# Engineering and Comparative Characteristics of Double Carbohydrate Binding Modules as a Strength Additive for Papermaking Applications

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In this study, four engineered proteins containing two family 1 and/or family 3 carbohydrate binding modules (CBMs) were constructed and expressed as soluble forms in *Escherichia coli*. Their binding performances and effect on paper's mechanical properties were comprehensively studied with the aim to design suitably engineered CBMs as novel biomaterials for use in the production of new cellulose materials. The recombinant engineered double CBMs exhibited obvious differences in their adsorption to different cellulosic substrates. The CBM3-GS-CBM3 was the most effective in enhancing paper mechanical properties in terms of folding endurance (27.4%) and tensile strength (15.5%) among the four engineered double CBMs, but it gave rise to only a slight increase in bursting strength (3.1%). On the other hand, CBM1-NL-CBM1 achieved a significant simultaneous increase in tensile strength (12.6%) and burst strength (8.8%), as well as folding endurance (16.7%). Unexpectedly, CBM3-GS-CBM1 and CBM3-NL-CBM1 had the lowest effective paper property improvement. The differences in types of CBMs and linker peptides in engineered double CBMs may contribute to the considerable differences in their cellulose binding and paper property modification. Our data suggested that CBM1-NL-CBM1 may provide a better upgrade of the secondary pulp, which makes it very suitable for fiber recycling. Meanwhile, CBM3-GS-CBM3 may have particular potential for paper manufacture requiring high folding endurance.

*Keywords: Engineered double CBMs; Carbohydrate binding modules (CBMs); Paper mechanical properties; Surface properties*

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## INTRODUCTION

Paper is a network of cellulose fibres. The dry strength of a three-dimensional cellulose fiber network depends on the strength of the individual fibers, the bonds between fibers, the number of bonds, and the distribution of the bonds between the fibers (Xu *et al*. 1999). Paper strength additives are widely used in paper manufacturing to improve paper quality, especially when low quality fibres such as recycled papers were used to produce paper with good properties. Most paper strength additives commercially available are natural, partially modified, or synthetic polyelectrolytes, for example, cationic starches, polyacrylamides (PAMs), polydiallyldimethylammonium chloride, and polyamideamine-epichlorohydrin (PAE). However, the adsorption of these ionic water-soluble polymer additives on the pulp fibres is not specific and is easily influenced by large amounts of inorganic ions and anionic trash in the furnish. Nonionic polymer systems using polyethylene oxides and phenol formaldehyde resins have been applied to

improve the fines' retention and drainage efficiency under industrial conditions, but they make nearly no contribution to paper strength enhancement (Yokota *et al*. 2008).

Most cellulose and hemicellulose-degrading enzymes have a two-domain structure that consists of a catalytic domain and carbohydrate binding module (CBM) connected by a linker region (Tomme *et al*. 1995; Shoseyov *et al*. 2006; Guillén *et al*. 2010). The main role of CBMs in most cellulases is to recognise and bind specifically to cellulose in both the crystalline and amorphous forms, which is necessary for the efficient hydrolysis of insoluble substrates by these enzymes (Linder and Teeri 1997).

The strong affinity that exists between CBMs and cellulose can be used in paper industry applications (Levy and Shoseyov 2002). Pala *et al*. (2001) reported that the fibres treated with a single family 1 CBM from *Trichoderma reesei* cellulases exhibited a simultaneous increase in drainability as well as strength properties of secondary paper pulps. Anionic polyacrylamide (A-PAM) conjugated with family1 CBM from *T. reesei* cellulases (CBM-A-PAM) showed good retention on pulp fibres, resulting in high tensile strength paper sheets (Yokota *et al*. 2008). However, the effectiveness of CBMs in pulp and paper properties improvement were not always consistent in literature. It was reported that a single CBM3b from endoglucanase Cel9B of *Paenibacillus barcinonensis* had a slight effect on this property (Cadena *et al*. 2010). Compared with single family 3 CBM from *Clostridium cellulovorans* scaffolding gene (*cbpA*), the double family 3 CBMs (CCP) showed a more significant improving effect on mechanical and surface properties of Whatman paper sheets, thus suggesting that double CBMs may be more attractive paper-modification materials (Levy *et al*. 2002). Due to wide differences in substrate specificities and binding affinities of different CBMs, further in-depth comparative study about the performance of different double CBMs in pulp and paper treatment should be necessary to develop double CBMs as novel biomaterials for application in this field.

Family 1 and 3 CBMs are distinctive in size and binding properties (Lehtiö *et al*. 2003). Family 1 CBM has the smallest compact structure with only 33 to 40 amino acids, and it binds reversibly with cellulose, while family 3 CBM is the largest in size and binds irreversibly with cellulose. In this study, the family 1 and 3 CBMs from *Volvariella volvacea* and *C. thermocellum* were selected for construction of engineered double CBMs (Poole *et al*. 1992; Ding *et al*. 2006). The cellulose binding properties and the effect of engineered double CBMs on the paper handsheets properties were evaluated. The purpose of this study was to further understand the performance of family 1 and 3 CBMs on paper properties improvement, specifically to understand whether the differences in CBM size, binding capacities, and linker peptides of engineered double CBMs cause different changes in paper properties, in order to design suitable double CBMs as strength additive for papermaking application.

