Molecular Engineering of Fungal GH5 and GH26 Beta-(1,4)-Mannanases toward Improvement of Enzyme Activity

Marie Couturier1,2, Julia Féliu1,2, Sophie Bozonnet3,4,5, Alain Roussel6, Jean-Guy Berrin1,2*

1 INRA, UMR1163, Laboratoire de Biotechnologie des Champignons Filamenteux, Polytech Marseille, Marseille, France, 2 Aix Marseille Université, Polytech Marseille, Marseille, France, 3 Université de Toulouse; INSA, UPS, INP, LISBF, Toulouse, France, 4 INRA, UMR792, Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France, 5 CNRS, UMR5504, Toulouse, France, 6 Architecture et Fonction des Macromolécules Biologiques, UMR7257, CNRS, Aix Marseille Université, Marseille, France

Abstract

Microbial mannanases are biotechnologically important enzymes since they target the hydrolysis of hemicellulosic polysaccharides of softwood biomass into simple molecules like manno-oligosaccharides and mannose. In this study, we have implemented a strategy of molecular engineering in the yeast Yarrowia lipolytica to improve the specific activity of two fungal endo-mannanases, PaMan5A and PaMan26A, which belong to the glycoside hydrolase (GH) families GH5 and GH26, respectively. Following random mutagenesis and two steps of high-throughput enzymatic screening, we identified several PaMan5A and PaMan26A mutants that displayed improved kinetic constants for the hydrolysis of galactomannan. Examination of the three-dimensional structures of PaMan5A and PaMan26A revealed which of the mutated residues are potentially important for enzyme function. Among them, the PaMan5A-G311S single mutant, which displayed an impressive 8.2-fold increase in \( k_{cat}/K_m \) due to a significant decrease of \( K_m \), is located within the core of the enzyme. The PaMan5A-K139R/Y223H double mutant revealed modification of hydrolysis products probably in relation to an amino-acid substitution located nearby one of the positive subsites. The PaMan26A-P140L/D416G double mutant yielded a 30% increase in \( k_{cat}/K_m \) compared to the parental enzyme. It displayed a mutation in the linker region (P140L) that may confer more flexibility to the linker and another mutation (D416G) located at the entrance of the catalytic cleft that may promote the entrance of the substrate into the active site. Taken together, these results show that the directed evolution strategy implemented in this study was very pertinent since a straightforward round of random mutagenesis yielded significantly improved variants, in terms of catalytic efficiency \( (k_{cat}/K_m) \).

Introduction

Hemicellulose is a combined designation of a diverse set of abundant non-crystalline carbohydrate polymers, among which mannan is the major component in softwoods [1] and are also present in certain plant seeds [2]. Mannans comprise molecules constituted either by a backbone of \(-1,4\)-linked D-mannose residues, known as mannan, or by a heterogeneous combination of \(-1,4\)-D-mannose and \(-1,4\)-D-glucose units, termed glucomannan. Both can be decorated with \(-1,6\)-linked galactose side chains, and these polysaccharides are referred to as galactomannan and glucomannan, respectively. According to Salminen [3] softwood glucomannans are incorporated into aggregates of cellulose, i.e., they are arranged in parallel with cellulose fibrils, to which they are tightly connected.

Mannans are hydrolyzed by the coordinated action of several types of glycoside hydrolases (GH) among which endo-\(-\beta\)-1,4 mannannases (EC 3.2.1.76) are the key enzymes that depolymerize the mannan backbone. They are encountered in families GH5, GH26 and GH113 in the CAZy database [4,5]. Beta-mannanases are useful in several industrial processes such as reduction of viscosity of coffee extracts [6] or biobleaching of softwood Kraft pulp [7] and it is now acknowledged that they will become increasingly important for the bio-refining of lignocellulose, especially from softwood biomass [8–11]. In the bio-refinery process, enzymatic hydrolysis of lignocellulosic biomass is one of the major bottlenecks due to the recalcitrance of the plant cell wall and the high cost of enzymes, mainly due to the fact that large amounts are required to breakdown lignocellulose to fermentable sugars [12–14].

