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ORIGINAL PAPER

Modification of paper properties using carbohydrate-binding module 3 from the *Clostridium thermocellum* CipA scaffolding protein produced in *Pichia pastoris*: elucidation of the glycosylation effect

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Abstract The carbohydrate-binding modules (CBMs) have emerged as an interesting alternative to enzymes for fibers modification, *e.g.* of pulp and paper. Glycosylation in CBMs is thought to have a key role in the improvement of cellulose fibers. Thus, in this work the non-glycosylated (CBM3mt) and glycosylated (CBM3wt) recombinant versions of CBM3 from *Clostridium thermocellum* CipA—both produced in *Pichia pastoris*—were studied. Binding assays showed that CBM3mt had a higher affinity for microcrystalline cellulose (Avicel) than CBM3wt. In addition, CBM3mt produced a much higher hydrophobization of Whatman paper than CBM3wt. However, the effects of the two CBM3s on pulp and paper were identical. The CBM3s did not affect the drainability of *Eucalyptus globulus* or a mixture of *E. globulus* and *Pinus sylvestris* pulps. On the other hand, both improved significantly strength-related properties of *E. globulus* papersheets, namely burst (up to 12 %) and tensile strength (up to 10 %) indexes. This is the first report showing the capacity of CBM3 from *C. termocellum* CipA to modify paper properties. The results showed that glycosylation did not influence the drainage of CBM3-treated pulps nor the properties of the produced papers. Thus, glycans in glycosylated CBM3 may not be related with fiber improvement, namely superior pulp drainage.

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Introduction

Cellulases and hemicellulases have long been applied in the pulp and paper industry for the modification of both fiber and paper properties (Bhat 2000). However, the hydrolytic activity of these enzymes often leads to the reduction of fiber strength and yield (Bhat 2000). To avoid these undesirable effects, the use of their carbohydrate-binding modules (CBMs) has emerged as an interesting alternative, as CBMs have been shown to modify pulp and paper properties independently from the catalytic domain (Kitaoka and Tanaka 2001; Pala et al. 2001; Yokota et al. 2009).

CBMs are non-catalytic modules present in several carbohydrate-active enzymes (CAZymes; Lombard et al. 2014) that exhibit high affinity for cellulose and potentiate the catalytic efficiency of the associated catalytic domains. Due to their independent fold and function, CBMs find application in different fields of biotechnology (Gomes et al. 2015; Oliveira et al. 2015). CBMs can be obtained from fungal and bacterial cellulases and hemicellulases through proteolysis (Lemos et al. 2000; Pinto et al. 2004a) or by recombinant DNA technology, mainly in fusion with a partner (reviewed in Oliveira et al. 2015). This last

method allows overcoming the purity limitations of the proteolysis approach and the fusion of CBMs with other biomolecules (Oliveira et al. 2015). Thus, in recent years, research has focused on the use of CBMs and CBM–CBM fusions obtained recombinantly from the bacterium *Escherichia coli* to enhance the mechanical properties of paper, particularly those related to paper strength (Cadena et al. 2010; Levy et al. 2002, 2004; Shi et al. 2014). Among the different recombinant CBMs tested, recombinant CBM3, originally from the *Clostridium thermocellum* scaffolding protein CipA, was reported not to affect pulp and paper properties (Machado et al. 2009). Nevertheless, when conjugated with polyethyleneglycol (PEG), recombinant CBM3 improved pulp drainability, without affecting the paper properties (Machado et al. 2009). This result was attributed to a stabilizing effect of PEG in fibers, driven by the high hydrophilic nature of that molecule, which was used to mimetize glycosylation in CBM3 (Machado et al. 2009). In fact, non-glycosylated CBMs have shown the opposite effect: they increase considerably the hydrophobicity of fibers (Levy et al. 2002) and reduce pulp drainage (Cadena et al. 2010). On the other hand, heavily glycosylated fungal CBMs were reported to improve fiber hydration and pulp drainage, presumably due to the presence of glycans, which are likely to be highly hydrated (Pinto et al. 2004b, 2006). CBMs with linked oligosaccharides are thought to stabilize the fibers due to steric effects, thus reducing the interfiber interactions in water suspensions (Pinto 2006). However, despite the several indications that glycosylation may be essential for the positive effects of CBMs in cellulose fibers (Machado et al. 2009; Pinto 2006), this subject has not yet been thoroughly studied.

Thus, this work aims at investigating the impact of glycosylation on the capacity of *C. thermocellum* CipA CBM3 to modify pulp drainability and paper properties (namely, wettability, bursting and tensile strengths, lengthening and tearing) in order to elucidate the effect of the glycans suggested in previous reports.

Materials and methods

Expression plasmids construction

The *cbm3wt* and *cbm3mt* genes were PCR amplified from pPCG vectors (Wan et al. 2011) with Vent DNA polymerase (Stratagene), using the primer forward 5′CCG **GAA TTC** CCA GTT TCT GGT AAC 3′ and the primer reverse 5′ CGG **GGTACC** TCA *ATG ATG ATG ATG ATG ATG* TGG TTC CTT ACC CCA AAC 3′. Restriction sites for *EcoR*I and *Kpn*I (in bold) were included in the forward and reverse primers, respectively, and a tail of six histidine codons (in italics) was included in the reverse primer before the stop codon (underlined) for recombinant protein purification by immobilized metal ion affinity chromatography (IMAC). The *cbm3wt* corresponds to the wild type gene with codons optimized for usage in *P. pastoris* (GenBank accession number HQ232851) and *cbm3mt* is a mutated version of the *cbm3wt* gene, in which three codons for asparagine (amino acid positions: 14, 68, and 124) were replaced with codons for glutamine (Wan et al. 2011). The PCR conditions used were: initial denaturation step at 95 °C for 2 min, followed by 30 cycles of amplification with 45 s of denaturation at 95 °C, 45 s of annealing at 60 °C and 1 min of elongation at 72 °C.

