Pseudomonas cellulose-binding domains mediate their effects by increasing enzyme substrate proximity

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To investigate the mode of action of cellulose-binding domains (CBDs), the Type II CBD from *Pseudomonas fluorescens* subsp. *cellulosa* xylanase A ($XYLA_{CBD}$) and cellulase E ($CELE_{CBD}$) were expressed as individual entities or fused to the catalytic domain of a *Clostridium thermocellum* endoglucanase (EGE). The two CBDs exhibited similar K_a values for bacterial microcrystalline cellulose ($CELE_{CBD}$, 1.62×10^6 M^{-1}; $XYLA_{CBD}$, 1.83×10^6 M^{-1}) and acid-swollen cellulose ($CELE_{CBD}$, 1.66×10^6 M^{-1}; $XYLA_{CBD}$, 1.73×10^6 M^{-1}). NMR spectra of $XYLA_{CBD}$ titrated with cello-oligosaccharides showed that the environment of three tryptophan residues was affected when the CBD bound cellohexaose, cellopentaose or cellotetraose. The K_a values of the $XYLA_{CBD}$ for C_6, C_5 and C_4 cello-oligosaccharides were estimated to be 3.3×10^2, 1.4×10^2 and 4.0×10^1 M^{-1} respectively, suggesting that the CBD can accommodate at least six glucose molecules and has a much higher affinity for insoluble cellulose than soluble oligosaccharides. Fusion of either the $CELE_{CBD}$ or $XYLA_{CBD}$ to the catalytic domain of EGE potentiated the activity of the enzyme against insoluble forms of cellulose but not against carboxymethylcellulose. The increase in cellulase activity was not observed when the CBDs were incubated with the catalytic domain of either EGE or XYLA, with insoluble cellulose and a cellulose/hemicellulose complex respectively as the substrates. *Pseudomonas* CBDs did not induce the extension of isolated plant cell walls nor weaken cellulose paper strips in the same way as a class of plant cell wall proteins called expansins. The $XYLA_{CBD}$ and $CELE_{CBD}$ did not release small particles from the surface of cotton. The significance of these results in relation to the mode of action of Type II CBDs is discussed.

INTRODUCTION

Most cellulases and xylanases derived from aerobic micro-organisms are modular proteins comprising both catalytic and non-catalytic domains, which are separated by highly flexible linker sequences that are often rich in hydroxyamino acids [1,2]. In most of these enzymes the non-catalytic domains constitute cellulose-binding domains (CBDs). Currently there are ten distinct types of CBD, with the Type II domain being the most common bacterial sequence [3].

Recently the three-dimensional structure of a Type II CBD from *Cellulomonas* exocellulase (Cex) was solved [4]. The domain comprises nine β-strands and has no α-helical structures. The polysaccharide-binding region is characterized by a flat surface containing three tryptophan residues, arranged linearly, which seem to have an important role in ligand binding, probably via stacking interactions with the pyranose rings of the glucose moieties. The affinity of the domain for crystalline cellulose is orders of magnitude higher than for soluble cello-oligosaccharides, probably because the binding interaction involves several chains in the ordered crystalline lattice of insoluble cellulose.

There is now substantial evidence to indicate that CBDs, including the Type II domains, potentiate the activity of cellulases against crystalline forms of cellulose, but not against highly accessible soluble substrates [3]. Furthermore studies by Din et al. [5,6] have demonstrated that the Type II CBD of *Cellulomonas fimi* endoglucanase A (CenA) disrupts the structure of highly crystalline cellulose. The binding domain also increases the activity of the catalytic domain of CenA against cotton fibres when the two domains are not covalently linked, suggesting that at least some of the potentiation effect is a result of the disruption of the ordered structure of the substrate, making it more accessible to enzyme attack.

In addition to cellulases, it is now apparent that several hemicellulases also contain Type II CBDs [7–10]. Recent studies [11] have shown that the activity of xylanase A (XYLA) and arabinoxylan:arabinofuranosidase C from *Pseudomonas fluorescens* subsp. *cellulosa* against cellulose/hemicellulose complexes is increased 2–3-fold by the respective endogenous CBDs, although no potentiation by the polysaccharide-binding domains was observed when the enzymes were evaluated with purified arabinoxylan as substrate. The precise mechanism by which CBDs increase hemicellulase activity against complex substrates remains to be elucidated.

Although the biochemical and biophysical properties of Type II CBDs from *Cellulomonas* enzymes, and the mechanism by which they increase cellulase activity, have been subject to extensive investigation [3,5,6,12], it is unclear whether these studies represent a good paradigm for Type II CBDs. For

Abbreviations used: ASC, acid-swollen cellulose; BMCC, bacterial microcrystalline cellulose; CBD, cellulose-binding domain; CD, catalytic domain; CELE, cellulase E; CenA, endoglucanase A; Cex, *Cellulomonas* exocellulase; EGE, endoglucanase E; XYLA, xylanase A.

