Fusion of family 2b carbohydrate-binding module increases the catalytic activity of a xylanase from *Thermotoga maritima* to soluble xylan

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Abstract A family 2b carbohydrate-binding module from *Streptomyces thermoviolaceus* STX-II was fused at the carboxyl-terminus of XynB, a thermostable and single domain family 10 xylanase from *Thermotoga maritima*, to create a chimeric xylanase. The chimeric enzyme (XynB-CBM2b) was purified and characterized. It displayed a pH–activity profile similar to that of XynB and was stable up to 90°C. XynB-CBM2b bound to insoluble birchwood and oatspelt xylan. Whereas its hydrolytic activities toward insoluble xylan and *p*-nitrophenyl-β-xylopyranoside were similar to those of XynB, its activity toward soluble xylan was moderately higher than that of XynB.

Key words: Xylanase; Thermostable; Carbohydrate-binding module; Chimeric enzyme

1. Introduction

Cellulose and xylan are β-1,4-linked polymers of glucopyranose and xylopyranose, respectively, and are the most abundant polysaccharides in the biosphere. The β-1,4-glycosidic bonds in these polymers are hydrolyzed by cellulases and xylanases, respectively. The molecular architectures of these enzymes are often complex. In general, they have a modular structure consisting of discrete structural and functional domains [1,2]. Many cellulases and xylanases contain a catalytic domain and one or more non-catalytic carbohydrate-binding modules (CBMs) [1,2]. CBMs are located either at the N- or C-terminal, or both. Currently, based on amino acid sequence similarities, 31 families of CBMs are described in the latest update of the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) [3]. Furthermore, these families have been grouped into three classes based on their structure, function and ligand specificities. Type A CBM binds to insoluble polysaccharides, type B binds to polysaccharides, and type C binds to mono- and disaccharides [4]. Within these types, a wide variation in binding specificity exists. For example, CBMs belonging to families 1, 2a, 3, 5, and 10 bind mainly to crystalline cellulose, while a preference for xylan is exhibited by members of families 2b, 4, 6, 13 and 22 [5–12]. Three-dimensional structures of members of several CBM families are now available from crystallographic as well as nuclear magnetic resonance (NMR) spectroscopic studies [2,5,11,13–19]. Most of these modules are formed exclusively from β-strands. Complementary to their substrates, type A CBMs exhibit a flat binding surface, whereas those of types B and C have binding grooves.

Several studies have shown that CBMs potentiate the catalytic activity of cellulases against crystalline substrates, and xylanases against cellulose/xylan complexes. However, these domains do not potentiate the activity of glycosyl hydrolases against soluble substrates [10,20,21]. The mechanism of the enhancements has CBM binding to polysaccharide to locate the catalytic domain close to the substrate [22,23]. Some CBMs in family IIa may disrupt the surface of cellulose fibers to increase the accessibility of the enzyme [24,25]. Addition of CBMs to single domain enzymes often increased their activity toward insoluble substrate. Maglione et al. [26] showed an increase in the specific activity of *Prevotella ruminicola* endoglucanase upon fusion with cellulose-binding domain from *Thermomonospora fusca* endoglucanase. Fusion of a family 6 CBM to *Ruminococcus albus* endoglucanase also displayed higher molar activity toward insoluble acid-swollen cellulose and ball-milled cellulose [27]. We recently demonstrated that addition of a family 6 CBM to *Bacillus halodurans* xylanase enhances activity toward insoluble xylan [28]. In all these studies, however, no activity enhancement was observed toward soluble substrates.

In the present study, a chimeric xylanase was constructed by fusing a family 2b CBM of xylanase II from *Streptomyces thermoviolaceus* (STX-II) [29] to the C-terminus of xylanase B (XynB) from *Thermotoga maritima* [30] to examine the effect of fusion on the catalytic activity of XynB toward soluble and insoluble xylan. XynB was chosen for this study because of its hyperthermophilic nature, whereas the choice of family 2b CBM from *S. thermoviolaceus* STX-II was based on its chain specificity and thermal stability [29]. Members of family 2b are known to bind specifically to xylan chains. In this paper, we show that the fusion of family 2b CBM to XynB enhances activity toward soluble but not insoluble xylan.