Despite the fact that mannanases are largely exploited in biotechnological applications, only a few studies have so far reported on the improvement of mannanase properties using molecular engineering [15,16]. Directed evolution is an important tool for improving critical traits of biocatalysts for industrial applications [17]. Recent advances in mutant library creation and high-throughput screening have greatly facilitated the engineering of biocatalysts but to date only few studies describe improvement of biomass-degrading enzymes using molecular evolution [18–20]. The major problem with most directed
evolution experiments on biomass-degrading enzymes is the setup of high-throughput assays [17,21,22] since it is problematic to measure enzyme activities towards insoluble (hemi)cellulosic substrates.

In this study, a random mutagenesis strategy was used to generate variants of two endo-mannanases from the ascomycete fungus Podospora anserina that belong to the glycoside hydrolase (GH) families GH5 and GH26 (PaMan5A and PaMan26A, respectively) in order to improve their activity towards galactomannan. To evaluate the activity of mannanase variants produced through anaerobic fermentation in Yarrowia lipolytica, variants were selected by screening in liquid cultures. The results were interpreted in the lights of the three-dimensional structures of both fungal mannanases [24].

Results and Discussion

PaMan5A and PaMan26A heterologous expression in Yarrowia lipolytica

The yeast Y. lipolytica was chosen as host to perform molecular engineering of PaMan5A and PaMan26A since a very high reproducibility in protein expression level was demonstrated [25]. The two genes encoding PaMan5A and PaMan26A were inserted into the Y. lipolytica expression vector in frame with the yeast preproLip2 secretion peptide under the control of the oleic acid-inducible promoter POX2 (Figure 1). Positive transformants selected on plates containing galactomannan were able to produce functional PaMan5A and PaMan26A enzymes to a level of 10.4±0.2 and 11.2±0.6 U.ml⁻¹ in shake flasks, respectively. The culture conditions enabling the secretion of wild-type PaMan5A and PaMan26A were miniaturized in 96-well plates as described in [26] to facilitate the set-up of the high-throughput screening procedure. The mean activities calculated from repeated experiments were 1.78±0.14 and 1.24±0.15 U.ml⁻¹ of culture for PaMan5A and PaMan26A, respectively (coefficient of variation [CV] of 7.7 and 12.2%, respectively), which is adequate for excluding false positives variants. The secretion yields of PaMan5A and PaMan26A in deep-well microplates were 6- and 9-fold lower than in shake flask cultures, which is probably due to a better oxygenation of cultures in flasks than in deep-well plates where the ratio volume of culture/volume of container was of 1/2 instead of 1/10. However, levels of mannanase production were in both cases sufficient to deploy a high-throughput screening campaign.

Construction of first-round mutagenesis libraries and screening

The objective of the directed evolution approach was to improve enzymatic activities of PaMan5A and PaMan26A, two endo-mannanases from Podospora anserina. The error-prone PCR mutagenesis round was carried out using the wild type genes as templates (GenBank HM357135 and HM357136 for paMan5a and paman26a, respectively) and with the conditions appropriate to reach a number of mutations in a range of 2 to 5 mutations per kb. Indeed, a very low number of mutations per kb (i.e., below one mutation per kb) would result in too many active variants with unchanged activity, and a high number of mutations per kb would result in too many inactive clones as reported in [17] (>70% inactive clones for a mutation rate of 10 mutation per kb). In our study, subcloning and sequence analysis of a representative set of mutated genes from each library revealed that the mutation rates were in the desired range (i.e. 2.6 and 4.5 mutations per kb for PaMan5A and PaMan26A, respectively). Figure 2 shows a summary of the libraries screening and the number of variants selected at each step. Y. lipolytica transformation yielded about 5,200 and 5,600 clones for PaMan5A and PaMan26A, respectively. As reported in other studies [20,27,28], 5,000 variants was considered as a reasonable size of library to identify mutants displaying improved characteristics. The first step of the screening consisted in the selection of active clones on solid medium containing AZO-dyed galactomannan. Since we were looking for an activity improvement that was expected to be rather modest, halo-producing clones could not be screened only by comparing the size of halos but required a second step of screening.