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example, it is possible that the properties of these domains are dependent on their enzymic and/or microbial origin. In addition, although Type II CBDs increase the activity of hemicellulases, it is unclear by which mechanism the domains elicit their effects. It is possible that CBDs loosen the structure of the plant cell wall by disrupting the cellulose fibrils, making the hemicellulose component more accessible to enzyme attack. Alternatively, as cellulose and hemicellulose are intimately associated in the plant cell wall, the CBD, by targeting cellulose, might simply maintain the enzyme in close proximity to its substrate, leading to increased polysaccharide hydrolysis. To address these questions we have investigated the properties of Type II CBDs from a xylanase and cellulase from *P. fluorescens* subsp. *cellulosa*. The data show that, in common with the corresponding domain from *C. fimi* Cex, the pseudomonad CBDs exhibit a much higher affinity for insoluble cellulose than for soluble cello-oligosaccharides. Furthermore both the cellulase and xylanase CBDs increase the activity of a cellulase against insoluble substrates. However, neither domain seemed to cause a significant disruption of either pure crystalline cellulose or the complete plant cell wall. In addition, the two binding domains did not potentiate the activity of either cellulase or xylanase catalytic domains, when present as descrete entities, suggesting that the *Pseudomonas* CBDs do not mediate their effects by disrupting the structure of either plant cell walls or crystalline cellulose.

MATERIALS AND METHODS

Microbial strains, plasmids and growth conditions

The *Escherichia coli* strains used in this study were XL1-Blue (Stratagene) and BL21 (DE3) pLysS (Novagen). The vectors used were pMTL22p [13], pET16b and pET32a (Novagen). Other plasmids, described previously, used in this study were as follows: pRVI.1 and pHGB2, which encode full-length *Clostridium thermocellum* endoglucanase E (EGE) and its catalytic domain (EGE_{CD}) respectively [14], pRS4 and pRS16, which encode full-length XYLA from *P. fluorescens* subsp. *cellulosa* [15] and its catalytic domain ($XYLA_{CD}$) respectively, and pC48, which encodes full-length cellulase E (CELE) from *P. fluorescens* subsp. *cellulosa* [16]. Plasmids constructed during the course of this study were as follows: pJR1, encoding the CBD of XYLA ($XYLA_{CBD}$); pJR2, encoding the CBD of CELE ($CELE_{CBD}$); pAC1, encoding EGE_{CD} fused to $XYLA_{CBD}$ (EGE_{CD}–$XYLA_{CBD}$) and pAC2, encoding EGE_{CD} fused to $CELE_{CBD}$ (EGE_{CD}–$CELE_{CBD}$). Media used to select and culture recombinant strains of *E. coli* were as described previously [17].

Construction of plasmids

To generate pJR1, which encodes the Type II CBD of XYLA, the region of the XYLA gene (*xynA*) between nucleotides 75 and 389 [18] was amplified from the plasmid pRS4 by PCR using the following primers: 5′-GCATATGGCACAAACAGCAACTTGCAG-3′ and 5′-CGGATCCTTAGGCCACAGATCCCGAGC-3′, which contained *Nde*I and *Bam*HI restriction sites respectively. The amplified DNA was restricted with *Nde*I and *Bam*HI and cloned into pET16b. To construct pJR2, the sequence of *Pseudomonas celE* (encoding CELE), between nt 1410 and 1738 [16], that encodes the Type II CBD of CELE was amplified by PCR from the plasmid pC48 with the following primers: 5′-GCATATGGGTCAATGCAGCTATACCGT-3′ and 5′-CGGATCCATACCTCCCGGCTCTGGATT-3′. The amplified DNA was restricted with *Nde*I and *Bam*HI and cloned into pET16b cleaved with the same restriction enzymes. The plasmids pAC1 and pAC2, which encode EGE_{CD}–$XYLA_{CBD}$ and EGE_{CD}–$CELE_{CBD}$, respectively, were generated as follows: the CBD-encoding regions of *xynA* and *celE* were amplified by PCR with the following pairs of primers: primer A, 5′-GCAAATATTTGTACGGTCAAACAGCAACTTGC-3′ and primer B, 5′-CCCAAGCTTTTAGGCCACAGATCCCGAGCA-3′ (reaction 1; $XYLA_{CBD}$); primer C, 5′-GCAAATATTTTGTACGGTCAAACAGCAACTTGC-3′ and primer D, 5′-CCCAAGCTTTTAATTGTTACAAACATTACC-3′ (reaction 2; $CELE_{CBD}$). Primers B and D both incorporated *Hin*dIII and translational stop signals at the 3′ ends of the amplified DNA. The 5′ region of the EGE gene, encoding the catalytic domain and the upstream proline/threonine 22-residue linker sequence was amplified from the plasmid pRVI.1 [14] with the following primers: primer E, 5′-CCGAGCTCAAAGAAATAAAAATCGGATGG-3′ and primer F, 5′-ACCGTACAAAATATTTGC-3′ (reaction 3; EGE_{CD}). The amplified DNA contains an *Sst*I restriction site at the 5′ end. To splice the EGE_{CD}- CBD-encoding DNA sequences together, the PCR product from reaction 3 was mixed with either reaction 1 or 2 and a further round of PCR was performed with primers B/E (reaction 4) and D/E (reaction 5). These subsequent PCR reactions spliced the CBD- and EGE-encoding sequences together because primer E was complementary to primers A and C. The PCR products from reactions 4 and 5 were restricted with *Hin*dIII and *Sst*I and cloned into pET32a to generate pAC1 and pAC2 respectively. The conditions used in the PCR reactions were as described previously [19]. A summary of the plasmids used in this study and the proteins they encode is given in Figure 1.