## EXPERIMENTAL

### Strains and Plasmids

*Escherichia coli* DH5α was used as the bacterial cloning host. *E. coli* origami (DE3) and BL21 (DE3) (Novagen) were used as hosts for the production of chimeric proteins. Plasmid pCR2.1 (Invitrogen) was used as cloning vector. Plasmid pET-32b (Novagen) was used for gene expression in *E. coli.*

## Construction of Genes Encoding Double CBMs

Four different genes containing double CBMs were constructed in this study (Fig. 1A): (1) *cbm3-gs-cbm1* consisting of family 3 CBM from *Clostridium thermocellum* YS cipB (CBM3, Genbank No. X68233.1) (Poole *et al*. 1992) and family 1 CBM from *Volvariella volvacea cbh 1-1*(CBM1, GenBank No. AY559102) (Ding *et al*. 2006) linked with $(GGGGS)_3$ peptide; (2) *cbm3-nl-cbm1* consisting of CBM3 and CBM1 linked with native linker from *cbh 1-1*; (3) *cbm3-gs-cbm3* consisting of double CBM3 linked with $(GGGGS)_3$ peptide ; and (4) *cbm1-nl-cbm1* consisting of double CBM1 linked with native linker from *cbh 1-1*.

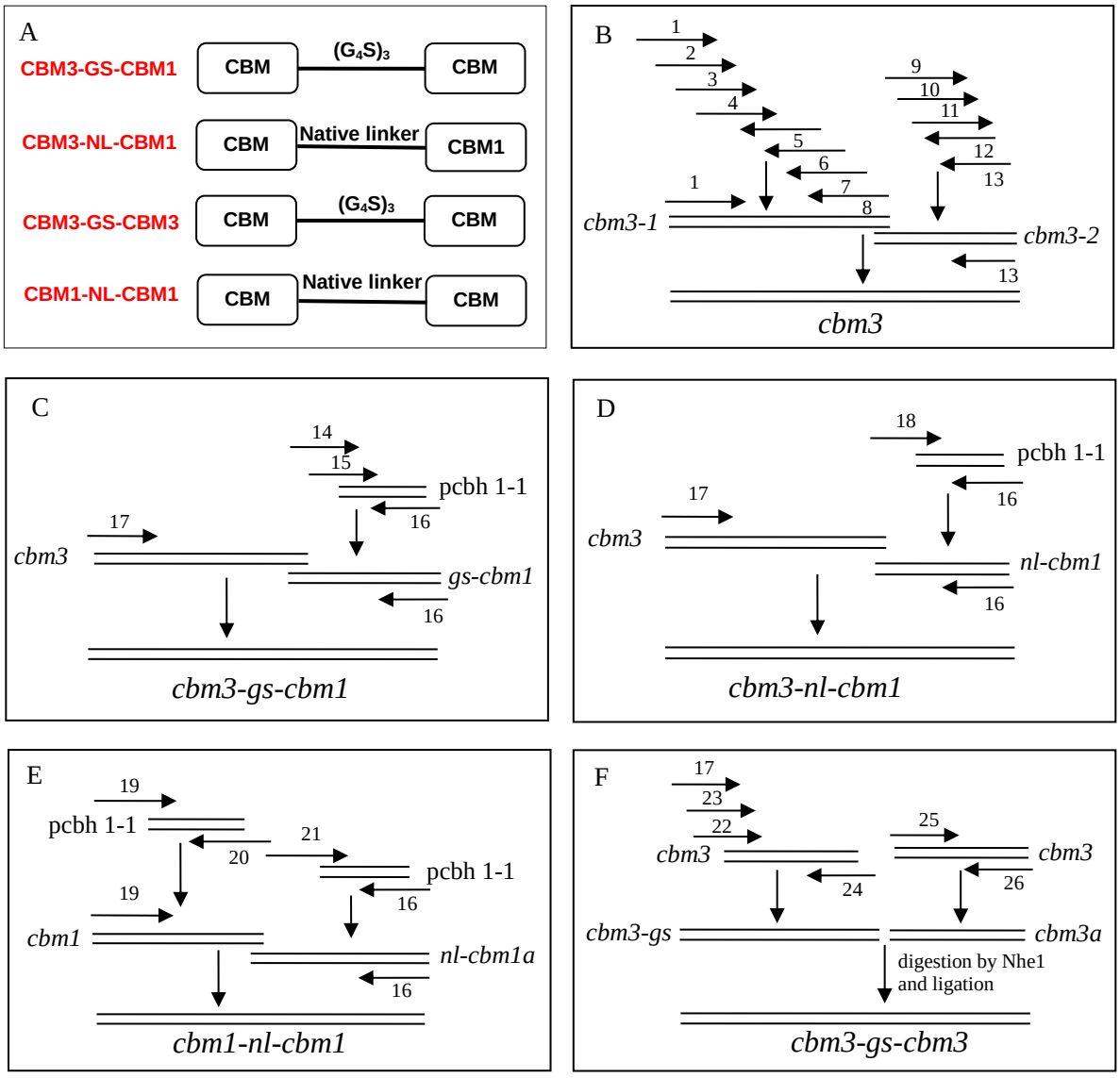


**Fig. 1**. Schematic structures of double CBMs (A), and PCR strategy for the amplification of single and double CBMs genes encoding for *cbm3* (B); *cbm3-gs-cbd1* (C); *cbm3-nl-cbm1* (D); *cbm1-nl-cbm1* (E); and *cbm3-gs-cbm3* (F).

