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The GH5 1,4-β-mannanase from *Bifidobacterium animalis* subsp. *lactis* BI-04 possesses a low-affinity mannan-binding module and highlights the diversity of mannanolytic enzymes

Johan Morrill¹, Evelina Kulcinskaja¹, Anna Maria Sulewska²,⁴, Sampo Lahtinen³, Henrik Stålbrand¹, Birte Svensson² and Maher Abou Hachem²*

**Abstract**

**Background:** β-Mannans are abundant and diverse plant structural and storage polysaccharides. Certain human gut microbiota members including health-promoting *Bifidobacterium* spp. catabolize dietary mannans. Little insight is available on the enzymology of mannan deconstruction in the gut ecological niche. Here, we report the biochemical properties of the first family 5 subfamily 8 glycoside hydrolase (GH5_8) mannanase from the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* BI-04 (BlMan5_8).

**Results:** BlMan5_8 possesses a novel low affinity carbohydrate binding module (CBM) specific for soluble mannan and displays the highest catalytic efficiency reported to date for a GH5 mannanase owing to a very high $k_{cat}$ (1828 ± 87 s⁻¹) and a low $K_m$ (1.58 ± 0.23 g · L⁻¹) using locust bean galactomannan as substrate. The novel CBM of BlMan5_8 mediates increased binding to soluble mannan based on affinity electrophoresis. Surface plasmon resonance analysis confirmed the binding of the CBM10 to manno-oligosaccharides, albeit with slightly lower affinity than the catalytic module of the enzyme. This is the first example of a low-affinity mannan-specific CBM, which forms a subfamily of CBM10 together with close homologs present only in mannanases. Members of this new subfamily lack an aromatic residue mediating binding to insoluble cellulose in canonical CBM10 members consistent with the observed low mannan affinity.

**Conclusion:** BlMan5_8 is evolved for efficient deconstruction of soluble mannans, which is reflected by an exceptionally low $K_m$ and the presence of an atypical low affinity CBM, which increases binding to specifically to soluble mannan while causing minimal decrease in catalytic efficiency as opposed to enzymes with canonical mannan binding modules. These features highlight fine tuning of catalytic and binding properties to support specialization towards a preferred substrate, which is likely to confer an advantage in the adaptation to competitive ecological niches.

**Keywords:** *Bifidobacterium*, Carbohydrate-binding module, Gut microbiota, Mannan, Probiotic bacteria, Surface plasmon resonance
Background

β-Mannans (hereafter mannans) are abundant polysaccharides playing diverse roles in plants including energy storage in seed endosperms, e.g. carob and guar seeds, legumes, coconuts and coffee beans [1], or structural support in the hemicellulose cell wall matrix [2], where they can constitute up to 25 % of the dry mass in softwood. The biochemical details of enzymatic mannan depolymerization have received increasing attention in recent years due to wide interest within the biofuel and biorefinery areas [3, 4]. Mannans, however, are also present in human nutrition, either as cell wall components in cereal grains and some fruits and vegetables such as kiwi, apple and tomato [5–8] or as common hydrocolloid food additives used as thickeners or to adjust texture [9, 10]. Mannans are not known to be hydrolyzed by human digestive enzymes and thus offer a potential resource to mannanolytic gut bacteria. The fermentation of guar gum (GG) galactomannan has been demonstrated in the human gut [11] and intake of partial hydrolysates of this polysaccharide stimulated the proliferation of Bifidobacterium spp. in humans [12] and mice [13]. However, insight into the microbial strategies and enzymes mediating mannan degradation in the human gut lags behind.

Mannans occur as insoluble crystalline polymers of β-1,4-linked mannosyl residues in seeds, such as ivory nut mannan (INM) or as soluble heteropolymeric glucomananns consisting of alternating β-1,4-linked glucosyl and mannosyl backbone e.g. in corns of Amorphophallus konjac. Mannan can be substituted to different degrees with α-1,6-linked galactosyl side-chains as in carob (locust bean) and guar gums and in glucomannan from softwood, which also has acetyl decorations [14]. Konjac glucomannan (KGM), locust bean (LBG) and GG galactomannans are the main mannans used as food additives [9, 10].

The concerted action of several backbone- and sidechain-degrading enzymes is required for depolymerization of mannans. The precise number of enzymatic activities varies with the substrate structure, but endo-β-1,4-mannanases (EC 3.2.1.78) that hydrolyze internal backbone β-1,4-linkages are in mannan degradation. β-Mannanases are assigned in glycoside hydrolase (GH) families 5, 26 and 113 in the Carbohydrate-Active enZYmes (CAZY) database [15]. Many bacterial mannanases cluster in subfamily 5 of GH5 (GH5_8) according to a phylogeny-based assignment [16]. Mannanases of GH5 employ a double displacement mechanism with the retention of anomeric configuration [17]. Some mannanases contain carbohydrate-binding modules (CBMs) that have been ascribed diverse roles including targeting enzymes to polysaccharides, increasing the local substrate concentration, or conferring processivity [18, 19].

The present study focuses on the widely utilized and clinically well-documented probiotic bacterium Bifidobacterium animalis subsp. lactis BI-04 [20]. Genome analysis identified a gene encoding a mannanase comprising a GH5 catalytic module joined to a CBM10. Members of CBM10 were previously found to bind to insoluble microcrystalline cellulose [21] and insoluble mannan [22]. Furthermore, we show that the GH5 mannanase (BlMan5_8) from Bifidobacterium animalis subsp. lactis BI-04, which is conserved within Bifidobacterium animalis subsp. lactis, displays the highest catalytic efficiency reported to date for a GH5 β-mannanase owing to a combination of very high $k_{cat}$ and low $K_m$. The CBM10 of this enzyme is the first described low-affinity mannan binding module and it forms a novel CBM10 subfamily together with close homologues. The distinct differences in the biochemical properties of this enzyme as compared to characterized β-mannanases from gut microbiota illustrate the diversity of mannan utilization strategies, which is likely to be important in adaptation to the highly competitive gut niche.

Methods

Carbohydrates

Cellotetraose, locust bean gum (LBG), microcrystalline cellulose (Avicel) and hydroxyethyl cellulose (HEC) are from Sigma-Aldrich (St. Louis, MO, USA); mannotriose (M₃), mannotetraose (M₄), mannopentaose (M₅), mannohexose (M₆), low-viscosity locust bean gum (LBG-lv), ivory nut mannan (INM) and konjac glucomannan (KGM) are from Megazyme (Bray, Ireland); guar gum (GG) is from Carl Roth (Karlsruhe, Germany). The compositions of the polysaccharides are listed in Additional file 1.

