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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Structural and biochemical analyses of glycoside hydrolase families 5 and 26 beta-(1,4)-mannanases from *Podospora anserina* reveal differences upon manno-oligosaccharides catalysis*

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*Running title : *Structural and biochemical characterization of two* P. anserina *mannanases*

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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.

SUMMARY

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 (*Pa*Man5A) and GH26 (*Pa*Man26A) 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 *Pa*Man5A displays a classical mode of action, *Pa*Man26A revealed an atypical hydrolysis pattern with the release of mannotetraose and mannose from mannopentaose resulting from a predominant binding mode involving the -4 subsite. The crystal structures of *Pa*Man5A and *Pa*Man26A were solved at 1.4Å and 2.85Å resolution, respectively. Analysis of the *Pa*Man26A structure supported strong interaction with substrate at the -4 subsite mediated by two aromatic residues W244 and W245. The *Pa*Man26A 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) catalyse the random hydrolysis of manno-glycosidic bonds in mannans 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.

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Several types of glycoside hydrolases[4] (GH) are required for complete degradation of mannans and endo-β-1,4 mannanases are the key enzymes. In the CAZy database (www.cazy.org; (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 $(\beta/\alpha)_8$-barrel protein fold, catalytic machinery and retaining double displacement mechanism (3,4,5). 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 catalyse transglycosylation reactions (6,7,8,9), while to date no evidence of transglycosylation has been reported for GH26 mannanases (10). β-mannanases are frequently encountered as modular enzymes. Indeed, some harbor CBM from families CBM1, CBM6, CBM10, CBM31, CBM35 (11,12). It is generally observed that the linker regions between catalytic module and CBM display a great deal of structural flexibility to maximize substrate accessibility, as it 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 3D structures from family GH5 are available: one from *Trichoderma reesei* (PDB 1QNO; (15)), one from the blue mussel *Mytilus edulis* (PDB 2C0H; (7)) and one from the tomato fruit *Solanum lycopersicum* (PDB 1RH9; (16)). Although several family GH26 endo-β-1,4 mannanases have also been characterized from different organisms (e.g., *Cellulomonas fimi* (17), *Cellvibrio japonicus* (18), *Pyromyces equi* (19)), only sparse studies have focused on GH26 endo-β-1,4-mannanases of fungal origin and the five 3D structures available *(B. subtilis*, PDB 2WHK, (20); *Bacillus subtilis* PDB 2QHA, (21); *C. fimi* PDB 2BVT, (17); *C. japonicus* PDB 1GVY (22) and PDB 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 mannotriose as end products (9, 17, 24, 25), and that their active site display generally 5 to 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, suggesting different biological role (20, 26).

The coprophilic fungus *Podospora anserina* has one of the largest fungal set of candidate enzymes for cellulose and hemicellulose degradation described to date and one of the highest numbers of carbohydrate-binding modules (CBMs) of all the fungal genomes available (27). In a previous study, comparative genomics was used which identified two mannanases from families GH5 (*Pa*Man5A) and GH26 (*Pa*Man26A) in the *Podospora 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 organisation 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 1.4Å and 2.85Å resolution, respectively.

EXPERIMENTAL PROCEDURES

Production and purification of PaMan5A and PaMan26A

*Pa*Man5A and *Pa*Man26A were produced in *P. pastoris* 2 liters 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 dialysed against the buffer used for the size exclusion chromatography (Hepes 20 mM pH 7.5, NaCl

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150 mM). The concentrated fraction was subsequently loaded onto a Superdex S200 HiLoad 16/60 column (Amersham, Buckinghamshire, UK). 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 Quickchange 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 (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 (http://www.cbs.dtu.dk/services/NetNGlyc/).
To remove N-linked glycans, purified enzymes were treated with PNGase F (NEB, Ipswich, MA, USA) under denaturing conditions according to manufacturer's instructions. Briefly, 10 µg of protein were incubated in 0.5% SDS and 40 mM DTT and boiled for 10 minutes for complete denaturation. Denaturated samples were subsequently incubated with 1,500 units of PNGase F in appropriate buffer for one hour at 37°C. Deglycosylated and control samples were analysed by SDS-PAGE (BioRad, Marnes-la-Coquette, France).

Analysis of end products release from polysaccharides
The activity of *Pa*Man5A and *Pa*Man26A was assayed towards glucomannan, galactomannan and linear mannan. Briefly, a 1% w/v solution was prepared in 50 mM Na-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 minutes. 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, USA) equipped with a carbo-Pac PA-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.

Hydrolysis product formation from oligosaccharides and determination of kinetic parameters
Products generated after hydrolysis of manno-oligosaccharides were analysed 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 (Chromeleon program, Dionex) and used to determine the amount of products released at different time points. All the assays were carried out in duplicates. The data were fitted to the equation of Matsui (29, 30), $k=\ln[S_0]/[S_t]$, where $k=(k_{cat}/K_m)[\text{enzyme}]\times\text{time}$ and $[S_0]$ and $[S_t]$ represent substrate concentration prior to the start of the reaction and at a specified time during the reaction, respectively.

Hydrolysis of M_5 and M_6 in $H_2{}^{18}O$
To determine and compare the hydrolytic cleavage patterns of M_5 and M_6 by *Pa*Man5A and *Pa*Man26A, HPAEC-PAD data on the hydrolysis products (as described above) was combined with the analysis of hydrolysis performed in $H_2{}^{18}O$ as described previously (31, 32). Each productive binding of M_5 or M_6 gives rise to two products (e.g. M_6 cleaved to either two molecules of M_3 or to M_5 and M_1 or to M_4 and M_2). Quantitative HPAEC-PAD analysis of one product per cleavage (M_3, M_4 and M_5, respectively) was used to calculate the relative frequency of the productive binding modes of M_5 and M_6 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 (^{16}O) and labeled (^{18}O) product (M_3, M_4 and M_5) was used. Reactions were performed at 8°C (low temperature was used to avoid spontaneous incorporation of ^{18}O, (31)) in $H_2{}^{18}O$ (93%, with a total of 7% $H_2{}^{16}O$ contamination (3% in the original $H_2{}^{18}O$ and

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4% from the enzyme and substrate stock solutions) containing 1 mM Na-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 plate for Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis. Matrix (DHB 10 mg.ml^{-1} in H_2O) was applied immediately to the sample and it was dried under warm air. Samples from 40 min and 60 min had sufficient product build up and the determined ratios were in good agreement (2-8% variation between the 40 min and the 60 min samples from each incubation). The data from 40 min samples was used.

MALDI-TOF MS data acquisition and analysis
MALDI-TOF MS spectra were recorded in positive reflector mode using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). The laser intensity was set at 5500, and 50 subspectra with 20 shots on each were accumulated from each sample spot. The program Data Explorer version 4.5 was used for analysis of the data. The relative frequencies of the different productive binding modes resulting in the same products were calculated from the relative areas of the monoisotopic peaks of ^{16}O- and ^{18}O-labeled products as previously described (31). Two corrections were made: one for the [M + 2] natural isotope peak (5.3 % of the monoisotopic peak for M_3, 8 % of the monoisotopic peak for M_4 and 11 % of the monoisotopic peak for M_5) of the light (^{16}O) species that overlaps with the heavy (^{18}O) peak, followed by another correction for the 7 % $H_2{}^{16}O$ contamination in the hydrolysis assays. M_1 was not detectable because of matrix suppression of low masses.

