Structural and Biochemical Analyses of Glycoside Hydrolase Families 5 and 26 β-(1,4)-Mannanases from Podospora anserina Reveal Differences upon Manno-oligosaccharide Catalysis

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Background: Fungal mannanases contribute to enzymatic degradation of lignocellulose.

Results: New fungal mannanases reveal striking differences in substrate specificities. A rigid linker tightly connects the family 26 glycoside hydrolase to its binding module.

Conclusion: Podospora anserina mannanases display differences in substrate binding modes, transglycosylation activity, and modular organization.

Significance: Information on the structure-function relationships of fungal mannanases is essential to improve the comprehension of biomass deconstruction.

The microbial deconstruction of the plant cell wall is a key biological process that is of increasing importance with the development of a sustainable biofuel industry. The glycoside hydrolase families GH5 (PaMan5A) and GH26 (PaMan26A) endo-β-1,4-mannanases from the coprophilic ascomycete Podospora anserina contribute to the enzymatic degradation of lignocellulosic biomass. In this study, P. anserina mannanases were further subjected to detailed comparative analysis of their substrate specificities, active site organization, and transglycosylation capacity. Although PaMan5A displays a classical mode of action, PaMan26A revealed an atypical hydrolysis pattern with the release of mannotetraose and manno- pentaoose resulting from a predominant binding mode involving the −4 subsite. The crystal structures of PaMan5A and PaMan26A were solved at 1.4 and 2.85 Å resolution, respectively. Analysis of the PaMan26A structure supported strong interaction with substrate at the −4 subsite mediated by two aromatic residues Trp-244 and Trp-245. The structure appended to its family 35 carbohydrate binding module revealed a short and proline-rich rigid linker that anchored together the catalytic and the binding modules.

Endo-β-1,4-mannanases (β-mannanases, E.C. 3.2.1.78) catalyze the random hydrolysis of mannoglycidosidic bonds in manns and heteromannans. These polysaccharides are the main components of hemicellulose in softwoods and are found in smaller amounts in angiosperms (1). Mannans comprise a backbone of β-1,4-linked d-mannose residues, known as mannan, or a heterogeneous combination of β-1,4-d-mannose and β-1,4-d-glucose units, termed glucomannan. Both can be decorated with α-1,6-linked galactose side chains, and these polysaccharides are referred to as galactomannan and galactoglucomannan, respectively.

Several types of glycoside hydrolases (GH)2 are required for complete degradation of mannans, and endo-β-1,4-mannanases are the key enzymes. In the CAZY database (2), β-1,4-mannanase activities are found in families GH5, GH26, and GH113. The three families belong to clan GH-A; they share the same (β/α)8-barrel protein fold, catalytic machinery, and retaining double displacement mechanism (3–5). Because of this retaining double displacement mechanism, some of these enzymes are able to perform transglycosylation in which a carbohydrate hydroxyl group can act as an acceptor molecule rather than water as is the case in hydrolysis. Transglycosylation thus leads to the synthesis of new glycosides or oligosaccharides longer than the original substrate. GH5 and GH113 mannanases have been described as able to catalyze transglycosylation reactions (6–9), whereas to date no evidence of transglycosylation has been reported for GH26 mannanases (10). β-Mannanases are frequently encountered as modular enzymes. Indeed, some harbor carbohydrate binding modules (CBMs) from families CBM1, CBM6, CBM10, CBM31, and CBM35 (11, 12). It is generally observed that the linker regions

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2 The abbreviations used are: GH, glycoside hydrolase; CBM, carbohydrate binding module; CD, catalytic domain; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; M1, mannose; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M10, mannopentaose; M11, mannohexaose; PaCBM35, P. anserina CBM35; PaMan5A, P. anserina GH5 mannanase A; PaMan26A, P. anserina GH26 mannanase A; Bistris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; BCMAn, B. subtilis z-2 mannanase.
between catalytic module and CBM display a great deal of structural flexibility to maximize substrate accessibility, as has been confirmed by the few crystal structures of bacterial modular enzymes (13, 14).

GH5 endo-β-1,4-mannanases, which are found in bacteria, fungi, animals, and plants are the most largely characterized family. To date only three eukaryotic endo-mannanase three-dimensional structures from family GH5 are available: one from *Trichoderma reesei* (PDB code 1QNO; Ref. 15), one from the blue mussel *Mytilus edulis* (PDB code 2C0H; Ref. 7), and one from the tomato fruit *Solanum lycopersicum* (PDB code 1RH9; Ref. 16). Although several family GH26 endo-β-1,4-mannanases have also been characterized from different organisms (*e.g.* *Cellulomonas fimis* (17), *Cellvibrio japonicus* (18), *Pirymyes equi* (19)), only sparse studies have focused on GH26 endo-β-1,4-mannanases of fungal origin, and the five three-dimensional structures available (*B. subtilis*, PDB code 2WHK (20); *B. subtilis* PDB code 2QHA, (21); *C. fimis* PDB code 2BVT (17); *C. japonicus* PDB code 1GVY (22); PDB code 2VX4 (23)) are all from bacteria and represent only catalytic domains (CDs).

The characterization of endo-β-1,4-mannanases biochemical properties and substrate specificities revealed that many release essentially mannobiose and mannntriose as end products (9, 17, 24, 25) and that their active site displays generally 5–6 subsites able to accommodate the substrate (10, 15). Although GH5 and GH26 mannanases share some characteristics, several studies revealed different modes of action. In particular, biochemical studies pointed to divergence in specificity between GH5 and GH26 bacterial mannanases, a suggesting different biological role (20, 26).