PCR products were digested with *EcoR*I and *Kpn*I (New England Biolabs) and inserted into the same restriction sites of the pPICZαA expression vector (Invitrogen), originating the plasmids pPICZαACBM3wt and pPICZαACBM3mt. Digested DNA fragments were gel purified using the Qiaquick Gel Extraction Kit (QIAgen) and ligated with T4 DNA ligase (Promega), according to the manufacturer's protocols. Plasmids were propagated and maintained in *E. coli* NZ5α (NZYTech). The sequence of the inserts in the plasmids was confirmed by sequencing (Eurofins MWG Operon) with the AOX1 primers pair (Invitrogen).

P. pastoris transformation and recombinant CBM3wt and CBM3mt production

The expression plasmids pPICZαACBM3wt and pPICZαACBM3mt were linearized using *Sac*I (New England Biolabs) and transformed into *P. pastoris* KM71H (Invitrogen) by electroporation, using Gene Pulse X-Cell (Bio-Rad), according to the protocol described in the EasySelect *Pichia* Expression kit manual (Invitrogen). Transformants were selected on YPD-sorbitol plates (2 % glucose, 2 % peptone, 1 % yeast extract and 1 M sorbitol) containing 100 μg/ml of Zeocin and incubated at 30 °C for 3 days. Insertions were confirmed by "colony PCR", as described elsewhere (Oliveira et al. 2008). Positive PCR

Table 1 Properties of the recombinant CBMs used in this work

CBM abbreviation	Potential N-glycosylation sites	Amino acid substitutions	Predicted MW (kDa)
CBM3wt	N14, N68, N124	None	18.7
CBM3mt	None	N14Q, N68Q, N124Q	18.6

transformants were inoculated on YPD plates containing 1000 μg/ml of Zeocin, for the selection of multicopy integrated strains.

The transformants that demonstrated the highest resistance to Zeocin were cultivated overnight in 200 ml of buffered minimal glycerol medium (BMG; 1 % glycerol, 100 mM potassium phosphate pH 6.0, 1.34 % yeast nitrogen base, 4×10^{-5} % biotin) at 30 °C and 200 rpm. After centrifugation (4000*g*, 10 min) and removal of BMG, cell pellets were suspended into 100 ml of fresh buffered minimal methanol medium (BMM; 0.5 % methanol, 100 mM potassium phosphate pH 6.0, 1.34 % yeast nitrogen base, 4×10^{-5} % biotin) and then incubated at 30 °C and 200 rpm during 4 days. To maintain induction, methanol was added every 24 h to a final concentration of 0.5 % (v/v). In order to improve the aeration in the culture, induction was carried out in baffled flasks covered with two layers of sterile gauze.

Recombinant CBM3wt and CBM3mt purification

The yeast cells were removed from the producing cultures by centrifugation (10 min at 4000*g* and 4 °C). The pH of the resulting supernatants was increased to 7.5 by adding 10 N NaOH to precipitate salts, which were removed by centrifuging twice for 10 min at 4000*g* and 4 °C. Then, supernatants were filtered through 0.45 μm pore size Polyethersulfone (PES) filters and concentrated using Vivaspin® 20 10,000 MWCO centrifugal filter devices (Sartorios). For buffer exchange, the concentrated fractions were repeatedly diluted with PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 2.68 g/l $Na_2HPO_4{\cdot}7H_2O$ and 0.24 g/l KH_2PO_4, pH 7.4) and concentrated again to a final volume of about 4 ml. The 25X concentrated supernatants from ten producing cultures were combined and loaded on a 5 ml Niquel Hi-Trap Column (GE Healthcare Life Sciences), previously equilibrated with 40 mM imidazole in 20 mM phosphate buffer, pH 7.4. Finally, the column was washed with the equilibration buffer and the recombinant proteins were eluted with 300 mM imidazole in 20 mM phosphate buffer, pH 7.4. After purification, elution buffer was exchanged with PBS buffer using PD10-columns (GE Healthcare Life Sciences). Concentrated supernatants and purified proteins were analyzed by SDS-PAGE using 12 % (w/v) acrylamide gels, followed by Coomassie Brilliant Blue staining. Predicted sizes for the recombinant CBMs are presented in Table 1. The concentration of each CBM was estimated from the absorbance at 280 nm using the molar extinction coefficient (35,410 M^{-1} cm^{-1}). Purified CBMs were stored at −20 °C until use in subsequent assays.

Deglycosylation of CBM3wt with Endoglycosidase H

To determine if the CBM3wt was N-glycosylated, this protein was digested with Endoglycosidase H according to the manufacturer's instructions (New England Biolabs). The digested protein was analysed by SDS-PAGE, as described above.

Glycosylated CBM3wt three-dimensional (3D) model building

Previous work by Wan et al. (2011) indicated that only the Asn68 of CBM3wt was N-glycosylated by *P. pastoris*, which is known to attach to recombinant proteins N-glycans containing predominantly 8–14 mannoses (Montesino et al. 1999; Blanchard et al. 2007). Based on this information, the 3D structure of the CBM3 from *Clostridium thermocellum* CipA deposited in the Protein Data Bank by Tormo et al. (1996) (PDB ID: 1NBC) was uploaded in GlyProt (Bohne-Lang and von der Lieth 2005), which was used to build a hybrid CBM3wt structure containing a high mannose-type N-glycan with 9 mannoses at the position Asn68. VMD (Humphrey et al. 1996) was used for visualization.

Cellulose-binding assays

In 2 ml eppendorf tubes, different concentrations of CBM3wt or CBM3mt (up to 1 mg/ml) were incubated with 10 mg of Avicel in 1 ml sodium acetate buffer (50 mM, pH 5.0) for 2 h at 5 °C, with continuous mixing by rotation. Then, Avicel was removed from the mixture by centrifuging for 10 min at 12,000*g* and 4 °C. Centrifugation and washing with sodium acetate buffer were repeated twice to ensure the removal of all unbound protein. The CBM concentration in the final supernatant was measured in a spectrofluorimeter (HORIBA Scientific) operated at an emission and excitation wavelengths of 344 and 275 nm, respectively. The spectrofluorimeter was calibrated using CBM solutions in which the protein concentration had been determined using the Bio-Rad Protein assay kit. Non-linear regression analysis was used to calculate the parameters of the Langmuir adsorption isotherm (Kim et al. 2001):

$$CBM_{Bound} = \frac{CBM_{Max} \cdot K_a \cdot CBM_{Free}}{1 + K_a \cdot CBM_{Free}}$$

where CBM_{Bound} is the molar amount of protein adsorbed per unit weight of cellulose, $[CBM]_{Free}$ is the molar protein concentration in the liquid phase at the adsorption equilibrium, CBM_{Max} is the maximum molar amount of protein adsorbed per unit weight of cellulose, and K_a is the adsorption equilibrium constant. Two independent assays were performed for each CBM concentration.