Transglycosylation analysis
Reactions were set up to aim for detection of transglycosylation products with MALDI-TOF-MS, similarly to what was done in (32). Five mM M_5 was incubated with 0.5 µM *Pa*Man5A at 40°C in 10 mM Na-acetate buffer pH 5 for 0-15 min. Samples (0.5 µl) were withdrawn at different time points and spotted directly onto a stainless steel MALDI plate. Matrix solution (DHB 10 mg.ml^{-1} 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 vapour diffusion method at 20°C. *Pa*Man5A 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 nl, 200 nl 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. *Pa*Man5A crystals belonged to the $P2_12_12_1$ space group with the following cell dimensions: a = 56.9Å, b = 58.0Å, c = 98.2Å and diffracted to 1.4Å resolution. X-ray diffraction data of a *Pa*Man5A crystal were collected at 100K at the European Synchrotron Research Facilities (ESRF, Grenoble, France) beam line ID29.
*Pa*Man26A was concentrated to ~ 26 mg.ml-1 in 20 mM Hepes pH 7.5, 150 mM NaCl buffer. Small *Pa*Man26A crystals were obtained in the conditions (i) 0.1 M Tris pH 7, 0.2 M Li_2SO_4, 1 M Potassium Sodium Tartrate and (ii) 0.1 M Imidazole pH 8, 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 $HgCl_2$. For cryoprotection, crystals were transferred in a solution containing 25% (v/v) glycerol, 1.5 M Li_2SO_4, 100 mM bis-Tris propane pH 7.4. The crystals belonged to the $P6_522$ space group with the following cell dimensions: a =b= 97.5Å, c = 268.7Å. 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 (ESRF, Grenoble, France). The best X-ray diffraction data were collected to 2.85Å resolution at the European Synchrotron Research Facilities (ESRF, Grenoble, France) beam line ID14-4.

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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 *Pa*Man5A was determined with the molecular replacement method using the AMoRe program (35) and the *T. reesei* GH5 mannanase coordinates (PDB entry 1QNO). The rotation function yielded one solution, and the translation function yielded a unique solution, with a correlation coefficient and an R_{factor} of 38.1 and 44.5%, respectively, for data between 10 and 4Å. After rigid body refinement, the correlation coefficient was 59.2% for an R_{factor} of 35.9%. After refinement using the programs Refmac (36) and Buster (37) the final crystallographic R_{factor} and R_{free} were 15.0 and 17.2%.
The structure of *Pa*Man26A was also determined with the molecular replacement method using the AMoRe program (35). The superposition of 4 structures of GH26 CDs (PDB code: 2QHA, 2BVT, 2VX4 and 2WHK) plus the homology model given 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_{factor} of 32.4 and 49.2%, respectively, for data between 10 and 4Å. After rigid body refinement, the correlation coefficient was 42.7% for an R_{factor} of 43.7%. A modified structure of the CBM35 from *Clostridium thermocellum* (PDB 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 *Pa*Man26A. 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_{factor} has decreased to 20.7% (R_{free} 25.8%). All representations of the structure in the figures were prepared with the program PyMOL (http://www.pymol.org/). Coordinates for the structure *Pa*Man5A and *Pa*Man26A have been deposited in the Protein Data Bank under the accession number 3ZIZ and 3ZM8, respectively.

RESULTS AND DISCUSSION

Hydrolytic activity of Pa*Man5A and* Pa*Man26A towards polysaccharides*

In a recent study, we showed that *Pa*Man5A and *Pa*Man26A displayed similar kinetic parameters toward a range of mannan substrates. To further compare the *P. anserina* mannanases, we measured the release of manno-oligosaccharides following hydrolysis of ivory nut mannan and carob galactomannan. Towards the end of the reaction, *Pa*Man5A 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). *Pa*Man26A 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. fimi Cf*Man26A and *C. japonicus Cj*Man26B released M_2 and M_1, (17, 26) and *B. subtilis* z-2 BCMan released M_2 and M_4 (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 Pa*Man5A and* Pa*Man26A towards oligosaccharides*
The capacity of *Pa*Man5A and *Pa*Man26A to hydrolyze a range of manno-oligosaccharides was evaluated by ionic chromatography to get further insights into their active site architecture (Figure 1). *Pa*Man5A had very low activity on M_3, higher activity on M_4 and cleaved M_5 and M_6 rapidly (Table 3). A decrease of k_{cat}/K_M was observed with decreasing degree of polymerization (DP). The relative k_{cat}/K_M of *Pa*Man5A on M_3, M_4, M_5, and M_6 were 1: 358: 1127: 1782. The increase of the DP from 4 to 5 (M_4 and M_5) resulted in a 3.1-fold increase in k_{cat}/K_M, suggesting that at least four subsites are required to achieve efficient hydrolysis. In contrast, *Pa*Man26A had no detectable activity on M_3, very low activity on M_4 and cleaved M_5 and M_6 rapidly. For *Pa*Man26A the relative k_{cat}/K_M on M_4, M_5, and M_6 were 1: 195: 365 with an increase of k_{cat}/K_M of 1.9-fold between M_5 and M_6 hydrolysis. These data suggest that *Pa*Man26A 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 *Pa*Man5A produced mainly M_3 with smaller amounts of M_2 and M_4, whereas M_6 hydrolysis by *Pa*Man26A produced mainly

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M_4 and M_2, with smaller amounts of M_5 and M_1, and without any M_3. M_5 hydrolysis by *Pa*Man5A yielded mainly M_2 and M_3, with small amounts of M_4 and M_1, whereas hydrolysis by *Pa*Man26A yielded only M_4 and M_1. M_4 hydrolysis by *Pa*Man5A yielded mainly M_2 with small amounts of M_1 and M_3, whereas *Pa*Man26A had low activity on M_4 and produced M_1, M_2 and M_3. *Pa*Man5A hydrolysed poorly M_3 yielding M_1 and M_2 and *Pa*Man26A had no detectable activity on M_3. Both mannanases had no detectable activity on M_2 and pNP-mannose even at high enzyme loading (data not shown). Again, *Pa*Man26A showed an atypical hydrolytic profile for a GH26 endo-mannanase compared to *Cf*Man26A and *Cj*Man26A. *Cj*Man26A hydrolyses M_4 rapidly and requires occupation of four subsites to achieve efficient hydrolysis (18), while *Cf*Man26A is less efficient towards M_4 and requires substrate binding at five subsites to achieve efficient hydrolysis (17). For *Pa*Man26A, the occupation by substrate of at least five subsites to achieve efficient hydrolytic activity is even more pronounced, with a dramatic increase in k_{cat}/K_M between M_4 and M_5.

