Three-dimensional structure of (1,4)- β -D-mannan mannanohydrolase from tomato fruit

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Abstract

The three-dimensional crystal structure of tomato (*Lycopersicon esculentum*) β -mannanase 4a (LeMAN4a) has been determined to 1.5 Å resolution. The enzyme adopts the $(\beta/\alpha)_8$ fold common to the members of glycohydrolase family GH5. The structure is comparable with those of the homologous *Trichoderma reesei* and *Thermomonospora fusca* β -mannanases: There is a conserved three-stranded β -sheet located near the N terminus that stacks against the central β -barrel at the end opposite the active site. Three noncanonical α -helices surround the active site. Similar helices are found in *T. reesei* but not *T. fusca* β -mannanase. By analogy with other β -mannanases, the catalytic acid/base residue is E204 and the nucleophile residue is E318. The active site cleft of *L. esculentum* β -mannanase most closely resembles that of the *T. reesei* isozyme. A model of substrate binding in LeMAN4a is proposed in which the mannosyl residue occupying the -1 subsite of the enzyme adopts the 1S_5 skew-boat conformation.

Keywords: 1,4- β -D-mannan mannanohydrolase; β -mannanase; mannan; ripening; *Lycopersicon esculentum*; tomato; glycoside hydrolase; glycohydrolase family GH5; $(\beta/\alpha)_8$ fold; TIM barrel; crystal structure; molecular dynamics

Softening of fleshy fruits during ripening is caused by the dissolution of pectin in the middle lamella that reduces cell-to-cell adhesion (Wakabayashi 2000) and also by the breakdown of the cell walls themselves (Fischer and Bennett 1991; Brummell and Harpster 2001). The cell wall is composed of cellulose microfibrils that are tethered by xyloglu-

cans in a matrix of pectic polysaccharides and structural proteins (Carpita and Gibeaut 1993). In addition, another type of polysaccharide that comprises a portion of the hemi-cellulosic matrix of the tomato fruit cell wall is mannan, most likely in the form of glucomannan (Tong and Gross 1988; Seymour et al. 1990). The enzyme 1,4- β -D-mannan mannanohydrolase (E.C. 3.2.1.78), or β -mannanase, is capable of cleaving this substrate within regions of consecutive mannose residues. The β -mannanase expressed in tomato fruit has been designated LeMAN4a (Bourgault and Bewley 2002b). The activity of LeMAN4a increases in the outer tissues of tomato fruits during ripening, and the enzyme is localized within the cell walls (Bewley et al. 2000; Bourgault et al. 2001). Currently, a cause-and-effect relationship between β -mannanase activity in fruits and ripening-associated softening is not clearly established. Plant β -mannanases are better characterized in terms of their role in seed germination (Nonogaki et al. 2000) and reserve mobilization during seedling growth (Bewley 1997).

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Abbreviations: LeMAN4a, *Lycopersicon esculentum* β -mannanase 4a; TrMAN, *Trichoderma reesei* β -mannanase; TlMAN, *Thermomonospora fusca* β -mannanase; PcMAN, *Pseudomonas cellulosa* β -mannanase; DFDM, 2-deoxy-2-fluoro-dinitrophenyl-mannotrioxide; MD, molecular dynamics; RMSD, root mean square deviation; MWCO, molecular-weight cutoff.

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While some researchers have focused on gaining an understanding of the role of β -mannanase in seed germination and fruit ripening, others have investigated its potential utility in biotechnological applications. There has been considerable interest in using β -mannanases from various sources as an additive in the bio-bleaching process for kraft pulp production (Tenkanen et al. 1997; Montiel et al. 2002) or as a livestock feed additive (Petty et al. 2002). In these applications it is desirable to improve the thermostability or change the pH optimum of the enzyme. In order to carry out the protein engineering required to achieve these goals, the three-dimensional structure of the enzyme must be elucidated. Prior structural studies have considered either bacterial (Hogg et al. 2001) or fungal (Hilge et al. 1998; Sabini et al. 2000) forms of the enzyme, whereas the current study is the first to investigate the structure of a β -mannanase from a plant source. This particular form of the enzyme may prove to be more suitable for some biotechnological applications, depending on the substrate or the conditions required for the treatment.