Water absorption assay

Rectangular pieces of Whatman paper no.1 (30 × 10 mm) were immersed for 2 h in 20 mM Tris base, pH 7.0, containing a fixed concentration of CBM3wt or CBM3mt. The CBM concentrations used were in the range 0.1–2 mg/ml. Afterwards, the papers were removed from the solutions and dried for approximately 24 h at room temperature. A drop of distilled water (3 µl) was applied onto the treated paper surface and the time for its complete absorption was measured in seconds using an optical Contact Angle System OCA (Dataphysics). The changes in drop shape over time were recorded with time lapses of 20 ms, and then analysed with SCA20 software. Paper treated in 20 mM Tris base, pH 7.0, was used as control. The assay was conducted twice for each CBM concentration and three measurements per each CBM3-treated paper were performed.

Treatment of paper pulps

Two industrial Portuguese paper pulps were used: elemental chlorine free (ECF) bleached *E. globulus* kraft pulp and a mixture of unbleached *E. globulus* and *P. sylvestris* (30:70) kraft pulp. *E. globulus* fibers were refined at 3000 revolutions in a PFI mill (ISO 5264/2) while the other pulp was used as supplied. Fibers were treated with the recombinant CBMs essentially as described in Machado et al. (2009). Briefly, the CBM3wt or CBM3mt was allowed to adsorb on the fibers by mixing vigorously the protein at the ratio of 2 or 4 mg per gram of pulp (relative to oven dry pulp, i.e. o.d.p.), using 10 g of fibers (relative to o.d.p.) in sodium acetate buffer (final volume of 400 ml), for 30 min at room temperature. Fibers were also treated in a similar way in the absence of the CBMs, to serve as control. After treatment, fibers were washed and filtered three times in sodium acetate buffer. Four independent assays were performed. Treated fibers were used to measure pulp drainability and to produce the laboratory handsheets.

Fluorescence microscopy

The adsorption of both recombinant CBMs (CBM3wt and CBM3mt) on *E. globulus* fibers was visualized by fluorescence microscopy using conjugates of CBMs with fluorescein isothiocyanate (FITC). FITC was dissolved in DMSO and allowed to react overnight with the CBM, at the ratio of 1:3 (CBM:FITC), at room temperature and with slow magnetic stirring. The FITC-CBM3 conjugates were purified using a PD-10 column (GE Healthcare Life Sciences), being eluted with PBS buffer. The incubation of the conjugated CBMs with the fibers was carried out using the conditions described in the previous subsection, but at an eppendorf scale and with fibers recovery by centrifugation (13,200 rpm for 5 min). Fibers used as control were treated in the presence of FITC only. Fibers were observed in a fluorescence microscope Olympus BX51 and the images were acquired with a colour camera Olympus DP72, using the Cell^B software (Olympus).

Analysis of pulp and paper properties

The Schopper-Riegler degree (°SR) method was used to measure pulp drainability and it was determined according to ISO 5267/1 standard method. Handsheet preparation and determinations of the paper properties were achieved according to the usual standard procedures: ISO 5636/3 1992 (F) (permeability); ISO 1924/2 1985 (F) (tensile strength); ISO 1947 (F) (tearing); ISO 2758 1983 (F) (bursting strength).

Results and discussion

Production and purification of CBM3wt and CBM3mt

Glycosylation in fungal CBMs has been suggested essential for the modification of paper pulps' properties, by improving fiber hydration (Machado et al. 2009; Pinto 2006). Glycosylation is the most common post-translational modification of proteins secreted by eukaryotic cells, occurring at asparagine residues in Asn-Xxx-Ser/Thr sequence motifs (N-linked) or serine and threonine residues (O-linked). The CBM3 from *C. thermocellum* CipA scaffolding protein has three potential N-glycosylation sites (Asn14, Asn68, and Asn124). Non-glycosylated CBM3, recombinantly produced in *E. coli*, was able to modify pulp properties only when modified by PEGylation (Machado et al. 2009). Thus, in this work we produced glycosylated CBM3 in the yeast *P. pastoris* to clarify the effect of glycans on CBM3's ability to modify the properties of cellulose fibers. A mutated version of CBM3 was also produced in the same host, in which the three potential N-glycosylation sites were completely removed, to serve as control (Table 1). Both glycosylated and non-glycosylated recombinant CBM3s were produced from *P. pastoris* in high amounts as soluble secreted proteins. After purification by IMAC, approximately 20 mg of pure recombinant CBM3s were obtained per liter of culture. In SDS-PAGE gels, the non-glycosylated version of the recombinant CBM3 (CBM3mt) presented a band with its calculated molecular weight (~18 kDa), while the putative glycosylated version (CBM3wt) presented a predominant smeared band with higher molecular weight (~35 kDa). Additional bands with lower molecular weights but equal or higher than 18 kDa were also observed, which may correspond to non-glycosylated or less glycosylated forms of CBM3wt, respectively. N-Glycosylation was confirmed by digestion with Endoglycosidase H (Endo H), which caused the reduction of all molecular weight bands to ~18 kDa, the predicted molecular weight of CBM3wt (Fig. 1). Wan et al. (2011) previously reported that only the Asn68 of CBM3wt was actually glycosylated when this CBM was produced in *P. pastoris* in fusion with the enhanced green fluorescent protein (EGFP). Therefore, considering that the N-glycans added by *P. pastoris* to other recombinant glycoproteins have been shown to generally contain two N-acetylglucosamines (0.4 kDa) plus 8–14 mannoses (1.3–2.3 kDa) (Montesino et al. 1999; Blanchard et al. 2007), N-glycosylation is estimated to account for around 10 % of the molecular weight of the recombinant CBM3wt produced in this yeast.

