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A comparative study between an endoglucanase IV and its fused protein complex Cel5-CBM6

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Abstract

The recombinant endoglucanase IV (Cel5; encoded by *egIV*) of *Ruminococcus albus* was compared with protein Cel5-CBM6 comprised of Cel5 fused at the C-terminus with the single-cellulose binding domain II (CBM6) of *Clostridium stercorarium* xylanase A, in order to improve its binding ability. Previous analyses using ball-milled cellulose had suggested that a cellulose binding domain of xylanase A could enhance cellulase activity, especially with insoluble substrates. Comparison of the catalytic activities of Cel5 and Cel5-CBM6 were determined using carboxymethylcellulose, Avicel, and filter paper as substrates. This study confirmed previous findings, and provided further evidence suggesting that Cel5-CBM6 exhibits enhanced activity with insoluble cellulose compared to native Cel5. However, its hydrolytic activity with soluble substrates such as carboxymethylcellulose was comparable to Cel5. For both cellulases, central linkages of cellulooligosaccharides (up to six glucose residues) were found to be the preferred points of cleavage. The rates of hydrolysis with both cellulases increased with cellulooligosaccharide chain length, and at least three consecutive glycosyl residues seemed to be necessary for hydrolysis to occur. Cel5-CBM6 showed a higher affinity for cellulose substrates than did Cel5, as demonstrated by transmission electron microscopy. Taken together, these results suggest that CBM6 increases the affinity of Cel5 for insoluble substrates, and this increased binding capacity seems to result in increased catalytic activity.

Keywords: Cellulase; Fusion protein; Enzyme–gold complex; Cellulose; Cellulose binding domain

1. Introduction

Enzymatic hydrolysis of cellulose is an environmentally benign technology for the transformation of insoluble carbohydrates into potentially valuable products. The cost-efficient use of cellulases to achieve cellulose hydrolysis requires a thorough knowledge of the enzyme and substrate used.

The rumen bacterium *Ruminococcus albus* is known to be an efficient degrader of plant cellulose [1,2]. Karita et al. [3] isolated a gene (*egIV*) encoding the endoglucanase enzyme Cel5 from *R. albus*. This enzyme belongs to family 5 of glycosyl hydrolases, and exhibits maximal activity with carboxymethylcellulose (CMC). To improve enzyme activity, gene *egIV* was fused to one of the genes encoding the cellulose binding domain (CBM) of *Clostridium stercorarium* xylanase A (XynA) [4]. The activity of that glucanase was shown to be influenced by its binding capacity to the inert substrate [5–7], and the presence of a binding domain was needed to greatly increase maximal activity of the enzyme with crystalline cellulose [8,9]. Hefford et al. [10] and Wilson [11] demonstrated that the beneficial effects of binding domains were negligible if a soluble cellulose substrate was used. Although enzyme adsorption plays an important role in cellulose degradation, the exact role played by cellulose binding domains in cellulose degradation has never been clearly defined. In addition, before this process can be described in detail, the mechanism of adsorption must be fully understood. The main reason

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for this lack of knowledge is that most research performed to date focussed on studying isolated, individual cellulase domains [12,13]. Since there are likely many factors at work, use of classical equilibrium analysis to study CBM's adsorption capabilities to the surface of cellulose will not yield an adequate explanation of the mechanism of cellulose degradation [14].

A better understanding of cellulase activity can be achieved through the simultaneous analysis of both hydrolytic performance and binding capacity. In an effort to find a causal relationship between the binding ability and the hydrolysis of cellulose, we have used two enzymes: endoglucanase IV (Cel5), which possesses one catalytic domain, and a recombinant two-domain enzyme (Cel5-CBM6), which possesses a catalytic domain and the single cellulose binding domain (CBM6) of *C. stercorarium* XynA [15].

This study was undertaken in order to investigate the biochemical properties and the mode of action of these two forms of cellulases on various cellulose substrates, and to demonstrate the importance of efficient binding for optimum activity. Thus, based on the biochemical data, we applied the enzyme–gold technique at the electron microscope level to substantiate the interaction between cellulase and substrates.

2. Materials and methods

2.1. Celluloses and chemicals

CMC (Sigma), Avicel (Fluka), Whatman No.1 filter paper and pulp (Kraft) were used as substrates. High-performance liquid chromatography (HPLC) grade oligosaccharides were purchased from Seikagaku Corporation.