All DNA manipulations for these genes construction were performed using standard protocols. The *cbm3* encoding for CBM3 was amplified by two-step primer extension PCR using thirteen codon optimised primers (1-13) and PrimeSTAR HS DNA polymerase (TaKaRa). Firstly, the fragments *cbm3-1* and *cbm3-2* were PCR-amplified using primers 1-8 and 9-13, respectively, then the full length of *cbm3* was amplified by overlapping PCR using primers 1 and 13, and *cbm3-1* and *cbm3-2* as template (Fig.1B). For gene *cbm3-gs-cbm1*, the fragment *gs-cbm1* encoding for CBM1 and $(GGGGS)_3$ linker was amplified by PCR using primers 14-16 and the pBluescript II KS-cbh1-1 (pcbh1-1) as template. Then the *cbm3-gs-cbm1* was amplified by overlapping PCR using primers 16 and17, and *cbm3* and *gs-cbm1* as the template (Fig. 1C). For *cbm3-nl-cbm1*, the fragment *nl-cbm1* encoding CBM1 and its native linker was amplified by PCR using primers 16 and 18, and the vector pcbh1-1 as template, then full length of *cbm3-nl-cbm1* was amplified by overlapping PCR using primers 16 and 17, and the fragments *cbm3* and *nl-cbm1* as template (Fig. 1D). Similarly, the fragments *cbm1* and *nl-cbm1a* were amplified respectively by PCR using primers 19 and 20, and 16 and 21, and using the vector pcbh1-1 as template, the full length of *cbm1-nl-cbm1* was then amplified by overlapping PCR using primers 16 and 19, and fragments *cbm1* and *nl-cbm1a* as template (Fig.1E). For *cbm3-gs-cbm3*, the fragment *cbm3-gs* and *cbm3a* with restriction site Nhe1 at 3'- and 5'-end respectively, were amplified by PCR using fragment *cbm3* as template, and primers 17, 22, 23, and 24 or primers 25 and 26, respectively, then digested with Nhe1 and ligated each other with ligase (Fig.1F). Each gene construct, containing two restriction endonuclease sites Nco 1 and Not 1 at 5' and 3' end respectively, was subcloned into pCR2.1. After being confirmed by sequencing, the generated recombinant plasmids were digested with Nco 1 and Not 1 and the excised products were cloned into expression vector pET32b (Novagen), previously digested with the same restriction enzymes to yield the constructs pET-32b-CBM3-GS-CBM1, pET-32b-CBM3-NL-CBM1, pET-32b-CBM3-GS-CBM3, and pET-32b-CBM1-NL-CBM1, respectively. These vectors carried a $T_7$ promotor and the resulting fusion proteins contained an N-terminal Trx•Tag and a C-terminal $His_6$-tag to facilitate soluble expression and purification. The oligonucleotides used for construction of the double CBMs genes were illustrated in Table 1.

## Expression of Four Double CBMs

Different optimal induction conditions were used to obtain soluble expression of engineered double CBMs. At first, the transformed *E. coli* cells were grown in Luria-Bertani medium containing Ampicillin (100 μg/mL) to an $A_{600}$ of 0.6 at 37 °C. Then induction was initiated by adding different concentration of isopropyl-β-D-thiogalactoside (IPTG) into cultures, and cells were continued to grow at different temperatures as described below. For CBM3-GS-CBM1 and CBM3-GS-CBM3, IPTG was added to a final concentration of 1 mM and induced at 30 °C. For CBM3-NL-CBM1, the concentration of IPTG was reduced to 0.8 mM and induced at 30 °C. For CBM1-NL-CBM1, the induction temperature and concentration of IPTG were decreased to 20° C and 0.4 mM respectively. After 6 h induction, the cells were harvested and re-suspended in 20 mM Tris buffer (pH 8.0), and disrupted by sonication. After centrifugation (10,000 g, 30 min), the crude soluble extracts were applied to a 1-mL HiTrap metal chelating column (Qiagen) charged with $Ni^{2+}$.

**Table 1.** Primers Used for the Construction of Double CBMs

| | | |
|---|---|---|
| 1 | CMB3F1： | GCGAACACCCCAGTCAGCGGCAATCTCAAAGTAGA ATTTTATAATAGCAATCCGT |
| 2 | BCBM3F2： | TTTATAATAGCAATCCGTCGGATACAACGAACAGTA TTAACCCGCAGTTTAAAGT |
| 3 | CBM3F3： | AACCCGCAGTTTAAAGTAACGAACACCGGCTCAAG TGCTATTGACTTGAGTAAAC |
| 4 | CBM3F4： | GTGCTATTGACTTGAGTAAACTGACCCTGCGCTACT ATTACACCGTGGACGGACA |
| 5 | CBNM3R1： | GGCGTGATCGCACCAAAATGTTTGATCTTTCTGTCC GTCCACGGTGTAATAGTAG |
| 6 | CBM3R2： | TATGCCATTATAGCTGCCATTCGAGCCAATGATTGC GGCGTGATCGCACCAAAAT |
| 7 | CBM3R3： | GCTCATTTTCACGAAAGTACCTTTAACGTTGCTCGT TATGCCATTATAGCTGCCA |
| 8 | CMB3R4： | GATCTCCAGATAGGTGTCCGCGTTATTAGTTGAGG AGCTCATTTTCACGAAAGTA |
| 9 | CBM3F21： | GGACACCTATCTGGAGATCAGCTTCACCGGGGGTA CGCTGGAACCTGGCGCCCAT |
| 10 | CBM3F22： | ACGCTGGAACCTGGCGCCCATGTCCAGATTCAAGG GCGCTTTGCGAAAAATGATT |
| 11 | CBM3F23： | AAGGGCGCTTTGCGAAAAATGATTGGTCTAACTACA CCCAGTCTAACGATTATAG |
| 12 | CBM3R21： | GATCCCATTCAACAAACTGCGAACGGGACTTGAAG CTATAATCGTTAGACTGGGT |
| 13 | CBM3R22： | CCCCAAACAAGCACACCATTTAAGTATGCAGTCACC TGATCCCATTCAACAAACT |
| 14 | CBM3(GGGGS)cele12CBMF1 | GAACCGGGAGGCTCAGTCGTGGGTGGCGGTGGCT CGGGCGGTGGTGGGTCGGGT |
| 15 | CBM3(GGGGS)cele12CBMF2 | GGCGGTGGTGGGTCGGGTGGCGGCGGATCCGCT GTCAGACCAAGGGGGT |
| 16 | cele12linkerCBMR1Not1 | CGAGTGCGGCCGCGATACACTGGCTGTACCATTG |
| 17 | CBM3 celc12CBMNco1 | GCGGTTCCATGGCGAACACCCCAGTCAGCGGC |
| 18 | cele12linkerCBMF1CBM3 | GAACCGGGAGGCTCAGTCGTGGGCACTACCTACAC TGGCGGTTCCGT |
| 19 | cele12CBMF1Nco1 | AAGGCCATGGCTGTTCAGACCAAGTGGGGT |
| 20 | cele12CBDR1 | GGAACCGCCAGTGTAGGTAGTGCCGATACACTGGC TGTACCATTGCCTTGGCGG |
| 21 | cele12linkerCBDF1 | GGCACTACCTACACTGGCGGTTCCGT |
| 22 | CBM3(GGGGS)CBM3-F1 | GAACCGGGAGGCTCAGTCGTGGGTGGCGGTGGCT CGGGCGGTGGTGGGTCGGGT |
| 23 | CBM3(GGGGS)CBM3-F2 | TCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCC GCGAACACCCCAGTCAGCGGC |
| 24 | CBM3 (GGGGS)CBM3Nhe1 | GCTATCGCTAGCGGATCCGCCGCCACCCG |
| 25 | CBM3 celc12CBMNhe1 | GATAAGCTAGC GCGAACACCCCAGTCAGCGGC |
| 26 | CBM3(GGGGS)CBM3Not1 | CGAGTGCGGCCGCCACGACTGAGCCTCCCGGTTC |