Impact of glycosylation on the binding properties of CBM3

Avicel (microcrystalline cellulose) was used to evaluate the capacity of CBM3wt and CBM3mt to bind insoluble forms of cellulose. The data presented in Fig. 2 reveal that the two CBMs bound efficiently to Avicel but had differences in their adsorption capacity, as shown by the analysis of the Langmuir adsorption isotherms. For CBM3mt, the CBM_{Max} and K_a values were equal to 6.72 μmol g^{-1} and 1.171 μM^{-1}, respectively, whereas the corresponding values for CBM3wt were 5.79 μmol g^{-1} and 1.581 μM^{-1}. This means that the glycosylated version of CBM3 (CBM3wt) has lower adsorption capacity (a reduction in the bound protein at saturation is noticeable in Fig. 2) and affinity for cellulose than the non-glycosylated version (CBM3mt). These results are in line

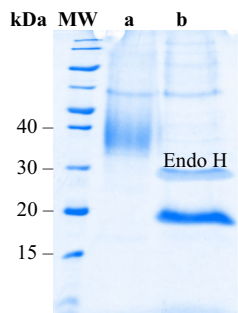

Fig. 1 SDS-PAGE analysis of CBM3wt before (*a*) and after (*b*) digestion with Endoglycosidase H (Endo H). MW, molecular weight standards

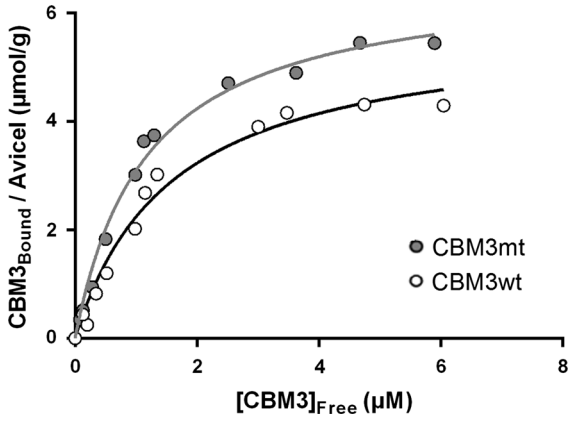

Fig. 2 Adsorption isotherms of CBM3mt and CBM3wt for Avicel. The values represent the average of two independent replicates

with those previously reported by Wan et al. (2011) for the binding of similar versions of CBM3, also produced in *P. pastoris* but in fusion with EGFP, to RAC (regenerated amorphous cellulose). The adsorption ability of CBM3wt-EGFP to RAC was 6–9 % lower than that obtained for CBM3mt-EGFP and non-glycosylated CBM3-EGFP produced in *E. coli* (Wan et al. 2011). Furthermore, the binding constant of CBM3wt-EGFP for RAC was 35 % lower than that determined for CBM3mt-EGFP, as observed in this work (Wan et al. 2011). The differences in the binding parameters of the two versions of CBM3 may be attributed to the presence of the glycans, which although not located in the binding site of this CBM (Tormo et al. 1996) possibly affect its conformation, and thus its accessibility to cellulose, as glycans are near the CBM3 binding face, according to the computational model shown on Fig. 3. Glycans may interfere with the binding of CBM3 to cellulose likely through a steric hindrance mechanism, since long glycans may hamper the interactions between the cellulose surface and the amino acid residues at the binding site (Bager et al. 2013; Chen et al. 2014). Indeed, large high mannose-type N-linked glycans (added by *P. pastoris*) near the binding face of a CBM2 were reported to have a detrimental effect on cellulose affinity (Boraston et al. 2001b). Still, it is worth of noting that glycans have been reported to produce different effects on CBMs: they may not affect (CBM2; Boraston et al. 2003), or they can either decrease (CBM2; Boraston et al. 2001a, b) or increase (CBM1; Chen et al. 2014; Taylor et al. 2012) the binding affinity of CBMs to cellulose, depending on the glycan localization and/or amount.

Effect of CBM3wt and CBM3mt on the wettability of Whatman paper

The effect of a range of concentrations of CBM3mt and CBM3wt on the surface properties of Whatman paper was analyzed by contact angles measurement. The results obtained are presented in Fig. 4, where it can be observed the different effects of CBM3mt and CBM3wt on the water adsorption time (WAT). The WAT of Whatman paper increased significantly with increasing CBM3mt concentrations (from 0.1 to 2 mg/ml), while only the higher CBM3wt concentrations tested (1.5 and 2 mg/ml) produced an effect similar to that observed with the lowest CBM3mt concentration (0.1 mg/ml). Thus, at least ten times more CBM3wt was needed to achieve the same moderate effect obtained with 0.1 mg/ml CBM3mt. At the highest concentration used (2 mg/ml), CBM3mt increased the Whatman paper WAT by 7.3-fold, and CBM3wt by 2.3-fold. Therefore, CBM3mt greatly reduced the wettability of papers but not CBM3wt. These results could be related to a different increase in the surface hydrophobicity of cellulose occupied by each CBM, as previously reported (Levy et al. 2002). Levy et al. (2002) showed that the treatment of Whatman paper with a recombinant CBM (originally from *C. cellulovorans*) and a double fusion of this CBM, both produced in *E. coli*, resulted in a moderate and high surface hydrophobicity increase, respectively. Moreover, as observed for CBM3mt, these effects were dependent on the CBM dosage, i.e. the hidrophobicity levels increased with increasing CBM concentrations. In this work, paper treated with non-glycosylated CBM3 (CBM3mt) was overall much more hydrophobic than paper treated with glycosylated CBM3 (CBM3wt). Thus, an effect of glycosylation is evident. Whilst CBM3 transforms the filter paper into a more water-repellent paper, the glycosylation seems to reduce that effect, i.e. it increases the hydration of treated fibers, as previously suggested (Machado et al. 2009; Pinto 2006).