β -Mannanase randomly hydrolyzes internal 1,4- β -D-mannopyranosyl linkages in mannans. In all characterized glycoside hydrolases, subsites exist for the binding of multiple sugar groups. These are numbered -4, -3, -2, -1, +1, and +2, from the nonreducing end to the reducing end of the polysaccharide (Davies et al. 1997). Cleavage occurs between the mannosyl residues occupying the -1 and +1 subsites. Family GH5 and family GH26 β -mannanases catalyze hydrolysis via a "retaining mechanism," whereby there is net retention of the configuration of the anomeric carbon atom of the -1 mannosyl group. Two key catalytic residues have been identified: an acid/base and a nucleophile. By analogy with related enzymes, these residues are E204 and E318, respectively, in LeMAN4a. Hilge and coworkers (1998) have described the retaining mechanism in detail. Briefly, the oxygen atom bridging the -1 and +1 mannosyl residues is protonated by the acid/base and the nucleophile forms a covalent bond with the C1' atom of the -1 mannosyl residue, releasing the reducing end. This results in the inversion of the C1' anomeric carbon atom. The acid/base residue then deprotonates a water molecule, and the resulting hydroxide ion attacks the glycoside-enzyme ester linkage, regenerating the nucleophile, reinverting the anomeric C1' atom, and releasing the nonreducing end of the substrate. These steps are illustrated in Figure 1.

The crystal structures of family GH5 β -mannanase from the fungi *Thermomonospora fusca* (TfMAN) (Hilge et al. 1998) and *Trichoderma reesei* (TrMAN) (Sabini et al. 2000) have been reported. The binding of mannotriose in the -4, -3, and -2 subsites of TfMAN has been reported, as well as the binding of mannobiose in the +1 and +2 subsites of TrMAN. The mannosyl groups adopted the energetically favorable 4C_1 configuration in all cases. A related bacterial enzyme (family GH26A) from *Pseudomonas cellulosa*

Figure 1. Schematic representation of proposed catalytic mechanism for LeMAN4a. The catalytic steps are described in the text.

(PcMAN) has also been described (Hogg et al. 2001). The crystal structures of this enzyme as a Michaelis complex with 2-deoxy-2-fluoro-dinitrophenyl-mannotriose (DFDM) and with 2-deoxy-2-fluoro-mannotriose moiety covalently linked to the catalytic nucleophile (E320) have also been determined (Ducros et al. 2002). In the Michaelis complex, the 2-deoxy-2-fluoro-mannosyl residue in the -1 subsite adopts the 1S_5 configuration. The complex with 2-deoxy-2-fluoro-mannotriose moiety covalently linked to the catalytic nucleophile (E320) represents a trapped reaction intermediate. The mannosyl residue adopts the 0S_2 configuration in this case. Ducros and coworkers (Ducros et al. 2002) concluded that the (-1) mannosyl residue follows a 1S_5 to $B_{2,5}$ to 0S_2 conformational itinerary from the Michaelis complex to the covalently linked intermediate.

Results

Following large-scale expression and cell disruption, SDS-PAGE analysis (data not shown) demonstrated that much of the LeMAN4a-Maltose Binding Protein fusion product remained insoluble. Extraction of the pellet using a high-salt buffer greatly increased the amount of soluble fusion protein. Affinity chromatography performed on the soluble extract using an Amylose resin yielded a highly enriched fusion protein that contained active enzyme when analyzed using a β -mannanase gel-diffusion assay. The fusion protein was then successfully cleaved using Factor Xa protease.

The success of the cleavage proteolysis was monitored by the disappearance of the 82-kDa fusion protein band and the appearance of two new bands at 40 and 42.5 kDa using SDS-PAGE. LeMAN4a (40 kDa) was then purified from the Maltose Binding Protein portion (42.5 kDa) of the fusion protein and other minor contaminants, using cation exchange chromatography. SDS-PAGE analysis of column fractions followed by Coomassie blue staining confirmed the purity of LeMAN4a (data not shown).