Productive binding mode of M_5 and M_6 by Pa*Man5A and* Pa*Man26A*

β-Mannanases usually bind oligomeric substrates in multiple productive binding modes that can generate identical products. The simplest example of this is M_3 hydrolysis in to M_2 and M_1, where mannose would be released from either the reducing end or the non-reducing end. In the former case M_3 binds productively from the -1 subsite to the +2 subsite and in the latter case from the -2 to the +1 subsite, following the established subsite nomenclature (41). As another example, from M_5, each of the products M_3 and M_4 respectively, can be produced by either of two binding modes (see scheme in Figure 2A). Binding of M_5 from subsite -2 to +3 or from subsite -3 to +2 both generates M_3 and binding from subsite -4 to +1 and from subsite -1 to +4 both generates M_4. Thus, product analysis using HPAEC-PAD data alone can not 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 M_3, M_4 and M_5 from the HPAEC-PAD data of M_5 and M_6 hydrolysis (Table 4) were used to calculate the relative molar distribution of these products (Table 4, values in brackets) and thus the frequencies of productive binding modes that give rise to these products could be estimated (Figure 2A, far left and far righ 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 M_3, M_4 or M_5), that was then used to estimate the relative frequency of the productive binding modes of M_5 and M_6 that give rise to these same products. The combined results of the HPAEC-PAD and MALDI-TOF-MS data are summarized in Figure 2A, showing the relative frequencies (%) of productive binding modes of M_5 and M_6. The calculation procedure is explained in Table S1. To exemplify, determined from HPAEC-PAD data (Table 4), 80% of the productive binding during the hydrolysis of M_5 by *Pa*Man5A generated M_3. MALDI-TOF analysis then determined the ratio between the two binding modes that give M_3. The analysis gave a ratio of M_3/M_3^{O18} of 1: 2.9 (Figure 2B), which shows that the enzyme binds M_5 preferably from subsite -3 to +2 to produce M_3, giving a 59% frequency of this binding mode (Figure 2A). Small amounts of M_1 and M_4 were also produced, and the ratio of M_4/M_4^{O18} was 1 : 0.7. Hydrolysis of M_6 by *Pa*Man5A produced mainly M_3 (55% binding frequency), but also smaller amounts of M_2 and M_4. The ratio of M_4/M_4^{O18} was 1: 3.2, which shows that *Pa*Man5A binds M_6 preferably from subsite -4 to +2 to produce M_4 (34% binding frequency). Hydrolysis of M_5 by *Pa*Man26A yielded M_1 and M_4 with a M_4/M_4^{O18} ratio of 1: 5.0 (Figure 2B), which shows that the enzyme binds M_5 preferently from subsite -4 to +1 (83% binding frequency, see Figure 2A). For hydrolysis of M_6, major product ratio analysis showed that the ratio of M_4/M_4^{O18} was 1: 10.8, which shows that *Pa*Man26A prefers to bind M_6 from subsite -4 to +2 (69% binding frequency). The ratio of the minor product M_5/M_5^{O18} was 1: 4.2, showing that the M_1 and M_5 are mainly produced without binding at the +2 subsite. Thus, these data reveal clear differences in the binding mode of the two *P. anserina* mannanases: the predominant mode of binding

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of M_5 and M_6 were significantly different (Figure 2A). *Pa*Man5A showed a classical pattern of hydrolysis products (M_3 and M_2 mainly were released from M_5) as described in several studies (*B. subtilis*, *C. japonicus*, *M. edulis*) whereas *Pa*Man26A showed release of M_4 and M_1 from M_5, which is unusual when compared to other GH26 endo-mannanases (*B. subtilis BC*Man, *C. fimi Cf*Man26A, *C. japonicus Cj*Man26A), suggesting an unusual arrangement of subsites in the catalytic center.

Transglycosylation ability

In order to detect potential transglycosylation ability of the two enzymes, they were incubated with M_5 as substrate. The resulting short time course study of the product formation clearly showed that *Pa*Man5A in addition to hydrolysis products also produces transglycosylation products with higher DP than the original substrate (Figure 3). *Pa*Man5A was able to transglycosylate yielding to oligosaccharide structures of up to a DP of 8 (n+1 to n+3), in good agreement with GH5 mannanases described before, *T. reesei* (n+1 to n+3) (31), and *A. nidulans* ManA (n+1 to n+3) and ManC (n+1 and n+2) (6). No transglycosylation products could be detected with *Pa*Man26A incubated with M_5 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 Pa*Man5A*

The crystal structure of *Pa*Man5A was solved in its free form. The crystal contained 1 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 *Pa*Man5A (Figure 4A) revealed a $(\beta/\alpha)_8$-barrel fold as expected for enzymes belonging to clan GH-A. When superimposed with *Tr*Man5A (PDB entry 1QNR) and *T. fusca* mannanase (PDB entry 3MAN) structures (Figure S1), the overall fold of *Pa*Man5A is very similar to that of *Tr*Man5A, with structural differences being confined mainly in the loop regions (Figure 4A) that have been defined as eight loops, loop 1 (residues 35 to 42), loop 2 (66 to 95), loop 3 (120 to144), loop 4 (177 to 184), loop 5 (213 to 232), loop 6 (252 to 258), loop 7 (287 to 289) and loop 8 (316 to 336). Compared to *Tr*Man5A that contains four disulfide bonds: C26-C29, C172-C175, C265-C272 and C284-C334, *Pa*Man5A contained only 3 disulfide bonds, i.e., C180-C184, C272-C279, C291-C342. Following N-deglycosylation of *Pa*Man5A using PNGaseF, no shift in the apparent molecular mass (46 kDa) was observed on SDS-PAGE compared to untreated sample (data not shown). This observation was in good agreement with NetGlyc predictions from the *Pa*Man5A primary sequence (no predicted N-glycosylation site) and analysis of the *Pa*Man5A crystallographic data that confirmed absence of glycosylation units.

The active site of *Pa*Man5A was clearly identified in the groove, with the two conserved catalytic glutamate residues (acid-base and nucleophile) positioned near the carboxy-terminal ends of β-strands four and seven of the $(\beta/\alpha)_8$ barrel (41), E177 and E283, respectively. Mutant E283A showed no catalytic activity for glucomannan, thus indicating that E283 should be the nucleophile. E177A had a specific activity of 0.47 $U.mg^{-1}$ towards glucomannan, which is roughly 100-fold lower than the wild-type enzyme (45 $U.mg^{-1}$), thus indicating that E177 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 *Pa*Man5A inactive mutants alone or in complex with its substrate has been obtained. Consequently, we performed comparative structural analysis of *Pa*Man5A with other GH5 mannanases complexes (*T. reesei* PDB 1QNO, *T. petrophila* PDB 3PZ9, and *S. lycopersicum* PDB 1RH9) to map the substrate binding subsites (Figure 4B). In the -1 and +1 subsites where the catalytic cleavage occurs, 7 out of 8 residues highly conserved in GH5 mannanases (44) are found in *Pa*Man5A, among which the catalytic residues E177 and E283 and R62, N176, H248, Y250, W315 (Figure 4B). *Pa*Man5A also has an arginine equivalent to R171 in the +2 subsite of *Tr*Man5A (15), which is semi-conserved among GH5 mannanases, and that was shown to play a significant role in the transglycosylation ability of *Tr*Man5A (32).