2. Materials and methods

2.1. Xylan

Xylans (from birchwood and oatspelt) were purchased from Sigma Chemicals (St. Louis, MO, USA). The xylans (200 mg) were stirred in water (20 ml) for 2 h at 25°C and then centrifuged. The supernatants

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were lyophilized to obtain soluble xylans, and the precipitates were dried under vacuum to obtain insoluble xylans.

2.2. Bacterial strains, plasmids and media

Plasmid vectors TM-xynB-TOPO-XL [30] and pXY87 [29] were used as templates for the amplification of full-length *T. maritima* XynB and *S. thermoviolaceus* STX-II CBM2b, respectively. A pDrive cloning vector was obtained from Qiagen (Hilden, Germany). Expression vector pET-28b(+) came from Novagen (Madison, WI, USA). *Escherichia coli* EZ competent cells and *E. coli* BL-21 CodonPlus-RIL competent cells (Stratagene, La Jolla, CA, USA) were used as host for cloning and expression, respectively. Luria–Bertani (LB) medium supplemented with kanamycin (50 μg/ml) was used for the cultivation of *E. coli* cells.

2.3. Construction, cloning and sequencing of chimeric gene

The plasmid DNA preparation, DNA electrophoresis and other basic DNA manipulations described in this paper were performed as described previously [31]. The chimeric gene was constructed using a three-step overlapping polymerase chain reaction (PCR) (Fig. 1). In the first step, full-length *xynB* and the gene segment encoding family 2b CBM and the linker (residues 348–416) from STX-II were amplified using primers 1, 2 and 3, 4, respectively (Table 1). Primers 1 and 4 had *Nco*I and *Sal*I sites for cloning into pET-28b(+) vector, whereas primers 2 and 3 had 20 bp overlapping regions, which were used as templates for self-priming in the second step of the overlapping PCR. For cloning into *Nco*I site of pET-28b(+) vector, Arg of XynB was mutated to a Glu residue. The second PCR step was self-priming and was carried out with equimolar concentrations of the two fragments obtained in the first PCR. In the last step, primers 1 and 4 were used to amplify the full-length chimeric gene (*xynB-CBM2b*). All three PCR steps consisted of denaturation at 98°C for 1 min, annealing at 55°C for 1 min, and primer extension at 68°C for 5 min with 25 cycles. All PCR steps were carried out with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using high fidelity KOD-plus DNA polymerase (Toyobo Biochemicals, Osaka, Japan). The chimeric gene was ligated to the TA cloning vector (pDrive cloning kit, Qiagen), and the plasmid was transformed into *E. coli* EZ cells. The plasmid *xynB-CBM2b*-pDrive was isolated from a positive clone, and DNA sequencing was done on both strands (ABI Prism 310 Genetic Analyzer, Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) to confirm the correct nucleotide sequence of the chimeric gene.

2.4. Construction of expression vector

The chimeric gene excised from *xynB-CBM2b*-pDrive with *Nco*I and *Sal*I was ligated to the pET-28b(+) vector using High T4 DNA ligase (Toyobo Biochemicals) and transformed into *E. coli* BL-21 CodonPlus-RIL competent cells. The expression construct (*xynB-CBM2b*-pET) from a positive colony was sequenced to verify the correct insertion of the open reading frame into the cloning site.

2.5. Expression and purification

For expression of the chimeric gene, recombinant *E. coli* BL-21 CodonPlus-RIL cells harboring *xynB-CBM2b*-pET were cultivated in LB broth supplemented with kanamycin (50 μg/ml) under shaking conditions at 25°C. In the exponential phase at $OD_{600} = 0.6$, expression was induced by adding isopropyl thiogalactoside (IPTG) to a final concentration of 0.6 mM, and incubation was continued overnight at 25°C. Cells were harvested by centrifugation, washed with 25 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer (pH 7.0), resuspended in the same buffer and disrupted by sonication. The supernatant obtained by centrifugation at $10\,000 \times g$ for 15 min contained the crude enzyme extract.

