted in lower kappa numbers and greater brightness than the kappa number and brightness of the control pulp. The xylanase treatment resulted in a lower kappa number (3.4 U) than the mannanase treatment (4.0 U). However, the lowest kappa number was obtained when the pulp was treated with a mixture of the two enzymes (3.1 U). The trend in the brightness levels of the pulps after the EO stage were similar. There was a 6.8% ISO unit increase in brightness when the enzyme mixture was used (from 53.9 to 60.7% ISO compared with the control pulp); however, only 0.7% ISO unit of this increase was due to the presence of the mannanase in the enzyme mixture.

DISCUSSION

The modular N-terminal domain-PT linker-CBD-PT linker-catalytic domain-PT linker-CBD structure of *C. cellulovorans* ManA is unique. No other enzymes with type IIIb CBDs that contain both internal and C-terminal CBDs in combination with multiple auxiliary domains have been described. Previously, Tomme et al. (45) and Tormo et al. (46) described a comprehensive classification scheme for CBDs. Family IIIb CBDs have been found predominantly in association with glycosyl hydrolase family 5 and 9 endoglucanases of *Bacillus*, *Clostridium*, and *Caldicellulosiruptor* species, either as internal domains or as C-terminal domains of multidomain peptides. It has been observed that family IIIc CBDs are always internal and are directly associated with bacterial family 9 glycosyl hydrolases and that they appear to be required to facilitate hydrolysis of crystalline cellulose by the family 9 catalytic domain (G. K. Farrington, M. D. Gibbs, P. Anderson, J. Eldredge, P. L. Bergquist, and D. P. Williams, submitted for publication). In all *Bacillus* enzymes, type IIIb CBDs occur as C-terminal domains that are separated from an N-terminal catalytic domain by a PT linker. In *Caldicellulosiruptor* strains, type IIIb CBDs occur as internal duplicated or triplicated domains that are separated from both N- and C-terminal catalytic domains by PT linkers.

Members of glycosyl hydrolase family 26 are β -mannanases,

while glycosyl hydrolase family 5 contains both endoglucanases and bacterial, fungal, and plant β -mannanases. Amino acid sequence alignments of members of family 5 rarely reveal levels of sequence identity greater than 20%, and as a result, members of family 5 have been classified in six subfamilies, subfamilies A1 to A6 (3, 27). Recently, a new alignment of the available bacterial and eukaryotic mannanase amino acid sequences resulted in description of two new subfamilies, subfamilies A7 (eukaryotic mannanases) and A8 (bacterial mannanases) (23). Members of subfamily A8 exhibit levels of sequence identity greater than 43%, while the levels of sequence identity for members of subfamily A7 and members of subfamily A8 are less than 20%. Subfamily A8 includes ManA of *Thermomonospora fusca* (23), ManA of *S. lividans* (1), ManA of *Vibrio* sp. (42), and the mannanase domain of CelC of *C. saccharolyticus* (33). Accordingly, based on amino acid sequence comparisons, the mannanase domain ManAd3 of *C. cellulovorans* is classified as a member of glycosyl hydrolase family 5, subfamily A8.

ManAd3 has a higher optimal temperature for activity (85°C) than most recombinant mannanases have. Thermostable β -mannanases belonging to families 5 and 26 have been described; enzymes of *C. saccharolyticus* (family 5) (7) and *D. thermophilum* Rt46B.1 (family 26) (18) have an optimal temperature for activity of 80°C, and there is no decrease in activity after incubation for 24 h at 70°C and after incubation for 16 h at 80°C, respectively. Recently, Duffaud et al. (13) described a β -mannanase of *Thermotoga neapolitana* 5068 which is optimally active at 92°C and has a half-life of 34 h at 85°C, but no sequence data for this enzyme is available yet.

ManAd3 of *C. cellulovorans* exhibited low specificity with pure ivory nut mannan and konjac gum glucomannan but high specificity with LBG galactomannan. The galactomannan specificity, however, decreased in the presence of the more substituted guar gum galactomannan, indicating that the hydrolysis yield depended on the degree of galactose substitution, as well as the nature of the substitution sugar in the mannan backbone. It has been reported that the degree of hydrolysis of galactomannan decreases with increasing substitution by galactose, which suggests that mannanase cleavage of the mannan backbone is obstructed by the sugar residue (30). Similarly, the recombinant family 5 ManA of *C. saccharolyticus* was found to exhibit high specificity with glucomannans and mannan, while the specificity decreased with the increase in galactose substitution in galactomannans. This result suggested that α -1,6-galactose branches may sterically hinder the mannanase, preventing enzyme-substrate binding (7).

It has been shown that using mannanases that act in association with xylanases enhances enzyme-aided bleaching of pulps obtained from modified kraft processes (10, 40). However, at this stage we do not have enough data to support the hypothesis that the activities of the two enzymes are synergistic. Additional experiments will be required to determine whether the beneficial effect of adding mannanase to xylanase could be

achieved simply by increasing the dose of xylanase. The same trend was observed with the brightness levels of the pulps after the D₁ and D₂ stages. The total yield of bleached pulp when the mannanase was used alone was about the same as the total yield of the control. However, when the enzyme mixture was used, the mannanase was responsible for a decrease in the yield of about 1.3%. The effects of both family 5 and family 26 mannanases on pulp bleaching have been examined (5, 39). Suurnäkki et al. (39) compared the effects of mannanases from *Trichoderma reesei* (family 26), *Aspergillus niger* (probably family 5), and *C. saccharolyticus* (family 5) on bleaching of *P. radiata* kraft pulp. These authors found that only the *T. reesei* mannanase effectively increased the bleachability of the pulp. The effectiveness of the *T. reesei* mannanase was attributed to the ability of this enzyme to solubilize the pulp glucomannan. In a similar study two family 26 mannanases, one from *C. saccharolyticus* Rt8.B4 and one from *D. thermophilum* Rt46B.1, were found to be ineffective in bleaching *P. radiata* kraft pulp (5).

It appears that mannanases may provide only marginal improvements as bleach-boosting agents but may have other uses in the pulp and paper industry (e.g., in fiber modification).

ACKNOWLEDGMENTS

This work was supported by grants from Macquarie University Research Grants Funds and by an Australian Research Council Small Grant.

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