2.2. Bacterial cellulase

Plasmid pRA12 carrying the *egIV* gene, and plasmid pCsCBDII carrying both the *egIV* gene and the gene encoding CBM6 from *C. stercorarium*, were used as described in Karita et al. [3,15]. Cel5 and Cel5-CBM6 were purified from *Escherichia coli* JM109 transformed with each plasmid. Enzyme extraction and purification were performed as previously described [3,15]. Purified endoglucanases Cel5 and Cel5-CBM6 were maintained at 4°C in 50 mM potassium phosphate buffer (pH 6.8) until used.

2.3. Protein analysis

Concentration of soluble protein in extracts was determined as described by Bradford [16] using bovine serum albumin as standard. Molecular mass of the purified enzymes was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 10% acrylamide separating gel. Gels were stained with 0.1% Coomassie brilliant blue in a solution containing 50% methanol and 15% acetic acid [17].

2.4. Cellulase activity

Endoglucanase activity was assayed using three substrates: CMC, Avicel and filter paper according to the modified procedure of Wood and Bhat [18] where the amount of reducing sugars liberated by the hydrolysis was determined using the dinitrosalicylic acid method. One unit (U) of activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars. In the case of CMC, 0.5 ml of diluted enzyme solution was incubated with 0.5 ml of a 1% solution of CMC in 100 mM phosphate buffer (pH 6.8) at 40°C for 30 min. Enzymatic hydrolysis of Avicel was performed as follows: a 1% (w/v) suspension of Avicel and 0.5 ml of diluted enzyme solution were incubated at 40°C for 24 h in a total volume of 2 ml. The reaction was centrifuged at 5000 rpm for 15 min, the supernatant was then removed and analyzed for the presence of reducing sugars. Assaying cellulolytic activity using filter paper as a substrate was performed by estimating the rate of release of reducing sugars. One milliliter of phosphate buffer 50 mM, pH 6.8, was mixed with 0.5 ml of enzyme suitably diluted in the same buffer and a 1×6 cm strip of Whatman No. 1 filter paper. The reaction mixture was then incubated at 40°C for 60 min, and the amount of reducing sugars was determined in the supernatant.

2.5. Hydrolysis of cellulooligosaccharides

Substrate specificity was evaluated by incubating each endoglucanase with cellulooligosaccharides (1% w/v in 10 mM phosphate buffer, pH 6.8) of specific lengths: cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6). One microliter of 2-mercaptoethanol and 35.6 μU of each purified enzyme were added to 1.5 ml of each cellulooligosaccharide solution and phosphate buffer (pH 6.8) to a total volume of 2.0 ml. The resulting mixture was incubated overnight at 37°C. The soluble sugars released from each substrate were analyzed by HPLC (Millipore Waters 600E) equipped with an REZEX RSO oligosaccharides column (Phenomenex). The elution solvent used was water, at a flow rate of 0.3 ml min^{-1}.

2.6. Electron microscopy

Samples (1 mm$\times$2 mm) of alfalfa stem, birch stem and filter paper were fixed for 2 h with 3% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2), rinsed with the same buffer, and then postfixed with 1% osmium tetroxide in a sodium cacodylate buffer for 1 h. They were then dehydrated in an ethanol series and embedded in Epon 812. Ultrathin sections were collected on formvar-

Table 1
Molecular properties and specific activity towards various substrates of endoglucanase IV (Cel5) and fusion protein (Cel5-CBM6)

Enzyme	Molecular properties		pH optimum	Specific activity (U mg^{-1})		
	MW	p*I*		CMC	Avicel	Filter paper
Cel5	36 000	4.8	7.0	0.21	0.014	0.004
Fusion protein	50 000	4.7	7.0	0.19	0.049	0.022

All the enzyme activity values are three times repeated mean values.

coated nickel grids and then prepared for cytochemical labelling.

The endoglucanase and fusion protein were individually complexed to colloidal gold according to Bendayan [19,20] with the exception that pH of the gold solution was pre-adjusted to 4.8 and 4.7, the isoelectric point of Cel5 and Cel5-CBM6, respectively. Labelling was accomplished by first floating the grids on a drop of 10 mM phosphate buffer (pH 7.0) containing 14 mM NaCl and 0.02% polyethylene glycol, for 5 min, and then transferring them to a drop of the enzyme–gold complex for 30 min at room temperature in a moist chamber. After washing them with phosphate-buffered saline (pH 7.0), the grids were rinsed with distilled water, and then contrasted with uranyl acetate and lead citrate. Examinations were carried out with a JEOL 1200 EX electron microscope. Sections incubated with either the bovine serum albumin–gold complex or the stabilized gold solution served as experimental controls.