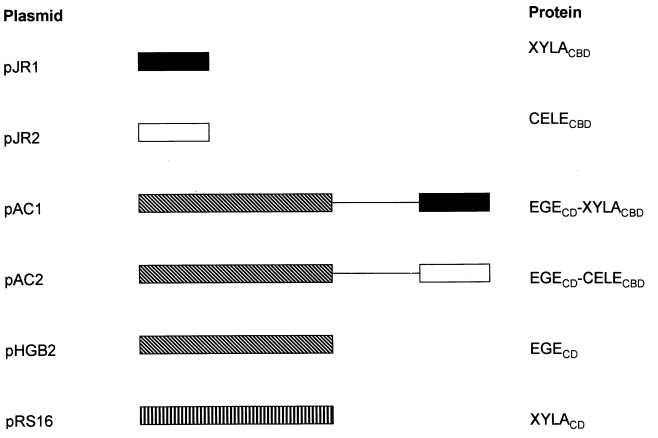

Figure 1 Molecular architecture of the proteins used in this study

The domains of the proteins are defined as follows: EGE_{CD} (diagonal hatching), proline/threonine linker sequence of EGE (line), $XYLA_{CBD}$ (filled rectangle), $CELE_{CBD}$ (open rectangle) and $XYLA_{CD}$ (vertical hatching). The plasmids encoding the various proteins are also defined.

Purification of expansins and recombinant proteins

Expansins were prepared from cucumber hypocotyls by the method of McQueen-Mason et al. [20]. EGE_{CD} was purified as described previously [18]. $XYLA_{CD}$ was purified as described previously [18]. EGE_{CD}–$XYLA_{CBD}$ and EGE_{CD}–$CELE_{CBD}$ were purified from *E. coli* harbouring either pAC1 or pAC2. Cultures were grown and cell-free extracts prepared (details available from the authors on request). The cell-free extracts were loaded on a 3 ml bed volume Talon™ metal-affinity column (Clontech). The column was washed with 1 vol. of 50 mM Tris/HCl buffer, pH 8.0 (used to resuspend bacterial cells before cell-free extract preparation), and then with 100 mM Tris/HCl buffer, pH 7.2,

until the A_{280} of the eluate was zero. The column was then equilibrated in 20 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$. Thrombin (Sigma Chemical Co.) was then added to a final concentration of 0.2 NIH units per mg of fusion proein, and digestion proceeded for 16 h at 22 °C. Pure EGE_{CD}–$XYLA_{CBD}$ and EGE_{CD}–$CELE_{CBD}$ were recovered in the eluate from the column; thioredoxin, which contains the His_{10} tag, remained bound to the Talon™ column. The CBDs of XYLA and CELE, expressed in pET16b, also contained His_{10} tags at their N-termini and thus could be purified by metal-affinity chromatography as follows: cultures (1 litre) of *E. coli* strains harbouring pJR1 and pJR2 were grown as described previously [19]. Cells recovered from the cultures were resuspended in 20 ml of Tris/HCl buffer, pH 8.0, and disrupted by sonication. Insoluble material was sedimented by centrifugation at 30000 ***g*** for 30 min at 4 °C. The CBD in the insoluble material was then solubilized by resuspending the pellet in 20 mM Tris/HCl buffer, pH 8.0, containing 100 mM NaCl and 8 M urea (buffer A) and incubating it at 37 °C for 30 min. After centrifugation, the CBD-containing supernatants were applied to a 5 ml bed volume Talon™ metal-affinity resin column. The column was washed with 5 vol. of buffer A and the CBDs were eluted with 15 ml of buffer A containing 100 mM imidazole. The CBDs were dialysed successively against 2 litres of 50 mM Tris/HCl buffer, pH 8.0, containing 4 M, 3 M, 2 M, 1 M and 500 mM urea respectively, then twice against 2 litres of 50 mM sodium phosphate buffer, pH 7.0. Finally, insoluble material was removed from the CBD preparations by centrifugation at 30000 ***g*** for 30 min, followed by filtration through a 0.2 μm filter (Amicon), and the CBD-containing supernatants stored at −20 °C.