Cloning

Bifidobacterium animalis subsp. lactis BI-04 chromosomal DNA [23] was used to clone the locus Balac_1450 (GenBank accession number ACS46797) encoding a β-mannanase of glycoside hydrolase family 5 subfamily 8 (GH5_8). The PCR-amplified gene fragment encoding the full-length mature β-mannanase lacking the N-terminal signal peptide (amino acid residues 1–29 as predicted by Signal P v.4.0, http://www.cbs.dtu.dk/services/SignalP/), referred to as BlMan5_8, was cloned within the Nhel and BamHI restriction sites in pET28a(+) (Novagen, Darmstadt, Germany) using a primer pair (Additional file 2) to yield the plasmid pET28a-BlMan5_8. This plasmid, encoding the enzyme fused to an N-terminal hexa-histidine purification tag, was transformed first into Escherichia coli TOP10 (Life Technologies, Grand Island, NY, USA). The presence of the insert was verified by sequencing and restriction analysis. The construct encoding the
truncated enzyme BlMan5_8-ΔCBM10 lacking the C-terminal CBM10, was located by site-directed mutagenesis in the middle of the linker sequence (G10-GGGSNSSGS
GNTGGN(S)TDDG531) between the catalytic and the binding modules, into a stop codon (QuickChange mutagenesis kit; Agilent Technologies, Santa Clara, CA, USA) and the primer pair in Additional file 2 to yield pET28a-BlMan5_8-ΔCBM10. The mutation was verified by full sequencing. The plasmids encoding BlMan5_8 and BlMan5_8-ΔCBM10 were transformed into E. coli BL21 (DE3) for production.

Enzyme production and purification
Production of BlMan5_8 and BlMan5_8-ΔCBM10 was carried out in 2 L baffled shake flasks. Overnight cultures were used to inoculate 1 L LB medium containing 10 mM glucose and 50 μg·mL⁻¹ kanamycin and grown at 30 °C to an OD₆₆₀ of 0.5. Protein expression was induced with 100 μM IPTG and growth was continued for 5 h. For BlMan5_8, the cells were harvested by centrifugation, resuspended in binding buffer (10 mM HEPES, 0.5 M NaCl, 10 % glycerol, 15 mM imidazole, pH 7.4) with added protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and lysed using a high pressure homogenizer, centrifuged and filtered through a 0.45-μm filter. For BlMan5_8-ΔCBM10, the cells were lysed using the BugBuster® Protein Extraction Reagent (Novagen), according to the manufacturer’s recommendations. Clarified BlMan5_8 and BlMan5_8-ΔCBM10 lysates were applied onto a 5-mL HisTrap HP (GE Healthcare, Uppsala, Sweden), washed with binding buffer, and eluted with a gradient formed with 400 mM imidazole in 10 mM HEPES, 0.5 M NaCl, 10 % glycerol, pH 7.4. Eluted fractions were analyzed by SDS-PAGE and for β-mannanase activity as described below. Pure fractions were pooled and concentrated (10 kDa cut-off ultrafiltration units; Amicon), and the histidine tag was cleaved off using human plasma thrombin (Calbiochem, San Diego, CA, USA) according to the manufacturer’s instructions. Cleaved enzymes were recovered after passing through a binding buffer pre-equilibrated 1 mL HisTrap HP column (GE Healthcare). The concentrated enzyme samples were further purified to electrophoretic homogeneity by chromatography on a HiLoad Superdex G75 26/60 column (GE Healthcare) eluted with 10 mM HEPES, pH 7.0 in 1.2 column volumes. The pure enzyme samples were concentrated as above and stored at 4 °C until further use.

Basic enzymatic properties and enzyme stability
β-Mannanase activity was measured towards 2.5 g·L⁻¹ LBG at 37 °C for 10 min in 400 μL standard assay buffer (40 mM phosphate-citrate buffer, pH 6.0, with 0.005 % (v/v) Triton X-100) with appropriately diluted enzyme. The substrate preparation and reducing end 3,5-dinitrosalicylic acid (DNS) assay were performed as previously reported [24, 25]. The pH dependence of activity was initially determined using 6.4 nM BlMan5_8 with LBG in 100 mM Britton-Robinson buffers at pH 2–10 and then refined in 40 mM phosphate-citrate at pH 5–7. To assess storage stability as function of pH, 1.28 μM BlMan5_8 was incubated at 4 °C for four days in 100 mM Britton-Robinson buffers at pH 2–10, thereafter diluted to 3.2 nM in standard assay buffer, and residual β-mannanase activity was determined as described above. The dependence of enzyme activity on temperature was determined for 6.4 nM BlMan5_8 with LBG in standard assay buffer, in the range 25–80 °C.

The conformational stability of 12.6 μM BlMan5_8 in 10 mM MES buffer, pH 6.5 was assessed using differential scanning calorimetry (DSC) between 15 °C and 90 °C at 1 °C·min⁻¹ in a MicroCal VP-DSC calorimeter. The enzyme was scanned twice with cooling and pre-equilibration in between to assess the reversibility of thermal transitions. The dialysis buffer was used as a reference and the reference and baseline corrected thermograms were analyzed using Origin7 with a DSC add-on provided with the instrument.

Substrate specificity
The soluble substrates LBG, LBG-lv, GG and KGM were prepared as previously described [25]. The insoluble substrates INM and Avicel were washed three times in water, twice for 2 h and once overnight, with a 15:1 volume to mass ratio, followed by 3 h wash in buffer. The specific activity of BlMan5_8 towards LBG-lv and KGM (2.5 g·L⁻¹, 10 min incubation), as well as GG (2.5 g·L⁻¹, 2.5 h incubation), INM (5 g·L⁻¹, 45 min incubation) and Avicel (10 g·L⁻¹, 3 h incubation) was measured using the standard activity assay.

Enzyme kinetics
LBG-lv (0.45–9.0 g·L⁻¹) was hydrolyzed by 1 nM BlMan5_8 or BlMan5_8-ΔCBM10 at 37 °C in standard assay buffer as measured by DNS (see above). The initial reaction rates were determined and the kinetic parameters $k_{cat}$ and $K_m$ were determined by fitting the Michaelis-Menten equation to the data using Prism 6 (GraphPad Software, La Jolla, CA, USA). Experiments were performed in duplicates.