The coprophilic fungus *Podospora anserina* has one of the largest fungal sets of candidate enzymes for cellulose and hemicellulose degradation described to date and on one of the highest numbers of CBMs of all the fungal genomes available (27). In a previous study comparative genomics were used that identified two mannanases from families GH5 (*Pa*Man5A) and GH26 (*Pa*Man26A) in the *P. anserina* genome (28). Investigation of the contribution made by each *P. anserina* mannanase to the saccharification of spruce demonstrated that they individually supplemented the secretome of the industrial *T. reesei* CL847 strain. The most striking effect was obtained with *Pa*Man5A that improved the release of total sugars by 28% and of glucose by 18% (28). In the present study *P. anserina* GH5 and GH26 mannanases were subjected to detailed comparative analysis of their substrate specificities, active site organization, and transglycosylation capacity. The three-dimensional structures of *Pa*Man5A and *Pa*Man26A linked to a CBM35 module were solved in their native form at 1.4 and 2.85 Å resolution, respectively.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of PaMan5A and PaMan26A**—*Pa*Man5A and *Pa*Man26A were produced in *P. pastoris* 2-liter cultures and purified as described previously in (28). Enzyme purification was completed by an additional size exclusion chromatographic step. After the nickel chelate purification step, the eluate containing *Pa*Man5A or *Pa*Man26A was concentrated using a Vivaspin with 10-kDa cut-off polyethersulfone membrane (Sartorius, Palaiseau, France) and dialyzed against the buffer used for the size exclusion chromatography (20 mM Hepes, pH 7.5, 150 mM NaCl). The concentrated fraction was subsequently loaded onto a Superdex S200 HiLoad 16/60 column (Amersham Biosciences). The fractions containing *Pa*Man5A or *Pa*Man26A were pooled and concentrated as described above.

**Construction of Site-specific Variants**—Site-directed mutagenesis was performed using the QuikChange kit (Stratagene), with primers listed in Table 1, according to the instructions of manufacturer. Using the wild-type *Pa*Man5A and *Pa*Man26A plasmids described in Couturier et al. (28), active-site variants were designed for each enzyme. Two single-site mutants were constructed for each enzyme: E177A and E283A for *Pa*Man5A and E300A and E390A for *Pa*Man26A. Transformation was performed in *P. pastoris*, and production and purification of enzyme variants were carried out as described above.

**Deglycosylation Assay—N-Glycosylation sites were predicted using the NetNGlyc 1.0 Server. To remove N-linked glycans, purified enzymes were treated with peptide *N*-glycosidase F (New England Biolabs, Ipswich, MA) under denaturing conditions according to the manufacturer’s instructions. Briefly, 10 µg of protein were incubated in 0.5% SDS and 40 mM DTT and boiled for 10 min for complete denaturation. Denatured samples were subsequently incubated with 1500 units of peptide *N*-glycosidase F in appropriate buffer for 1 h at 37 °C. Deglycosylated and control samples were analyzed by SDS-PAGE (Bio-Rad).

**Analysis of End Products Release from Polysaccharides**—The activity of *Pa*Man5A and *Pa*Man26A was assayed toward glucomannan, galactomannan, and linear mannan. Briefly, a 1% w/v solution was prepared in 50 mM sodium acetate buffer, pH 5.2. The assay was performed by incubating 75 µg of enzyme with 90 µl of 1% w/v substrate solution or suspension at 40 °C for 30 min. After hydrolysis, mono- and oligo-saccharides were analyzed using high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) (ICS 3000; Dionex, Sunnyvale, CA) equipped with a carbo-PacPA-1 analytical column (250 × 4 mm). 10-µl samples of enzymatic reactions were stopped by the addition of 90 µl of 100 mM NaOH before injection (5 µl) into the HPAEC system. Elution was carried out in 130 mM NaOH using a 25-min linear gradient program from 100% A (130 mM NaOH) to 60% A and 40% B (NaOAc, 500 mM; NaOH, 130 mM). All the assays were carried out in triplicate.

**TABLE 1**

| Primer | Nucleotide sequence 5' to 3' |
|--------|----------------------------|
| E177Aforward | GGGAACTTGCCAACCGGCAAGCGAGTCGAAGGG |
| E177Abrverse | CCTTGGACCTGGGCGGGTCGTGAGGAGGAGTCGAAGGG |
| E283Aforward | CCGTGTTGGGGGATAGGAGGAGGAGTGATAGG |
| E283Abrverse | CCAACCACCCCGGGCAGTGAGGGAGGGTGGTTGG |
| E320Aforward | CGACCCACCCCGGGCAGTGAGGGAGGGTGGTTGG |
| E320Abrverse | GATGAGGGAGGGTGGTTGGGCCTGAGGGGTTGG |
| E177Aforward | GGCGAGCGCGCGGCGAGGGGTTGGTTGG |
| E177Abrverse | GGCGACACCACCCGAGGGGTTGGTTGG |

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Structural and Biochemical Characterization of Two Mannanases

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Hydrolysis Product Formation from Oligosaccharides and Determination of Kinetic Parameters—Products generated after hydrolysis of manno-oligosaccharides were analyzed using HPAEC-PAD as described above. 20 μl of suitably diluted enzyme were incubated at 40 °C for various time lengths with 180 μl of 100 μM substrate in 50 mM acetate buffer, pH 5.2. Calibration curves were plotted using β-1,4-manno-oligosaccharides as standards from which response factors were calculated (Chromelone program, Dionex) and used to determine the amount of products released at different time points. All the assays were carried out in duplicate. The data were fitted to the equation of Matsui (29, 30), 

$$k = \ln(S_0)/[S_i]$$

where $k = (k_{cat}/K_m)[enzyme] \times time$, and $[S_0]$ and $[S_i]$ represent substrate concentration before the start of the reaction and at a specified time during the reaction, respectively.