Effect of CBM3wt and CBM3mt on paper pulps

The effect of the glycosylated CBM3 (CBM3wt) and non-glycosylated CBM3 (CBM3mt) on the drainability

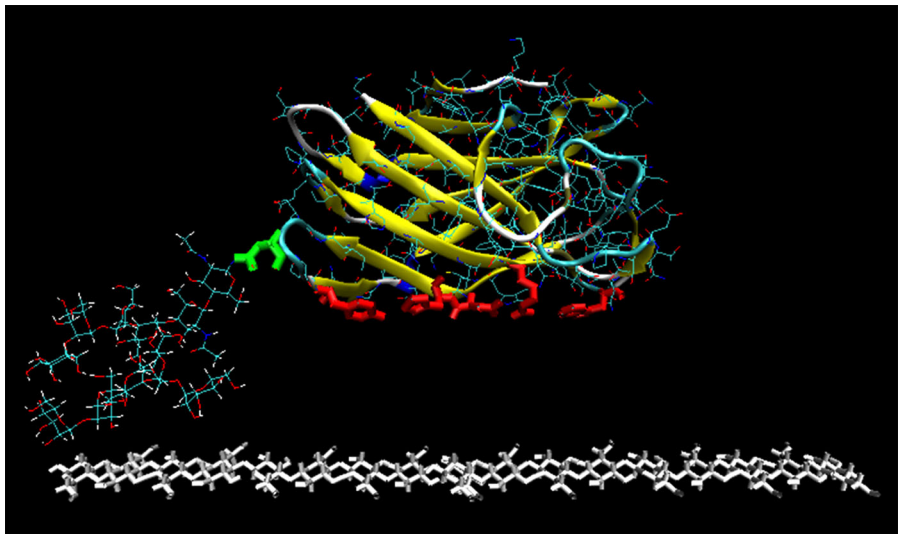

Fig. 3 Side view of the molecular model of CBM3wt carrying a high mannose-type N-glycan at the position Asn68 (shown in *green*) aligned above the top layer of a cellulose slab (shown in *white*). The amino acid residues described to interact with cellulose (Tormo et al. 1996) are shown in *red*

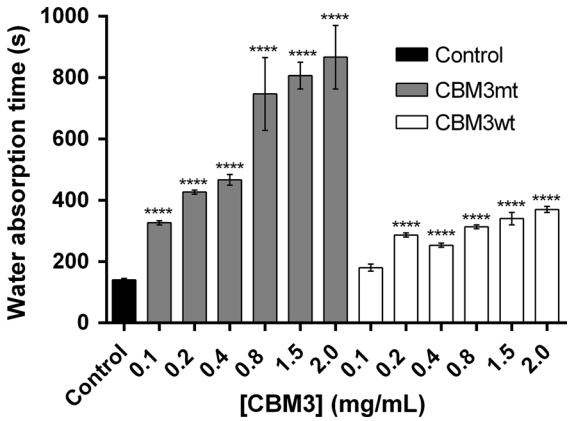

Fig. 4 Water adsorption time of Whatman paper treated with CBM3mt and CBM3wt at different concentrations (measured by contact angles). The control refers to paper treated with 20 mM Tris base, pH 7.0. The *bars* represent the mean ± SEM of two independent replicates. The statistical significance of the differences observed between the control and each condition was analyzed using a *t*-test. ****$P < 0.0001$

of bleached kraft pulp of *E. globulus* and unbleached kraft pulp containing eucalyptus and pine (30:70) was analyzed by measuring the Shopper-Riegler degree (°SR). Pulp drainability is the capacity of paper pulp to drain water. A reduction in the °SR values indicates a better water drainage. Enhancements in the drainage section may have an important positive impact on the global performance of the papermaking process, allowing relevant energy savings. The data revealed no reduction of the °SR values of fibers treated with CBM3mt, nor with the CBM3 glycosylated version, CBM3wt (Table 2). This result corroborates a previous work, in which the same CBM3 from *C. thermocellum*, but produced in *E. coli* and used at a lower dosage than here (1 mg/g o.d.p.), was also unable to modify the drainability of *E. globulus* or *P. sylvestris* pulps with a similar refining degree as we used in the *E. globulus* pulp (Machado et al. 2009).

Although CBM3mt and CBM3wt introduce distinct hydrophobicity levels on paper fibers (Fig. 4), no differences were detected between the °SR values of pulps treated with these CBMs (Table 2). Thus, glycosylation did not affect the drainability of the tested pulps. These results suggest that the modification of fiber drainage by CBM action may not be related with the hydration degree of fibers, contrarily to what was previously hypothesized. Glycosylated CBMs obtained by proteolysis of *T. reesei* cellulases were shown to be capable of improving the drainability of paper pulps (Pala et al. 2001; Pinto et al. 2004b). Pinto et al. (2004b) showed that the adsorption of a fungal CBM to pulp and paper produced a reduction of the °SR values to a maximum of 15 %, a result that has also been described for secondary fibers (Pala et al. 2001). It was suggested that the binding of the CBM to the fiber surface leads to a modification of the surface/interfacial properties of the fibers, therefore affecting the technical properties of the pulp and paper in a positive way. Drainage improvement was attributed to the dispersion of fiber fines, allowing the water to more freely flow between the fibers. According to Pinto (2006) the oligosaccharides attached to the

Table 2 Shopper-Riegler index (°SR) of paper pulps treated with CBM3mt and CBM3wt

Pulp	Shopper-Riegler index (°SR)		
	Control	CBM3mt	CBM3wt
E. globulus	29.0 ± 0.0	28.5 ± 0.3	28.3 ± 0.3
E. globulus and *P. sylvestris* (30:70)	53.4 ± 0.6	53.3 ± 0.4	52.7 ± 0.7

CBMs were applied at the ratio of 2 mg CBM/g o.d.p. The control refers to fibers treated with sodium acetate buffer. The values represent the average ± SEM of two independent replicates, each one performed in duplicate

CBMs (up to 30 % of the CBMs molecular weight) presumably have high water affinity and are capable of stabilizing the fibers, thereby reducing the interfiber interactions and contributing to a better water drainage. It is worth of noting that these CBMs were later described as having glycosylated linkers that alone fully bind to cellulose (Payne et al. 2013). Thus, it is not clear if the observed effect of these CBM-linker domains is due to the glycosylation of CBM, linker, or both. On the other hand, Machado et al. (2009) showed that recombinant CBM3 produced in *E. coli* could only improve the drainability of paper pulps (by reducing the °SR value up to 15.7 %) when modified with PEG, which as glycosylation is likely to be highly hydrated, thus reinforcing the idea of the oligosaccharide stabilizing effect. However, our results suggest that the modification of pulp drainage by the CBMs may be dependent on other factors rather than glycosylation, such as the CBM itself, i.e. different CBMs may cause distinct effects. In fact, reduction of pulp drainability by CBM action has also been reported in the literature. Cadena et al. (2010) showed that recombinant CBM3b produced in *E. coli*, originally from *Paenibacillus barcinonensis* endoglucanase Cel9B, increased drainage resistance of totally chlorine free kraft pulp from *E. globulus* by up to 9 %, without altering the water retention value in fibers. Therefore, the role of CBMs in the modification of pulp drainage may be CBM-specific and thus needs further elucidation.