Rod-like crystals of length 0.3 mm were obtained from condition 9 of crystal screen I: 30% (w/v) PEG 4000, 0.1 M sodium citrate (pH 5.6), 0.2 M ammonium acetate. These crystals diffracted to 1.5 Å using synchrotron radiation. Data processing and reduction indicated that the crystal lattice was primitive orthorhombic. Statistics for the X-ray data are given in Table 1. Systematically absent reflections indicated space group $P2_12_12_1$.

Molecular replacement with *T. reesei* β -mannanase (Protein Data Bank [PDB] entry 1QNP) was successful. The molecular replacement solution corresponded to the highest peaks in the rotation (4.94σ) and translation (18.15σ) functions. The second highest rotation and translation function peaks were 4.00σ and 11.18σ , respectively. The initial R_{factor} of the molecular replacement model was 54.1% ($R_{\text{free}} = 54.6\%$). As refinement progressed, differences between LeMAN4a and 1QNP were observed in electron density maps and modeled into the crystal structure. Toward the end of the refinement, water molecules were evident in the electron density maps and included in the model. One spherical peak in the electron density maps was too high to be explained by the presence of water and was interpreted as a chloride ion, based on the height of the peak and surrounding positive charges. The final model consists of residues 30–399, 409 water molecules, and one chloride ion. Statistics for the model are given in Table 2. A ribbon diagram of the structure and the electron density in the active site is shown in Figure 2.

Apart from TfMAN and TrMAN, structural homologs of LeMAN4a identified in the FSSP database include human β -glucuronidase, cellulase CelC from *Acidothermus cellulolyticus*, and cellulase CelC from *Clostridium thermocel-*

Table 2. Refinement statistics

Non-Hydrogen Atoms:	
Protein	3143
Ions	1
Water	409
R-factor (%)	18.2
R-free (%)	20.5
RMSD values	
Bonds (Å)	0.009
Angles (°)	1.224
Chiral centers (Å ³)	0.074
Torsion angles (°)	6.423
Planar restraints (Å)	0.006
B-factors (bonded atoms)	1.229
Ramachandran plot	
Core region	91.7%
Allowed region	7.7%
Additionally allowed region	0.3%
“Disallowed” region	0.3%

lum. The data are summarized in Table 3. The root mean square deviation (RMSD) over C α atoms between the various β -mannanases are given in Table 4. The structure-based sequence alignment of LeMAN4a, TrMAN, and TfMAN is shown in Figure 3.

A model of LeMAN4a bound to mannopentaose was constructed based on crystal structures of available β -mannanase/substrate complexes and subject to 1-nsec molecular dynamics (MD) simulation. In the course of the simulation, the enzyme and substrate underwent slight structural rearrangements due to the initial relaxation of the structure. However, after 100 psec the system reaches equilibrium (Fig. 4A,B). Key distances crucial for catalysis were monitored throughout: The nucleophile E318O ϵ to mannosyl residue C1' distance, maintained at 3.5 ± 0.25 Å, and the E204H ϵ to mannosyl residue O1 distance (Fig. 4C). The latter is more variable, due to rotations about the X2 dihedral angle to adopt a more energetically favorable position. The interactions of the mannopentaose model with LeMAN4a are illustrated in Figure 5.