Hydrolysis of M₅ and M₆ in H₂¹⁸O—To determine and compare the hydrolytic cleavage patterns of M₅ and M₆ by PaMan5A and PaMan26A, HPAEC-PAD data on the hydrolys products (as described above) was combined with the analysis of hydrolysis performed in H₂¹⁸O as described previously (31, 32). Each productive binding of M₅ or M₆ gives rise to two products (e.g. M₅ cleaved to either two molecules of M₃ or to M₅ and M₁ or to M₅ and M₃). Quantitative HPAEC-PAD analysis of one product per cleavage (M₅, M₄, and M₃, respectively) was used to calculate the relative frequency of the productive binding modes of M₅ and M₆ that give rise to these products. Each of these products can further be produced by either of two binding modes, and to distinguish between these two modes, the ratio of non-labeled (¹⁶O) and labeled (¹⁸O) product (M₅, M₄, and M₃) was used. Reactions were performed at 8 °C (low temperature was used to avoid spontaneous incorporation of ¹⁸O (31)) in H₂¹⁸O (93%, with a total of 7% H₂¹⁶O contamination (3% in the original H₂¹⁸O and 4% from the enzyme and substrate stock solutions) containing 1 mM sodium acetate buffer, pH 5, 0.8 mM substrate, and 0.1 μM enzyme. Samples (0.5 μl) were withdrawn at different time points (0–60 min) and spotted directly on a stainless steel MALDI plate. Matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in water) was applied (0.5 μl), and the samples were dried under warm air. Data acquisition and analysis was performed as described above.

Protein Crystallization, Data Collection, and Processing—All crystallization trials were carried out by the vapor diffusion method at 20 °C. PaMan5A was concentrated to 8 mg/ml⁻¹ in 20 mM Hepes, pH 7.5, 150 mM NaCl buffer. Initial crystallization trials were performed using Wizard and MDL screens (Qiagen) on a cartesian robot. For each condition, three drops (100 nl of screen buffer + 100, 200, and 300 nl of protein) were formed. Optimization was then carried out by varying the pH and the concentration of precipitant. The final crystallization conditions were Tris 0.1 M pH 8.5, 0.2 M sodium acetate, 30% PEG 4000. Glycerol was used at a concentration of 25–30% as the cryoprotectant in the subsequent data collection stage. PaMan5A crystals belonged to the P2₁₂₁₂₁ space group with the cell dimensions $a = 56.9$ Å, $b = 58.0$ Å, and $c = 98.2$ Å and diffracted to 1.4 Å resolution. X-ray diffraction data of a PaMan5A crystal were collected at 100K at the European Synchrotron Research Facilities (ESRF, Grenoble, France) beam line ID29.

PaMan26A was concentrated to ~26 mg/ml⁻¹ in 20 mM Hepes, pH 7.5, 150 mM NaCl buffer. Small PaMan26A crystals were obtained in the conditions (i) 0.1 M Tris pH 7, 0.2 M Li₂SO₄, 1 M potassium sodium tartrate and (ii) 0.1 M imidazole, pH 8.1, 0.1 M potassium sodium tartrate, 0.2 M NaCl, both conditions of the Wizard screen. The best crystals were obtained after optimization in a solution containing 0.1 M Tris, pH 7, 0.2 M NaCl, 0.8 M potassium sodium tartrate, 1 mM HgCl₂. For cryoprotection, crystals were transferred in a solution containing 25% (v/v) glycerol, 1.5 M Li₂SO₄, 100 mM Bistris propane, pH 7.4. The crystals belonged to the P6_322 space group with the following cell dimensions: $a = b = 97.5$ Å, $c = 268.7$ Å. Several x-ray diffraction data sets were collected on beam line Proxima1 at the French synchrotron SOLEIL (Saint-Aubin, France) and on beam lines ID14-4 and ID29 at the European Synchrotron Research Facilities. The best x-ray diffraction data were collected to 2.85 Å resolution at the European Synchrotron Research Facilities beam line ID14-4.

All the data sets were processed with the programs XDS (33) and SCALa (34). The data collection statistics are summarized in Table 2.

Structure Determination and Refinement—The structure of PaMan5A was determined with the molecular replacement method using the AMoRe program (35) and the T. reesei GH5 mannanase coordinates (PDB code 1QNO). The rotation function yielded one solution, and the translation function yielded a
unique solution, with a correlation coefficient and an $R_{\text{factor}}$ of 38.1 and 44.5%, respectively, for data between 10 and 4 Å. After rigid body refinement, the correlation coefficient was 59.2% for an $R_{\text{factor}}$ of 35.9%. After refinement using the programs Refmac (36) and Buster (37), the final crystallographic $R_{\text{factor}}$ and $R_{\text{free}}$ were 15.0 and 17.2%.

The structure of PaMan26A was also determined with the molecular replacement method using the AMoRe program (35). The superposition of four structures of GH26 CDs (PDB codes 2QHA, 2BVT, 2VX4, and 2WHK) plus the homology model generated by the Phyre server (38) has been used as an ensemble search model for molecular replacement. The rotation function yielded one solution, and the translation function yielded a unique solution, with a correlation coefficient and an $R_{\text{factor}}$ of 32.4 and 49.2%, respectively, for data between 10 and 4 Å. After rigid body refinement, the correlation coefficient was 42.7% for an $R_{\text{factor}}$ of 43.7%. A modified structure of the CBM35 from Clostridium thermocellum (PDB code 2W47) with most of the loops deleted was located manually in the difference Fourier electron density map and was used as a starting point to build the CBM domain of PaMan26A. After performing several cycles of refinement using Refmac (36) and Buster (37) programs and manual replacement and building on the graphic display with the Turbo-Frodo program (39), the $R_{\text{factor}}$ has decreased to 20.7% ($R_{\text{free}}$ 25.8%). All representations of the structure in the figures were prepared with the program PyMOL. Coordinates for the structure PaMan5A and PaMan26A have been deposited in the Protein Data Bank under the accession number 3ZIJ and 3ZM8, respectively.