Enzyme, ligand binding and protein assays

Xylanase activity against both soluble substrates and cellulose/hemicellulose complexes was determined as described previously [11]. The activities of the derivatives of EGE against cellulosic substrates were determined as follows [21]: all assays were performed at 50 °C in 50 mM phosphate/12 mM citrate buffer, pH 6.5, using a substrate concentration of 2% (w/v), which is 10-fold the K_m of the enzyme for soluble cellulosic substrates [18]. The amount of enzyme and time of the reactions were as follows for each substrate: carboxymethylcellulose, 10 min reactions with 150 ng of EGE; acid-swollen cellulose (ASC), 1 h reactions with 1 μg of EGE; Avicel, 2.5 h reactions with 4 μg of EGE; bacterial microcrystalline cellulose (BMCC), 16 h reactions with 17 μg of EGE; filter paper (Whatman no. 1), up to 24 h with 50 μg of EGE; cotton, 54 h reactions with 50 μg of enzyme. Reaction rates for all substrates except cotton were determined by measuring the release of reducing sugar [22]. Release of carbohydrate from cotton fibres was determined by the phenol/sulphuric acid method of Dubbois et al. [23]. The binding of CBDs to different forms of cellulose was performed as described by Creagh et al. [12] except that incubations were for only 1 h and unbound protein solutions were not filtered before measuring A_{280}. Each CBD (0.5–25 μM in 50 mM phosphate buffer, pH 7.0) was mixed with either 0.2 mg of BMCC or 0.5 mg of ASC, to a final aqueous volume of 650 μl. Protein concentration was determined by the method of Pace et al. [24], and the purity of protein preparations was assessed by SDS/PAGE [25].

Biophysical properties of cellulose and plant cell walls

The capacity of the isolated CBDs to release microfibrils from cotton was determined as described by Din et al. [5]. The extensibility of plant cell walls was determined with the Creep assay as described previously [20]. Briefly, isolated hypocotyl wall specimens were held between a fixed and a mobile clamp (5 mm between clamps) and subjected to a constant tensile force of 20 g. Extension was monitored by a displacement transducer attached to the mobile clamp and recorded by a microcomputer. Wall specimens were bathed in 50 mM sodium acetate, pH 4.5, or 50 mM sodium phsophate, pH 7.0, for approx. 10 min before CBD or cucumber expansin was added to a final concentration of 2 mg/ml or 40 μg/ml respectively. The strength of wetted strips (4 mm × 10 mm) of Whatman 3MM chromatography paper was measured with an Instron 91-51-17M extensometer with a 10 N load cell. Before measurement, paper strips were soaked for 30 min in 50 mM sodium acetate, pH 4.5, or 50 mM sodium phosphate, pH 7.0, containing CBD or expansin at 2 mg/ml or 40 μg/ml respectively. The strips were then held between two clamps with approx. 5 mm between each and extended at a constant rate of 5 mm/min until the paper broke; the maximum stress generated was recorded. Controls consisted of paper strips soaked in buffered solutions without added proteins.

NMR spectroscopy

$XYLA_{CBD}$ was concentrated to 700 μM in 500 μl of 50 mM sodium phosphate buffer, pH 7.0, containing 10% (v/v) ^2H_2O. NMR spectra were recorded with a Bruker AMX 500 spectrometer and the ^1H chemical shifts were referenced to an internal standard of 3-trimethylsilyl-2,2,3,3,-d_4-propionate at 0.00 p.p.m. Experiments were acquired at 303 K with a resolution of 8192 complex points over a sweep width of 12500 Hz. All data were processed with a Gaussian or sine-bell window convolution with FELIX v2.30 (Biosym Technologies, San Diego, CA, U.S.A.). The binding affinity of $XYLA_{CBD}$ for a series of cello-oligosaccharides was measured by following the shift of two tryptophan NHϵ signals with the addition of increasing ligand concentration dissolved in an identical buffer solution. Analysis of the data and fitting of the chemical shift change to a standard equation for a saturation isotherm was performed with EXCEL v5.0 (Microsoft Corporation).