For kinetic analysis of M₅ hydrolysis, 0.25 nM BlMan5_8 was incubated with 0.25–10 mM M₅ at 37 °C in standard assay buffer. Samples were withdrawn at 0, 5, 10, 15 and 20 min, and boiled for 5 min. The hydrolysis products of M₅ were analyzed by HPAEC-PAD using a DX-500 system (Thermo Scientific Dionex, Sunnyvale, CA, USA). A CarboPac™ 100 column and guard column
were used with a mobile phase of 78 mM sodium hydroxide at a flow rate of 1.0 mL·min⁻¹. The decrease in M₅ concentration is stoichiometrically equivalent to the increase in concentrations of degradation products. These concentrations were calculated and used to determine $k_{cat}$ and $K_m$ as described [26].

**Mannan hydrolysis action patterns**

The product profiles for 5.3 nM $B\!\!L\!\!Ma\!\!n5_8$ or $B\!\!L\!\!Ma\!\!n5_8$-ΔCBM10 towards 2.5 g·L⁻¹ INM or LBG in standard assay buffer at 37 °C at 0 min, 30 s, 5, 15 and 30 min, as well as 2 and 24 h, were analyzed by HPAEC-PAD (ICS-5000 system; Thermo Scientific Dionex, Sunnyvale, CA, USA). A CarboPac™ PA-200 column and guard column were used with a mobile phase of 78 mM sodium hydroxide and a linear gradient of 0–50 mM sodium acetate at a flow rate of 0.5 mL·min⁻¹.

**Binding to polysaccharides and oligosaccharides**

Qualitative screening of binding of $B\!\!L\!\!Ma\!\!n5_8$ and $B\!\!L\!\!Ma\!\!n5_8$-ΔCBM10 to soluble polysaccharides was analysed using affinity electrophoresis as previously described [27]. The electrophoretic separation of 2 µg of either protein was performed at 4 °C and 45 V for 20 h in 50 mM Tris-Borate, pH 8.7 and in 12 % polyacrylamide gels without (control) or with 0.1, 0.5 or 2.5 g·L⁻¹ KGM, as well as 2.5 g·L⁻¹ hydroxethyl cellulose (HEC). Quantitative binding analysis of $B\!\!L\!\!Ma\!\!n5_8$ and $B\!\!L\!\!Ma\!\!n5_8$-ΔCBM10 to LBG-lv was done using affinity electrophoresis [27, 28] at 100 V for 4 h in gels without (control) or with 0.05, 0.10 or 0.25 g·L⁻¹ LBG-lv, as described above. The apparent dissociation constant ($K_d$) for LBG-lv was calculated by nonlinear regression using Prism 6 (GraphPad Software, La Jolla, CA, USA). Binding to insoluble INM and Avicel was measured by adding 50 µl of appropriately diluted enzyme to 400 µl of 0 (negative control), 0.5, 5, 50 or 100 g·L⁻¹ of either substrate suspended in standard assay buffer. The slurry was incubated at 4 °C for 1 h with gentle shaking, centrifuged at 16 000 x g for 10 min and the mannanase activity in the supernatant was assayed as described above.

Surface plasmon resonance (SPR) analysis was performed using a Biacore T100 (GE Healthcare) to assess binding to manno-oligosaccharides and cellotetraose. Enzymes diluted to 40 µg·mL⁻¹ in 10 mM sodium acetate, pH 3.9, were immobilized onto a CM5 chip (GE Healthcare) using a standard amine coupling protocol to a density of 3999 and 2879 response units (RU) for $B\!\!L\!\!Ma\!\!n5_8$ or $B\!\!L\!\!Ma\!\!n5_8$-ΔCBM10, respectively. Binding analysis was performed in triplicates at 25 °C in 10 mM MES, 150 mM NaCl, pH 6.0, 0.005 % (v/v) P20 (GE Healthcare) running buffer at 30 µL·min⁻¹ with association and dissociation times of 60 and 30 s, respectively, at seven concentrations of $M_4$ (0.5–25 mM), $M_5$ (0.05–25 mM), $M_6$ (0.05–12.5 mM) and cellotetraose (0.05–25 mM). A one-binding-site model was fit to the blank and reference cell-corrected steady state response data to determine the dissociation constant $K_d$ and the saturation binding level $R_{max}$, which was used to estimate the molar stoichiometry ($n$) [29] of oligosaccharide binding to immobilized enzymes that retain binding activity.

**Action pattern analysis using mannopentaose**

For analysis of the productive binding modes of $M_5$, enzymatic reactions were carried out in 18O labeled water (H218O 97 % pure from Sigma-Aldrich, St. Louis, MO, USA), as described [26, 30] at 8 °C with 1 mM $M_5$ and 20 nM $B\!\!L\!\!Ma\!\!n5_8$ in 1 mM sodium phosphate, pH 6.0 in 93 % H218O. Samples were incubated for 0–24 h and analyzed with MALDI-TOF MS. Aliquots of 0.5 µL were withdrawn at each time point, spotted directly on a MALDI plate, covered with 0.5 µL matrix (10 g·L⁻¹ 2,5-dihydroxybenzoic acid (DHB) in water) and dried under warm air. This analysis combined with HPAEC-PAD quantification of $M_4$ and $M_5$ products of $M_5$ hydrolysis was used to determine the frequency of productive binding modes of $M_5$ as previously described [26].

**Transglycosylation product formation from $M_5$**

For analysis of transglycosylation 5 mM $M_5$ was incubated with 1.6 nM $B\!\!L\!\!Ma\!\!n5_8$ in standard assay buffer for 0–24 h. Samples were analyzed with MALDI-TOF MS as described above.

**Sequence analyses and homology modeling**

The sequence of $B\!\!L\!\!Ma\!\!n5_8$ was analyzed to identify catalytic and ancillary modules using dbCAN [31], a CAZy-based database [15] for automated carbohydrate-active enzyme annotation. The sequence of the $B\!\!L\!\!Ma\!\!n5_8$ catalytic module was aligned with 69 sequences listed as GH5_8 in CAZy [15], as well as 12 characterized β-mannanases from GH5 subfamilies 7, 8 and 10, using MAFFT 7 [32]. The CBM10 of $B\!\!L\!\!Ma\!\!n5_8$ was aligned with 83 predicted CBM10 sequences retrieved using dbCAN, after removing two sequences that appeared fragmented and three sequences that displayed E-values above 0.02. These sequences were aligned using MAFFT 7 and a phylogenetic tree was calculated using the unweighted pair group method with arithmetic mean using standard settings accessed on the CBRC server (http://mafft.cbrc.jp/alignment/server/phylogeny.html) and rendered using Dendroscope 3 [33]. The crystal structure of the *Thermobifida fusca* mannanase *TjMan5A* (PDB ID: 2MAN) [34] served as template to model the 39 % amino acid sequence identical catalytic module of $B\!\!L\!\!Ma\!\!n5_8$, using MODELLER [35]. The homology model was evaluated with MolProbity [36], showing that 93.29 %
of all residues were in Ramachandran favored regions. The model had a clash score of 72.89, defined as the number of unfavorable (≥0.4 Å) steric overlaps per 1000 atoms [37]. Evaluation with ProQ [38] gave a predicted LGscore of 5.903 and a predicted MaxSub of 0.266, indicating good model quality.