Table 1. Mannanase activity of selected Y. lipolytica variants.

| Enzyme | Activity (U.ml⁻¹) | CV (%) | Activity Improvement (%) |
|--------|------------------|--------|--------------------------|
| PaMan26A wt | 1.24±0.15 | 12.2 | - |
| PaMan26A-P140L/D416G | - | - | 147 |
| PaMan5A wt | 1.78±0.14 | 7.7 | - |
| PaMan5A-V256L/G276V/Q316H | - | - | 46 |
| PaMan5A-W36R/I195T/V256A | - | - | 9 |
| PaMan5A-K139R/Y223H | - | - | 20 |
| PaMan5A-G311S | - | - | 11 |

The mannanase activity was measured at 40°C in sodium acetate buffer 50 mM, pH 5.2 using 1% (w/v) galactomannan. The coefficient of variation (CV) was defined as the ratio of the standard deviation to the mean and was calculated for each of the wild-type enzymes. wt, wild type.

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was performed using our in-house robotic platform with about 2,000 and 1,500 mutants for PaMan5A and PaMan26A, respectively (Figure 2). Since the screening capability of this high-throughput system is 15 plates per day, ~1,400 variants could be analysed in one day and the defined screening job (3,500 variants) was finished within 3 days. Among the best-performing clones that were further tested to confirm the enhancement of activity in liquid cultures, we finally selected four mutants of PaMan5A and one mutant of PaMan26A that displayed improved activity beyond the CV of wild-type strain expressing wild-type activity towards galactomannan of 147% compared to the strain expressing wild-type PaMan26A that corresponded to 12 CV.

Mutant production in *P. pastoris* and biochemical characterization

For mutant enzymes production, we used the *P. pastoris* expression system because (i) it yields higher expression levels than *Y. lipolytica* [29] and (ii) wild-type PaMan5A and PaMan26A were already successfully expressed to high yields in *P. pastoris* [10]. The ratio of mannanase production yields obtained in the culture medium with *Y. lipolytica* and *P. pastoris* was roughly 1:10. All of the mutant enzymes were successfully produced in *P. pastoris* with yields of approximately 1 g per liter of culture and purified taking advantage of the (His)6-tag. SDS-PAGE analysis of the purified mutants compared to the wild type enzymes revealed similar apparent molecular masses (data not shown). Isoelectrofocusing analysis revealed that all of the mutants displayed similar pIs compared to wild-type enzymes except for the PaMan5A-W36R/I195T/V256A mutant that exhibited an increase of 0.3 unit (Figure S1). This increase is likely to be due to the incorporation of an arginine residue. Circular dichroism analysis of all selected mutants was also carried out to check the relative content of secondary structures in each mutant. Differences were not observed between variants and parental enzymes, suggesting that the folding of mutants were similar to the parental enzymes (data not shown).

### Table 2. Kinetic constants of wild-type enzymes and selected variants toward galactomannan, mannohexaose (M6) and mannopentaose (M5).

| Enzyme          | Galactomannan | M6     | M5     |
|-----------------|---------------|--------|--------|
|                 |   | $k_{cat}$ (min$^{-1}$) | $K_m$ (mg.ml$^{-1}$) | $k_{cat}/K_m$ (mg$^{-1}$.ml.min$^{-1}$) | $k_{cat}/K_m$ (mg$^{-1}$.ml.min$^{-1}$) |
| PaMan26A wt     | 2.4±0.3       | 3356±159 | 1413±155 | 7.6×10$^5$ | 3.8×10$^5$ |
| 26-P140L/D416G* | 2.1±0.2       | 3860±80  | 1849±148 | 1.0×10$^6$ | 7.8×10$^5$ |
| PaMan5A wt      | 11.5±1.5      | 1674±138 | 147±19  | 3.0×10$^6$ | 9.2×10$^5$ |
| S-V256L/G276V/Q316H | 7.8±1.6     | 1493±167 | 197±38  | ND    | ND     |
| S-W36R/I195T/V256A* | 16.2±2.5   | 4199±438 | 263±41  | ND    | ND     |
| S-K139R/Y223H*  | 6.7±0.5       | 1655±60  | 248±17  | 2.7×10$^6$ | 6.8×10$^5$ |
| S-G311S*        | 1.5±0.4       | 1781±134 | 1247±292| 3.4×10$^6$ | 1.2×10$^6$ |