The protein was eluted with a linear gradient of 0 to 300 mM imidazole containing 0.5 M NaCl and 20 mM Tris-HCl (pH 8.0). The eluted protein fractions were dialysed against PBS buffer (NaCl 137 mmol/L, KCl 2.7 mmol/L, $Na_2HPO_4$ 10 mmol/L, and $KH_2PO_4$ 2 mmol/L, pH 8.0) to remove salts and imidazole. The expression of engineered double CBMs was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%, SDS-PAGE). The protein concentration was determined by measuring the absorbance at 280 nm and calculated with the extinction co-efficients ($\varepsilon$(CBM3-GS-CBM1)=68255$M^{-1}cm^{-1}$, $\varepsilon$(CBM3-NL-CBM1)=69745$M^{-1}cm^{-1}$, $\varepsilon$(CBM3-GS-CBM3)=85300$M^{-1}cm^{-1}$, and $\varepsilon$(CBM1-NL-CBM1)=52700$^{-1}cm^{-1}$).

## Binding Properties Assay

The binding of the engineered double CBMs to cellulosic substrates were assayed in Eppendorf tubes containing different concentrations of recombinant proteins (0 to 2.0 mg) and cellulosic substrates including Avicel (PH-101, Sigama) and filter paper (Whatman No.1, Maidstone, UK) with a concentration of 10 mg/mL in 1 mL of buffer (50 mM potassium phosphate, pH 7.0). Incubation was done at 4 °C with constant shaking in inverting shaker (150 rpm). After 1 h, cellulosic substrates were removed by centrifugation (13,000 g, 30min, 4 °C), and the free protein concentration left in the supernatant was determined. The bound protein concentrations were calculated by subtracting free enzyme concentrations from total enzyme concentrations. Each experiment was done in triplicate. The equilibrium association constants and target binding capacity were determined by nonlinear regression of bound versus free protein concentrations to Langmuir isotherm based on the equation as follows,

$$[B] = \frac{[N_0]K_a[F]}{1+K_a[F]} \quad (1)$$

where $[N_0]$ is the concentration of the total available binding sites in the absence of ligand (μmol/g cellulose), $[F]$ the concentration of free (unbound) protein (μmol/L),$[B]$ the concentration of bound protein(μmol/g cellulose), and $K_a$ the binding constant (μmol/L)

## Effect of Double CBMs on Paper Mechanical Properties

At first, eucalyptus and pine kraft pulps were refined using a Valley beater to 50 ºSR (Schopper-Riegler index, ºSR) and 37 ºSR, respectively. The refining process in the Valley beater follows ISO 5264-1:1979. Then, the pulp mixture containing eucalyptus and pine kraft pulp in a ratio of 9:1 was suspended in the PBS buffer at 0.5% consistency and disintegrated for 10 min. After the disintegration step, the recombinant proteins (2.5 mg per gram of dried fibres) were added and the mixture was continuously slowly mixed at 22 to 25 ºC for 30 min. Handsheet preparation (with a nominal grammage of 70 g/$m^{-2}$) and determinations of the pulp and paper properties were achieved according to the usual standard procedures: burst strength (ISO2758), tensile strength (ISO 1924/2), folding endurance (ISO 5626), and sheet density (ISO 534). Pulp samples treated under identical conditions in the absence of engineered double CBMs were used as controls.

## Water Absorption Assay

Water absorption was assayed by measuring the absorption time of a water drop in the treated papers, as described by Levy *et al.* (2002). A drop of distilled water (3 μL) was pipetted onto the engineered double CBMs treated paper surface; the changes in drop

shape over time were recorded with time lapses of 20 ms using an optical contact angle meter, CAM2000 (KSV Instruments, Helsinki, Finland), and then analysed frame by frame with the GBX software (Windrop, GBX, France). At least five measurements per sample were carried out.