Structure of Pa*Man26A catalytic module*

The structure of *Pa*Man26A was successfully solved using molecular replacement. The search model was composed of the superimposition of four structures of bacterial

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mannanases (PDB code: 2QHA, 2BVT, 2VX4 and 2WHK). The final structure comprising 443 residues was refined at 2.85Å resolution. The overall structure of *Pa*Man26A CD revealed a $(\beta/\alpha)_8$-barrel fold (Figure 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 (E300 and E390) positioned at the end of the $(\beta/\alpha)_8$ barrel, and several aromatic residues forming the subsites of catalytic cleft. Mutant E390A showed no catalytic activity for glucomannan, indicating that E390 should be the nucleophile. E300A had a specific activity of 0.33 $U.mg^{-1}$, which is roughly 200-fold lower than the wild-type enzyme (65 $U.mg^{-1}$), thus indicating that E300 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, N268, which is located in the CD on the external side of the barrel. As modeled from the electron density, 2 β-1,4 linked N-acetylglucosamine (GlcNAc) units are attached to this N-glycosylation site. N-deglycosylation of *Pa*Man26A using PNGaseF was associated with a 2-3 kDa shift in the apparent molecular mass on SDS-PAGE compared to untreated sample (data not shown). These results confirm that *Pa*Man26A is N-glycosylated and are in agreement with the NetNGlyc prediction (one predicted N-glycosylation site at position N268).

Several regions are highly conserved between *Pa*Man26A and other GH26 mannanases from *B. subtilis* z-2 (PDB 2QHA), *B. subtilis* subsp. *bacillus* (PDB 2WHK), *C. japonicus* (PDB 1GW1 and 2VX6), and *C. fimi* (PDB 2BVT; Figure S2) as shown superimposition of *Pa*Man26A and *B. subtilis* z-2 (PDB 2QHA) structures (Figure 5A). The central β-barrel and most of the surrounding α-helices are superimposable between *Pa*Man26A and *B. subtilis* z-2 while loop regions are dramatically different. Indeed, *B. subtilis* enzyme exhibits a flat surface with a shallow dish shaped active center whereas *Pa*Man26A displays large loops that form a deep cleft. According to the three dimensional structure of *Pa*Man26A, 8 loops are involved in the binding of the substrate to the active site: loop 1 (171 to 174), loop 2 (195 to 208), loop 3 (230 to 266), loop 4 (301 to 314), loop 5 (342 to 346), loop 6 (362 to 368), loop 7 (392 to 396) and loop 8 (413 to 425). The most striking difference stands in loop 2 that contains four aromatic residues (W244, W245, F248 and Y249) and is nine amino-acids longer than *B. subtilis* z-2 (PDB 2QHA), *B. subtilis subsp. bacillus* (PDB 2WHK), *C. japonicus* (PDB 2VX6) and *C fimi* (PDB 2BVT) mannanases. A shorter loop 2 does not allow interaction with the substrate at the glycone binding subsites in the case of *B. subtilis* z-2 (PDB 2QHA). The -1 and +1 subsites of *Pa*Man26A are quite similar to homologous enzymes with the conserved residues H299, W305, F306, Y362, W413 (Figures 5B and 5C). As described for *Cf*Man26A and *Cj*Man26A, *Pa*Man26A Y362 is probably involved in a hydrogen bond with the catalytic nucleophile E390, while *Pa*Man26A W305 and W413 could play a role as aromatic platforms to stabilize mannopyrannose rings at the +1 and -1 subsites, respectively (Figure 5C). In the -2 subsite of BCMan, binding is not favorable because of steric hindrance due to the position of Y40 (21). In the case of *Cf*Man26A, the two aromatic F123 and Y124 residues that are superimposed with *Pa*Man26A F248 and Y249, stabilize the interaction with a mannose unit at the -2 subsite (Figure 5C).

Our experimental data indicate that *Pa*Man26A displays strong interactions at the -4 subsite. Indeed, *Pa*Man26A was poorly active toward M_4 probably due to the formation of an unproductive complex between -4 and -1 subsites. We further analyzed the -4 subsite in the *Pa*Man26A structure and identified two aromatic residues, W244 and W245, located in loop 2 that could stabilize mannopyrannose rings in the -4 subsite (Figures 5B and 5C). As *Pa*Man26A, *Cf*Man26A 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 -4 were described as making a minor contribution to binding (17). In *Pa*Man26A, there is no equivalent to the F42, F325 and Q329 *Cf*Man26A -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, *Cf*Man26A produces M_2 and M_3 as

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major products from M_5 with only minor amount of M_1 and M_4 (31).

Pa*Man26A modular organization*

*Pa*Man26A harbors a family 35 CBM at its N-terminal end, and the closest characterized enzyme is *Humicola insolens* β-mannanase (GenBank AAQ31840, (46)) with 78% amino-acid identity. Following a BlastP search using *Pa*Man26A 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 *Pa*Man26A CBM35, only one CBM35 module binding to galactan has been characterized to date in a *Phanerochaete chrysosporium* exo-β-1,3-galactanase (47). We previously suggested that the N-terminal CBM35 module of *Pa*Man26A displayed dual binding specificity toward xylan and mannan (28) and the phylogenetic analysis performed by (48) clustered *Pa*CBM35 in the subfamily II that is proposed to target β-1,4 mannan.

While 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 *Pa*Man26A is short without any glycosylation sites while modular fungal GHs usually display long and highly glycosylated linkers. The *Pa*Man26A linker sequence was rich in proline residues, i.e., it contains 4 prolines (P132, P134, P135 and P140) out of 12 residues that may confer rigidity to the modular enzyme (Figure 6A). The linker starts on residue S130 at the end of the last β-strand of the N-terminal CBM domain. Only 2 residues (A131 and P132) 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. R133 and H136 side chains make hydrogen bond with D382, N374 and Q404 while the side chain of I138 fits into a hydrophobic cavity made of R159 (aliphatic part of the side chain), Y162 and M385. The CBM and the catalytic module are thus in close association thanks to the embedded linker (Figure 6B) and it may explain why attempts to express the catalytic module alone were unsuccessful (data not shown). Alignment of *Pa*Man26A-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 L58 and L103 on the surface of the CBM35 domain stands in front of a cluster of hydrophobic residues (A402, Y403 and L399) 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.

Pa*CBM35 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 *Clostridium thermocellum* (PDB code 2W1W, (50)), with a Z-score of 18.6. Its superimposition with *Pa*CBM35 shows that 57 Cα out of 125 Cα (45%) have equivalent positions in both molecules, with the distance between the superimposed Cα atoms being < 1Å. The main differences occur in the loops connecting the beta-strands as shown in Figure 6C. A metal ion is present, which 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 *Pa*CBM35. A platform of three aromatic residues (F87, W117 and W119) was observed at the surface of the *Pa*CBM35 (Figure 6B). These residues are aligned with the *Pa*Man26A catalytic cleft suggesting that they could play a role in substrate binding.