The kinetic parameters were determined at 40°C in sodium acetate buffer 50 mM, pH 5.2 as described in the Methods section. Paired t test was used to compare the kinetic parameters of mutants versus native enzyme. The difference was considered statistically significant when p<0.05 (*). wt, wild type. ND: not determined. doi:10.1371/journal.pone.0079800.t002
Kinetic parameters of mutants toward galactomannan

Each mutant was characterized using High Performance Anion Exchange Chromatography – Pulsed Amperometric Detection (HPAEC-PAD) to assess its ability to hydrolyze galactomannan and manno-oligosaccharides: mannopentaose (M5) and mannohexaose (M6) (Table 2). Regarding the M5 and M6 hydrolysis, \textit{PaMan26A-P140L/D416G} exhibited 100% and 30% increase of $k_{cat}/K_M$ toward M5 and M6, respectively. \textit{PaMan5A-V256L/G276V/Q316H} and \textit{PaMan5A-W36R/I195T/V256A} exhibited same hydrolysis profile as \textit{PaMan5A} at 10 min or 20 min manno-oligosaccharide hydrolysis (data not shown) and therefore $k_{cat}/K_M$ were considered unchanged and not determined. \textit{PaMan5A-K139R/Y223H} revealed a decrease of $k_{cat}/K_M$ of about 35% and 11% respectively. \textit{PaMan5A-G311S} displayed an increased $k_{cat}/K_M$ of about 37% and 12% towards M5 and M6, respectively.

Regarding the $K_M$ apparent values, all of the mutants displayed improved apparent affinity for galactomannan. Although turn-over numbers of almost all of the mutant enzymes were similar to wild-type enzymes, \textit{PaMan5A-W36R/I195T/V256A} mutant displayed a 2.5-fold increase of turn-over toward galactomannan resulting in an overall catalytic efficiency improved by 1.8-fold. In terms of catalytic efficiency, the best-performing mutant was the \textit{PaMan5A-G311S} mutant for which the single amino-acid substitution led to a 8.2-fold increase in $k_{cat}/K_M$ due to a drastic improvement of $K_M$ whereas the $k_{cat}$ remained unchanged. Other \textit{PaMan5A} mutants exhibited also to a lower extent increased $k_{cat}/K_M$ towards galactomannan, i.e., between 32 and 79% improvement. The \textit{PaMan26A-P140L/D416G} mutant, which is the unique mutant selected from the 5,600 \textit{PaMan26A} variants screened, displayed an improved $k_{cat}/K_M$ of approximately 30%.

All of the mutants were subjected to a paired t-test in comparison with the corresponding native enzyme. Three out of four \textit{PaMan5A} mutants and the \textit{PaMan26A} mutant revealed $p$-values below 0.05 for galactomannan hydrolysis and were therefore considered statistically different from their native counterpart (Table 2). Only sparse studies have previously led to the identification of mutants displaying such increase in catalytic efficiency. For example, several endoglucanase mutants with $k_{cat}/K_M$ improvement between 15 and 80% were generated after three rounds of mutagenesis [26]. Song \textit{et al} [20] identified several xylanase mutants with $k_{cat}/K_M$ towards xylan increased in a range of 20 to 50% after several rounds of evolution.
Structure-function relationships of identified variants