Discussion

LeMAN4a, like other known β -mannanase structures, adopts the canonical (β/α)₈ fold (Fig. 2A). The protein features a roughly V-shaped groove that, by analogy with the other β -mannanase structures, binds mannan. Interspersed with the conserved secondary structure elements that define this fold (designated $\beta 1$, $\alpha 1$, $\beta 2$, $\alpha 2$, etc.) are some additional β -strands and α -helices (designated αA , βA , etc.) (Figs. 2, 3). Three strands, βA , βB , and βC , located near the N terminus, form a β -sheet that stacks against the central β -barrel, at the end opposite the active site. These structural elements are also present in TrMAN and TfMAN (Figs. 2,

Table 1. Crystallographic data statistics for LeMAN4a

Space group	$P2_12_12_1$
Unit cell (Å, °)	$a = 59.5$, $b = 74.5$, $c = 79.0$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	100–1.50 (1.55–1.50) ^a
R-factor	0.080 (0.748)
I/ σ (I)	12.13 (1.19)
Completeness (%)	98.4 (99.7)
No. of measured reflections	230,329 (20,768)
No. of unique reflections	56,178 (5613)
Redundancy	4.1 (3.7)

^a Numbers in parentheses pertain to the highest-resolution bin.

Figure 2. (A) Wall-eyed stereo-view ribbon diagram of the structure of LeMAN4a. The canonical $(\beta/\alpha)_8$ fold secondary structure elements are colored green (β -strands) and blue (α -helices). Additional secondary structure elements are colored cyan (β -strands) and red (α -helices) and are labeled in some cases. The catalytic glutamate residues are shown as ball-and-stick structures. (B) Wall-eyed stereo view of the final $2mF_o - DF_c$ electron density map, contoured at 1σ , around the active-site.

3). Following strand $\beta 1$ is a short helix (αA) unique to LeMAN4a. Another small β -sheet (βD and βE) is present in both the LeMAN4a and *T. reesei* enzymes between strand $\beta 2$ and helix $\alpha 2$. The three noncanonical helices (αA , αB , and αC) surround the active site (Fig. 2A).

Two residues in the LeMAN4a crystal structure had “disallowed” Ramachandran angles: N31 and D136. N31, near the N terminus, is built in two conformations and seems to have considerable mobility about its Φ angle based on the electron density map. The outlying Ramachandran angles for this residue are probably a reflection of the flexibility of the polypeptide chain at that juncture and appears to have little structural significance. Excellent electron density was observed for D136, which trails the conserved active site residue W135. The strained conformation of D136 may be important for the correct orientation of W135. The Ram-

achandran angles of D136 remain strained throughout the MD simulation.

LeMAN4a shows the greatest structural similarity with TrMAN (Tables 3, 4; Fig. 3), sharing many common active site residues and structural motifs. One significant difference, however, is in the loop containing F138 in LeMAN4a. This loop shows a large degree deviation in the superposed structures, despite similar amino-acid sequences (Fig. 3). The result is that although Y117 in TrMAN forms part of the -3 subsite, the sequence alignment equivalent residue in LeMAN4a, F138, is not part of the active site and is instead stacked on to helix αB . Structurally, Y117 in TrMAN is

Table 3. DALI search of FSSP Top hits with LeMAN4a

Enzyme	E.C.	Species	PDB	RMSD, NR ^a
β -mannanase	3.2.1.78	<i>T. reesei</i>	1QNO 2.1	325
β -glucuronidase	3.2.1.31	<i>H. sapiens</i>	1BHG 2.4	261
Cellulase	3.2.1.4	<i>A. cellulolyticus</i>	1ECE 2.8	270
Cellulase	3.2.1.4	<i>C. thermocellum</i>	1CEO 2.7	253
β -mannanase	3.2.1.78	<i>T. fusca</i>	1BQC 3.1	255

^a Number of equivalent residues over which RMSD was calculated.

Table 4. Comparison of β -mannanase structures

	<i>P. c</i>	<i>T. f</i>	<i>T. r</i>	<i>L. e</i>
<i>L. esculentum</i>	2.080 ^a	1.822	1.339	370 ^c
	153 ^b	187	282	
<i>T. reesei</i>	2.284	1.825	349	
	105	181		
<i>T. fusca</i>	1.990	302		
	103			
<i>P. cellulosa</i>	353			

P.c., *P. cellulosa*; *T.f.*, *T. fusca*; *T.r.*, *T. reesei*; *L.e.*, *L. esculentum*.

^a RMSD.

^b Number of residues used in comparison.