2.7. Quantitative evaluation of gold labelling

Cel5 and Cel5-CBM6 binding to cellulose was estimated by direct observation of the labelling density. Using picture-processing shareware on scanned micrographs, the number of gold particles observed over a specified cell area on 15 fields, randomly chosen from serial sections at a magnification of 10 000×, was counted. Dividing the number of gold particles by the total surface area gave the density of labelling. Statistical evaluation was carried out by means of a *t*-test.

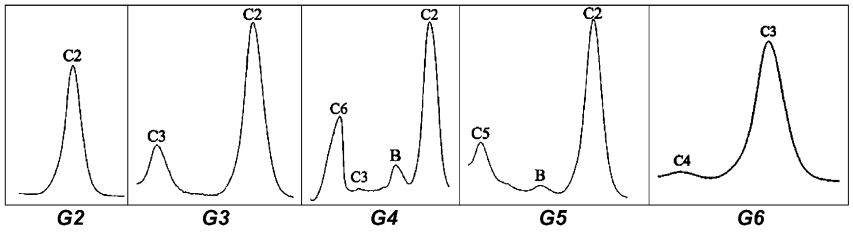
3. Results and discussion

3.1. Properties of Cel5 and Cel5-CBM6

Cel5 and Cel5-CBM6 were purified to homogeneity and determined by SDS–PAGE. One band was found for each protein (36 kDa and 50 kDa, respectively), which is in good agreement with their theoretical mass (35.766 kDa and 49.958 kDa, respectively). Table 1 presents their molecular properties, substrate specificity, and specific activity. The isoelectric points (p*I*) were derived from the amino acid sequences encoding each enzyme. Both forms of cellulase exhibited similar specific activity towards soluble cellulose (CMC). This supports the hypothesis that the catalytic domain of Cel5-CBM6 is fully active but does not improve Cel's hydrolysis activity with soluble substrates. The activity of the two enzymes with insoluble substrates such as Avicel and filter paper was significantly different. Compared to the native recombinant enzyme, the CBM6-containing fusion protein yield an approximately four-fold higher concentration of reducing sugar under the same assay conditions. Our results confirmed other findings [4,6,21,22], without proving any one of three hypotheses underlying the increased activity seen by fusion proteins when hydrolyzing insoluble substrates. It is thought that foreign binding domains (such as CBMs) either (i) create new catalytic activity [4]; or (ii) disrupt cellulose structure, which leads to exposure of new sites [6,22]; or (iii) anchor a higher amount of catalytic domains on the substrate surface [22]. We tried to address this last point with a cytochemical study, described later.

Table 2
HPLC profiles of cellulooligosaccharides by Cel5 and the fusion protein Cel5-CBM6

HPLC profile



HPLC profiles after 8 h enzyme treatment.