Results

Basic properties of \( B\)l Man\(_5\) _8_

\( B\)l Man\(_5\) _8_ is predicted to be an extracellular modular \( \beta\)-mannanase comprising a catalytic module of glycoside hydrolase family 5 subfamily 8 (GH5_8) joined to a C-terminal carbohydrate-binding module of family 10 (CBM10) by a typical linker sequence plausibly starting from Gly\(_{380}\) to Gly\(_{381}\) (see sequence in Methods). The position of the truncation (Ser\(_{380}\)) was chosen as to avoid destabilisation of the catalytic module due to possible loss of interactions with the first few residues in the linker. The full-length \( B\)l Man\(_5\)A and the truncated \( B\)l Man\(_5\) _8_-\( \Delta\)CBM10 were produced and purified to electrophoretic homogeneity. Both enzyme forms migrated in accord with their theoretical molecular mass of 37.9 kDa and 33.8 kDa, respectively. The lack of activity towards microcrystalline cellulose (Avicel) and the high activity towards mannans (Table 1) confirmed the \( \beta\)-mannanase specificity of \( B\)l Man\(_5\) _8_.

\( B\)l Man\(_5\) _8_ has highest activity at pH 6.0 and retains approximately 75 % of its maximal activity between pH 5 and 7 using LBG as a substrate (Additional file 3A). After incubation for 4 days at 4 °C, the enzyme retained 100 % of its initial activity at pH 8.0, and approximately 75 % at pH 6.0 (Additional file 3B). \( B\)l Man\(_5\) _8_ displayed highest \( \beta\)-mannanase activity at 55 °C and pH 6.0 (Additional file 4A), corresponding to an Arrhenius activation energy of 69.5 kJ · mol\(^{-1}\) (Additional file 4B). The unfolding temperature of \( B\)l Man\(_5\) _8_ was determined to 55.9 °C by differential scanning calorimetry (DSC) analysis asserting the structural integrity of the enzyme. The unfolding thermogram featured a single transition (Additional file 5), suggesting that the unfolding of the two modules of \( B\)l Man\(_5\) _8_ is overlapping.

**Table 1** Specific activity of \( B\)l Man\(_5\) _8_ towards soluble and insoluble polysaccharides

| Substrate | Specific activity (kat · mol\(^{-1}\)) | Specific activity (U · mg\(^{-1}\)) |
|-----------|--------------------------------------|-----------------------------------|
| LBG       | 1213                                 | 1920                              |
| LBG-lv    | 872                                  | 1380                              |
| GG        | n.d.*                                | n.d.*                             |
| KGM       | 1592                                 | 2520                              |
| INM       | 76                                   | 120                               |
| Avicel    | n.d.*                                | n.d.*                             |

* Not detected

Substrate specificity and kinetics

Activity of \( B\)l Man\(_5\) _8_ was highest towards the soluble KGM followed by LBG and low-viscosity LBG (LBG-lv) (Table 1). Activity towards insoluble INM was only 6 % of that on LBG, and no activity towards GG could be measured (Table 1). The apparent kinetic parameters for \( B\)l Man\(_5\) _8_ and \( B\)l Man\(_5\) _8_-\( \Delta\)CBM10 were determined for LBG-lv (Table 2; Additional file 6A) and marginal increases in \( K_m\) and \( k_{cat}\) were observed as a result of deleting the CBM10. Apparent kinetic parameters were also determined towards mannopentaose (M\(_5\)) for \( B\)l Man\(_5\) _8_ (Table 2; Additional file 6B). The enzyme displayed no activity towards mannotriose (M\(_3\)), and very low activity towards mannotetraose (M\(_4\)), but was highly active on larger manno-oligosaccharides as analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (data not shown).

Product profiles, transglycosylation and productive binding frequency analysis

The hydrolysis profiles of LBG and INM by \( B\)l Man\(_5\) _8_ and \( B\)l Man\(_5\) _8_-\( \Delta\)CBM10 were analyzed using HPAEC-PAD. Both enzyme variants produced M\(_3\), M\(_4\) and M\(_5\) as the main initial hydrolysis products from both LBG and INM, along with some mannobiose (M\(_2\)) (Fig. 1). The main products of \( B\)l Man\(_5\) _8_ from M\(_3\) were M\(_2\), M\(_3\) and M\(_4\). The formation of 10-fold higher M\(_4\) compared to mannose (M\(_1\)) (Fig. 2) is indicative of transglycosylation activity, which was supported by the detection of mannohexaose (M\(_6\)), mannoheptaose (M\(_7\)) and mannooctaose (M\(_8\)) transglycosylation products by mass-spectrometry following incubation with M\(_3\) (Fig. 3). All four productive M\(_3\) binding modes were observed, albeit with higher frequency (66 %) for binding modes where the larger part of the substrate was bound at the aglycone binding subsites (Fig. 4).

Binding to polysaccharides and oligosaccharides

Based on retardation in affinity electrophoresis, \( B\)l Man\(_5\) _8_ bound to LBG-lv (Additional files 7–8) with an apparent \( K_d\) of 0.31 g · L\(^{-1}\), while the affinity of \( B\)l Man\(_5\) _8_-\( \Delta\)CBM10 was too low to be determined due to lack of change in relative retardation as a function of increasing the LBG concentration. Neither the full-length nor the truncated enzyme displayed binding to soluble hydroxyethyl cellulose (HEC), insoluble cellulose (Avicel) or insoluble mannann (INM) in this analysis.

SPR is a particularly powerful tool to quantify low affinity interactions as observed above for \( B\)l Man\(_5\) _8_ binding to mannan. Ideally, CBM binding analysis would be carried out on an isolated CBM. In our case, however, the small size of the CBM10 and the presence of only a single lysine residue (used for random amine coupling
or biotinylation), renders the immobilization of the protein to sufficient levels challenging. To circumvent this limitation and avoid using this single lysine residue for immobilization with risk of affecting the function of the module, the binding analysis was done using immobilized full-length enzyme and BlMan5_8ΔCBM10 to evaluate the contribution of the CBM10 to binding. Only the full-length enzyme showed affinity towards M₄ based on the SPR analysis, which suggested that the CBM10 mediated binding to M₄ (Table 3). The apparent binding stoichiometry of both M₅ and M₆ was approximately twice for the full-length enzyme as compared to the isolated catalytic module (Table 3), reflecting binding of these ligands to both the GH5 catalytic module and the CBM10. No binding to cellotetraose was measured with either enzyme variant.