^c Number of residues in structure.

Figure 3. Structure based sequence alignment of family GH5 β -mannanases. Numbering applies to the LeMAN4a sequence. The secondary structure elements of LeMAN4a are labeled *above* the sequences. The acid/base (E) and nucleophile (N) are highlighted. Secondary structure as determined by DSSP is indicated. Uppercase letters (boxed) indicate regions of structural homology. Shaded letters are implicated in substrate recognition.

substituted for H92 in LeMAN4a, which is in a loop following strand β 2. It performs a similar structural role as part of the -3 subsite.

A nonproline *cis*-peptide bond is present between residues W360 and N361. This *cis*-conformation is also present in the TfMAN and TrMAN structures. It appears to be essential for the correct conformation of W360, which forms part of the -1 subsite.

Bourgault and Bewley (2002b) have shown that the penultimate L398 residue is important for catalytic activity. In the crystal structure, this residue engages in packing interactions with the strand β 1 and C-terminal end of helix α 8. Deletion of L398 and S399 residues would appear to result in exposure of hydrophobic packing residues. This could destabilize the protein, leading to a great reduction in enzyme activity.

Stabilization of the sugar in the $+1$ subsite through ring-stacking interactions appears to be very important for glycoside hydrolase function: All family GH5 glycosylhydrolases studied have a tryptophan residue that forms part of the $+1$ or $+2$ subsites or that overlaps the two. In LeMAN4a, this residue is W135, structurally analogous to W114 in TrMAN (Fig. 3). This residue occurs after strand β 3 in amino acid sequence in both enzymes. In TfMAN, however, the equivalent residue is W167, located after strand β 5 in that enzyme. The ring system of W167 is coplanar with those of W135 and W114 when the three structures are superposed. Thus, in our model of the Michaelis complex, W135 engages in a ring-stacking interaction with the $+1$ mannosyl (Fig. 5).

Several glycoside hydrolase crystal structures have been reported, some in complex with substrate analogs in order to study the conformational changes in the sugar moiety bound

Figure 4. RMSD of LeMAN4a backbone atoms (gray line) and all non-hydrogen atoms (black line) (A) and RMSD of the nonhydrogen atoms of the mannopentaose moiety (B) with respect to the starting structure over the time-course of the MD simulation. (C) E318O ϵ to mannan-C1' distance (black line) and E204He to mannan-O1 distance (gray line).

in the -1 subsite throughout the catalytic cycle. Substrate distortion has been observed in endoglucanases from families GH5 (Davies et al. 1998), GH7 (Sulzenbacher et al. 1996), and family GH20 chitinase (Tews et al. 1996). In these enzymes, the sugar residue occupying the -1 subsite adopts the 1S_3 or similar 4E configuration prior to hydrolysis. The conformation is thought to change to 4H_3 in the transition state before adopting a 4C_1 conformation as the covalently linked intermediate. This was shown not to be the case in family GH26A β -mannanase, for which a 1S_5 to $B_{2,5}$ to 0S_2 conformational itinerary for the Michaelis complex to transition state to covalent enzyme-substrate intermediate has been proposed (Ducros et al. 2002). This different itinerary allows the 2' hydroxyl group to remain pseudoequatorial over the course of the formation of the enzyme-sugar intermediate.

To test the possibility that the sugar residue occupying the -1 subsite of LeMAN4a also adopts the 1S_5 conformation in the Michaelis complex, a 1-nsec MD simulation of a LeMAN4a/mannopentaose complex was performed. The bound position of the substrate was reconstructed from TrMAN/mannobiose, TfMAN/mannotriose, and PcMAN/DFDM crystal structures. In this simulation, the mannosyl residue in the -1 subsite was given the 1S_5 configuration.