Examination of the recently solved three-dimensional structures of PaMan3A and PaMan26A [24] revealed that some of the mutated residues are potentially important for enzyme function. The localization of all of the mutated residues of the PaMan3A mutants revealed that five mutated residues [W36R, K139R, Y223H, V256A/L, and Q316H] out of eight are located within the active site cleft and three mutations are located inside the enzyme core ([I195T, G276V and G311S]). Three mutations were identified in the PaMan5A-W36R/I195T/V256A mutant, among which W36 and V256 that are located close to the catalytic site (Figure 3A) and I195T is located in the enzyme core (not shown). The W36 residue side chain lies at the bottom of the active site cleft and may contribute to the −4 subsite. Interestingly, the Val256 does not seem to be directly involved in the subsite organization of PaMan5A, but mutation of this residue was found in two distinct variants (PaMan5A-V256L/G276V/Q316H and PaMan5A-W36R/I195T/V256A), indicating a possible hot spot for enzyme improvement to further explore. Concerning the PaMan5A-K139R/Y223H mutant, analysis of the manno-oligosaccharides produced upon hydrolysis of M3 and M4 revealed a potential modification of substrate binding (Figure 4). Indeed, the wild type PaMan5A is known to produce mainly M3 from M6, but mutation of this residue were found among GH5 mannanases within the start of the β-strand and it should be noted that the last residue of this strand is the W315 residue (Figure 3B), which shapes the −2 subsite. The G311 residue is in close vicinity with a compact hydrophobic core made of residues F20, I60, I115, F170, F244, L280 and L313 (Figure 3C). It is difficult to determine the effect of this single substitution from a structural point of view, although we can hypothesize that the hydroxyl group of the serine residue may contribute to the modification of the β-strand by interacting with the surrounding residues although circular dichroism analysis of the PaMan5A-G311S mutant revealed a similar profile compared to the parental enzyme, suggesting that its folding was similar to the parental enzyme (data not shown). Therefore the single mutation PaMan5A-G311S does not seem to modify the overall architecture of the enzyme but a slight movement of the β-strand could move the W315 residue at the surface of the enzyme and explain the decreased Kₘ.

One of the most remarkable findings of this study is the activity increase of the PaMan26A-P140L/D416G mutant that contained only two mutations, i.e., one in the linker region (P140L) and one at the entrance of the active site (D416G) (Figure 5). The region from residue R133 to residue N141, which may be considered as the end of the linker, is tightly bound to the catalytic domain thanks to a dense network of hydrogen bonds and hydrophobic interactions [24]. Moreover, the PaMan26A linker sequence contains four proline residues (P132, P134, P135 and P140) out of 12 residues that may confer rigidity to the modular enzyme [24]. In the PaMan26A-P140L/D416G mutant, the P140 residue was substituted by a leucine residue, probably resulting in a decrease of linker rigidity that could partially explain the improved kcat/Kₘ. The D416 amino-acid substitution is located at the edge of the catalytic cleft (Figure 5). The favourable mutation consisted in the removal of the carboxylic group since the D416 amino-acid was substituted by a glycine residue. We suggest that the lack of the carboxylic acid side chain may promote the entrance of the substrate into the active site.

Conclusions

Activity improvement of fungal carbohydrate-active enzymes has typically met many obstacles, owing primarily to the lack of efficient expression systems and high-throughput screening methodologies. In this study, molecular engineering of two fungal mannanases was undertaken using random mutagenesis in the yeast T. lipolytica. All of the selected mutants highlighted improved characteristics when compared to wild-type enzymes, thus validating the approach of mutagenesis and screening that were employed. Examination of the three-dimensional structures of PaMan5A and PaMan26A revealed several hot spots (i) around the active site of both mannanases, (ii) in the core region of PaMan5A and (iii) in the linker region of PaMan26A. The directed evolution strategy implemented in this study was very successful since a straightforward round of random mutagenesis yielded significantly improved variants that would have been
difficult to predict using rational site-directed mutagenesis and structure-guided design.

Methods

Plasmids, strains and culture conditions

The growth media and culture conditions for the \textit{Y. lipolytica} JMY1212 strain (Ura\textsuperscript{−}, Leu\textsuperscript{+}, ΔAEP, Suc\textsuperscript{+}) have been previously described \cite{30}. Briefly, the \textit{Y. lipolytica} JMY1212 strain was cultured in YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) at 28°C, 130 rpm in baffled flasks or in Petri dishes containing YPD supplemented with agar (15 g/l). The plasmid used for expression in \textit{Y. lipolytica} was JMP61 that displayed the \textit{POX2} promoter for induction by oleic acid \cite{31} and the Zeta recombination platform for controlled monocopy integration in \textit{Y. lipolytica} genome. Subcloning of parental genes was performed using the TOPO TA kit (Invitrogen, Cergy-Pontoise, France) and TOP10 \textit{E. coli} competent cells (Invitrogen).