The enzyme was purified by Ni-nitrilotriacetic acid (NTA) agarose chelate chromatography followed by ion-exchange chromatography on MonoQ performed at room temperature. Crude extract (10 ml) was applied to 4 ml of Ni-NTA agarose resin (Qiagen) equilibrated with 50 mM MOPS buffer (pH 7.0) containing 25 mM imidazole and 300 mM NaCl. The enzyme was eluted with a linear gradient of 25–250 mM imidazole in 50 mM MOPS buffer (pH 7.0) containing 300 mM NaCl. The active fractions were pooled, dialyzed overnight at 4°C against 5 mM MOPS (pH 7.0) and then applied onto a MonoQ column, which was previously equilibrated with 50 mM MOPS buffer (pH 7.0). The protein was eluted with a linear gradient of 0–500 mM NaCl at a flow rate of 0.5 ml/min. The active fractions were pooled and dialyzed overnight against 5 mM MOPS buffer (pH 6.2) and subjected to a second round of ion-exchange chromatography on MonoQ. The active fractions were pooled and dialyzed against 50 mM MOPS buffer (pH 6.2), and the dialyzed enzyme was used for the enzyme assays.

2.6. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 12% acrylamide gels [32], and the proteins were visualized by staining with Coomassie brilliant blue R-250. Benchmark protein ladder (Invitrogen, USA) was used as the protein standard.

2.7. Enzyme assay

Xylanase activity was determined by measuring the reducing sugar released from the soluble birchwood xylan at 50°C for 15 min. The assay mixture consisted of 0.5% birchwood xylan in 50 mM MOPS buffer (pH 6.2). Appropriately diluted enzyme was added to the reaction mixture to a final volume of 200 μl. The reaction mixture was incubated at 50°C for 15 min, and the reaction was terminated by adding 200 μl of Somogyi reagent (Wako Pure Chemicals, Osaka, Japan) and placing the mixture immediately in a boiling water bath for 15 min. After it cooled, Nelson reagent (200 μl, Wako Pure Chemicals) and water (400 μl) were added, and the absorbance at 520 nm was measured. One unit of xylanase activity corresponded to 1 μmol of reducing sugar as xylose/min under the conditions described for the assay. The K_m and k_{cat} values were determined using substrate concentrations ranging from 0.15 to 4 g/l of soluble xylan. Activity towards *p*-nitrophenyl-β-xylopyranoside was measured at 30°C by the rate of *p*-nitrophenol released during substrate cleavage in 50 mM MOPS buffer (pH 6.2). The increase in absorbance was measured at 405 nm using a spectrophotometer (Model DU640, Beckman, USA) with a temperature-controlled cell holder. Initial rates were determined at six different concentrations ranging from approximately 0.5 to 2.0 times the K_m value. The kinetic parameters and their standard errors were calculated using a non-linear regression analysis program 'Grafit' [33].

2.8. Effect of temperature and pH

The temperature optimum was determined under standard assay conditions by incubating the reaction mixtures at 30–100°C. Thermal stability was determined by incubating the enzyme for 30 min at different temperatures. After it cooled on ice for 10 min, residual activity was determined using a standard assay. For determining the optimum pH for enzyme activity, a standard assay mixture in 50 mM of the following buffers was used: citrate (pH 2.18–4.21), sodium acetate (pH 3.76–5.73), 2-(*N*-morpholino)-ethanesulfonic acid (MES; pH 5.15–7.13), MOPS (pH 6.22–8.2), phosphate (pH 6.22–8.22), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES; pH 6.54–8.54), Tris(hydroxymethyl) aminomethane hydrochloride (Tris; pH 7.20–9.00), 3-(cyclohexylamino)-1-ethanesulfonic acid (CHES; pH 8.17–10.14) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 9.44–11.39). The pH stability was determined by preincubating the enzyme in the above-mentioned buffers for 30 min at 70°C. Enzyme activity was then measured under the standard assay conditions.

2.9. Binding assays

Binding assays were carried out in 2 ml glass screw-cap vials. Each vial contained 480 μl of 50 mM MOPS buffer (pH 6.2), 2 mg of insoluble xylan and 20 μl (~4.0 μg protein) of enzyme. The vials were incubated with slow rotation for 1 h on ice. The binding substrate was pelleted by centrifugation (5 min, 15 000 rpm) and the supernatants were tested for xylanase activity or protein content. Each experiment included controls without binding substrate and without enzyme. The xylanase activity determined in the sample without substrate was defined as 100%.