This structural configuration introduces a kink into the mannan chain and helps it to fit into the V-shaped active site cleft of LeMAN4a. After an initial equilibration period of ~ 200 psec, the mannopentaose group stabilizes with a RMSD of ~ 1.2 with respect to its initial modeled configuration (Fig. 4B). The protein also equilibrates rapidly (Fig. 4A). The slight upward drift of the RMSD of the protein throughout the simulation appears to be caused by movement of flexible surface loops and side chains as they explore the available conformational space. This has no effect on the active site residues, which remain close to their initial positions. The MD simulation thus provides a glimpse at how LeMAN4a could bind its substrate. The interactions of mannopentaose with LeMAN4a in the MD simulation are summarized in Figure 5. The 2' hydroxyl group of the -1 mannosyl group engages in a hydrogen bonding interaction with N203, which is strictly conserved in all family GH5 glycoside hydrolases (Sabini et al. 2000). Mutation of the equivalent residue in *Erwinia chrysanthemi* cellulase results in a total loss of catalytic activity (Bortoli-German et al. 1995). If the mannosyl group occupying the -1 subsite was not to adopt the 1S_5 configuration, the 2' OH group would no longer be pseudoequatorial and would not be able to engage with N203. The nucleophile E318O ϵ to C1' distance after equilibration (3.5 ± 0.25 Å) is very similar to the equivalent distance in the PcMAN/DFDM Michaelis complex (3.33 Å), and is maintained throughout the latter part of the MD simulation to within a small margin (Fig. 4C). E318O ϵ makes a nucleophilic attack on the mannosyl C1' carbon atom to form the glycoside-enzyme covalent intermediate (Fig. 1). The E204He to mannosyl residue O1 distance is more variable (Fig. 4C) because of rotations about the X2 side-chain dihedral angle. This flexibility may be

Figure 5. Schematic representation of the interactions of mannopentaose with LeMAN4a in the 1-nsec MD simulation of the complex. Hydrogen bonds are indicated as dashed lines; ring-stacking interactions are indicated as semitransparent lines drawn from the protein (thin end) to the sugar ring (thick end). The scissile bond in the substrate is indicated ($\Rightarrow$). The carbon atoms in the substrate (1' to 6') are labeled. The subsites each sugar molecule binds in (-3 to $+2$) are indicated.

required because E204 must contact water and abstract a hydrogen ion (Fig. 1) as well as protonate the mannosyl residue O1 oxygen atom.

Based on these results, we propose a binding model for mannan to LeMAN4a in which the mannosyl residue occupying the -1 subsite in family GH5 β -mannanases follows a conformational itinerary similar to PcMAN, and that is consistent with structural data of other family GH5 β -mannanases.

Materials and methods

Cloning, expression, and protein purification

The *LeMAN4a* cDNA (GenBank AY046588) was obtained using the mRNA for β -mannanase from ripening tomato fruit and cloned into the pMAL fusion protein expression vector (New England Biolabs) as described in Bourgault and Bewley (2002b). Large-scale fusion-protein expression was performed according to manufacturer's instructions using the protease-deficient *Escherichia coli* strain BL21 (Stratagene). Twenty milliliters of an overnight culture were used as a seed to inoculate 2 L Luria-Bertani medium supplemented with 2 g/L glucose and 100 μ g/mL ampicillin. The culture was grown at 37°C with aeration at 120 rpm to an OD₆₀₀ of $\sim$ 0.5, at which time expression was induced by the addition of 6 mL of 0.1 M isopropylthio- β -D-galactoside. Following continued growth for an additional 2 h, the cells were harvested by centrifugation at 4000g for 20 min. Cell pellets were resuspended in 100 mL ice-cold column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA) containing 50 μ g/mL leupeptin to inhibit proteolysis. To aid in cell lysis, cell suspensions were frozen overnight at -20°C , thawed in cold water, and divided into 12 mL aliquots. The cells were disrupted by sonication and then spun at 9000g for 20 min at 4°C. The pellets were frozen, and the supernatants were combined, frozen at -20°C , and retained for purification of the fusion protein. A 12% (w/v) SDS-PAGE gel was run to analyze samples of uninduced and induced crude cell lysates and cell-free crude extract to check for fusion protein expression and completeness of cell disruption. It was determined that the yield of soluble fusion protein was somewhat low. Therefore an additional extraction step was performed on the insoluble material. The pellets were resuspended in 25 mL column buffer containing 500 mM NaCl, to improve the solubility of the fusion protein. The resuspended pellets were sonicated and centrifuged as above and the supernatant retained.