RESULTS

Affinity of $XYLA_{CBD}$ and $CELE_{CBD}$ for cellulose and cello-oligosaccharides

To investigate the properties of Type II *Pseudomonas* CBDs, we have expressed the Type II CBDs from *Pseudomonas* XYLA and CELE and purified them by metal-affinity chromatography (Figure 2). The affinity of these domains for different forms of cellulose was analysed by binding-isotherm measurements in accordance with the model described previously [26] by using the relationship:

$$[B] = [N_0]\,K_a[F]/(1 + K_a[F])$$

where [B] is the bound CBD concentration (mol/g of cellulose), [F] is the equilibrium concentration of unbound CBD (mol/l), $[N_0]$ is the concentration of binding sites on the cellulose surface (mol/g of cellulose) and K_a is the equilibrium association constant (l/mol). Non-linear regression of the isotherm data gives values for $[N_0]$ and K_a, from which the relative equilibrium constant (K_r) can be obtained with the following equation:

$$K_r = [N_0]\,K_a$$

A typical binding-isotherm curve is shown in Figure 3. Although the data seem to fit a two-site binding model slightly better than a one-site model, we have chosen to use a one-site model for our

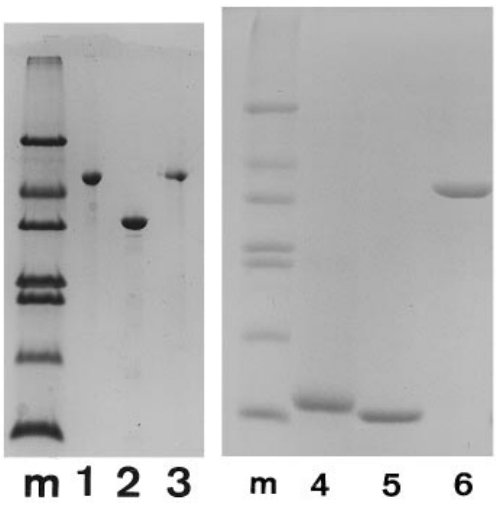

Figure 2 SDS/PAGE of proteins used in this study

The proteins used in this study were purified as described in the Materials and methods section and subjected to SDS/PAGE. The lanes contained the following proteins: lane 1, EGE_{CD}–$CELE_{CBD}$; lane 2, EGE_{CD}; lane 3, EGE_{CD}–$XYLA_{CBD}$; lane 4, $CELE_{CBD}$; lane 5, $XYLA_{CBD}$; lane 6, $XYLA_{CD}$; lanes M, Sigma low-molecular-mass markers. Samples in lanes 1–3 and 4–6 were run on 10% (w/v) and 12.5% (w/v) polyacrylamide gels respectively.

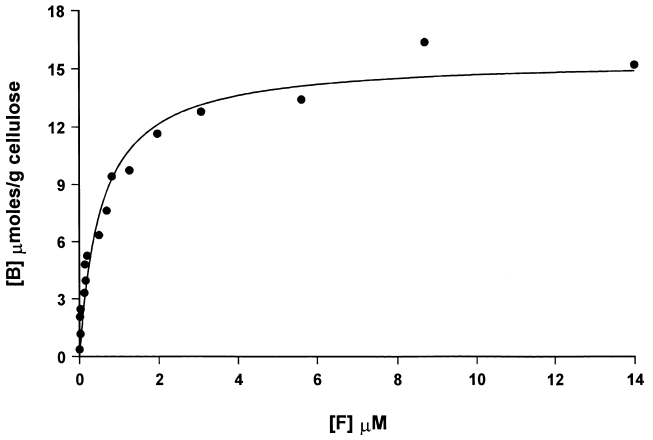

Figure 3 Binding isotherm for $XYLA_{CBD}$ on BMCC

Adsorption measurements were performed at 30 °C in 50 mM phosphate buffer, pH 7.0, as described in the Materials and methods section.

Table 1 Affinity of $CELE_{CBD}$ and $XYLA_{CBD}$ for different forms of cellulose

Enzyme domain	Cellulose	K_r (l/g)	$10^{-6} \times K_a$ (M^{-1})	Saturation (μmol/g)
$CELE_{CBD}$	BMCC	23.5	1.62	14.5
	ASC	14.3	1.66	8.6
$XYLA_{CBD}$	BMCC	28.3	1.83	15.5
	ASC	15.7	1.73	9.1

analysis because the identity of the possible weak binding site is unknown. The weak binding site could be on the cellulose surface, but it is equally likely that it is a protein–protein interaction between bound and free CBD molecules (at high concentrations the CBDs are known to interact). The tabulated data, shown in Table 1, are derived from measuring the initial slopes of the binding isotherms (Figure 3) where the possible weak binding is not influencing CBD–cellulose interactions. The results indicate that the affinities of the two domains for either BMCC or ASC were not significantly different, and were broadly similar to the Type II CBD from *C. fimi* Cex [27].