3. Results and discussion

3.1. Construction, expression and purification of chimeric xylanase

S. thermoviolaceus STX-II has a modular architecture composed of two distinct domains (Fig. 1). Residues 1–234 corre-

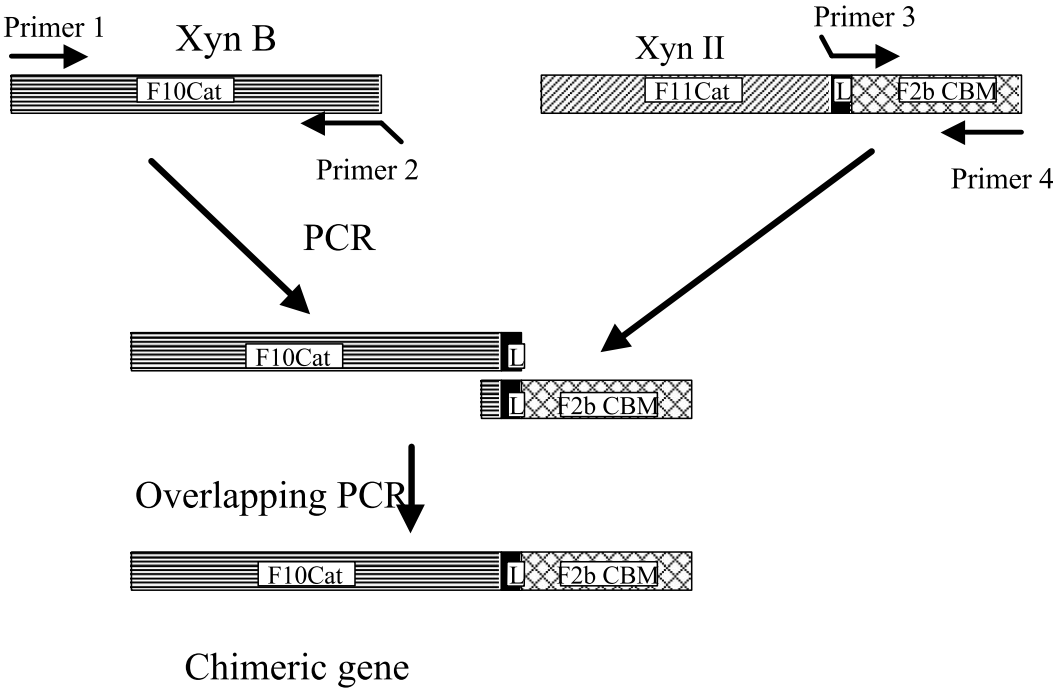

Fig. 1. Strategy for the construction of the chimeric enzyme (XynB-CBM2b). F10Cat, family 10 catalytic domain of XynB; F11Cat, family 11 catalytic domain of STX-II; L, linker region of STX-II; F2b CBM, family 2b CBM of STX-II.

spond to a xylanase catalytic domain belonging to glycoside hydrolase family 11. Downstream of the catalytic domain (residues 246–335) is a family 2b CBM. The interdomain region is a typical polyglycine linker sequence. *T. maritima* XynB, on the other hand, is a single domain enzyme with a catalytic domain belonging to glycosyl hydrolase family 10.

In order to examine the effect of addition of a specific CBM on *T. maritima* XynB catalytic activity, we constructed a chimeric xylanase containing a family 2b CBM fused at the C-terminus of *T. maritima* XynB (XynB-CBM2b). Specific primers matching the approximate border of the regions coding for CBM2b (with linker) and catalytic domain were selected on the basis of the nucleotide sequence of the XynB and STX-II genes. The gene segments were amplified by PCR and were fused, using an overlapping PCR as described in Section 2. The chimeric gene was cloned and expressed in *E. coli* BL-21 CodonPlus-RIL under the control of the inducible phage T7 promoter of vector pET-28b(+). SDS–PAGE analysis of cell lysate indicated the production of a protein of molecular mass 52.5 kDa as the major protein. The apparent molecular mass of overexpressed protein as seen by SDS was consistent with that of the calculated molecular mass of 52 553 Da, based on the deduced amino acid sequence. The overexpressed protein displayed xylanase activity, indicating that fusion of the enzyme with the binding domain did not result in misfolding, at least not in the catalytic domain part of the chimeric xylanase. XynB-CBM2b expressed was purified from cell lysate by Ni-NTA chelate chromatography followed by ion-exchange chromatography on MonoQ. A single band on SDS–PAGE indicated its purity to homogeneity (Fig. 2).