In order to verify the adsorption of CBM3mt and CBM3wt on *E. globulus* fibers, these fibers were treated with FITC-labeled CBMs and observed by fluorescence microscopy. This also allowed characterizing the surface distribution. Representative images for the binding of each CBM are shown in Fig. 5. The *E. globulus* fibers appear to be completely covered by the CBM3mt (Fig. 5b) and CBM3wt (Fig. 5c), which is notable taking into account the short period of incubation (30 min) and the vigorous mixing applied. Furthermore, the adsorption of both CBMs on *E. globulus* fibers was heterogeneous: the CBMs were preferentially concentrated around the fiber's extremities and in some middle regions, as indicated by the brighter fluorescence in these places (Fig. 5d, e and f). This is a common observation for the binding of CBMs to cellulose fibers with different crystalline properties and/or surface areas. Pinto et al. (2006) reported that the distribution of glycosylated fungal CBMs (labeled with FITC) on Whatman CF11 fibers (crystalline cellulose) was not uniform, but concentrated instead around the fiber's extremities, which are characterized by disordered packing of the microfibrils (non-uniformity) that have a higher surface area available for CBM adsorption. Since CBM3 can bind amorphous and crystalline cellulose (Gao et al. 2014), although with different affinities (Hong et al. 2008), it is able to recognize both cellulose surfaces in *E. globulus* fibers. Our results may also indicate a higher concentration of adsorbing sites for CBM3 on *E. globulus* fibers extremities or higher affinity of CBM3 for these places.

Effect of CBM3wt and CBM3mt on *E. globulus* papersheets

The effect of CBM3wt and CBM3mt on the paper burst and tensile strengths, lengthening and tearing were studied, according to standard procedures. Bleached *E. globulus* pulp was treated with each CBM as described in the materials and methods section and used to produce the handsheets. Both recombinant CBM3s improved significantly the burst and tensile strengths of *E. globulus* papersheets, while lengthening and tearing were not significantly affected ($P > 0.05$) (Fig. 6). Previously, CBM3 was

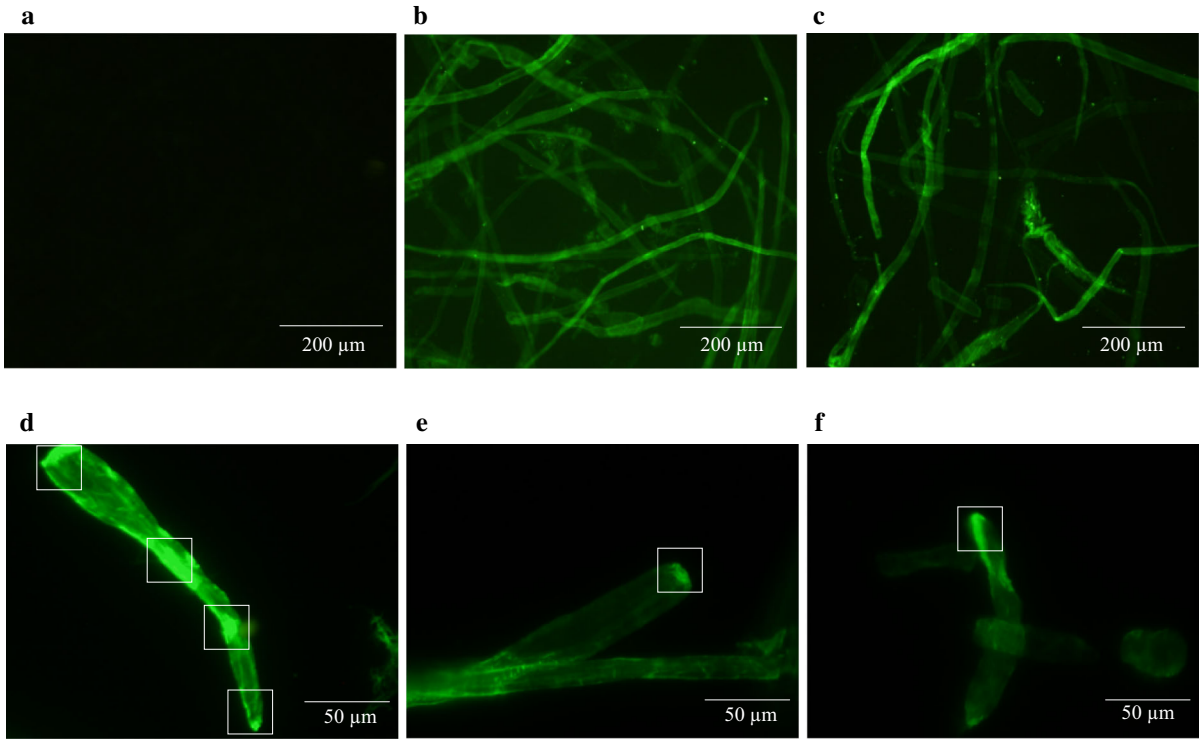

Fig. 5 Fluorescence microscopy of *E. globulus* fibers treated with FITC-labeled CBM3mt (**b**, **f**) and CBM3wt (**c**, **d**, **e**). Conjugated CBMs were applied at the ratio of 4 mg CBM/g o.d.p. In the control (**a**) (fibers treated with sodium acetate buffer containing FITC), no fluorescence was detected, as expected. The *white squares* indicate zones with higher brightness. The images were obtained using the same exposure time (200 ms)

demonstrated not to have any relevant effect on the properties of papers made of *E. globulus* or *P. sylvestris* pulps treated with this CBM (Machado et al. 2009). However, in this work CBM3 was applied in *E. globulus* pulps at a higher ratio (4 mg/g o.d.p.) than previously (1 mg/g o.d.p.). Furthermore, we observed smaller effects for the CBM3 ratio of 2 mg/g o.d.p. than for the ratio of 4 mg/g o.d.p. (data not shown), which altogether indicates that CBM3 affects the paper properties in a dose dependent manner.