Conclusions

The *P. anserina* CAZome (the genome-wide inventory of CAZymes) includes three genes encoding β-(1,4)-mannanases (www.cazy.org): 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

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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 *P. anserina*'s 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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FOOTNOTES

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[4]The abbreviations used are: CBM, Carbohydrate-Binding Module; CD, Catalytic Domain; DHB, 2,5-dihydroxybenzoic acid; DP, degree of polymerization; GH, Glycoside Hydrolase; HPAEC-PAD, High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection; MALDI-TOF-MS, Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry, M_1, mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; M_5, mannopentaose; M_6, mannohexaose *Pa*CBM35, *Podospora anserina* CBM35; *Pa*Man5A, *Podospora anserina* GH5 mannanase A; *Pa*Man26A, *Podospora anserina* GH26 mannanase A

FIGURE LEGENDS

FIGURE 1: Progress curve of the manno-oligosaccharides generated by *Pa*Man5A and *Pa*Man26A after the hydrolysis of manno-oligosaccharides.
The recombinant enzymes were incubated with 100 µM manno-oligosaccharides in acetate buffer pH 5.2 at 40°C. The quantity of mannose (open diamond), M_2 (open squared), M_3 (full circle), M_4 (full triangle), M_5 (full diamond), and M_6 (full squares) produced during the course of the reaction was quantified using HPAEC-PAD. The concentrations of enzymes used were *Pa*Man5A (A) 18.2 nM with M_6, (B) 18.2 nM with M_5 and (C) 60 nM with M_4; *Pa*Man26A (D) 15 nM with M_6 and (E) 30 nM with M_5.

FIGURE 2: Relative frequency of the productive modes of binding of manno-oligosaccharides to *Pa*Man5A and *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 M_5 and M_6 using MALDI-TOF-MS analysis of ^{18}O-labeled products, allowing to distinguish further between binding modes (numbers in bold). The arrow indicates the mannosidic bond to be cleaved. * = reducing end of oligosaccharide. The -4 and +3 dashed subsites are only present in *Pa*Man26A and *Pa*Man5A, respectively. ND = not detected. B. MALDI-TOF-MS spectra of M_5 hydrolysis by *Pa*Man5A and *Pa*Man26A, showing enlarged parts of the spectra with the M_3 product formed by *Pa*Man5A at a ratio of 1: 2.9 of M_3/M_3^{O18} (left) and the M_4 product formed by *Pa*Man26A at a ratio of 1: 5.0 of M_4/M_4^{O18} (right). The peaks in the spectra correspond to the monoisotopic masses of sodium adducts $[M + Na]^+$ of the manno-oligosaccharides.

FIGURE 3: **MALDI-TOF-MS analysis of the transglycosylation product formation from M_5 during 1-10 min by *Pa*Man5A.**
Peaks in the spectra correspond to monoisotopic masses of sodium adducts [M + Na]+ of manno-oligosaccharides: M_2: m/z 365.1, M_3: m/z 527.1, M_4: m/z 689.2, M_5: m/z 851.2, M_6: m/z 1013.3, M_7: m/z 1175.3 and M_8: m/z 1337.3. The enlarged part in each spectrum corresponds to approximately 3 % of the relative intensity.

FIGURE 4: Crystal structure of *Pa*Man5A.

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A: Superposition of *Pa*Man5A (green) and *Tr*Man5A (yellow) structures. The two views are related by a rotation of approximately 90° about the vertical axis. B: Surface view of the catalytic cleft of *Pa*Man5A 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 *Pa*Man5A to map the substrate-binding subsites.

FIGURE 5: Crystal structure of *Pa*Man26A.
A: Superposition of *Pa*Man26A catalytic module (green) and *Bc*Man (orange) structures. The two views are related by a rotation of approximately 90° about the vertical axis. B: Surface view of the catalytic cleft of *Pa*Man26A 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 *Pa*Man26A to map the substrate-binding subsites. C: Organization of the glycone binding subsites in *Pa*Man26A (yellow) compared *with C. fimi* (cyan).

FIGURE 6: Views of Modular architecture of *Pa*Man26A.
A: Ribbon diagram of *Pa*Man26A catalytic (blue) and CBM (green) domains. The proline-rich linker is shown in stick format. B. Molecular surface representation of *Pa*Man26A structure with the catalytic domain in blue, the *Pa*CBM35 domain in green and the linker in purple. The three aromatic residues present at the surface of the *Pa*CBM35 domain are shown in yellow. C: Superposition of *Pa*CBM35 domain (green) and *C. thermocellum* CBM35 (orange). The calcium ion is represented by a blue sphere.

TABLES

Table 1: Mutagenesis primers used in the study

Primer	Nucleotide sequence 5' to 3'[a]
E177AFor	GGGAACTTGCCAACGCGCCCAGGTGCAAGGG
E177ARev	CCCTTGCACCTGGGCGCGTTGGCAAGTTCCC
E283AFor	CCGTGTTTGTTGGAGGCGTATGGGTATGAGAGTGATAGG
E283ARev	CCTATCACTCTCATACCCATACGCCTCCAACAAACACGG
E320AFor	GGAGACCTCTTCACGCCGCGGAGGGTGGTTGG
E320ARev	CCAACCACCCTCCGCGGCGTGAAGAGGTCTCC
E410AFor	GATGATTGCTGCTGCAGCCGTTGGCGCTGCC
E410ARev	GGCAGCGCCAACGGCTGCAGCAGCAATCATC

[a]Modified codons are underlined

Table 2: Data collection and model refinement statistics of *Pa*Man5A and *Pa*Man26A

	*Pa*Man5A	*Pa*Man26A
Data collection		
Wavelength (Å)	0.97914	1.00648
Space group	$P2_12_12_1$	$P6_522$
a, *b*, *c* (Å)	56.87, 57.90, 97.86	97.49, 97.49, 268.72
Resolution (Å)[a]	30-1.4 (1.48-1.4)	50-2.85 (3.0-2.85)
Unique reflections[a]	62,519 (8,848)	18,575 (2,627)
Multiplicity[a]	8.4 (8.3)	11.8 (11.8)
Completeness (%)[a]	97.3 (95.7)	99.9 (100.0)
I/σ[a]	18.7 (3.4)	25.6 (4.6)
R_{merge} (%)[a,b]	8.6 (67.8)	7.6 (65.2)
Refinement and model quality		
Resolution (Å)	30-1.4	30-2.85

Reflections	59,254	18,483
R_{cryst}/R_{free} (%)[c]	15.0/17.2	20.6/25.7
Number of atoms	3,193	3,676
Protein/carbohydrate moiety	2,745/0	3,493/28
Water/solvent/ion	434/14/0/	143/12/2
Average B-factors (Å^2)		
Protein/carbohydrate moiety	10.6	68.2/888
Water/solvent/ion	28.5/10.6	63.3/85.1/92.1
Rmsd[d]		
Bond (Å)	0.010	0.008
Angle (°)	1.02	1.04
Ramachandran plot (%)		
Most favoured regions	98.0	95.5
Additionally allowed regions	2.0	4.3
Outlier regions	0	0.2
PDB accession code	3ZIZ	3ZM8

[a] Values in parentheses are for the highest-resolution shell.
[b] $R_{merge}=\Sigma_{hkl}(\Sigma_i|I_{hkl}-\langle I_{hkl}\rangle|)/\Sigma_{hkl}|\langle I_{hkl}\rangle|$.
[c] $R_{cryst}=\Sigma_{hkl}||Fo|-|Fc||/\Sigma_{hkl}|Fo|$; R_{free} is calculated for 5% of randomly selected reflections excluded from refinement.
[d] Root mean square deviation from ideal values.