### Table 2

| Data collection and model refinement statistics of PaMan5A and PaMan26A | PaMan5A | PaMan26A |
|---------------------------------------------------------------|---------|----------|
| Wavelength (Å)                                                | 0.97914 | 1.00648  |
| Space group                                                  | P2$_1$/2$_1$ | P6$_2$ |
| $a$, $b$, $c$ (Å)                                             | 56.37, 57.90, 97.86 | 97.49, 97.49, 268.72 |
| Resolution (Å)$^a$                                           | 30.1-4 (1.8-4) | 50-2.85 (3-2.85) |
| Unique reflections$^a$                                        | 52.519 (8.848) | 18.575 (2.02) |
| Multiplicity$^a$                                              | 8.4 (8.3) | 11.8 (11.8) |
| Completeness (%)$^a$                                         | 97.3 (95.7) | 99.9 (100.0) |
| $I/\sigma(I)$                                                 | 18.7 (3.4) | 25.6 (4.6) |
| $R_{\text{merge}}$ (%)                                        | 8.6 (67.8) | 7.6 (65.2) |

$^a$ Values in parentheses are for the highest resolution shell.

### Results and Discussion

Hydrolytic Activity of PaMan5A and PaMan26A toward Polysaccharides—In a recent study we showed that PaMan5A and PaMan26A displayed similar kinetic parameters toward a range of mannan substrates. To further compare the $P$. anserina mannanases, we measured the release of manno-oligosaccharides after hydrolysis of ivory nut mannan and carob galactomannan. Toward the end of the reaction, PaMan5A yielded mainly M$_2$ and M$_3$ and smaller amount of M$_1$ (data not shown), consistent with other GH5 mannanases such as T. reesei (40). PaMan26A produced mainly M$_4$ and smaller amounts of M$_1$, M$_2$, and M$_3$ (data not shown). In other GH26 mannanases, different profiles have been observed; C. fimii CjMan26A and C. japonicus CjMan26B released M$_2$ and M$_1$ (17, 26); and B. subtilis BCMan released M$_2$ and M$_1$ (21). The nature of oligosaccharide products released upon mannan hydrolysis confirms (i) the endo-mode of action of the two enzymes and (ii) differences between the two enzymes in substrate binding.

Hydrolytic Activity of PaMan5A and PaMan26A toward Oligosaccharides—The capacity of PaMan5A and PaMan26A to hydrolyze a range of manno-oligosaccharides was evaluated by ion chromatography to get further insights into their active site architecture (Fig. 1). PaMan5A had very low activity on M$_6$, higher activity on M$_4$, and cleaved M$_3$ and M$_6$ rapidly (Table 3). A decrease of $k_{\text{cat}}/K_m$ was observed with decreasing degree of polymerization. The relative $k_{\text{cat}}/K_m$ values of PaMan5A on M$_2$, M$_3$, M$_4$, and M$_6$ were 1:358:1127:1782. The increase of the degree of polymerization from 4 to 5 (M$_4$ and M$_5$) resulted in a 3.1-fold increase in $k_{\text{cat}}/K_m$, suggesting that at least four subsites are required to achieve efficient hydrolysis. In contrast, PaMan26A had no detectable activity on M$_5$, very low activity on M$_4$, and cleaved M$_5$ and M$_6$ rapidly. For PaMan26A the relative $k_{\text{cat}}/K_m$ values on M$_4$, M$_5$, and M$_6$ were 1:195:365 with an increase of $k_{\text{cat}}/K_m$ of 1.9-fold between M$_5$ and M$_6$ hydrolysis. These data suggest that PaMan26A requires at least five subsites to achieve maximum manno-oligosaccharide hydrolysis efficiency.

The nature of the hydrolysis products yielded from manno-oligosaccharides (summarized in Table 4) also revealed striking differences between the $P$. anserina mannanases. M$_6$ hydrolysis by PaMan5A produced mainly M$_1$ with smaller amounts of M$_2$ and M$_3$, whereas M$_4$ hydrolysis by PaMan26A produced mainly M$_5$ and M$_6$, with smaller amounts of M$_1$ and M$_2$ and without any M$_4$. M$_2$ hydrolysis by PaMan5A yielded mainly M$_4$ and M$_5$, with small amounts of M$_1$ and M$_3$, whereas PaMan26A had low activity on M$_4$ and produced M$_1$, M$_2$, and M$_3$. PaMan5A poorly hydrolyzed M$_3$, yielding M$_1$ and M$_2$, and PaMan26A had no detectable activity on M$_4$. Neither mannanase had detectable activity on M$_5$ and 4-nitrophenyl-mannose even at high enzyme loading (data not shown). Again, PaMan26A showed an atypical hydrolytic profile for a GH26 endo-mannanase compared with CjMan26A and CjMan26B. CjMan26A hydrolyzes M$_4$ rapidly and requires occupation of four subsites to achieve efficient hydrolysis (18),
whereas CfMan26A is less efficient toward M4 and requires substrate binding at five subsites to achieve efficient hydrolysis (17). For PaMan26A, the occupation by substrate of at least five subsites to achieve efficient hydrolytic activity is even more pronounced, with a dramatic increase in $k_{\text{cat}}/K_m$ between M4 and M5.