The effects of CBM3wt and CBM3mt on burst and tensile strengths were identical. As it can be seen in Fig. 6, CBM3mt increased the burst strength index (by 12 %), whereas the effect of CBM3wt on this property was smaller (7 % increase), comparing to control. In addition, CBM3mt and CBM3wt significantly increased the tensile strength index, in about 10 % and 8 %, respectively. Although CBM3wt apparently causes a less marked increase on these two properties, the effects obtained with this CBM were not statistically different from those observed with CBM3mt. Thus, glycosylation is not essential for the modification of these paper properties by CBM3. Possibly as a result of increased fiber bonding, and thus paper strength, the air permeability of the papersheets was reduced by the recombinant CBM3s (by up to 8 %; data not shown). These results suggest that this CBM3 can crosslink *E. globulus* fibers in the cellulose web, increasing the amount of consolidated fibers in papersheets, thereby substantially raising air resistance, as previously shown for other CBM3 (Cadena et al. 2010).

Paper strength enhancement has been previously reported in some works using recombinant CBM-based approaches, in which other bacterial CBMs from family 3 have also been employed (Cadena et al. 2010; Levy et al. 2002, 2004; Shi et al. 2014). Among different double CBM fusions tested, recombinant CBM3-GS-CBM3 produced in *E. coli* (CBM3 from *C. thermocellum* CipB linked by the (G4S)3 linker), was the most effective in enhancing paper mechanical properties in terms of folding endurance (27.4 %) and tensile

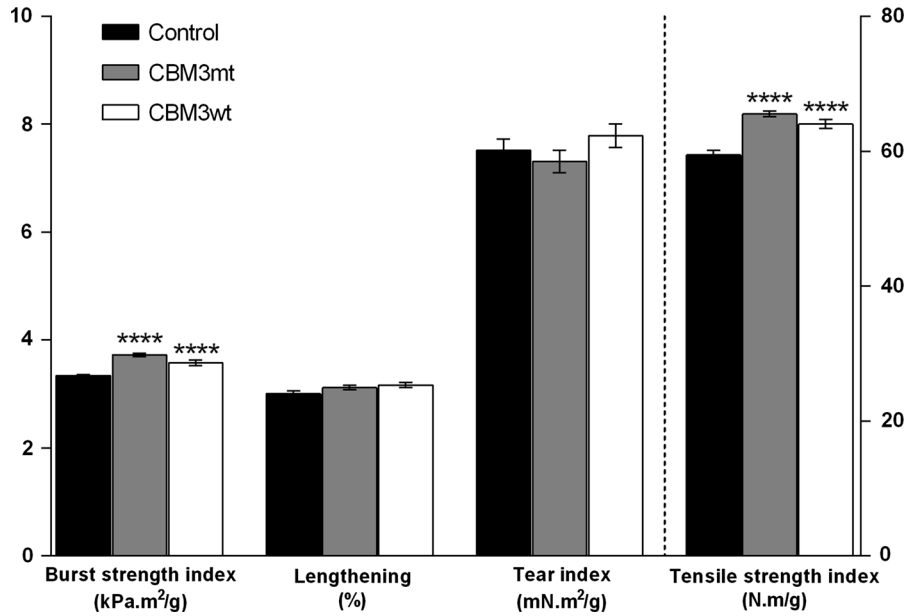

Fig. 6 Effect of CBM3mt and CBM3wt on the mechanical properties of *E. globulus* handsheets produced from pulp treated with these CBMs at the ratio of 4 mg CBM/g o.d.p. The control refers to pulp treated with sodium acetate buffer. The *bars* represent the mean ± SEM of four independent experiments. The statistical significance of the differences observed between the control and each condition was analyzed using a *t*-test. ****$P < 0.0001$

strength (15.5 %), but led to a slight increase in bursting strength (3.1 %), using a mixture of eucalyptus and pine kraft pulp in a ratio of 9:1 treated with 2.5 mg of CBM/g o.d.p. (Shi et al. 2014). Concerning the use of single CBMs, recombinant CBM3b, originally from *P. barcinonensis* endoglucanase Cel9B, increased tensile (4 %) and burst (6 %) indexes, using TCF kraft pulp from *E. globulus* treated with 2 mg of CBM/g o.d.p. (Cadena et al. 2010). In addition, recombinant CBM3b decreased tear index up to 7 % (Cadena et al. 2010). In this work, CBM3mt also slightly decreased tear index (Fig. 6), but this was not a statistically significant result. This work shows that a higher CBM ratio results in higher paper strength enhancement.

Conclusions

In this work, the effect of two different versions of CBM3 from *C. thermocellum* CipA produced in *P. pastoris* (glycosylated and non-glycosylated) on pulp and paper properties was evaluated to determine the importance of glycosylation in fiber modification. Characterization of these recombinant CBM3s revealed that glycosylation reduced the affinity of CBM3 for cellulose, as well as its hydrophobization effect on the surface of paper fibers. Both non-glycosylated and glycosylated CBM3 did not modify the drainability of the studied paper pulps but improved the burst and tensile strengths of the produced papers in a similar way. Therefore, glycosylation did not change these different effects of CBM3. On the other hand, this yet unknown capacity of CBM3 to enhance paper strength-related properties was found to be dependent on the CBM dosage used, being more pronounced at higher dosages.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Bager R, Johansen JS, Jensen JK, Stensballe A, Jendroszek A, Buxbom L et al (2013) Protein conformational change delayed by steric hindrance from an N-linked glycan. J Mol Biol 425:2867–2877

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Blanchard V, Gadkari RA, Gerwig GJ, Leeflang BR, Dighe RR, Kamerling JP (2007) Characterization of the N-linked

oligosaccharides from human chorionic gonadotropin expressed in the methylotrophic yeast *Pichia pastoris*. Glycoconj J 24:33–47

Bohne-Lang A, von der Lieth CW (2005) GlyProt: in silico glycosylation of proteins. Nucleic Acids Res 33:W214–W219