To investigate the affinity of $XYLA_{CBD}$ for cello-oligosaccharides, three approaches were employed; (1) the binding of the CBD to BMCC was analysed in the presence of cellohexaose; (2) the capacity of cellohexaose to elute the CBD from BMCC was determined; (3) NMR spectra of the domain were recorded in the presence of increasing concentrations of cello-oligosaccharides to determine the K_a of the CBD for the soluble ligands. There was no significant difference in the binding of $XYLA_{CBD}$ to BMCC in the presence or absence of cellohexaose, and the oligosaccharide did not elute the CBD from crystalline cellulose even at saturating concentrations of the sugar (results not shown). Analysis of the low-field region of the $XYLA_{CBD}$ NMR spectrum initially revealed four narrow signals, which were shown by two-dimensional NMR methods to correspond to two backbone amide protons and four of the five expected tryptophan side-chain NHϵ protons (results not shown). On titration of the protein with increasing concentrations of oligosaccharides, three of the tryptophan side-chain signals exhibited a downfield shift, with two of the peaks moving approx. 0.1 p.p.m. and the third 0.04 p.p.m. over the course of the experiments (Figure 4). The shift in the two overlapping tryptophan peaks (corresponding to resonance B in Figure 4) during the titration with various oligosaccharides followed a typical saturation curve for ligand binding; the third peak could not be analysed because of spectral overlap. The affinity constant for the binding was estimated from a plot of the change in chemical shift against ligand concentration by using a method analogous to that of Johnson et al. [28]. The results showed that the K_a values for cellohexaose, cellopentaose and cellotetraose were 3.3×10^2, 1.4×10^2 and 4.0×10^1 respectively (results not shown). It is therefore apparent that the affinity of the CBD for cellohexaose was approx. 1/5000 that for insoluble cellulose. This explains the inability of the short oligosaccharide to affect the binding of $XYLA_{CBD}$ to, or the elution of the domain from, BMCC as noted above. As the oligosaccharides decreased in length, their affinity for the XYLA domain diminished, suggesting that the CBD binding region could accommodate at least six sugar residues. Collectively, these results show that $XYLA_{CBD}$ binds much more tightly to insoluble cellulose than to soluble oligosaccharides and therefore suggests that the domain interacts with more than one cellulose chain within the crystalline lattice.

Previous studies showed that CBDs derived from enzymes with at least some cellulase activity increase enzyme activity against crystalline substrates [29,30]. To investigate whether the CBD of an enzyme that exhibited no detectable cellulase activity could enhance the activity of a heterologous cellulase against insoluble cellulose, the CBD of XYLA was fused to the catalytic domain of EGE. The CBD from CELE was also fused to the *Clostridium* cellulase to confirm that a cellulase-derived CBD enhanced the catalytic activity of EGE against recalcitrant substrates. The two hybrid enzymes and the isolated catalytic domain of EGE were purified to apparent homogeneity (Figure 2), and the capacity of the three enzymes to hydrolyse different forms of cellulose was evaluated. Both hybrid proteins exhibited increased activity against crystalline (BMCC and cotton fibres),

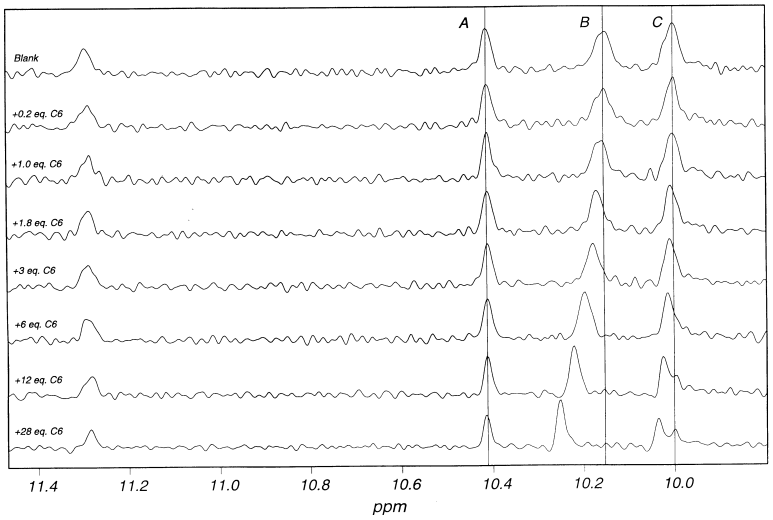

Figure 4 NMR spectra of $XYLA_{CBD}$ in the presence of increasing amounts of cellohexaose

The $XYLA_{CBD}$ (0.7 mM) was titrated with increasing molar equivalents of cellohexaose. The vertical lines mark the initial positions of four tryptophan resonances. Signal B is composed of two overlapping tryptophan NHϵ resonances. Signals B and C shifted to low field on the addition of cello-oligosaccharides.