## RESULTS AND DISCUSSION

### Expression of Double CBMs

Four kinds of genes encoding double CBMs, differing in types of CBMs and linker peptides, were constructed in this study to demonstrate the variety in their binding capacities for different cellulosic substrates and their effects on pulp and paper mechanical properties. SDS-PAGE analysis of total cell proteins of *E. coli* cells transformed with the individual construct showed successful expression of all engineered double CBMs, and their molecular sizes were in good agreement with those deduced from the nucleotide sequences (Fig. 2A, B, C, and D).

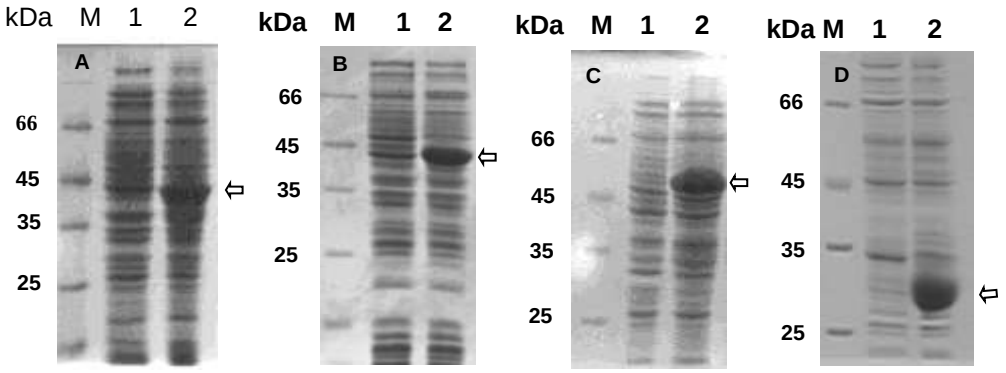


**Fig. 2.** SDS-PAGE analysis of expression of the CBM3-GS-CBM1(A), CBM3-NL-CBM1(B), CBM3-GS-CBM3(C), and CBM1-NL-CBM1(D) in *E. coli.* M: Protein marker; lane 1: before induction; lane 2: after induction. Arrows indicate the target protein bands.

It was found that different optimal concentrations of IPTG and temperatures were needed to obtain soluble expression of each specific protein. The engineered double CBMs including CBM3-GS-CBM1, CBM3-NL-CBM1, and CBM3-GS-CBM3 were partially expressed as soluble forms in the supernatant of cytoplasmic fraction when the recombinant *E. coli* were induced by 1 mM and 0.8mM IPTG at 30 ºC, respectively (data not shown). In contrast, CBM1-NL-CBM1 formed inclusion bodies in *E. coli* when it was expressed at above induction temperatures; soluble expression of CBM1-NL-CBM1 was obtained when the concentration of ITPG and induction temperature were reduced to 0.4 mM and 20 ºC, respectively (data not shown).

Based on the findings just noted, the soluble engineered double CBMs CBM3-GS-CBM1, CBM3-NL-CBM1, and CBM3-GS-CBM3 were produced by inducing the cells at 30 ºC, while soluble CBM1-NL-CBM1 was produced by growing the cells at 20 ºC. All soluble engineered double CBMs were further purified from the supernatant of cell lysate of *E. coli* with Ni-NTA chelating affinity column chromatography and used in the following experiment. The purity of engineered double CBMs was confirmed on SDS-PAGE, as shown in Fig. 3.

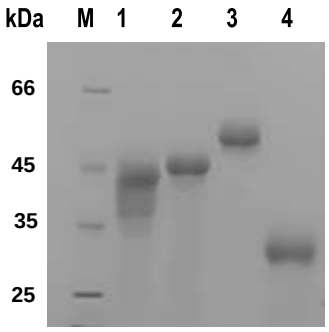


**Fig. 3.** SDS-PAGE of purified four engineered double CBMs. M: Protein marker; 1: CBM3-GS-CBM1; 2: CBM3-NL-CBM1; 3: CBM3-GS-CBM3; 4: CBM1-NL-CBM1

## Binding Properties of Double CBMs on Insoluble Cellulosic Substrates

The binding isotherms of four engineered double CBMs towards insoluble cellulosic substrates, including Avicel and filter paper, are shown in Fig. 4. The binding isotherm showed that the binding curves of all engineered double CBMs to Avicel and filter paper were non-linear. The one binding site Langmuir isotherm model is widely used to describe the binding kinetic properties of various cellulase−cellulose systems (Guo and Catchmark 2013). In both cases, experimental data fitted well to one binding site Langmuir model kinetics with satisfactorily high relation coefficients ($R^2$>0.98). Thus, the equilibrium association constants ($K_a$) and target binding capacity ($N_0$) were determined by nonlinear regression of bound versus free protein concentrations expressed as a Langmuir isotherm (Table 2). The equilibrium constant ($K_a$) is an index for estimating the binding affinity, where higher values of the $K_a$ are indicative of higher binding affinity of the double CBMs for the specific substrate. From Table 2, it seems that the adsorption of CBMs to cellulose is greatly influenced by the cellulose structure and type of CBMs. The $K_a$ value for adsorption of double CBMs to cellulose decreases with the decreased crystalline contents in cellulose substrates. The equilibrium constant values estimated for all double CBMs on crystalline cellulose Avicel were higher than the corresponding values on filter paper, which contains the both crystalline and amorphous cellulose, indicating that both CBM1 and CBM3 have a higher binding affinity to crystalline cellulose than amorphous cellulose. The $K_a$ value of CBM3-GS-CBM3 was over 4-fold higher than CBM1-NL-CBM1 (0.281 *vs.* 0.060) to Avicel, suggesting CBM3 showed much higher binding affinity to crystalline cellulose than that of CBM1. Values of binding capacity ($N_0$) provide information concerning the maximum number of substrate sites that are available for adsorption. The $N_o$ values of all four double CBMs on filter paper were much higher than those estimated for Avicel. These higher values were probably due to that the filter paper had more accessible surface area per unit mass available to CBMs than does Avicel, which is consistent with previous reports (Morag *et al*. 1995). On another hand, the protein size is also a key variable in determining binding capacity, since the $N_o$ values of all four double CBMs on Avicel decreased with increasing molecular weight. However, the trends of the double CBMs for filter paper are inconsistent with those for Avicel, indicating some factor other than the molecular weight that might be responsible for this phenomenon (Bothwell *et al*. 1997).