Table 3: Catalytic efficiencies of *Pa*Man5A and *Pa*Man26A on manno-oligosaccharides

	k_{cat}/K_M (M^{-1} min^{-1})	
Substrate	*Pa*Man5A	*Pa*Man26A
M_6	2.9×10^6	2.4×10^6
M_5	1.9×10^6	1.3×10^6
M_4	5.9×10^5	6.4×10^3
M_3	1.6×10^3	ND

ND: Not determined due to low activity.

Table 4: Hydrolysis products released by *Pa*Man5A and *Pa*Man26A from manno-oligosaccharides

			Products (µM)				
	Enzyme loading (nM)	Substrate	M_1	M_2	M_3	M_4	M_5
***Pa*Man5A**	60	M_4	11	49	21	-	-
	18.2	M_5	2	32	20 (80*)	5 (20*)	-
	18.2	M_6	n.d.	12	34 (**55***)	14 (45*)	n.d.
***Pa*Man26A**	30	M_5	34	n.d.	n.d.	43 (100*)	-
	15	M_6	13	28	n.d.	33 (75*)	11 (25*)

Products were quantified using HPAEC-PAD and are expressed in µM. Incubation was carried out for 30 minutes at 40°C; n.d., not detected. *The values in brackets represent the relative molar distribution (%) between the products M_3, M_4 and M_5 from each of the M_5 and M_6 incubations, which were used to estimate the relative frequency of productive binding modes yielding these products (presented in Figure 2A). One product (M_3, M_4 or M_5) per productive binding was used for calculations, thus only half of the produced M_3 (bold) from M_6 incubations was accounted for (two molecules of M_3 are produced from each molecule of M_6).