Sequence analyses and homology modeling
BlMan5_8 contains the seven amino acids that are conserved in family GH5 [39] (Additional file 9). The homology model of BlMan5_8 revealed that residues interacting with the substrate in subsites −4 through −2 are conserved between BlMan5_8 and TfMan5A [34] (Fig. 5). There are also several aromatic residues in the putative aglycone binding region of BlMan5_8 including Trp¹⁹⁶ and Trp²²⁵ which are invariant in GH5.8 as well as Trp²⁰⁰ which is conserved in 77 % of GH5_8 sequences (Additional file 10).

The CBM10 of BlMan5_8 and close homologues thereof, which are exclusively joined to β-mannanase catalytic modules, populate a distinct clade in the CBM10 phylogenetic tree (Fig. 6A). This clade is distinguished by unique sequence features, including substitution of a tyrosine residue conserved in other CBM10s as well as an insertion of five residues (Fig. 6B).

Discussion
The human gastrointestinal tract comprises one of the most densely populated ecological niches in nature, with bacterial counts exceeding 10¹¹ cells · g⁻¹ content [40]. Since most dietary glycans are indigestible to humans, their metabolism plays an instrumental role in shaping the composition of the gut microbiota [41]. To date, the GH26 mannanases from Bacteroides fragilis [42] and Bifidobacterium adolescentis [28] are the only described β-mannanases from the gut niche. In this study we report the biochemical properties of the first GH5 mannannase from the Bifidobacterium genus.

**Table 2** Kinetic parameters for BlMan5_8 on LBG-lv and M₅

| Substrate | Enzyme             | ⁰kₕcat (s⁻¹) | M₅ (g · L⁻¹) | ⁰kₕcat/M₅ (L · s⁻¹ · g⁻¹) |
|-----------|--------------------|--------------|--------------|--------------------------|
| LBG-lv    | BlMan5_8           | 1828 ± 87     | 1.58 ± 0.23 g · L⁻¹ | 1157 ± 177 L · s⁻¹ · g⁻¹ |
|           | BlMan5_8-ΔCBM10    | 2005 ± 179    | 1.75 ± 0.47 g · L⁻¹ | 1146 ± 324 L · s⁻¹ · g⁻¹ |
| M₅        | BlMan5_8           | 97 ± 2        | 1.09 ± 0.11 mM  | 89 ± 9 s⁻¹ · mM⁻¹       |

BlMan5_8 is an exceptionally potent enzyme optimized for efficient depolymerization of soluble mannan

The preferred substrates for BlMan5_8 are soluble glucomannan and galactomannan with low degree of galactosyl substitution (Table 1). BlMan5_8 displays 19-fold higher ⁰kₕcat on LBG as compared to M₅ (Table 2), suggesting that substrate contacts beyond five mannosyl residues contribute to catalytic turnover, similarly to other mannanases [43]. The catalytic efficiency of BlMan5_8 on LBG is the highest measured to date for a GH5 mannanase owing to both a very high ⁰kₕcat and a low ⁰Kₘ (Additional file 11). Notably, the ⁰Kₘ value for BlMan5_8 towards soluble mannan is one of the lowest amongst bacterial mannanases, which is likely to be an important advantage in the adaptation to the highly competitive gut ecological niche. The ⁰Kₘ value in part reflects substrate affinity, which appears higher at the

![Fig. 1](image-url) Initial products from INM and LBG. Manno-oligosaccharide products formed after 30 s of a INM and b LBG hydrolysis by BlMan5_8 as measured using HPAEC-PAD analysis. The chromatograms from BlMan5_8-ΔCBM10 (I), BlMan5_8 (II) and the substrate before hydrolysis (III)
aglycone-accommodating region of the active site cleft as evident from the productive binding frequency of M5 (Fig. 3). Substrate affinity at aglycone subsites has been shown to contribute to lowering \( K_m \) and increasing transglycosylation [30, 44]. This is in line with the high transglycosylation activity of \( Bl\)Man5_8, reflected by the disproportionate liberation of M4 relative to M1 ratio using M5 as a substrate (Fig. 2), which is attributed to secondary hydrolysis of transglycosylation products of larger degree of polymerization (Fig. 3). Interestingly, sequence comparison and homology modeling revealed that the putative aglycone binding regions of \( Thermobifida fusca \) GH5_8 mannanase (TfMan5A) [34] and \( Bl\)Man5_8 are lined with conserved aromatic residues, including Trp196 and Trp200 (Additional file 10; Fig. 5). Indeed, the position and conservation of these residues correlate with the favored productive binding at the aglycone region (Fig. 4).

The CBM10 of \( Bl\)Man5_8 binds soluble mannan with low affinity and represents a new clade within CBM10

The CBM10 of the modular \( Cj\)Xyn10A xylanase from \( Cellvibrio japonicus \), which binds tightly to Avicel, is the only structurally characterized CBM10 member [21]. The Man5A \( \beta \)-mannanase from the same organism, which possesses a C-terminal tandem repeat of two CBM10 modules (\( Cj\)Man5A-CBM10-1-CBM10-2), was also shown to bind both Avicel and insoluble mannan (INM) [22]. Deletion of CBM10-2 of \( Cj\)Man5A, which is a close homolog to the counterpart from \( Cj\)Xyn10A, resulted in a severe reduction of affinity to Avicel and INM [22], which hinted that the CBM10-1 was not efficiently mediating binding to the above insoluble substrates. Similarly, the CBM10 of \( Bl\)Man5_8, which

| Enzyme        | Substrate | \( K_d \) (mM) | \( n^a \) |
|---------------|-----------|---------------|---------|
| \( Bl\)Man5_8 | M4        | 5.1 ± 0.8     | 0.78    |
|               | M5        | 3.7 ± 0.3     | 0.94    |
|               | M6        | 0.58 ± 0.05   | 0.92    |
| Cellotetraose | n.d.      |               | –       |
| \( Bl\)Man5_8-ΔCBM10 | M4  | n.d.         | –       |
|               | M5        | 1.5 ± 0.3     | 0.36    |
|               | M6        | 0.24 ± 0.042  | 0.53    |
| Cellotetraose | n.d.      |               | –       |