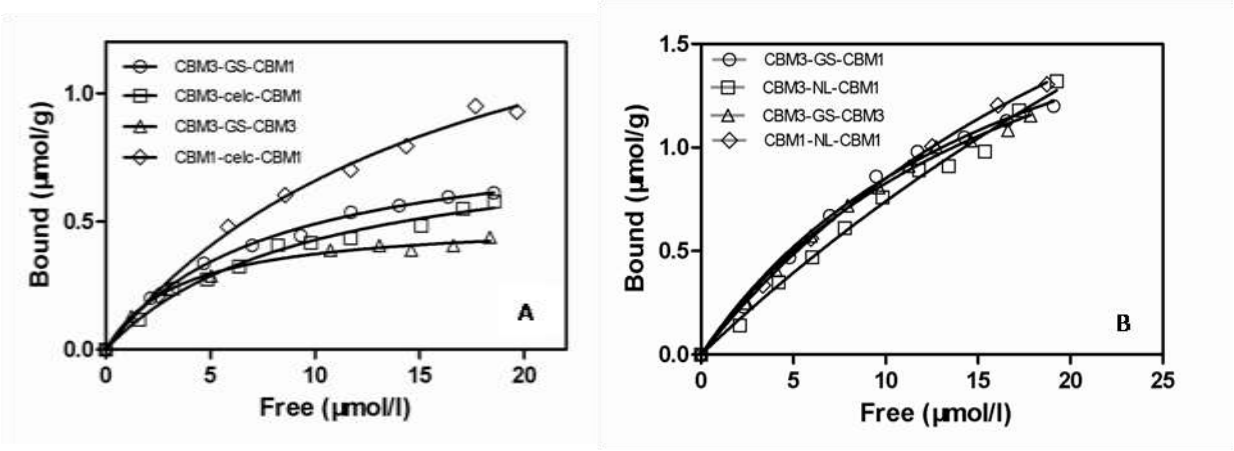


**Fig. 4.** Binding isotherms of four engineered double CBMs to Avicel (A) and Whatman filter paper (B).

**Table 2.** Binding Parameters for Double CBMs to Avicel and Filter Paper

| Best-fit values / proteins | $N_0$ (µmol/g) | | $K_a$ ( µmol$^{-1}$ ) | | Goodness of Fit ($R^2$) | |
|---|---|---|---|---|---|---|
| | Avicel | Filter paper | Avicel | Filter paper | Avicel | Filter paper |
| CBM3-GS-CBM1 | 0.864 | 2.351 | 0.131 | 0.057 | 0.9959 | 0.9955 |
| CBM3-NL-CBM1 | 0.839 | 5.835 | 0.104 | 0.015 | 0.9881 | 0.9915 |
| CBM3-GS-CBM3 | 0.505 | 2.355 | 0.281 | 0.054 | 0.9951 | 0.9967 |
| CBM1-NL-CBM1 | 1.760 | 3.515 | 0.060 | 0.032 | 0.9943 | 0.9998 |

Both family 1 and 3 CBMs, though distinctive in size, belong to type A CBMs, which have a flat or platform-like hydrophobic surface composed of aromatic residues responsible for binding with the flat surfaces of crystalline polysaccharides such as cellulose or chitin. The binding properties of single family 1 and 3 CBMs have been well studied, but the binding behaviour of double CBMs to cellulosic substrates is still not completely understood. Linder *et al*. (1996) postulated a two-step model to explain the binding behaviour of the double family 1 CBMs. In the first step, either of the domains binds with an affinity equal to that of a single domain. In the second step, which is referred to as the unimolecular step, the binding affinity is influenced by its proximity to the surface and steric constraints exerted by the linker (Linder *et al*. 1996). The considerable differences in binding properties of two engineered double CBMs CBM3-GS-CBM3 and CBM1-NL-CBM1 may be mainly attributed to the distinctive in size and innate binding specificity of different carbohydrate-binding modules (CBMs) (Boraston *et al*. 2004). The peptide linkers are important for fusion protein domains conformation (George and Heringa 2002). The flexible $(GGGGS)_3$ peptide was often used as synthetic linker to separate functional domains of bi- or multifunctional fusion proteins or chimeric antibodies (Lu *et al*. 2008).

In this study, the $(GGGGS)_3$ linker and native linker were used in a fusion of two CBMs. Similar equilibrium constant ($K_a$) and binding capacity ($N_0$) were observed for CBM3-GS-CBM1 and CBM3-NL-CBM1 to Avicel, indicating that both linkers were effective in separating the bi-domains. But there were obvious differences imparted to the filter paper, suggesting that linker peptides may have some effect on the binding properties of engineered double CBMs to especially for cellulosic materials containing amorphous cellulose.