Table 1
Primers used in the construction of the chimeric gene

1.	5′-**CCATGG**AAATATTACCTTCTGTGTT-3′
2.	5′-CACCGCCTTTTCTTTCTTCTATCTTTTTCTCC-3′
3.	5′-AGAAAGAAAAGGCGGCGGTGGTGGCGG-3′
4.	5′-**GTCGAC**GCCGACGGAGCAGCTGAC-3′

Restriction enzyme sites are in bold letters. 10 bp of the overlapping regions are underlined.

3.2. *Effect of pH and temperature on activity and stability of chimeric xylanase*

Grafting of CBMs has been shown to alter the activity and thermal stability of the catalytic domain. Fusion of CBM4 to the feruloyl acetyl esterase (FAE) domain of *Clostridium stercorarium* XynZ was shown to result in a drastic decrease in the thermal stability of the FAE domain [34]. On the other hand, Karita et al. [27] reported improvement in the thermal stability of *R. albus* endoglucanase upon fusion with a family 6 CBM. The influences of pH and temperature on the activity and stability were therefore investigated. Chimeric xylanase displayed an optimum pH of 6.2 at 50°C, and it was stable

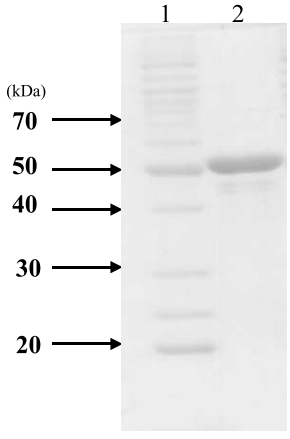

Fig. 2. SDS–PAGE analysis of purified XynB-CBM2b. Lane 1, 10 kDa protein marker; lane 2, purified XynB-CBM2b.

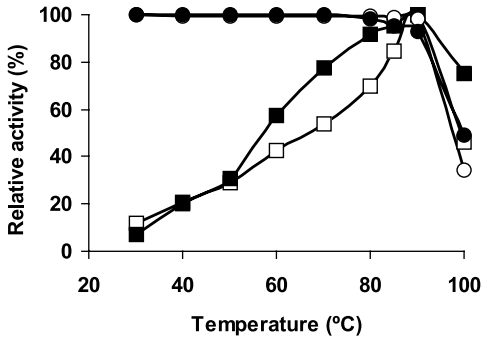

Fig. 3. Temperature optimum (squares) and thermal stability (circles) of XynB (open symbols) and XynB-CBM2b (solid symbols).

in the pH range 5.0–11.0 at 70°C (data not shown). Fig. 3 shows the temperature optimum and thermal stability profiles of chimeric and parental enzyme. As can be seen, XynB-CBM2b displayed the identical temperature–activity profile as that of XynB, both showing a temperature optimum at 90°C and stability up to 90°C. These results together with pH–activity profile indicate that artificial connection of the CBM2b to XynB has no effect on the structure and function of the catalytic domain.

3.3. Binding of chimeric xylanase to insoluble xylan

The ability of XynB-CBM2b to bind insoluble xylan was studied by incubating the protein with insoluble birchwood and oatspelt xylan and determining the residual xylanase activity/protein concentration in the filtrate (unbound protein). The results showed that XynB-CBM2b bound to the insoluble xylan, whereas XynB was unable to adhere to these polymers (Table 2). This result indicates that the affinity of the XynB-CBM2b towards insoluble polysaccharide is due to the presence of CBM and not the catalytic domain. Table 2 also shows difference in the relative adsorption values for birchwood and oatspelt xylan, adsorption being higher in the case of oatspelt xylan than birchwood. The difference in the binding affinity is probably due to the differences in the composition of the two polysaccharides. In the case of birchwood xylan, the backbone is decorated with more 4-*O*-methyl-α-D-glucuronopyranosyl residues (acidic) [35]. α-L-Arabinofuranosyl residues (neutral) are common side chains in oatspelt xylan.