Construction of JMP61-\textit{paman5a} and JMP61-\textit{paman26a} plasmids and yeast transformation

\textit{PaMan5a} and \textit{PaMan26A} genes (\textit{paman5a} [HM357135] and \textit{paman26a} [HM357136], respectively) were amplified with primers listed in Table 3, starting from the pPICZ\textit{\textalpha}-\textit{paman5a} and the pPICZ\textit{\textalpha}-\textit{paman26a} plasmids \cite{10}. Insertion of both genes in the JMP61 expression vector was performed using \textit{BanHI} and \textit{AavII} restriction sites. \textit{Y. lipolytica} Zeta competent cells were prepared using the lithium acetate method as described in le Dall \textit{et al} \cite{32}. Competent cells were immediately transformed with 500 ng \textit{NotI}-linearized recombinant plasmid combined with 25 μg of salmon sperm DNA by heat shock at 39°C for 10 minutes and immediately recovered in 1.2 ml of 100 mM lithium acetate. Transformants were plated on YNB agar (1.7 g/l YNB, 10 g/l glucose, 5 g/l ammonium chloride, 2 g/l casamino acids, in 50 mM sodium–potassium phosphate buffer, pH 6.8, 17 g/l agar) and incubated at 28°C for 48 hours.

Construction of mutagenesis libraries by error-prone PCR

Three PCR were carried out for each gene as shown in Figure 1 with primers listed in Table 3. Mutagenic PCR (PCR1) were performed using GeneMorph II Random Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions using primers JMP61EvDF and PCR2r for \textit{paman5a} and PCR1dtmutCBM-F and PCR2r for \textit{paman26a}. The PCR contained 500 ng of parental gene, 200 μM dNTP, 0.25 μM primers and 2.5 units of Mutazyme. The PCR were made up to 50 μl and incubated at 95°C for 5 min and then at 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and 30 sec for 40 cycles followed by 5 min at 72°C. PCR2 was carried out using HiFi polymerase (Invitrogen) to amplify the zeta platform without

Figure 5. Structural view of \textit{PaMan26A} (PDB 3ZM8) exhibiting substituted amino-acids. The central panel shows a surface view of the entire \textit{PaMan26A} structure, which is composed of a carbohydrate binding module (CBM) belonging to the CBM35 family in cyan, a linker in violet and a catalytic domain belonging to the GH26 family in green. The two catalytic glutamate residues, E300 and E390, are coloured in red. The two substituted amino-acids, P140 and D416, are labelled and coloured in yellow. The top view represents the surface view of the catalytic cleft of \textit{PaMan26A} rotated about 90° along the horizontal axis with mannotriose modelled into the −2 to −4 subsites. The structure of GH26 from \textit{C. fimi} in complex with mannotriose was superimposed on the top of the structure of \textit{PaMan26A} to map the substrate-binding subsites. The bottom view displays the \textit{PaMan26A} linker (from residue 131 to residue 141) in stick representation. The molecule has been rotated of about 90° along the horizontal axis and in the opposite direction compared to the top view. The proline residues of the linker are labelled.

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introducing any mutations. Primers used were PCR1d and GH5EvDRev for paman5a and PCR1d and GH26EvDRev for paman26a. PCR products were gel-extracted using Gel-Extraction kit (Qiagen, Courtaboeuf, France) and a third overlap PCR (PCR3) was subsequently carried out using gel-extracted products of PCR1 and PCR2 to rebuild the whole zeta fragment containing mutant genes. The primers used for PCR3 were PCR1dL and PCR2rL for the two constructions. The overlap PCR was carried out using i-Star Max II DNA polymerase (Qiagen, Courtaboeuf, France) and a third overlap PCR product was gel-extracted using Gel-Extraction kit (Qiagen, Courtaboeuf, France) and a third overlap PCR was subsequently carried out using i-Star Max II DNA polymerase (Intron Biotechnology, Boca Raton, FL).