Table 2 Catalytic activity of EGE_{CD} and its derivatives

The discrete CBDs were incubated with EGE_{CD} in a molar ratio of 1:1. Catalytic activities against ASC, BMCC and Avicel are relative to the activity of the respective enzymes against CMC. The activity of the enzymes against cotton is relative to the activity of EGE_{CD}. Abbreviation: n.a., no discrete CBD was added. The values in parentheses are S.E.M.

		Relative catalytic activity				
Enzyme	CBD	CMC	ASC	Avicel	BMCC	Cotton
EGE_{CD}	n.a.	1.0 (6.1×10^{-2})	1.25×10^{-2} (7.0×10^{-4})	8.96×10^{-4} (9.4×10^{-5})	1.92×10^{-5} (1.4×10^{-6})	1.00 (0.2)
EGE_{CD}	$XYLA_{CBD}$	1.0 (4.2×10^{-2})	1.03×10^{-2} (1.0×10^{-3})	9.45×10^{-4} (5.2×10^{-5})	2.06×10^{-5} (1.3×10^{-6})	0.94 (0.3)
EGE_{CD}	$CELE_{CBD}$	1.0 (3.1×10^{-2})	1.16×10^{-2} (3.4×10^{-4})	8.72×10^{-4} (4.8×10^{-5})	2.18×10^{-5} (7.0×10^{-7})	0.97 (0.1)
EGE_{CD}–$CELE_{CBD}$	n.a.	1.0 (1.3×10^{-1})	2.80×10^{-2} (2.1×10^{-3})	2.05×10^{-3} (5.1×10^{-5})	9.74×10^{-5} (3.8×10^{-7})	2.05 (0.2)
EGE_{CD}–$XYLA_{CBD}$	n.a.	1.0 (1.5×10^{-1})	4.97×10^{-2} (5.5×10^{-3})	2.86×10^{-3} (3.7×10^{-7})	1.04×10^{-4} (3.1×10^{-5})	2.51 (0.2)

partly crystalline (Avicel) and amorphous (ASC) cellulose compared with EGE_{CD}, but against soluble forms of the polysaccharide the activities of the three enzymes were similar (Table 2). These results suggest that *Pseudomonas* CBDs, in common with the corresponding *Cellulomonas* domains, potentiate cellulase activity against crystalline substrates, although it is interesting to note that, unlike the *C. fimi* CenA and Cex binding domains [29,30], the XYLA and CELE CBDs also increased the activity of EGE against ASC. The results also demonstrate that CBDs derived from enzymes with no endogenous cellulase activity are able to enhance the activity of cellulases.

Do *Pseudomonas* CBDs disrupt the structural integrity of plant structural polysaccharides?

To evaluate whether *Pseudomonas* Type II CBDs, derived from both cellulases and hemicellulases, disrupt the structure of the plant cell wall and crystalline cellulose, the corresponding domains from CELE and XYLA were incubated with plant cell walls and with filter paper strips, and their effect on the integrity of these macromolecules was monitored by the Creep and the Instron assays. As a positive control these experiments were also performed with a group of plant proteins termed 'expansins'. Expansins seem to induce cell wall extension by binding to cellulose microfibrils and disrupting the hydrogen bonds between the microfibrils and the hemicelluloses that coat them [31]. The two *Pseudomonas* Type II CBDs at both pH 7.0 and 4.5 had no effect on the degree to which plant cell walls could be extended by a constant force (Creep assay), or on the maximum load required to break paper strips in the Instron assay (results not shown). In contrast, expansins from cucumber hypocotyls increased the extensibility of plant cell walls by 370 μm and decreased the force required to break filter paper strips by 33 % (results not shown). It has been shown [5,6,29] that *Cellulomonas* CBDs release small microfibril particles from cotton, supporting the view that these binding domains disrupt the structure of crystalline cellulose. Incubation of either $XYLA_{CBD}$ or $CELE_{CBD}$ with cotton did not result in the release of small particles (results

not shown). Collectively, the small particle release experiments, together with the Instron and Creep assays, indicated that neither of the *Pseudomonas* CBDs caused a significant disruption of either the complete plant cell wall or crystalline cellulose.