## Effect of Double CBMs on Paper Mechanical Properties

The effects of four engineered double CBMs on paper mechanical properties were determined by measuring the mechanical properties of the treated papers, as shown in Fig. 5. The mean values of tensile strength, folding endurance, and burst strength were calculated from at least 10 measurements.

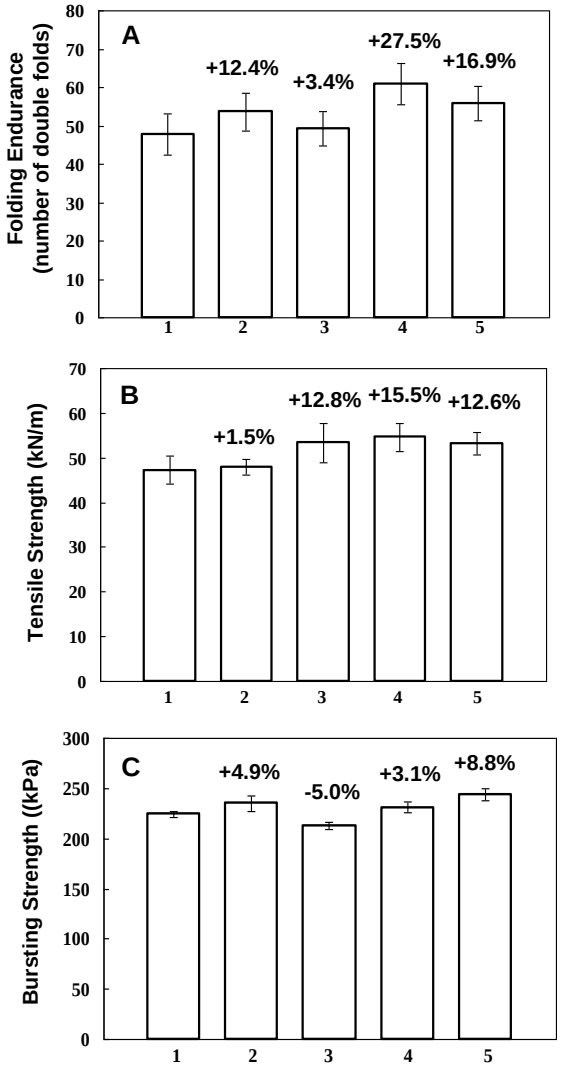


**Fig. 5.** The effect of four engineered double CBMs on the paper's folding endurance (A), tensile strength (B), and bursting strength (C). 1, control paper; 2-5, papers treated with CBM3-GS-CBM1, CBM3-NL-CBM1, CBM3-GS-CBM3, and CBM1-NL-CBM1, respectively.

The folding endurance values of the papers treated with CBM3-GS-CBM1, CBM3-NL-CBM1, CBM3-GS-CBM3, and CBM1-NL-CBM1 had 12.3%, 3.34%, 27.4%, and 16.7% increases compared with control. The increases of 1.5%, 12.8%, 15.5%, and 12.6% for the tensile strength were also observed for the CBM3-GS-CBM1, CBM3-NL-CBM1, CBM3-GS-CBM3, and CBM1-NL-CBM1 treated papers respectively. Smaller increases in burst strength, with values of 4.9%, 3.1%, and 8.8%, were detected for the CBM3-GS-CBM1, CBM3-GS-CBM3, and CBM1-NL-CBM1 treated papers respectively, but a 5.0% decrease in burst strength was observed for CBM3-NL-CBM1 treated paper.

The CBM3 from *Clostridium thermocellum* CipB is a very well characterised family 3 CBM with respect to its binding properties (Poole *et al*. 1992; Morag *et al*. 1995). Its binding properties with common substrates were found to be very similar to that of the CBM3 from *C. cellulovorans* YS cipB, though their sequences have 50% identity. It has been demonstrated that the recombinant double family 3 CBMs (CCP) from *C. cellulovorans* significantly improved paper tensile strength and water-repellent properties by increasing the inter fibre bonding between the cellulose chains in the formed paper (Levy *et al*. 2002). However, one limitation with this experiment is that the paper treatment was not carried out according to the currently used standard papermaking method. In normal papermaking research, strength-enhancing polymers are adsorbed on cellulose fibres. Paper is made from the fibres instead of exposing the existing fibre-fibre joints of Whatman filter paper to protein solution. In this study, we comprehensively evaluated the effect of four engineered double CBMs containing both CBM1 or/and CBM3 on paper properties according to standard papermaking method. Our research clearly demonstrated engineered double CBMs have potential in improving the paper mechanical properties, specifically folding strength and tensile strength compared with control sample under standard papermaking procedure. However, the effects may be relied on the kinds of the CBMs and linker peptides. The CBM3-GS-CBM3 displayed the most effective in improving the treated papers properties among these four engineered double CBMs in terms of folding strength and tensile strength, but only slightly increasing in bursting strength. CBM1-NL-CBM1 showed less effective for enhancing the folding endurance and tensile strength but effective for enhancing the bursting strength. In both treatments, the differences were statistically significant ($P<0.05$). Unexpectedly, both CBM3-GS-CBM1 and CBM3-NL-CBM1 revealed the lowest effective for paper properties improvement, suggesting homologous hybrid double CBMs are more suitable than heterologous double CBMs as a strength additive for papermaking applications.