**Productive Binding Mode of M5 and M6 by PaMan5A and PaMan26A**

Mannanases usually bind oligomeric substrates in multiple productive binding modes that can generate identical products. The simplest example of this is M3 hydrolysis to M2 and M1, where mannose would be released from either the reducing end or the non-reducing end. In the former case M3 binds productively from the 2 subsite to the 1 subsite and in the latter case from the 1 to the +1 subsite, following the established subsite nomenclature (41). As another example, from M5, each of the products M3 and M4, respectively, can be produced by either of two binding modes (see the scheme in Fig. 2A). Binding of M5 from subsite 2 to +3 or from subsite 3 to +2, both, generates M3, and binding from subsite 4 to +1 and from subsite -1 to +4, both, generates M4. Thus, product analysis using HPAEC-PAD data alone cannot distinguish between binding modes giving the same products. However, this can be achieved when the HPAEC-PAD product analysis (as in previous paragraph) is combined with in situ product isotope labeling using $^{18}$O-labeled water followed by mass spectrometric analysis as shown previously (31, 32).

Relative quantities of the produced M3, M4, and M5 from the HPAEC-PAD data of M5 and M6 hydrolysis (Table 4) were used to calculate the relative molar distribution of these products (Table 4, values in parentheses), and thus the frequencies of productive binding modes that give rise to these products could be estimated (Fig. 2A, far left and far right column). MALDI-TOF-MS analysis was conducted to determine the ratio of non-labeled ($^{16}$O) and labeled ($^{18}$O) species of each product (light versus heavy M3, M4, or M5), which was then used to estimate the relative frequency of the productive binding modes of M5.

**TABLE 3**

| Substrate | $k_{\text{cat}}/K_m$ (PaMan5A) | $k_{\text{cat}}/K_m$ (PaMan26A) |
|-----------|-------------------------------|-------------------------------|
| M6        | $2.9 \times 10^5$             | $2.4 \times 10^6$             |
| M5        | $1.9 \times 10^5$             | $1.3 \times 10^6$             |
| M4        | $5.9 \times 10^5$             | $6.8 \times 10^3$             |
| M3        | $1.6 \times 10^3$             | ND*                           |

*Not determined due to low activity.*
and M₆ that give rise to these same products. The combined results of the HPAEC-PAD and MALDI-TOF-MS data are summarized in Fig. 2A, showing the relative frequencies (%) of productive binding modes of M₅ and M₆. The calculation procedure is explained in supplemental Table S1. To exemplify, determined from HPAEC-PAD data (Table 4), 80% of the productive binding during the hydrolysis of M₅ by \textit{Pa}Man₅A generated M₃. MALDI-TOF analysis then determined the ratio between the two binding modes that give M₃, giving a 59% frequency of this binding mode (Fig. 2A). Small amounts of M₁ and M₄ were also produced, and the ratio of M₄/M₄⁰¹₈ was 1:0.7. Hydrolysis of M₆ by PaMan5A produced.

| Enzyme     | Enzyme loading | Substrate | M₁ | M₂ | M₃ | M₄ | M₅ |
|------------|----------------|-----------|----|----|----|----|----|
| \textit{Pa}Man₅A | 60^NM       | M₅        | 11 | 49 | 21 | 20 (80°) | 5 (20°) |
|            | 18.2         | M₅        | 2  | 32 | 20 (80°) | 5 (20°) |
|            | 18.2         | M₆        | ND | 12 | 34 (55°) | 14 (45°) | ND |
| \textit{Pa}Man₂₆A | 30          | M₅        | 34 | ND | ND | 43 (100°) | ND |
|            | 15           | M₆        | 13 | 28 | ND | 33 (75°) | 11 (25°) |

* The values in parentheses represent the relative molar distribution (%) between the products M₂, M₄, and M₅ from each of the M₅ and M₆ incubations, which were used to estimate the relative frequency of productive binding modes yielding these products (presented in Fig. 2A). One product (M₃, M₄, or M₅) per productive binding was used for calculations; thus, only half of the produced M₃ (bold) from M₆ incubations was accounted for (two molecules of M₃ are produced from each molecule of M₆).

**TABLE 4**

Hydrolysis products released by \textit{Pa}Man₅A and \textit{Pa}Man₂₆A from manno-oligosaccharides

Products were quantified using HPAEC-PAD and are expressed as μM. Incubation was carried out for 30 min at 40 °C; ND, not detected.

**FIGURE 2.** Relative frequency of the productive modes of binding of manno-oligosaccharides to \textit{Pa}Man₅A and \textit{Pa}Man₂₆A. A, the numbers represent the percentages of binding in each binding mode. These were calculated from the quantitative product analysis using HPAEC-PAD (numbers to the far left and far right, obtained from Table 4) followed by a detailed analysis of the hydrolytic cleavage patterns of M₅ and M₆ using MALDI-TOF-MS analysis of ^¹⁸O-labeled products. The arrow indicates the mannosidic bond to be cleaved. *, reducing end of oligosaccharide. The −4 and +3 dashed subsites are only present in \textit{Pa}Man₂₆A and \textit{Pa}Man₅A, respectively. ND, not detected. B, MALDI-TOF-MS spectra of M₃ hydrolysis by \textit{Pa}Man₅A and \textit{Pa}Man₂₆A show enlarged parts of the spectra with the M₃ product formed by \textit{Pa}Man₅A at a ratio of 1:2.9 of M₃/M₃⁰¹₈ (left) and the M₄ product formed by \textit{Pa}Man₂₆A at a ratio of 1:5.0 of M₄/M₄⁰¹₈ (right). The peaks in the spectra correspond to the monoisotopic masses of sodium adducts [M + Na]^+. The structures and Biochemical Characterization of Two Mannanases
mainly M3 (55% binding frequency) but also smaller amounts of M2 and M4. The ratio of M4/M4\textsuperscript{O18} was 1:3.2, which shows that PaMan5A binds M4 preferably from subsite −4 to +2 to produce M4 (34% binding frequency). Hydrolysis of M5 by PaMan26A yielded M4 and M5 with a M4/M5\textsuperscript{O18} ratio of 1:5.0 (Fig. 2B), which shows that the enzyme binds M5 preferentially from subsite −4 to +1 (83% binding frequency, see Fig. 2A). For hydrolysis of M5, major product ratio analysis showed that the ratio of M5/M5\textsuperscript{O18} was 1:10.8, which shows that PaMan26A prefers to bind M5 from subsite −4 to +2 (69% binding frequency). The ratio of the minor product M3/M3\textsuperscript{O18} was 1:4.2, showing that M1 and M5 are mainly produced without binding at the +2 subsite. Thus, these data reveal clear differences in the binding mode of the two M. anserina mannanases; the predominant modes of binding of M5 and M6 were significantly different (Fig. 2A). PaMan5A showed a classical pattern of hydrolysis products (M1 and M2 mainly were released from M5) as described in several studies (B. subtilis, C. japonicus, M. edulis), whereas PaMan26A showed release of M4 and M1 from M5, which is unusual when compared with other GH26 endo-mannanases (B. subtilis BCMan, C. fimi CJMan26A, C. japonicus CJMan26A), suggesting an unusual arrangement of subsites in the catalytic center.