PCR3 products were subsequently transformed into Y. lipolytica as described above and plated onto YNB in QTrays plates (Intron Biotechnology, Boca Raton, FL).

PCR3 products were subsequently transformed into Y. lipolytica as described above and plated onto YNB in QTrays plates (Intron Biotechnology, Boca Raton, FL). The Ura-positive Y. lipolytica transformants obtained from mutagenesis libraries were subsequently gridded on YNB agar medium containing oleic acid (1% v/v, Sigma) and Azo-galactomannan (0.2% w/v, Megazyme) using a QPixII colony picker (Genetix, Molecular Devices, Sunnyvale, CA, USA). The plates were further incubated at 28°C for 48 hours. Mannanase activity was visualized as clear halos around colonies within a blue background.

Liquid culture-based screening. Mannanase-positive colonies were picked on OmniTrays (Nunc, Thermo Fisher Scientific, Courtaboeuf, France) containing YPD agar and incubated for 48 hours at 328°C. Further screening was performed as described in [26]. Briefly, 96-well preculture plates containing 200 μl of each preculture were transferred in 1 ml of YTO medium (10 g/l yeast extract, 20 g/l% w/v tryptone, 2% oleic acid, in 50 mM phosphate buffer, pH 6.8) in 96-deep-well plates. Cultures were further incubated at 28°C with shaking at 800 rpm. After 4 days induction, the supernatants containing mutant enzymes were recovered by centrifugation (10 min, 3500 rpm) and endo-mannanase activity towards galactomannan was determined from DNS assay as described before [23]. Briefly, 10 μl of culture supernatant were incubated with 190 μl of 1% (w/v) galactomannan in 50 mM sodium phosphate buffer (pH 5) at 40°C. 80 μl of reaction mixture was recovered and reaction was terminated by addition of same volume of dinitrosalicylic acid reagent at 1% (w/v) in 96 well PCR plates. Samples were heated at 95°C for 10 min and DO260 was measured relative to a mannose standard curve (1 to 20 mM).

Wild-type and mutant enzymes large-scale production

For heterologous production of PaMan5A and PaMan26A mutant proteins, the selected genes were amplified from genomic DNA using GH5-InFuFOR and GH5-InFuR for PaMan5A and GH26CBM-InEuFOR and GH26CBM-InEuRev for PaMan26A, respectively, listed in Table 3 and transferred into the pPICZαA plasmid using InFusion kit (Clontech, Takara). Resulting plasmids were transformed into P. pastoris and protein productions and purification were carried out as described before [10].

Biochemical and biophysical characterization

Protein concentration was determined by using the Bio-Rad protein assay kit with bovine serum albumin as standard (Bio-Rad, Marne-la Coquette, France) and UV absorbance at 280 nm. SDS-PAGE was performed in 10% (w/v) polyacrylamide gel (Bio-Rad) using a Pharmacia LMW electrophoresis calibration kit (GE Healthcare, Buc, France). Native IEF was carried out at 4°C in the Bio-Rad gel system, using pI standards ranging from 4.45 to 8.2. IEF gel was coloured with IEF staining solution (0.04% Coomassie Blue R250, 0.05% Cocein Scarlett, 10% acetic acid, 27% isopropanol).