Boraston AB, McLean BW, Guarna MM, Amandaron-Akow E, Kilburn DG (2001a) A family 2a carbohydrate-binding module suitable as an affinity tag for proteins produced in *Pichia pastoris*. Protein Expr Purif 21:417–423

Boraston AB, Warren RAJ, Kilburn DG (2001b) Glycosylation by *Pichia pastoris* decreases the affinity of a family 2a carbohydrate-binding module from *Cellulomonas fimi*: a functional and mutational analysis. Biochem J 358:423–430

Boraston AB, Sandercock L, Warren RA, Kilburn DG (2003) O-glycosylation of a recombinant carbohydrate-binding module mutant secreted by *Pichia pastoris*. J Mol Microbiol Biotechnol 5(1):29–36

Cadena EM, Chriac AI, Pastor FIJ, Diaz P, Vidal T, Torres AL (2010) Use of cellulases and recombinant cellulose binding domains for refining TCF kraft pulp. Biotechnol Prog 26:960–967

Chen LQ, Drake MR, Resch MG, Greene ER, Himmel ME, Chaffey PK et al (2014) Specificity of O-glycosylation in enhancing the stability and cellulose binding affinity of Family 1 carbohydrate-binding modules. Proc Natl Acad Sci USA 111:7612–7617

Gao SH, You C, Renneckar S, Bao J, Zhang YHP (2014) New insights into enzymatic hydrolysis of heterogeneous cellulose by using carbohydrate-binding module 3 containing GFP and carbohydrate- binding module 17 containing CFP. Biotechnol Biofuels 7:24

Gomes D, Rodrigues AC, Domingues L, Gama FM (2015) Cellulase recycling in biorefineries: is it possible? Appl Microbiol Biotechnol 99:4131–4143

Hong J, Yea X, Wang Y, Zhang YH (2008) Bioseparation of recombinant cellulose-binding module-proteins by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Anal Chim Acta 621:193–199

Humphrey W, Dalke A, Schulten K (1996) VMD—visual molecular dynamics. J Molec Graphics 14:33–38

Kim DW, Jang YH, Kim CS, Lee NS (2001) Effect of metal ions on the degradation and adsorption of two cellobiohydrolases on microcrystalline cellulose. Bull Korean Chem Soc 22:716–720

Kitaoka T, Tanaka H (2001) Novel paper strength additive containing cellulose-binding domain of cellulase. J Wood Sci 47:322–324

Lemos MA, Teixeira JA, Mota M, Gama FM (2000) A simple method to separate cellulose-binding domains of fungal cellulases after digestion by a protease. Biotechnol Lett 22:703–707

Levy I, Nussinovitch A, Shpigel E, Shoseyov O (2002) Recombinant cellulose crosslinking protein: a novel paper-modification biomaterial. Cellulose 9:91–98

Levy I, Paldi T, Shoseyov O (2004) Engineering a bifunctional starch-cellulose cross-bridge protein. Biomaterials 25:1841–1849

Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495

Machado J, Araujo A, Pinto R, Gama FM (2009) Studies on the interaction of the carbohydrate binding module 3 from the *Clostridium thermocellum* CipA scaffolding protein with cellulose and paper fibres. Cellulose 16:817–824

Montesino R, Nimtz M, Quintero O, Garcia R, Falcón V, Cremata JA (1999) Characterization of the oligosaccharides assembled on the *Pichia pastoris*–expressed recombinant aspartic protease. Glycobiology 9:1037–1043

Oliveira C, Felix W, Moreira RA, Teixeira JA, Domingues L (2008) Expression of frutalin, an alpha-D-galactose-binding jacalin-related lectin, in the yeast *Pichia pastoris*. Protein Expr Purif 60:188–193

Oliveira C, Carvalho V, Domingues L, Gama FM (2015) Recombinant CBM-fusion technology: applications overview. Biotechnol Adv 33:358–369

Pala H, Lemos MA, Mota M, Gama FM (2001) Enzymatic upgrade of old paperboard containers. Enzyme Microb Tech 29:274–279

Payne CM, Resch MG, Chen L, Crowley MF, Himmel ME, Taylor LE 2nd et al (2013) Glycosylated linkers in multimodular lignocellulose-degrading enzymes dynamically bind to cellulose. Proc Natl Acad Sci USA 110:14646–14651

Pinto R (2006) Production of cellulose-binding domains by proteolysis; studies on the adsorption and modification of cellulose fibres. Ph.D. thesis, University of Minho, Braga

Pinto R, Moreira S, Mota M, Gama M (2004a) Studies on the cellulose-binding domains adsorption to cellulose. Langmuir 20:1409–1413

Pinto R, Amaral E, Costa AP, Gama FM, Duarte AP (2004b) Improving papermaking with cellulose-binding domains. CIADICYP 2004: congresso Iberoamericano de Investigacion en Cellulose y Papel, vol. 2004. Córdoba, Spain, pp 303–305. ISBN 84-7498-504-8

Pinto R, Carvalho J, Mota M, Gama M (2006) Large-scale production of cellulose-binding domains. Adsorption studies using CBD-FITC conjugates. Cellulose 13:557–569

Shi XR, Zheng F, Pan RH, Wang J, Ding SJ (2014) Engineering and comparative characteristics of double carbohydrate binding modules as a strength additive for papermaking applications. Bioresources 9:3117–3131

Taylor CB, Talib MF, McCabe C, Bu L, Adney WS, Himmel ME et al (2012) Computational investigation of glycosylation effects on a family 1 carbohydrate-binding module. J Biol Chem 287:3147–3155

Tormo J, Lamed R, Chirino AJ, Morag E, Bayer EA, Shoham Y et al (1996) Crystal structure of a bacterial family-III cellulose-binding domain: a general mechanism for attachment to cellulose. EMBO J 15:5739–5751

Wan W, Wang DM, Gao XL, Hong J (2011) Expression of family 3 cellulose-binding module (CBM3) as an affinity tag for recombinant proteins in yeast. Appl Microbiol Biotechnol 91:789–798

Yokota S, Matsuo K, Kitaoka T, Wariishi H (2009) Retention and paper-strength characteristics of anionic polyacrylamides conjugated with carbohydrate-binding modules. Bioresources 4:234–244