Transglycosylation Ability—To detect potential transglycosylation ability of the two enzymes, they were incubated with M6 as substrate. The resulting short time course study of the product formation clearly showed that PaMan5A, in addition to hydrolysis products, also produces transglycosylation products with higher degree of polymerization than the original substrate (Fig. 3). PaMan5A was able to transglycosylate yielding to oligosaccharide structures of up to a degree of polymerization of 8 (n + 1 to n + 3), in good agreement with GH5 mannanases described before, T. reesei (n + 1 to n + 3) (31), and Aspergillus nidulans ManA (n + 1 to n + 3) and ManC (n + 1 and n + 2) (6). No transglycosylation products could be detected with PaMan26A incubated with M6 in the same experimental conditions, which is consistent with some other family GH26 mannanases that have been described as non transglycosylating enzymes (10).

Structure of PaMan5A—The crystal structure of PaMan5A was solved in its free form. The crystal contained one monomer in the asymmetric unit, and light-scattering experiments indicated that the protein is a monomer in solution (data not shown). The overall structure of PaMan5A (Fig. 4A) revealed a (β/α)\textsubscript{8}-barrel fold as expected for enzymes belonging to clan GH-A. When superimposed with TrMan5A (PDB code 1QNR) and Thermomonospora fusca mannanase (PDB code 3MAN) structures (supplemental Fig. S1), the overall fold of PaMan5A is very similar to that of TrMan5A, with structural differences being confined mainly in the loop regions (Fig. 4A) that have been defined as eight loops: loop 1 (residues 35–42), loop 2 (66–95), loop 3 (120–144), loop 4 (177–184), loop 5 (213–232), loop 6 (252–258), loop 7 (287–289), and loop 8 (316–336). Compared with TrMan5A, which contains four disulfide bonds, Cys-26—Cys-29, Cys-172—Cys-175, Cys-265—Cys-272, and Cys-284—Cys-334, PaMan5A contained only three disulfide bonds, i.e. Cys-180—Cys-184, Cys-272—Cys-279, and Cys-291—Cys-342. After N-deglycosylation of PaMan5A using peptide N-glycosidase F, no shift in the apparent molecular mass (46 kDa) was observed on SDS-PAGE compared with untreated sample (data not shown). This observation was in good agreement with NetGlyc predictions from the PaMan5A crystallographic data that confirmed absence of glycosylation units.

The active site of PaMan5A was clearly identified in the groove, with the two conserved catalytic glutamate residues (acid-base and nucleophile) positioned near the C-terminal...
ends of β-strands four and seven of the (β/α)_8 barrel (41), Glu-177 and Glu-283, respectively. Mutant E283A showed no catalytic activity for glucomannan, thus indicating that Glu-283 should be the nucleophile. E177A had a specific activity of 0.47 units/mg toward glucomannan, which is roughly 100-fold lower than the wild-type enzyme (45 units/mg), thus indicating that Glu-177 should be the acid-base catalytic residue. These results are in agreement with other homologous GH5 enzymes where catalytic residues have been determined (42, 43). Despite several attempts, no structure of PaMan5A inactive mutants alone or in complex with its substrate has been obtained. Consequently, we performed comparative structural analysis of PaMan5A with other GH5 mannanases complexes (T. reesei PDB code 1QNO, Thermotoga petrophila PDB code 3PZ9, and S. lycopersicum PDB code 1RH9) to map the substrate binding subsites.

Structure of PaMan26A Catalytic Module—The structure of PaMan26A was successfully solved using molecular replacement. The search model was composed of the superimposition of four structures of bacterial mannanases (PDB codes 2QHA, 2BVT, 2VX4, and 2WHK). The final structure comprising 443 residues was refined at 2.85 Å resolution. The overall structure of PaMan26A CD revealed a (β/α)_8-barrel fold (Fig. 5A) as expected for enzymes belonging to clan-GHA. The active site was clearly identified in the groove, with the two conserved catalytic glutamate residues (Glu-300 and Glu-390) positioned at the end of the (β/α)_8 barrel and several aromatic residues forming the subsites of catalytic cleft. Mutant E390A showed no catalytic activity for glucomannan, indicating that Glu-390 should be the nucleophile. E300A had a specific activity of 0.33 units/mg, which is roughly 200-fold lower than the wild-type enzyme (65 units/mg), thus indicating that Glu-300 should be the acid-base catalytic residue. These results are in agreement with other homologous GH26 enzymes where catalytic residues have been determined (45).