| Table 3. List of primers used in the study. |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Gene                | PCR step                       | Primer name                          | Sequence (5’>3’)                        |
|---------------------|--------------------------------|--------------------------------------|--------------------------------------|
| paman5a             | integration in JMP61           | GH5JMP61F 5’                          | TTTGGATCCCCCTCCCCAAAGCAAA            |
|                     |                                | GH5JMP61R 3’                          | TTTCTAGCCCTACGGCCGGAGAAGCATT          |
| mutagenesis PCR (PCR1) | JUMP61EvDF                     | PCR1r                                | GCAGAAGCGATTCCGCGATCC               |
|                     |                                | PCR2r                                | GGAGTTCCCGCCACCC                    |
| zeta platform PCR (PCR2) | PCR1d                          | GH5EvDRev                            | GATCCCCACGGGAATTGC                  |
|                      |                                | GH5EvDRev                            | GGAGTTCCCGCCACCC                    |
| integration in pPICZαA |                                | GH5-InFuFOR                          | GCTGATCCGACTCTCCCCAAAGCAAAAGC        |
|                     |                                | GH5-InFuR                            | GAGTTCTTGGTACCCGAGAAGCAT            |
| paman26a            | integration in JMP61           | JUMP61-CBMGH26-F                      | TTTGGATCCAGCCTTGGTAAAGC             |
|                     |                                | GH26-JMP61-Rev                       | TTTCTAGCCAACTCTCCCACTCCCCTGAAT      |
| mutagenesis PCR (PCR1) |                                | PCR1dtmutCBM-F                       | TTTCAACCTCAAACCCCCAC                 |
|                      |                                | PCR2r                                | GAGTTCTTGGGAGAAGCAT                 |
| zeta platform PCR (PCR2) |                                | PCR1d                                | GATCCCCACGGGAATTGC                  |
|                      |                                | GH26EvDRev                           | GAGTTCTTGGGAGAAGCAT                 |
| integration in pPICZαA |                                | GH26CBM-InFuFOR                      | GCTGAGCTCAGATCCCCTTGAAAGCCTCGT      |
|                     |                                | GH26InFuR                            | GAGTTCTTGGGAGAAGCAT                 |
| common              | overlap PCR (PCR 3)            | PCR1dL                               | CCGCTGGCGAGAAGCGGGTTAGGGAAGCAT      |
|                      |                                | PCR2rL                               | CCGCAGGGCTGGCTGGGAGAAGCAT           |

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Characterization and kinetic properties of individual enzymes

Determination of kinetic parameters on galactomannan was performed using DNS activity assay. Unless otherwise indicated, assay mixtures contained substrate and suitably diluted enzyme in sodium acetate buffer 30 mM, pH 5. Briefly, 1 μg of enzyme was mixed with 190 μl of galactomannan (Megazyme International, Wicklow, Ireland) using a range of substrate concentration from 1 to 20 mg.ml⁻¹ (eight concentrations) and incubated at 40°C for 5 minutes. Reactions were performed in triplicate independent experiments. The reaction was stopped by the addition of 300 μl of 1% DNS reagent and samples were heated at 95°C for 10 minutes. DO₅₄₀ was measured relative to a mannose standard.

The kinetic parameters were estimated using weighted nonlinear squares regression analysis with the Grafit software (Erithacus Software, Horley, UK).

Analysis of initial sugar release by HPAEC-PAD and kinetic parameters measurement

Monosaccharide and oligosaccharides generated after hydrolysis of manno-oligosaccharides (M₅ and M₆, Megazyme) were analysed using HPAEC-PAD. 10 μl of suitably-diluted enzyme were incubated at 40°C for various time lengths with 190 μl of 1 mM substrate in 50 mM acetate buffer pH 5.2. At each time point, 10 μl of reaction mixture were recovered and the reaction was terminated by the addition of 90 μl of 180 mM NaOH. For HPAEC analysis, 10 μl were injected and elution was carried out as described in [10]. Calibration curves were plotted using β-1,4-mannooligosaccharides as standards from which response factors were calculated (Chromleq program, Dionex) and used to estimate the amount of products released in test incubations. All the assays were carried out at least in duplicates. The specificity constants were calculated using the Matsui equation [24,33].

Supporting Information

**Figure S1** Isoelectrofocusing analysis of *Pa*Man5A and *Pa*Man26A variants. 1: pl marker (values are on the left); 2: *Pa*Man26A wild-type; 3: *Pa*Man26A-P140L/D416G; 4: *Pa*Man5A wild-type; 5: *Pa*Man5A-V256L/G276V/Q316H; 6: *Pa*Man5A-W36R/I195T/V256A; 7: *Pa*Man5A-K139R/Y223H; 8: *Pa*Man5A-G311S. (EPS)

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Author Contributions

Conceived and designed the experiments: MC JGB. Performed the experiments: MC JF SB. Analyzed the data: MC AR JGB. Contributed reagents/materials/analysis tools: JGB. Wrote the paper: MC JGB.

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