The contents of a 15-mL bottle of Amylose resin (New England Biolabs) were poured into a 15 $\times$ 1-cm-internal-diameter standard liquid chromatography column and equilibrated with eight column volumes of column buffer. The crude cell extract and high-salt pellet extract were thawed in cold water and combined, and the protein concentration was determined using the Pierce BCA protein assay reagent. The extract was diluted to a protein concentration of 2.5 mg/mL with ice-cold column buffer and applied to the column at a flow rate of $\sim$ 0.5 mL/min. Following sample application, the Amylose affinity resin was washed with 12 column volumes of column buffer to remove unbound proteins. The fusion protein was eluted from the resin by the application of column buffer plus 10 mM maltose and collected in ten 4-mL fractions. Aliquots (10 μ L) of each column fraction were run on a 12% (w/v) SDS-PAGE gel and stained with Coomassie blue dye. An assay gel

for β -mannanase activity (Bourgault and Bewley 2002a) was also run on all column fractions to confirm those fractions showing the presence of a fusion protein on the SDS-PAGE gel contained active enzyme.

SDS-PAGE analysis showed that fractions 2 to 5 each contained substantial fusion protein, and the enzyme assay confirmed that these same fractions also exhibited an appreciable amount of β -mannanase activity. These fractions were combined and concentrated to $\sim$ 1.5 mg/mL total protein using a 15 mL Centricon concentrator with a 30 kDa MWCO (molecular-weight cutoff) membrane (Amicon). The highly enriched fusion protein was cut at the N terminus of the β -mannanase portion of the protein by the addition of the protease Factor Xa (New England Biolabs) and incubation at room temperature for 3 d (when cleavage was complete). The cleaved fusion protein mixture was then placed in 6–8-kDa MWCO dialysis tubing and dialysed in 3 $\times$ 2 L changes of 20 mM HEPES–NaOH (pH 7.2). This was performed to remove the NaCl, change the buffer to one suitable for cation exchange chromatography and adjust the pH so that the enzyme had a positive charge.

Cation exchange chromatography was performed using an UNO-S6 column attached to a Bio-Rad Duo-Flow Liquid Chromatography System running with Bio-Logic software version 3.02. Starting buffer (buffer A) was 20 mM HEPES–NaOH (pH 7.2), and the gradient buffer (buffer B) was 20 mM HEPES–NaOH (pH 7.2) plus 500 mM NaCl. Chromatography was carried out at room temperature in two separate, but identical, 5 mL loadings with a constant flow rate of 1 mL/min. Following sample injection, the column was washed with an additional 5 mL of buffer A, after which the linear gradient of buffer B increased to 100% over 40 mL. This was followed by 2 mL 100% buffer B, a gradient of 100% to 0% buffer B over 1 mL and then isocratic flow of buffer A for 10 mL to re-equilibrate the column. Column fractions of 1.5 mL were collected, labeled, stored at 0°C, and analyzed by 12% (w/v) SDS-PAGE to assess purity. Gradient fractions containing pure β -mannanase were pooled, concentrated, and desalted using a 10-kDa MWCO Centricon column with 20 mM HEPES–NaOH (pH 7.2).

Crystallization and data collection

Purified LeMAN4a (8 mg/mL) was subjected to crystallization trials. The hanging drop vapor diffusion technique was employed, using 24-well trays (Flow Laboratories) and siliconized coverslips (Hampton Research). Three hundred micrometers of each solution in Crystal Screen I (Hampton Research) were pipetted into each well. One microliter of protein solution was mixed with 1 μ L of the well solution in all cases. The crystallization trays were allowed to equilibrate at room temperature.