3.4. Effect of family 2b CBM on catalytic activity of XynB

The function of CBMs in cellulases is clear. One is to increase the effective enzyme concentration at the surface of insoluble substrates through their affinity for cellulose. Another is to loosen the crystalline structure of cellulose and supply substrates that are easily hydrolyzable. The function of CBMs in xylanases is more ambiguous, although they might attach/secure the enzymes on cellulose in plant cell walls, resulting in the increase of substrate (xylan) concentrations around the enzyme. Since the family 2b CBMs have affinity for soluble xylan chains, we presumed that CBM2b has an important role in xylan hydrolysis, and we compared the hydrolytic activities of XynB-CBM2b and XynB toward soluble and insoluble substrates. Though the binding ability of XynB-CBM2b to insoluble xylan at 50°C was not directly confirmed because the insoluble xylan was readily hydrolyzed in the presence of the catalyst, the grafted CBM2b was considered to be intact because the binding ability of XynB-CBM2b at 0°C did not decrease after 30 min of incubation at 50°C.

Table 2
Adsorption of XynB and XynB-CBM2b to insoluble xylans

	XynB	XynB-CBM2b
Birchwood xylan	2	31
Oatspelt xylan	2	71

Numbers represent the percentage of the bound enzyme.

Table 3
Activities of XynB and XynB-CBM2b on the hydrolysis of various substrates

	XynB	XynB-CBM2b
Soluble birchwood xylan		
K_m (%)	0.21	0.28
k_{cat} (s^{-1})	33	57
Insoluble birchwood xylan		
v at 5 g/l (s^{-1})	0.97	1.05
p-Nitrophenyl-β-xylopyranoside		
K_m (mM)	9.4	9.9
k_{cat} (s^{-1})	2.8	2.2

The specific activities of the purified XynB and XynB-CBM2b were 37 and 43 U/mg, respectively, when assayed with soluble birchwood xylan as a substrate. It is worth noting that the specific activity on a per weight basis of XynB-CBM2b is higher than XynB despite its molecular mass (52.5 kDa) being higher than that of the parental enzyme (42 kDa). This suggests that addition of a specific CBM, in this case a family 2b, increases activity toward soluble xylan. When initial rates of reaction were measured at 50°C in various concentrations of birchwood xylan, there was a clear difference in the k_{cat} values of the parental and chimeric enzymes – the hydrolytic activity of XynB-CBM2b was greater than that of XynB (Table 3). On the other hand, when insoluble birchwood xylan was used as substrate, no significant difference in hydrolytic activities was observed (Table 3), suggesting that the family 2b CBM has no role in the hydrolysis of insoluble xylan. The increase in the catalytic activity of XynB toward soluble xylan upon fusion with a CBM is somewhat puzzling, since most of the CBMs studied to date are known to enhance catalytic activity only toward insoluble substrate. Members of families 2b, 6, 13, and 22, though, bind soluble chains, but they enhance the catalytic activity only toward insoluble substrates. We determined the kinetic parameters for *p*-nitrophenyl-β-xylopyranoside, a substrate for which family 2b CBMs have no affinity. XynB exhibits exceptionally higher activity toward *p*-nitrophenyl-β-xylopyranoside [30]. The results in Table 2 showed that the kinetic parameters of the parental and chimeric enzyme for *p*-nitrophenyl-β-xylopyranoside were not significantly different, indicating that the addition of the CBM did not affect the real catalytic efficiency. The increase in the activity to a soluble polymeric compound is not readily explained. A possible explanation of the results may be that the addition of a binding domain somehow increased the ratio of productive enzyme–substrate complex.

It is generally believed that substrate-binding domains are not required for the degradation of soluble substrates. Our results, however, indicate that at least in the case of soluble chain-binding CBMs, this assumption should not be taken for

granted, because these CBMs clearly interact with soluble glycan chains.

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