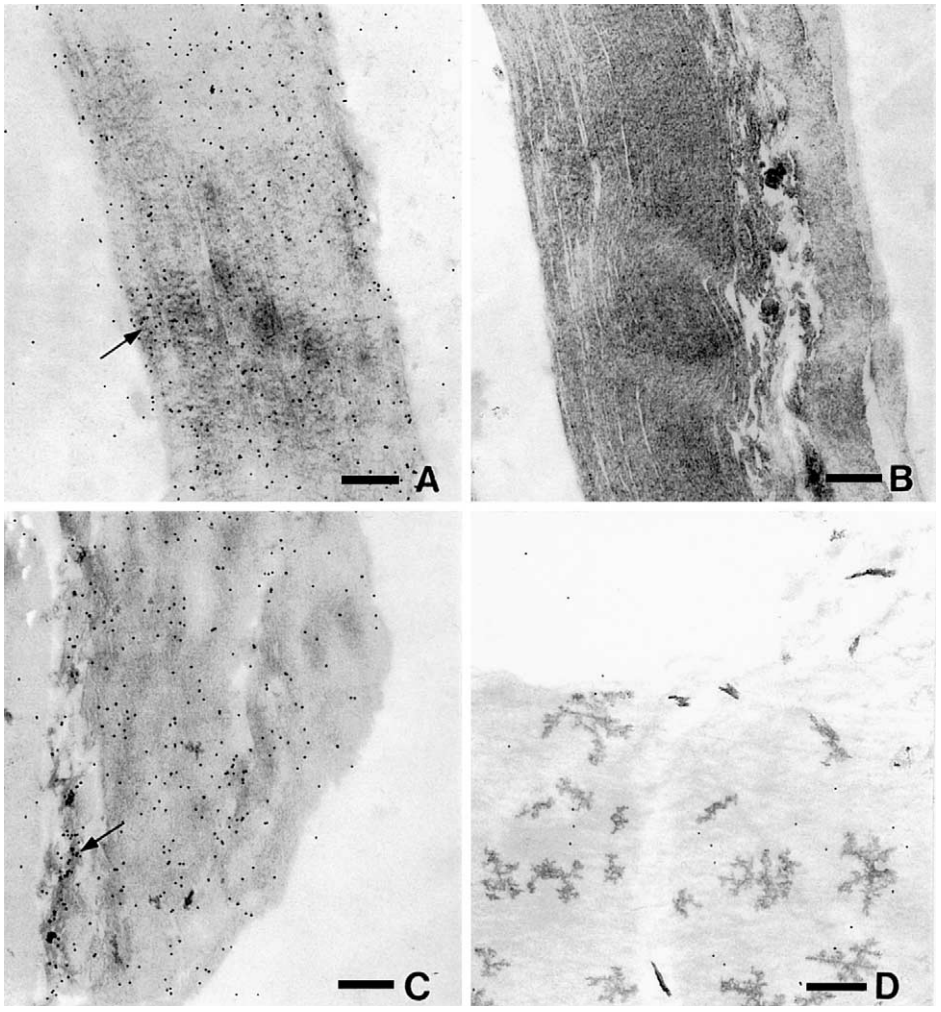


Fig. 1. Transmission electron micrographs of filter paper (A,B) and pulp (C,D) samples. Sections were incubated with Cel5-CBM6–gold (A,C) and Cel5–gold (B,D) complexes to localize cellulose. Note that gold particles are more numerous over more grayish areas (A,C, arrows). Scale bars: 500 nm.

3.2. Hydrolysis of cellulooligosaccharides

The two enzymes also displayed a similar mode of action on cellulooligosaccharides. The degradation patterns for Cel5 and Cel5-CBM6 are summarized in Table 2. Both Cel5 and Cel5-CBM6 lacked activity with G2. However, they partially hydrolyzed G3. G4 was hydrolyzed into G2 as the main reducing sugar, indicating a preferential cleavage of bond 2. The HPLC chromatogram also revealed the formation of smaller quantities of G3 and G5, which was also reported with EGI from *R. albus* [23]. This suggests that these cellulases (EGI, Cel5, and Cel5-CBM6) can transfer G1 to G4 to form G5, while G2, cleaved from G4, can react with the latter in a G2–enzyme complex transglucosylation reaction to form G6. G6 can then be further degraded into two G3 units. These results indicate that both forms of cellulase not only possess an endo-type degrading activity but also have an ability to transfer β-glycosyl units. This transferring activity has also been reported for an endo-β-1,4-glucanase from *Clostridium josui* [24]. When both Cel5 and its fusion protein, Cel5-CBM6, were incubated with G5, complete hydrolysis occurred overnight. It was degraded mainly to G2 and G3, suggesting that bonds 2 and 3 were preferentially cleaved. Reaction with G6 took only 15 h. G3 was the predominant reducing sugar formed. Since some G2 and G4 were also present, one can assume that bonds 2 and 4 were also cleaved by the enzymes. The relative proportion of the products indicates that the central linkages were the preferred points of cleavage of the cellulooligosaccharides, and that both endoglucanases participate in the hydrolysis of cellulose by random cleavage of internal glycosidic linkages.