Crystals were snap-frozen at 100 K using an Oxford 600 series cryostream (Oxford). X-ray diffraction data were collected using Beamline 14-BM-C at the Advanced Photon Source (wavelength 0.900 Å) equipped with a Quantum-4 area detector (Area Detector Corp. of America) and processed using the HKL package (Otwinowski and Minor 1997).

Structure solution and refinement

The coordinates (PDB code 1QNP; Sabini et al. 2000) of *T. reesei* β -mannanase were used as the search model for molecular replacement as implemented in MOLREP (Vagin and Teplyakov 1997). The correct molecular replacement solution was readily evaluated, and the coordinates were subjected to eight cycles of refinement in

REFMAC (Murshudov et al. 1997) and manual building in O (Jones et al. 1991). Hydrogen atoms were added and refined in their riding positions during refinement, and bulk solvent correction based on Babinet's principle was applied. $2mF_o - DF_c$ and $mF_o - DF_c$ electron density maps for manual building were calculated using the weighted phase and structure factor data produced by REFMAC. A set of reflections (5% of the total) were set aside and used for cross-validation of refinement protocols.

Structure analysis

Structural homologs of LeMAN4a in the FSSP database (Holm and Sander 1994a) were identified using the DALI server (Holm and Sander 1994b). Structures of family GH5 β -mannanases were aligned using LSQMAN (Kleywegt 1999). Secondary structure elements were identified using DSSP (Kabsch and Sander 1983). A structure-based sequence alignment of LeMAN4a with TrMAN and TfMAN was calculated using STAMP (Russell and Barton 1992).

MD simulations

To explore substrate binding, MD simulations of LeMAN4a were performed with mannopentaose modeled into the putative -3 to +2 subsites (i.e., a Michaelis complex). The coordinates of mannopentaose from the crystal structure of TrMAN/mannobiose complex (Sabini et al. 2000) were used to model the mannosyl groups in the +1 and +2 subsites. The crystal structure of TfMAN/mannotriose complex (Hilge et al. 1998) was used to model the mannosyl residues in the -3 and -2 subsites. The -1 mannosyl was positioned using the crystal structure of PcMAN/DFDM complex (Ducros et al. 2002). As in that complex, the -1 mannosyl group was given the 1S_5 configuration. All other sugar residues were given the 4C_1 configuration. Crystallographically positioned water molecules were included in the MD simulation. Water molecules that clashed sterically with the mannopentaose model were removed prior to simulations.

All MD simulations were run in NAMD 2.5 (Kalé et al. 1999). WHATCHHECK (Hooft et al. 1996) was used to determine which imidazole nitrogen atoms of histidine residues should be protonated prior to simulations. The CHARMM22 force-field (MacKerell et al. 1998) was used for protein parameters and the Carbohydrate Solution Force Field (Kuttel et al. 2002) was used for mannan parameters. The TIP3P parameters (Jorgensen et al. 1983) were used for the water molecules. All mannan/ β -mannanase models were placed in a box of water molecules using the SOLVATE package in the program VMD (Humphrey et al. 1996). Prior to MD calculations, all models were subjected to 10,000 steps of energy minimization to relieve geometric strain and close intermolecular contacts. A 1-nsec simulation was run using periodic boundary conditions, with constant pressure and temperature (1 bar at 298 K) applied using the Nosé-Hoover Langevin Piston algorithm (Nosé 1984; Hoover 1985). The integration time-step was 1 fsec in all cases. A switching function was used to smooth long-range interactions to zero between 8.5 Å and 10 Å. The presence of periodic boundary conditions allowed the use of the Particle Mesh Ewald algorithm to accelerate long-range electrostatic calculations. As an indication of the reliability of the MD simulation of the Michaelis complex, the distance of the nucleophile O ϵ to the C1' atom of the (-1) mannosyl residue was monitored throughout. This distance is significant insofar as the O ϵ atom must be kept in close proximity to the sugar C1' atom in order to make a nucleophilic attack upon it.

Accession numbers

The crystallographic coordinates have been deposited with the PDB, accession number 1RH9.

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