Electron density was observed for two carbohydrate sugar residues at one glycosylation site, Asn-268, which is located in

FIGURE 4. Crystal structure of PaMan5A. A, superposition of PaMan5A (green) and TrMan5A (yellow) structures is shown. The two views are related by a rotation of −90° about the vertical axis. B, shown is a surface view of the catalytic cleft of PaMan5A with mannotriose modeled in the −2 and −3 subsites and mannobiose modeled in the +1 and +2 subsites. The structures of GH5 from T. reesei and T. fusca in complex with mannobiose and mannotriose, respectively, were superimposed on the top of the structure of PaMan5A to map the substrate binding subsites.

FIGURE 5. Crystal structure of PaMan26A. A, superposition of PaMan26A catalytic module (green) and BCMan (orange) structures. The two views are related by a rotation of −90° about the vertical axis. B, shown is a surface view of the catalytic cleft of PaMan26A with mannotriose modeled in the −2 to −4 subsites. The structure of GH26 from C. fimi in complex with mannotriose was superimposed on the top of the structure of PaMan26A to map the substrate-binding subsites. C, shown is the organization of the glycone binding subsites in PaMan26A (yellow) compared with C. fimi (cyan).
the CD on the external side of the barrel. As modeled from electron density, 2 β-1,4-linked N-acetylglucosamine (GlcNAc) units are attached to this N-glycosylation site. N-Deglycosylation of PaMan26A using peptide N-glycosidase F was associated with a 2–3-kDa shift in the apparent molecular mass on SDS-PAGE compared with untreated sample (data not shown). These results confirm that PaMan26A is N-glycosylated and are in agreement with the NetNGlyc prediction (one predicted N-glycosylation site at position Asn-268).

Several regions are highly conserved between PaMan26A and another GH26 mannanases from \textit{B. subtilis} z-2 (PDB code 2QHA), \textit{C. subtilis} subsp. \textit{bacillus} (PDB code 2WHK), \textit{C. japonicus} (PDB codes 1GW1 and 2VX6), and \textit{C. fimi} (PDB code 2BV7; supplemental Fig. S2) as shown in the superimposition of PaMan26A and \textit{B. subtilis} z-2 (PDB code 2QHA) structures (Fig. 5A). The central β-barrel and most of the surrounding α-helices are superimposable between PaMan26A and \textit{B. subtilis} z-2, whereas loop regions are dramatically different. Indeed, \textit{B. subtilis} enzyme exhibits a flat surface with a shallow dish-shaped active center, whereas PaMan26A displays large loops that form a deep cleft. According to the three-dimensional structure of PaMan26A, 8 loops are involved in the binding of the substrate to the active site: loop 1 (171–174), loop 2 (195–208), loop 3 (230–266), loop 4 (301–314), loop 5 (342–346), loop 6 (362–368), loop 7 (392–396), and loop 8 (413–425). The most striking difference stands in loop 2 that contains four aromatic residues (Trp-244, Trp-245, Phe-248, and Tyr-249) and is nine amino acids longer than \textit{B. subtilis} z-2 (PDB code 2QHA), \textit{B. subtilis} subsp. \textit{bacillus} (PDB code 2WHK), \textit{C. japonicus} (PDB code 2VX6), and \textit{C. fimi} (PDB code 2BV7) mannanases. A shorter loop 2 does not allow interaction with the substrate at the glycone binding subsites in the case of \textit{B. subtilis} z-2 (PDB code 2QHA). The −1 and +1 subsites of PaMan26A are quite similar to homologous enzymes with the conserved residues His-299, Trp-305, Phe-306, Tyr-362, Trp-413, Ser-415, and Gln-369. As described for \textit{CfMan26A} and \textit{CjMan26A}, PaMan26A Tyr-362 is probably involved in a hydrogen bond with Asp-382, Asn-374, and Gln-404, whereas PaMan26A Trp-305 and Trp-413 could play a role as aromatic platforms to stabilize mannoypyrannose rings at the +1 and −1 subsites, respectively (Fig. 5C). In the −2 subsite of BCMAn, binding is not favorable because of steric hindrance due to the position of Tyr-40 (21). In the case of \textit{CjMan26A}, the two aromatic Phe-123 and Tyr-124 residues that are superimposed with PaMan26A F248 and Tyr-249 stabilize the interaction with a mannose unit at the −2 subsite (Fig. 5C).

Our experimental data indicate that PaMan26A displays strong interactions at the −4 subsite. Indeed, PaMan26A was poorly active toward M4 probably due to the formation of an unproductive complex between −4 and −1 subsites. We further analyzed the −4 subsite in the PaMan26A structure and identified two aromatic residues, Trp-244 and Trp-245, located in loop 2 that could stabilize mannoypyrannose rings in the −4 subsite (Fig. 5, B and C). As PaMan26A, \textit{CfMan26A} active site also contains four glycone binding subsites, but experimental results provided evidence for the existence of a strong −3 subsite, and residues involved in the −4 subsite were described as making a minor contribution to binding (17). In PaMan26A, there is no equivalent to the Phe-42, Phe-325, and Gln-329 \textit{CfMan26A} −3 subsite residues. The lack of a strong −3 subsite and the presence of a strong −4 subsite in the structure is in agreement with our experimental results that suggest a predominant substrate binding mode involving the −4 subsite. Lacking a strong −4 subsite, \textit{CfMan26A} produces M4 and M3 as major products from M3 with only minor amounts of M1 and M4 (31).