\( ^a \) Estimated stoichiometry of binding to the immobilized protein

\( ^b \) Not detected
belongs to the same subgroup as the CBM10-1 of CjMan5A, did not bind to Avicel or HEC. By contrast, this CBM10 conferred increased affinity to soluble mannan based on larger relative retardation in affinity electrophoresis of the full-length BlMan5_8 compared to the truncated BlMan5_8-ΔCBM10. Remarkably, the affinity of BlMan5_8 for soluble galactomannan is 35–67-fold lower than that reported for GH26 mannanases BaMan26A from Bifidobacterium adolescentis (BaMan26A) [28] and CfMan26A from Cellulomonas fimi [45] possessing typical mannan-specific CBM23 modules. Binding analysis using SPR confirmed the mannan specificity and low affinity of the CBM10 of BlMan5_8, as deletion of this module considerably reduced the apparent stoichiometry of binding to M₅ and M₆ (Table 3). BlMan5_8-ΔCBM10 moreover displayed about two-fold higher affinity to M₅ and M₆ than the full-length enzyme, suggesting that the CBM10 had lower affinity than the catalytic module. The CBM10 of BlMan5_8 is a novel low-affinity CBM that binds manno-oligosaccharides with $K_d$ values in the mM range, in contrast to canonical moderate-affinity mannan-binding CBMs displaying $K_d$ values in the low μM range [46, 47]. Low-affinity starch-binding CBMs, conferring more dynamic binding, have been identified in plastidial α-amylases and starch-regulatory glucan water dikinases [48, 49].

The low affinity of the CBM10 is consistent with its very modest impact on the kinetic parameters of LBG hydrolysis (Table 2). The trend of increased $K_m$ and $k_{cat}$ upon CBM deletion is similar to other enzymes acting on soluble substrates, but the magnitude of these changes is much larger with typical CBMs than for the CBM10 of BlMan5_8 [50, 51].

The CBM10 of BlMan5_8 clusters exclusively with counterparts from GH5 and GH26 mannanases and segregates in the phylogenetic tree from sequences resembling the cellulose-specific CBM10 from CjXyn10A (Fig. 6; Additional file 12) also occurring in xylanases, cellulases and other carbohydrate-active enzymes as well as mannanases (Additional file 12). The aromatic binding residue Tyr⁸ in the cellulose-binding site of CBM10 of CjXyn10A [21] is substituted to smaller residues (Fig. 6, Additional file 9) in the BlMan5_8 CBM10 subfamily, that possesses a unique insertion preceding the substrate binding residue Trp²² and lacks the two conserved cysteines Cys⁵ and Cys³⁸ (Fig. 6) suggesting the loss of one disulfide bridge compared to the clade represented by the CBM10 of CjXyn10A (Additional file 13). Altogether, the loss of the Tyr⁸ aromatic stacking platform combined with significant differences in the loop length close to the binding Trp²² provide a possible explanation for the low affinity of the CBM10 from BlMan5_8 compared to CjXyn10A. Additionally, the loss of a disulfide bridge may elicit flexibility and/or structural changes that affect the biochemical properties of the BlMan5_8 CBM10 and its close homologs. Assigning the effects of these differences requires further structural and mutational analyses of this clade of CBM10.

Mannan utilization in the gut and the biological implication of BlMan5_8 activity

The concentration and residence time of mannan in the gastrointestinal tract is expected to be highly dynamic
and dependent on nutritional intake and gut microbiota composition. The presence of mannose utilization pathways in human gut-adapted taxa, including the major gut commensal genus *Bacteroides*, reflects an evolutionary adaptation to the dietary intake of this glycan. Recently, a novel pathway for the utilization of mannose involving a putative extracellular mannobiose-forming GH26 exo-mannanase from *Bacteroides fragilis* (*Bf* Man26A) was reported [42]. The mannobiose released by this enzyme is taken up by specialized ion symporters and subsequently degraded and metabolized intracellularly by an epimerase and a phosphorylase. This GH26 enzyme does not possess a CBM, and was speculated to act on mannose fragments produced by other β-mannanases from the same organism. The extracellular putatively cell-attached GH26 mannanase from *Bifidobacterium adolescentis* (*Ba* Man26A) hydrolyzes mannan to mainly mannotriose [28]. This modular enzyme, featuring a GH26 catalytic module joined to a C-terminal tandem repeat of CBM23 mannan-binding modules, binds tightly to LBG (K<sub>d</sub> = 8.8 mg·L<sup>-1</sup>) [28]. The deletion of the tandem CBM23 repeat abolishes measurable binding and yields an enzyme with very high...
$K_m$ (21.3 g L$^{-1}$), reminiscent of typical canonical moderate-affinity CBMs that decrease apparent $K_m$ values of the enzymes they are attached to [50, 51]. This CBM-mediated decrease in $K_m$ however, is often associated with a penalty of reduced $k_{cat}$ [50, 51]. Such effects are very different in case of BlMan5$\_8$, where the CBM10 makes little contribution to the low $K_m$. The justification for maintaining a CBM, albeit with lower affinity, could be a trade-off to increase the substrate-binding affinity of the enzyme (as observed from retardation electrophoresis data) and thus lower the $k_{cat}/k_{off}$ of the polymeric substrate, while minimizing the energy barrier pertaining to anchoring of the substrate tightly to the CBM. This may maintain proximity of the substrate to the enzyme, which has been shown to promote additional hydrolysis events following the initial enzyme-substrate encounter in cellulases [53] due to a higher $k_{cat}/k_{off}$ ratio. The optimization of substrate affinity through a low-affinity CBM is likely to offer a different adaptation solution to the fierce competition prevalent in the gut niche as opposed to the higher substrate affinity, but decrease in catalytic rates associated with canonical CBMs.

Conclusions
In conclusion, BlMan5$\_8$, which is conserved in the B. animalis subsp. lactis displays a different modular organization, product profile and substrate preference than characterized β-mannanases from gut bacteria. BlMan5$\_8$ is the first characterized GHS β-mannanase from the gut niche, which moreover possesses a novel low-affinity soluble-mannan-specific CBM10 not previously described in β-mannanases. This CBM may have evolved to increase the available substrate binding surface, while imparting less reduction in the catalytic rate typically observed for canonical moderate affinity CBMs. These unique substrate-binding properties highlight the diversity of mannan utilization strategies. Further studies are required to assess how these differences and the interplay of extracellular enzymes and transport systems contribute to establishing the hierarchy of mannan degradation in the gut niche.