3.3. Cytochemical study

Naturally occurring Cel5 lacks a cellulose binding domain whereas fusion protein Cel5-CBM6 has one [3,15]. It was previously shown [25] that the ability of cellulase to degrade cellulose is related to its ability to bind to the

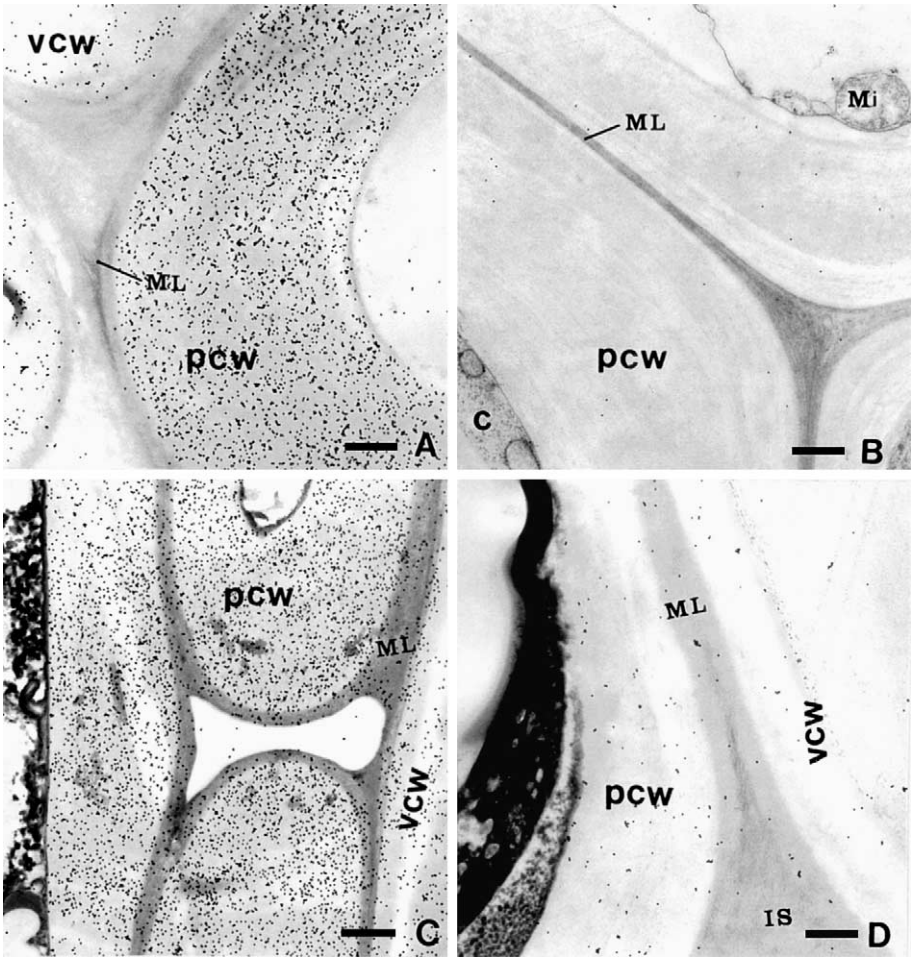


Fig. 2. Transmission electron micrographs of alfalfa (A,B) and birch (C,D) stem samples. Sections were incubated with Cel5-CBM6–gold (A,C) and Cel5–gold (B,D) complexes to localize cellulose. A: Parenchyma cell walls (pcw) are more intensely labelled than the vessel cell walls (vcw); few gold particles are seen over the middle lamella (ML). B: The parenchyma cell walls (pcw) are only faintly labelled and the cytoplasm is nearly devoid of labelling. Mi: mitochondrion; C: chloroplast. C: An intense labelling is present over the parenchyma cell walls (pcw). A few gold particles are also seen over the middle lamella (ML). D: A less intense labelling is seen over cell walls and middle lamella (ML), and the intercellular space (IS) is free of labelling. Scale bars: 500 nm.

substrate. In addition, some high affinity CBMs enhance the hydrolytic capacity of cellulases for insoluble cellulose, even though the native enzyme exhibits no activity towards insoluble cellulose alone [5,26]. This leads one to expect differences in the adsorption properties of the two enzymes: selective binding, recognition pattern or adsorption capacity. Since most information available today is derived from enzymatic digestion experiments [27,28] and does not constitute a formal proof, we applied an enzyme–gold technique on a variety of substrates: filter paper, pulp, and native cellulosic materials (alfalfa and birch stem). To better evaluate the binding capacity of each enzyme, a quantitative analysis of the gold labelling was carried out.