\textit{PaMan26A} Modular Organization—PaMan26A harbors a family 35 CBM at its N-terminal end, and the closest characterized enzyme is \textit{Humicola insolens} β-mannanase (GenBank$^\text{TM}$ AAQ13840 (46)) with 78% amino acid identity. After a BlastP search using the PaMan26A amino acid sequence, it is interesting to note that all related bacterial and fungal sequences harbor a CBM35 module at their N terminus. In fungi, in addition to PaMan26A CBM35, only one CBM35 module binding to galactan has been characterized to date in a \textit{Phanerochaete chrysosporium} exo-β-1,3-galactanase (47). We previously suggested that the N-terminal CBM35 module of PaMan26A displayed dual binding specificity toward xylan and mannan (28), and the phylogenetic analysis was performed by Correia et al. (48) clustered \textit{PaCBM35} in the subfamily II that is proposed to target β-1,4-mannan.

Although the structures of fungal GH bearing a CBM are generally determined separately, this is the first intact structure that allows visualization of the juxtaposition of the CBM35 module relative to the GH26 CD. The linker region of PaMan26A is short without any glycosylation sites, whereas modular fungal GHs usually display long and highly glycosylated linkers. The PaMan26A linker sequence was rich in proline residues, \textit{i.e.} it contains 4 prolines (Pro-132, -134, -135, and -140) of 12 residues that may confer rigidity to the modular enzyme (Fig. 6A). The linker starts on residue Ser-130 at the end of the last β-strand of the N-terminal CBM domain. Only two residues (Ala-131 and Pro-132) have no interaction with the rest of the molecule. The region from residue R133 to residue N141, which may be considered as the end of the linker, is tightly bound to the CD. Arg-133 and His-136 side chains make hydrogen bond with Asp-382, Asn-374, and Gln-404, whereas the side chain of Ile-138 fits into a hydrophobic cavity made of Arg-159 (aliphatic part of the side chain), Tyr-162, and Met-385. The CBM and the catalytic module are thus in close association thanks to the embedded linker (Fig. 6B), and it may explain why attempts to express the catalytic module alone were unsuccessful (data not shown). Alignment of PaMan26A-CBM35 with 60 microbial GH26 mannanases sequences bearing a CBM35 module revealed that they all display a short linker region (12–14 residues) rich in proline residues (data not shown).

The CBM35 domain comes into contact with the CD through hydrophobic interactions. Indeed a hydrophobic patch comprising Leu-58 and Leu-103 on the surface of the CBM35 domain stands in front of a cluster of hydrophobic residues (Ala-402, Tyr-403, and Leu-399) of the CD. The rationale of the tight modular association of bacterial and fungal GH26-CBM35 mannanases will need further work to gain insights into their function.
PaCBM35 Domain—The CBM35 domain overall structure consists of 2 antiparallel sheets consisting of 4 and 5 antiparallel β-strands, respectively. The two sheets are packed in a β-sandwich conformation enclosing a highly hydrophobic core. The closest structural homologue found using the DALI server (49) is a CBM35 from *C. thermocellum* (PDB code 2W1W (50)), with a Z-score of 18.6. Its superimposition with PaCBM35 shows that 57 Ca of 125 Ca (45%) have equivalent positions in both molecules, with the distance between the superimposed Ca atoms <1 Å. The main differences occur in the loops connecting the β-strands as shown in Fig. 6C. A metal ion is present that has been modeled as calcium based on its coordination geometry exclusively with oxygen atoms. A calcium ion is also present at a similar location in the structure of the CBM35 domain from *C. thermocellum* (50). However, the second calcium evidenced in all of the other CBM35s and involved in carbohydrate recognition (50) is not conserved in PaCBM35. A platform of three aromatic residues (Phe-87, Trp-117, and Trp-119) was observed at the surface of the PaCBM35 domain (Fig. 6). These residues are aligned with the PaMan26A catalytic cleft, suggesting that they could play a role in substrate binding.

![Figure 6. Views of Modular architecture of PaMan26A. A, shown is a ribbon diagram of PaMan26A catalytic (blue) and CBM (green) domains. The proline-rich linker is shown in stick format. B, shown is a molecular surface representation of PaMan26A structure with the catalytic domain in blue, the PaCBM35 domain in green, and the linker in purple. The three aromatic residues present at the surface of the PaCBM35 domain are shown in yellow. C, shown is superposition of the PaCBM35 domain (green) and *C. thermocellum* CBM35 (orange). The calcium ion is represented by a blue sphere.](image-url)
Conclusions—The *P. anserina* CAZome (the genome-wide inventory of CAZymes) includes three genes encoding β-(1,4)-mannanases: two GH5 mannanases without CBM (including *Pa*Man5A) that both belong to the GH5 subfamily 7 (51) and one GH26 mannanase bearing a CBM35 (i.e. *Pa*Man26A) with affinity for hemicellulosic polysaccharides (28). Based on our kinetic analysis, we can conclude that *Pa*Man5A and *Pa*Man26A are complementary in terms of hydrolysis profile and could act in synergy to deconstruct mannan polysaccharides. Indeed, *Pa*Man26A produces larger manno-oligosaccharides that could be processed by *Pa*Man5A. In *C. japonicus*, a bacterium also producing both GH5 and GH26 β-mannanases, the catalytic modules of GH5 mannanases were linked to various CBMs, whereas GH26 mannanases were found as single CD (25). Therefore, it has been suggested that GH26 mannanases were involved in degradation of storage tissues, whereas GH5 mannanases harboring cellulose-specific CBMs were involved in degradation of plant cell wall (20, 26). It is interesting to note that the *P. anserina* mannanase system does not seem to fit with this model, suggesting a difference in the strategies to degrade mannan between these two microbes.

Together with our previous studies on *P. anserina* CAZymes (28, 52, 53), the present findings give more insights into the *P. anserina* enzymatic machinery for the deconstruction of plant cell wall polysaccharides. This knowledge is essential to design tailor-made biocatalysts, which can then be used in the biofuel and bioprocessing industries.

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