The dry strength of a three-dimensional cellulose fibre network depends on the strength of the individual fibres, the bonds between fibres, the number of bonds, and the distribution of the bonds between the fibres (Xu *et al*. 1999). Long and flexible fibres provide high folding endurance. It was reported that the interfacial system of fibre-water-fibre, and after drying, fibre-air-fibre, may be affected by the single CBM treatment due to their adsorption to the fibre surface, therefore influencing the pulp and paper technical properties (Ciolacu *et al*. 2010; Pala *et al*. 2003). It was postulated that the large crosslinking molecules can reinforce the fibre-to-fibre bonds, thus resulting in a marked increase in dry strength (Xu *et al*. 1999; Ciolacu *et al*. 2010). The action of the double cellulose-binding domains on the fibres is still incompletely understood, but it has been hypothesized that the double CBMs (CCP) can efficiently crosslink between fibers

in the cellulose web. This leads to improvement of paper mechanical properties, to a higher extent when compared to single CBM (Levy and Shoseyov 2002). In this study, the significant improvement of folding endurance, together with tensile strength by CBM3-GS-CBM3 was achieved, which further supported this hypothesis (Levy *et al*. 2002). High folding endurance is a requirement for some papers, such as bond, ledger, currency, map, blueprint, and record papers. Our data suggested that CBM3-GS-CBM3 containing double engineered family 3 CBMs fused with a flexible $(GGGGS)_3$ linker may be particularly useful for such paper manufacture. In contrast, the CBM1-NL-CBM1, the smallest in molecular size among the four engineered double CBMs, was also revealed to be quite attractive in pulp treatment. It seems that, besides the fibres bridging effect of double CBMs as suggested before (Levy *et al*. 2002), the difference in modification of fibre surface/interfacial properties by different CBMs may also contribute to paper properties to some extent. When CBM1-NL-CBM1 was applied, it was possible to achieve a significant simultaneous increase in pulp tensile strength and burst strength, as well as folding strength, compared to the other engineered double CBMs. Together with its true reversibility to cellulose, CBM1-NL-CBM1 may provide a better upgrade of the secondary pulp, which makes it very suitable for fibre recycling by addition of the double CBMs to recovered fibers. The significant improvement of folding endurance by CBM3-GS-CBM3 might also be partially attributed to the flexible $(GGGGS)_3$ linker of engineered double CBMs on the surface of the paper. This might also explain the better performance of CBM3-GS-CBM1 treated paper in folding endurance compared to CBM3-NL-CBM1. However, further studies are needed to elucidate the effect of the linker region in double CBMs on paper properties in the future.

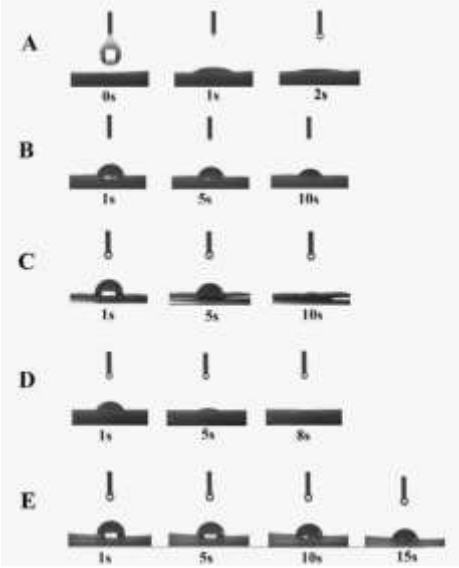


**Fig. 6.** The effect of double CBMs on the wettability of treated papers: A. control paper; B. CBM3-GS-CBM1 treated paper; C. CBM3-NL-CBM1 treated paper; D. CBM3-GS-CBM3 treated paper; E. CBM1-NL-CBM1 treated paper

## Effect of Double CBMs on Wettability

The wettability of CBM3-GS-CBM1, CBM3-NL-CBM1, CBM3-GS-CBM3, and CBM1-NL-CBM1 treated papers was significantly reduced compared to control paper (Fig. 6). However, the decreases in the wettability of the treated paper in the present study were much less than the cellulose crosslinking protein-CCP, probably due to the

different method of paper treatment. The decrease in the wettability of treated paper was thought to be caused by an increase in the surface hydrophobicity of Whatman cellulose filter paper occupied by CBM moieties (Levy *et al*. 2002). Machado *et al*. (2009) reported that adsorption of CBMs led to a substantial coverage of the fibres, and a surface coating corresponding to 0.8 and 1.5 layers of proteins was found in CBM-PEG conjugate treated fibres. In our experiment, some engineered double CBMs may have intruded into the inner layers of paper and resulted in a smaller amount of engineered CBMs on the paper surface compared to a previous study, which was expected to lead to less modification of the surface wettability.

## CONCLUSIONS

1. Four engineered double carbohydrate binding modules (CBMs) containing family 1 or/and 3 CBMs were successfully expressed in *E. coli* as soluble proteins under induced conditions. They displayed obviously different binding capacities and affinity towards crystalline cellulose. CBM1-NL-CBM1 displayed the highest binding capacities to Avicel, followed by CBM3-GS-CBM1, CBM3-NL-CBM1, and CBM3-GS-CBM3.
2. CBM3-GS-CBM3 was the most effective in improving the folding endurance and tensile strength of treated papers among the four engineered double CBMs, but only slightly effective for burst strength improvement. On the other hand, CBM1-NL-CBM1 achieved a significant simultaneous increase in pulp tensile strength and burst strength, as well as folding endurance, compared with the other engineered double CBMs.
3. The CBD1-NL-CBD1 also significantly reduced the wettability of treated papers compared to control paper, following by CBM3-GS-CBM1, CBM3-NL-CBM1, and CBM3-GS-CBM3.
4. Elaborately engineered double CBMs were found to have high potential as novel biomaterials for paper property improvement. CBM1-NL-CBM1 may provide a better upgrade of the secondary pulp, which makes it very suitable for fibre recycling. Meanwhile, CBM3-GS-CBM3 may have particular potential for the manufacture of paper requiring high folding endurance.

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