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Figure 1

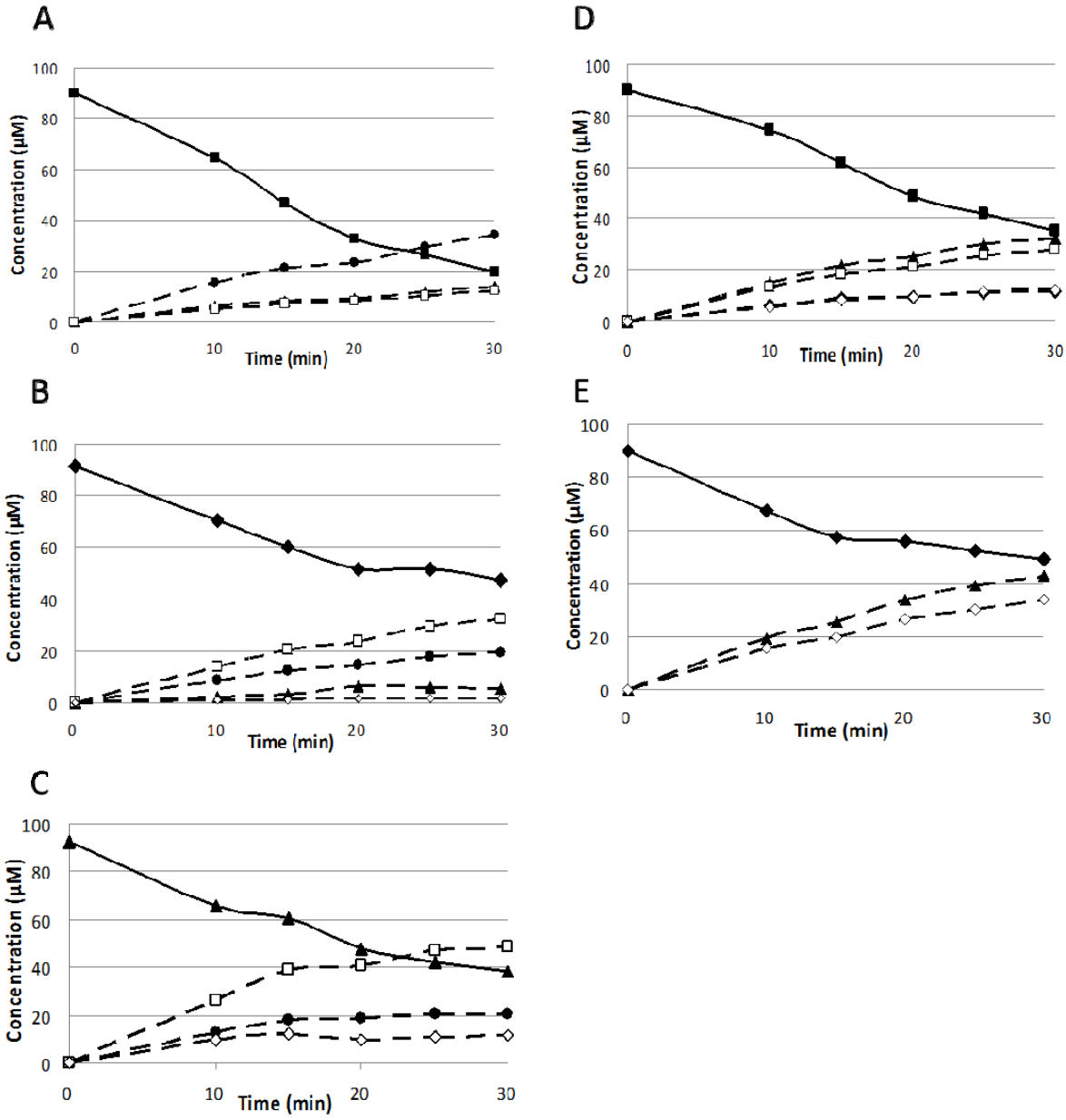

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Figure 2

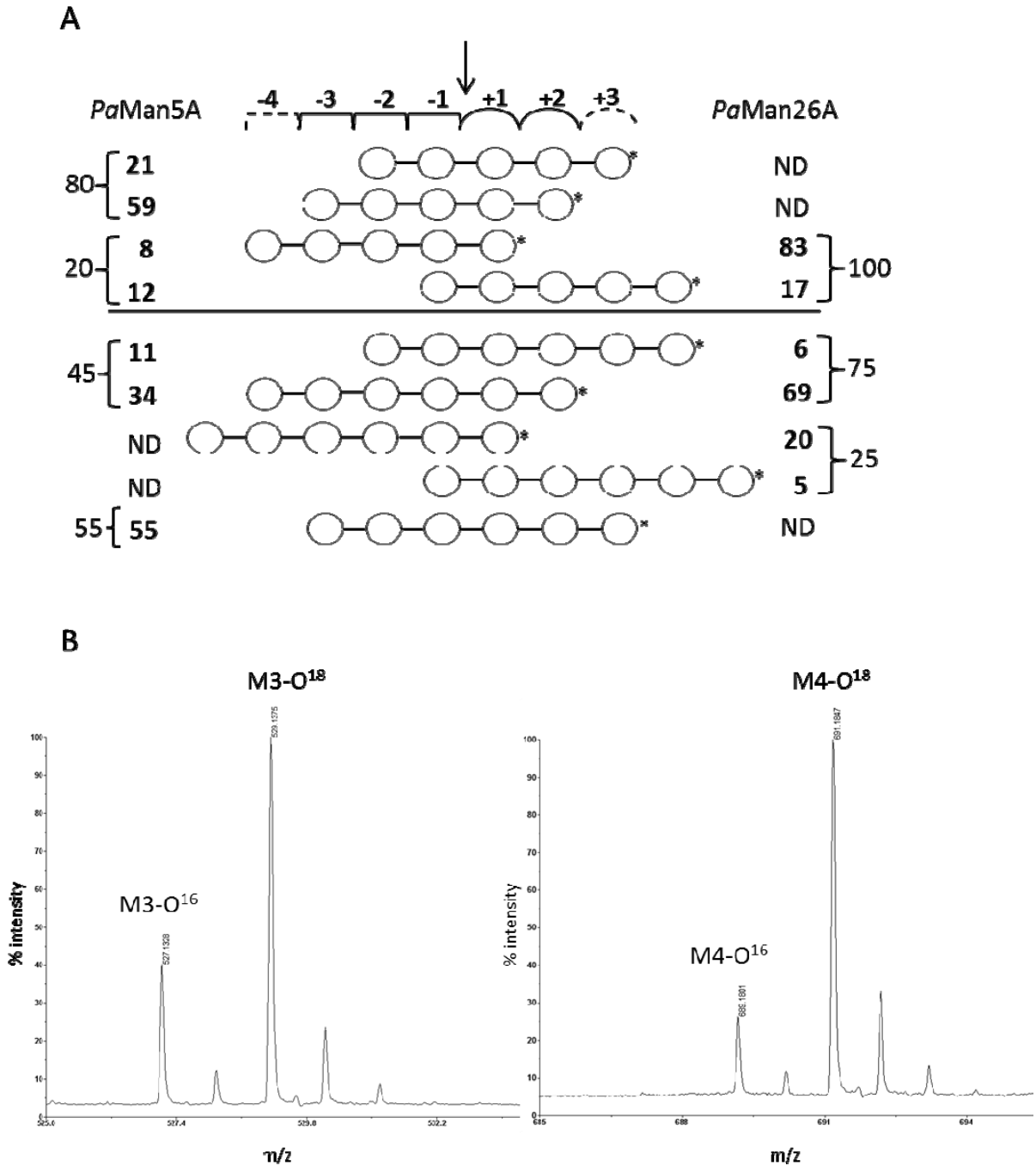

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Figure 3