Table 3
Quantitative evaluation of the distribution of the enzyme (Cel5 or Cel5-CBM6)–gold complex (gold particles per μm^2 cellulose substrates)

	Cel5	Cel5-CBM6	*P*
Filter paper	1.6 ± 2.4	30.4 ± 6.4	< 0.001 (S)
Pulp	0.8 ± 1.1	25.2 ± 11.5	< 0.001 (S)
Alfalfa	1.4 ± 2.6	107.0 ± 33.3	< 0.001 (S)
Birch	6.8 ± 7.4	85.1 ± 15.3	< 0.001 (S)

Data are expressed as the means of 10–15 fields. *P*, probability value of a test statistic; S, significant.

The incubation of the Cel5-CBM6–gold complex on filter paper and pulp resulted in intensive labelling (Fig. 1A,C), whereas only a sparse labelling was observed with Cel5 (Fig. 1B,D). The labelling intensities were over 10–20 times higher for Cel5-CBM6 than Cel5 (Table 3). This increase in labelling intensity suggests that CBM6 played an essential role in binding to cellulose. There was no marked difference in the distribution of gold labelling on either substrate for both enzymes.

When thin sections of plant substrates such as alfalfa and birch stems were treated with either Cel5–gold complex (Fig. 2B,D), or Cel5-CBM6–gold (Fig. 2A,C) complex, their distribution was mostly within the plant cell

walls and vessel walls. Middle lamellae were labelled only in the case of birch (Fig. 2C,D). Cell cytoplasm was not labelled (Fig. 2A–D). In all sections examined, a more intense labelling was present over parenchyma cell walls, compared to vessel walls. This result reflects differences in the organization of these cell types. As was seen for filter paper and pulp, labelling with Cel5-CBM6–gold complex was always stronger than with Cel5–gold complex (Table 3). These results confirmed Cel5-CBM6 has a higher cellulose adsorption capacity compared to Cel5.

Several lines of genetic information [29,30] of *Ruminococcus* enzymes showed the existence of cellulose binding domain or multidomain structure, and an earlier adhesion-defective mutant study [31] demonstrated that the binding process of cellulose can be an important step for subsequent cellulose degradation. However, additional single-domain enzymes without cellulose binding domain have also been reported [3]. Thus, we can speculate the *Ruminococcus* enzymes may have different mechanisms with/without cellulose binding domain. Thus, this hybrid enzyme research is a very useful approach providing understanding of the mechanism of substrate binding and hydrolysis.

The present results show that the Cel5-CBM6 complex binds with various cellulose substrates increasing their activity on insoluble celluloses. Artificial annexation of CBM from *Thermomonospora fusca* endoglucanase to *Prevotella ruminicola* CMCase increased the activity of parental enzyme against insoluble cellulose [21] but CBM derived from *Pseudomonas fluorescens* hemicellulases to heterologous endoglucanases did not improve their activities against insoluble celluloses [32]. The relationship between cellulose and CBM seems to be influenced by the relationship between the organisms themselves. The Cel5 of *R. albus* and the CBM6 of *C. stercorarium* xylanase A are active with quite distinct substrates, namely cellulose and xylan, yet the binding domain of Cel5-CBM6 shows a greater general function. Although CBM6 is a domain originating from an enzyme with definite xylolytic activity, it performed efficient cellulose binding when linked to Cel5. This is consistent with a previous report [27].

The enzyme–gold method also demonstrated that Cel5-CBM6 displays a higher labelling affinity with natural cellulosic materials (Fig. 2) compared to processed materials (filter paper and pulp; Fig. 1). These results support those of Roger et al. [33], and Din et al. [21] who reported that purified matrices, such as the crystalline cellulose in filter paper and pulp, are poorer substrates for endocellulase. In addition, the presence of by-products involved in the chemical treatment during the production of these materials may hinder some cellulosic binding sites. Further work will be necessary to explain the differences in the labelling of various cellulosic substrates by Cel5-CBM6.

In conclusion, our results show that a binding domain, CBM6, fused to an endoglucanase, Cel5, enhances the hydrolysis of insoluble cellulose by increasing the attachment of the enzyme to its surface. It is also worth noting that Cel5 and Cel5-CBM6 have comparable hydrolytic properties with soluble substrates, and that Cel5-CBM6 has additional desirable enzymatic properties.

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