Additional files

Additional file 1: Composition of the polysaccharide substrates used in this study [56]. (PDF 50 kb)
Additional file 2: Primers used for cloning of the full-length (BlMan5$\_8$) and generation of the truncated (BlMan5$\_8\_\Delta$CBM10) constructs. (PDF 49 kb)
Additional file 3: pH optimum (A) and stability (B) for BlMan5$\_8$ activity towards LBG. pH stability is measured as relative activity after 4 days of storage in Britton-Robinson buffers of pH 2-10. Grey error bars represent deviation between duplicate samples. (TIF 1113 kb)
Authors' contributions
SL. provided genomic DNA for Bifidobacterium animalis subsp. lactis BI-04. JM, EK, A.M.S. and M.A.H. performed the experiments. JM, EK, HS, BS, and M.A.H. planned experiments, interpreted the data and wrote the paper. All authors have read and approved the final version of the paper.

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References
1. Scheller HV, Uldovsk P. Hemicelluloses. Annu Rev Plant Biol. 2016;61(1):263–89. doi:10.1146/annurev-arplant-040215-034815.
2. Lundqvist J, Teleman A, Junel L, Zacchi G, Dahlman O, Tjerneld F, et al. Isolation and characterization of galactoglucomannan from spruce (Picea abies). Carbohydr Polym. 2002;48:29–39.
3. Cherubini F. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conv Manage. 2012;79:174–82.
4. Yamabhai M, S S-U, W S, Haltrich D. Mannan biotechnology: from biofuels to health. Crit Rev Biotechnol. 2014:1–11 [Epub ahead of print].
5. Schröder R, Nicolas P, Vincent SJF, Fischer M, Reymond S, Redgwell RJ, et al. Carbohydrate-binding modules from a thermostable S. thermosaccharolyticus putrefaciens strain. Carbohydr Res. 2013;362:36–45.
6. Prakash R, Johnston SL, Boldingh HL, Redgwell RJ, Atkinson RG, Melton LD, et al. Mannans in tomato fruit are not depolymerized during ripening by a marcomannase. J Plant Physiol. 2012;169:16–23.
7. Ray S, Vigouroux J, Quémener B, Bonnin E, Laylae M. Novel and diverse fine structures in LCI-OIMSO extracted apple hemicellulose. Carbohydr Polym. 2014;98:46–57.
8. Rodríguez-Gacio MC, Iglesias-Fernández R, Carboneiro P, Matilla AJ. Softening-up mannan-rich cell walls. J Exp Bot. 2012;63(11):3976–87.
9. Gallagher E, Gormley TR, Arendt EK. Recent advances in the formulation of gluten-free cereal-based products. Trends Food Sci Techn. 2004;15:43–52.
10. Prajakat VS, Jani GK, Moradally NG, Randeria NP, Nagar BJ. Locust bean gum: a versatile biopolymer. Carbohydr Polym. 2013;94:814–21.
11. Tomlin J, Read NW, Edwards CA, Duerrden BI. The degradation of guar gum by a faecal incubation system. Br J Nutr. 1998;65:481–6.
12. Okubo T, Ishihara N, Takahashi H, Fujisawa T, Mujo K, Yamamoto T. Effects of partially hydrolyzed guar gum intake on human intestinal microflora and its metabolism. Biosci Biotechnol Biochem. 1994;58:1364–9.
13. Berger K, Fälck P, Linninge C, Nilsson U, Asling L, Grey C, et al. Cereal byproducts have prebiotic potential in mice fed a high-fat diet. J Agric Food Chem. 2014;62(32):1619–78. doi:10.1021/jf502343v.
14. Wilforf S, Sundberg K, Tenkanen M, Holmbohm B. Spruce-derived mannan - A potential raw material for hydrocolloids and novel advanced materials. Carbohydr Polym. 2008;72:197–210.
15. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZY) in 2013. Nucleic Acids Res. 2014;42:D490–DS.
16. Aspeborg H, Coutinho P, Wang Y, Bruner H, Henrissat B. Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5). BMC Evol Biol. 2012;12(1):186.
17. Rye CS, Withers SC. Glycoside mechanisms. Curr Opin Chem Biol. 2000;4:573–80.
18. Gilbert HJ, Knop JP, Boraston AB. Advances in understanding the molecular basis of plant cell wall polysaccharide recognition by carbohydrate-binding modules. Curr Opin Struct Biol. 2013;23(5):690–77.
19. Motan M, Thomsen P, Rendl S. Arabinose binding studies. Biochim Biophys Acta. 1997;94:433–41.
20. Painea D, Carcano D, Leyer G, Darguy S, Aylanakis MA, Simoneau G, et al. Effects of seven potential probiotic strains on specific immune responses in healthy adults: A double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol. 2008;53:107–13.
21. Ponsy T, Säbbö L, Ngy T, Croz L, Simpson PJ, Williamson MP, et al. Trp22, Trp24, and Trp7 play a pivotal role in the binding of the family 10 cellulose-binding module from Pseudomonas xylanase A to insoluble ligands. Biochim Biochem Acta. 2000;1498:985–91.
22. Hogg D, Pell G, Dupree P, Goubert F, Martin-Orué SM, Armand S, et al. The modular architecture of Cellulibio h jenus mannanases in glycoside hydrolase families 5 and 26 to their differences in domain organization. Gradation Biochem J. 2009;371:1027–43.
23. Barranguet R, Britzinski EP, Traeger LL, Loquasto JR, Richards M, Horvath PC, M A, et al. Comparison of the complete genome sequences of Bifidobacterium animalis subsp. lactis DSM 10140 and B4-04. J Bacteriol. 2009;191:4144–51.
24. Stålsbrand H, Sika-aho M, Tenkanen M, Valkila L. Purification and characterization of two β-mannanases from Trichoderma reesei. J Biotechnol. 1993;36(3):229–42. http://dx.doi.org/10.1016/0168-1656(93)90055-R.
25. Diókiprimer A, Nakai H, Gottfredsen CH, Baumann MJ, Nakai N, Abou Bachem M, et al. Recombinant production and characterization of two related GHS endo-β-1,4-mannanases from Aspergillus nidulans FGSC A4 showing distinctly different transglycosylation capacity. Biochim Biophys Acta. 2011;1814(12):1720–9.
26. Hekmat O, Lo Leggo L, Rosengren A, Kamarauskaitė J, Kolenova K, Stålsbrand H. Rational engineering of mannosyl binding in the distal glucose subunits of Cellulomonas fimii endo-β-1,4-mannanase: mannosyl binding promoted at subsite –2 and demoted at subsite –3. Biochemistry. 2010;49(23):4884–96. doi:10.1021/bi100097f.
27. Abou Bachem M, Nordberg Karlsson E, Bartonove-Roxá E, Rahgotttina S, Simpson PJ, Gilbert HJ, et al. Carbohydrate-binding modules from a thermostable Rhodothermus marinus xylanase: cloning, expression and binding studies. Biochem J. 2000;345:53–60.
28. Kulcinskaja E, Rosengren A, Ibrahim R, Kolenova K, Stålsbrand H. Expression and characterization of a Bifidobacterium adolescentis beta-mannanase carrying mannin-binding and cell association motifs. Appl Environ Microbiol. 2013;79(1):33–40. doi:10.1128/aem.02118-12.
29. Eby M, Fredslund F, Vujicic-Zagar A, Svensson B, Storboom DJ, Abou HM. Structural basis for arabinoylxylo-oligosaccharide capture by the probiotic Bifidobacterium animalis subsp. lactis BI-04. Mol Microbiol. 2013;90:1100–12.
30. Rosengren A, Häggplund P, Anderson L, Pavan-Orozco P, Peterson-Wulf R, Nernicks W, et al. The role of subsite +2 of the Trichoderma reesei β-mannanase TrMMaSA in hydrolysis and transglycosylation. Biocat Biotransform. 2012;30(3):338–52. doi:10.3109/10242422.2012.674726.
31. Yin Y, Mao X, Yang JC, Chen X, Mao F, Xu Y. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 2012;40:445–51.
32. Katch K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30:772–80.
33. Huson DH, Scornavacca C. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst Biol. 2012;61:1061–7.
34. Hilge M, Gloor SM, Rypniewski W, Sauer O, Heightman TD, Zimmermann W, et al. High-resolution native and complex structure of thermostable β-mannanase from Thermomonospora fusca - substrate specificity in glycosyl hydrolase family 5. Structure. 1998;6:1433–44.
35. Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, et al. Comparative protein structure modeling using MODELLER. Curr Protoc Protein Sci. 2007;Ch 2Unit 2–9.

36. Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D Biol Crystallogr. 2010;66:12–21.

37. Word JM, Lovell SC, LaBean TH, Taylor HC, Zalis ME, Presley BK, et al. Visualizing and quantifying molecular goodness-of-fit: small-probe contact dots with explicit hydrogen atoms. J Mol Biol. 1999;285:1711–33.

38. Cristobal S, Zemla A, Fischer D, Rychlewski L, Elofsson A. A study of quality measures for protein threading models. BMC Bioinformatics. 2001;2,5.

39. Lo Leggio L, Larsen S. The 1.62 Å structure of Thermus aquaticus endoglucanase: completing the structural picture of subfamilies in glycoside hydrolase family 5. FEBS Lett. 2002;523:103–8.

40. Walter J, Ley R. The human gut microbiome: ecology and recent evolutionary changes. Annu Rev Microbiol. 2011;65:411–29.

41. Koropatkin N, Cameron E, Martens E. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol. 2012;10:323–35.

42. Kawaguchi K, Semoura T, Ito T, Taia T, Ito H, Wasaki J, et al. The mannosibiose-forming exo-mannanase involved in a new mannan catabolic pathway in Bacillus fragilis. Arch Microbiol. 2014;196:17–23.

43. Le Nours J, Anderson L, Stoll D, Stålbönd H, Lo LL. The structure and characterization of a modular endo-1,4-β-mannanase from Cellulomonas fimi. Biochemistry. 2005;44:12700–8.

44. Rosen gren A, Reddy SK, Sjöberg JS, Aurelius O, Logan D, Kolenová K, et al. An Aspergillus nidulans β-mannanase with high transglycosylation capacity revealed through comparative studies within glycoside hydroxylase family 5. Appl Microbiol Biotechnol. 2014;98:241:10091–104. doi:10.1007/s00253-014-5871-8.

45. Stoll D, Boraston A, Stålbönd H, Mclean BW, Kilburn DG, Warren RAJ. Mannanase Man26A from Cellulomonas fimi has a mannan-binding module. FEBS Microbiol Lett. 2003;183:265–9.

46. Boraston AB, Revett TJB, CM, Nurizzo D, Davies GJ. Structural and thermodynamic dissection of specific mannann recognition by a carbohydrate binding module. TmCBM27. Structure. 2003;11:665–75.

47. Mizutani K, Arai M, Watanabe M, Inagaki F. Influence of a mannan binding family 32 carbohydrate binding module on the activity of the appended mannanase. Appl Environ Microbiol. 2012;78:4781–7.

48. Christiansen C, Abou Hachem MA, Glaring MA, Viksa-Nielsen A, Sigurkjøld BW, Svensson B, et al. A CBM20 low-affinity starch-binding domain from glucon, water dikinase. FEBS Lett. 2009;583:1159–63.

49. Glaring MA, Baumann MJ, Abou Hachem M, Nakai H, Nakai N, Santelia D, et al. Starch-binding domains in the CBM45 family – low-affinity domains from glucon, water dikinase and α-amylase involved in plastidial starch metabolism. FEBS J. 2011;278:1715–85.

50. Santos CR, Paiva JH, Morca ML, Neves JL, Navarro RZ, Kota J, et al. Dissecting structure-function-stability relationships of a thermostable GH5-CBM3 cellulase from Bacillus subtilis 168. Biochem J. 2012;441:95–104.

51. Wang Y, Yuan H, Wang J, Yu Z. Truncation of the cellulose binding domain improved thermal stability of endo-beta-1,4-glucanase from Bacillus subtilis JA18. Biosensour Technol. 2009;102:3465–9.

52. Raghithamina S, Simpson PJ, Saibb L, Nagy T, Gilbert HJ, Williamson MP. Solution structure of the CBM10 cellulose binding module from Pseudomonas xylanase A. Biochemistry. 2000;39:978–84.

53. Payne CM, Jiang W, Shirts MR, Himmel ME, Crowley MF, Beckham GT. Glycoside hydrolase processivity is directly related to olosaccharide binding free energy. J Am Chem Soc. 2013;135:18831–9.

54. Kumagai Y, Kawakami K, Uraji M, Hatanaka T. Binding of bivalent ions to constitutive mannan-binding lectin. Glycobiology. 2010;20:775–82.

55. Armand S, Andrews SR, Charnock SJ, Gilbert HJ. Influence of the aglycone region of the substrate binding cleft of Pseudomonas xylanase 10A on catalysis. Biochemistry. 2001;40:7404–9.

56. Katsuraya K, Okuyama K, Hatanaka K, Oshima R, Sato T, Matsuzaki K. Constitution of konjac glucomannan: chemical analysis and 13C NMR spectroscopy. Carbohydr Polym. 2003;53:183–9.

57. Do BC, Dang TT, Berrin JG, Haltrich D, To KA, Sigilloit JC, et al. Cloning, expression in Pichia pastoris, and characterization of a thermostable GH5 mannan endo-1,4-β-mannosidase from Aspergillus niger 8K01. Microbiol Cell Fact. 2009;8:59.