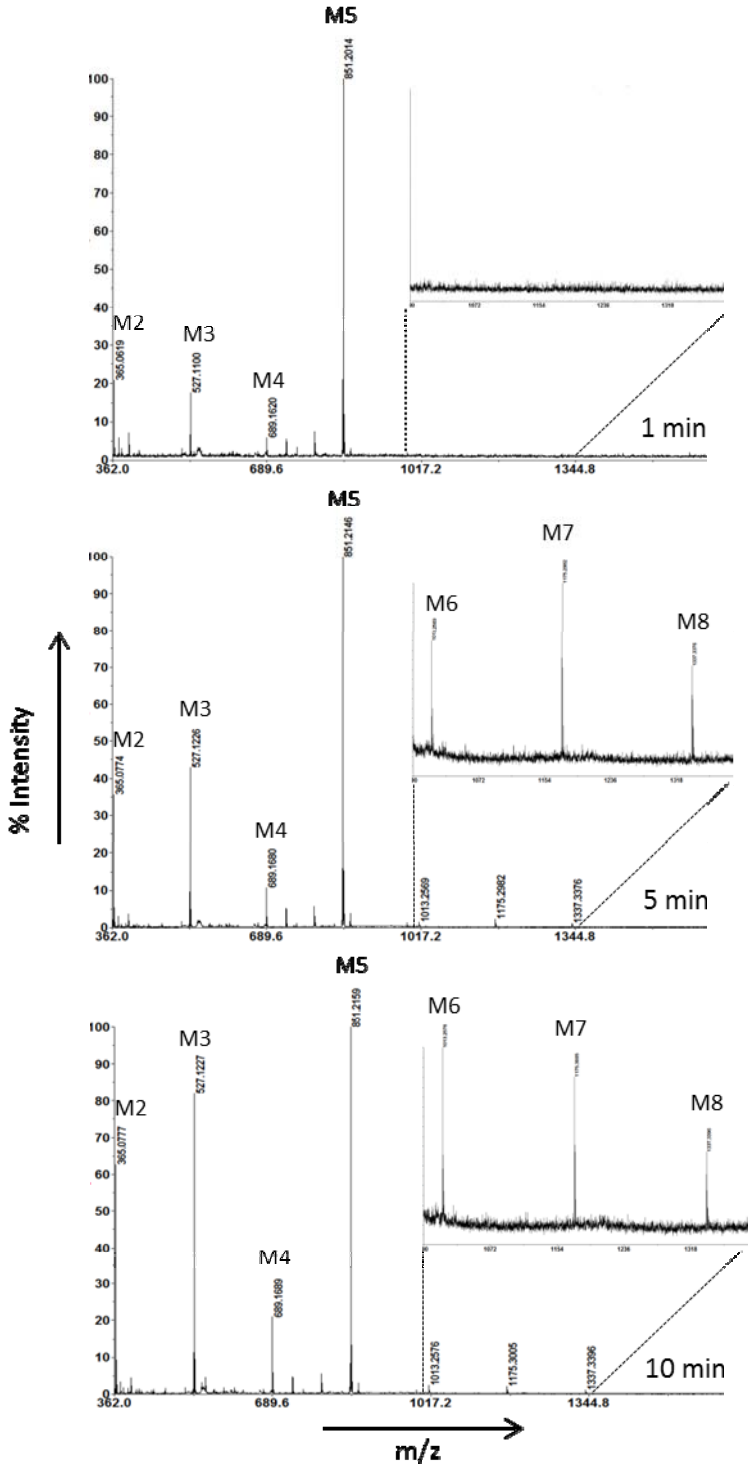

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Figure 4

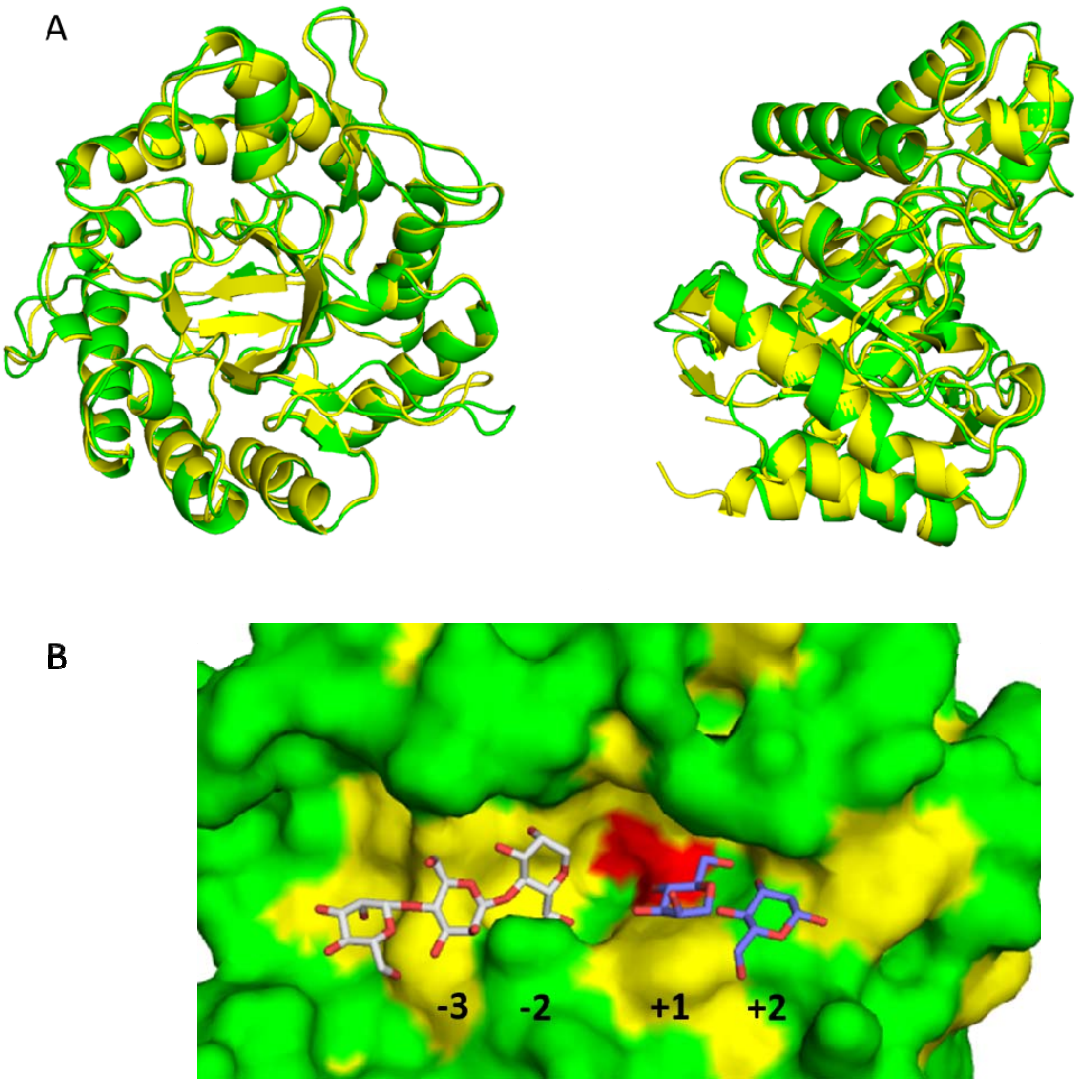

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Figure 5

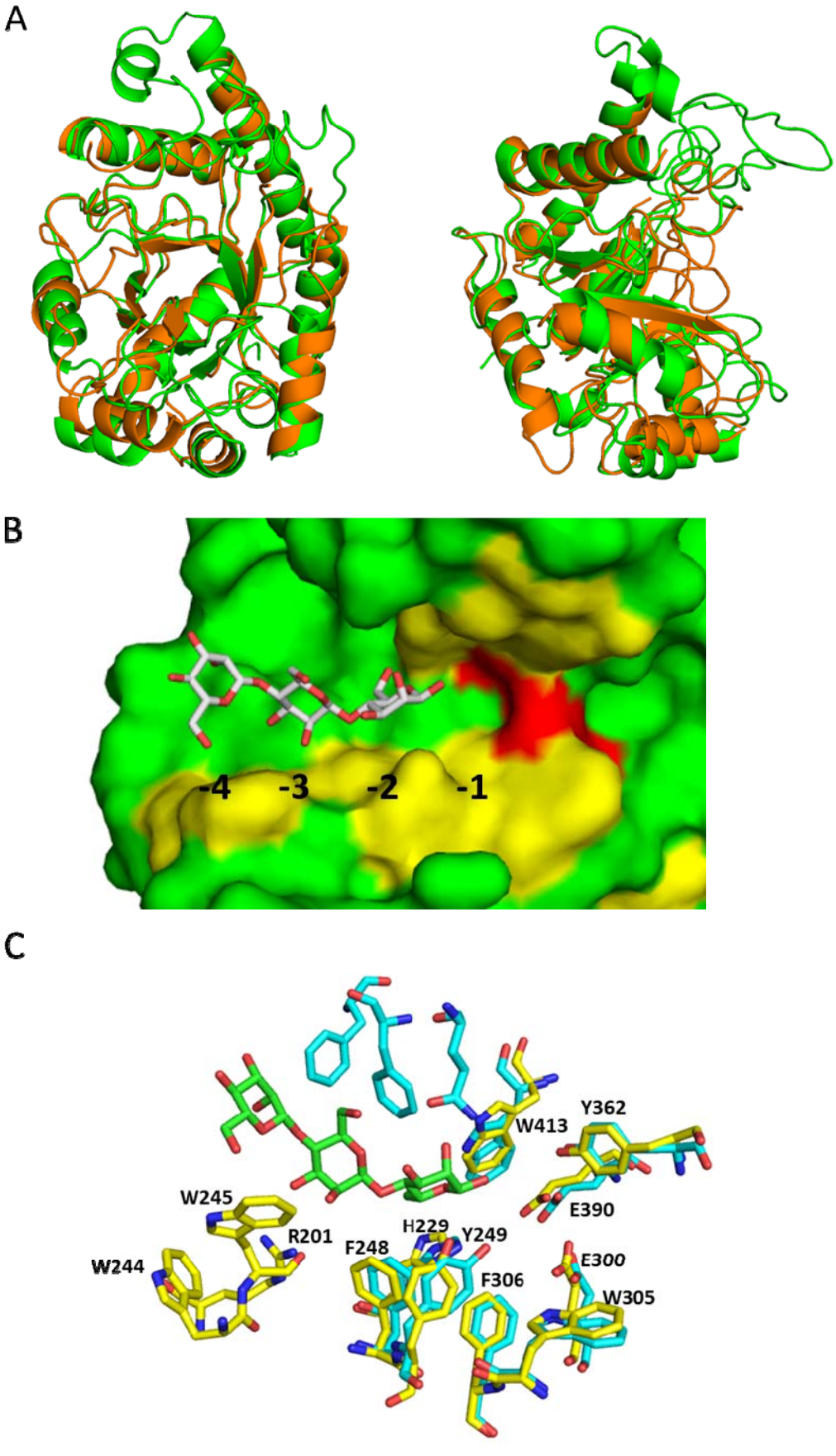

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Figure 6