A previous study [6] showed that the Type II CBD from *C. fimi* CenA enhanced cellulase activity against cotton fibres, despite the absence of a covalent linkage between the two domains. To investigate whether $XYLA_{CBD}$ and $CELE_{CBD}$ increased the activity of plant cell wall hydrolases, when present as discrete entities, the capacity of the catalytic domain of XYLA and EGE to hydrolyse cellulose/hemicellulose complexes and crystalline cellulose respectively was evaluated in the presence or absence of the *Pseudomonas* CBDs. The results showed that $XYLA_{CBD}$, when not covalently attached to $XYLA_{CD}$, did not potentiate xylanase activity at a $XYLA_{CBD}$:$XYLA_{CD}$ molar ratio that ranged from 0.4 to 10 (results not shown). Similarly, the two *Pseudomonas* CBDs did not increase the activity of EGE, when the domains were discrete entities, at a molar ratio of CBD to catalytic domain of 1:1, against either BMCC, Avicel, ASC, carboxymethyl cellulose or cotton fibres (Table 2). Increasing the ratio of CBD to catalytic domain up to 4:1 also had no effect on the activity of the cellulase against cotton (results not shown). In addition, we incubated filter paper with EGE and the two discrete CBDs at molar ratios that ranged from 1:0.5 to 1:30 (filter paper saturated with CBD at 1:30) for 2, 6, 12 and 24 h. No potentiation of either the rate or extent of reducing sugar release by EGE was observed (results not shown). These results suggest that the *Pseudomonas* CBDs do not mediate their positive effects on plant cell wall hydrolases by disrupting the structure of the target substrates, and supports the view that these domains do not cause a significant perturbation of the structure of either pure crystalline cellulose or the plant cell wall.

DISCUSSION

The focus of this study was to assess whether the properties of Type II CBDs vary according to their enzyme and/or microbial origin. To address this question we have investigated the properties of Type II CBDs from *P. fluorescens* subsp. *cellulosa* CELE and XYLA.

Results presented here clearly showed that *Pseudomonas* Type II CBDs, in common with the Type II CBD from *C. fimi* Cex, bound tightly to BMCC and ASC but exhibited a much lower affinity for cello-oligosaccharides. NMR spectra of $XYLA_{CBD}$ titrated with cello-oligosaccharides show that the chemical shifts of three of the tryptophan residues change. This is similar to results obtained by Xu et al. [4], who showed that the chemical shifts of two tryptophan residues in Cex CBD (identified as Trp-54 and Trp-72) change significantly on titration with cello-hexaose, whereas a third (Trp-17) shifted to a smaller extent. Xu et al. argued that as Trp-54 and Trp-72 were exposed on the surface of the CBD and mutation of the corresponding residues in the Type II CBD of *C. fimi* CenA [32] led to a substantial decrease in affinity for cellulose, the changes in chemical shift were probably a reflection of a direct interaction of the ligand with the aromatic residues. In view of the sequence similarity between the CBDs of XYLA and Cex, which implies similar structures, and the fact that in XYLA Trp-13, Trp-49 and Trp-66 are analogous to the solvent-exposed residues Trp-17, Trp-54 and Trp-72 respectively in the Cex CBD, it is tempting to speculate that the tryptophan residues involved in ligand binding are conserved in the XYLA and Cex CBDs. The affinity of $XYLA_{CBD}$ for the cello-oligosaccharides increased with increasing chain length, although the difference in K_a between cello-hexaose and cellopentaose was relatively small. These results suggest that the binding site of $XYLA_{CBD}$ can accommodate at least six glucose moieties. Currently it is unclear whether the third surface tryptophan, whose chemical shift was only slightly perturbed by cello-oligosaccharide addition, has an important role in ligand binding. Because Type II CBDs bind much more tightly to insoluble forms of cellulose, it is possible that these domains, in addition to binding to a single linear cellulose chain, interact with other adjacent cellulose molecules in the microfibril structure, and the third tryptophan has an important role in these other interactions.

Although Type II CBDs from cellulases increase the activity of homologous and heterologous catalytic domains against insoluble cellulose [3,16,29,30,33], it is unclear whether the corresponding binding domains from hemicellulases also enhance cellulase activity against insoluble substrates. Cex, an enzyme with xylanase and cellulase activity, contains a CBD that has a pivotal role in the activity of the enzyme against crystalline cellulose. It is therefore possible that the primary function of the Cex CBD is to increase the activity of the enzyme against cellulose, and thus it might exhibit atypical properties compared with CBDs from hemicellulases that display no cellulase activity. To assess whether the CBD from a true hemicellulase could increase the activity of an endoglucanase by the same amount as a CBD from a cellulase, the capacities of the Type II CBDs from *Pseudomonas* XYLA (which has a carboxymethylcellulase activity less than 1/50000 that of its xylanase [34]), and CELE, a typical endoglucanase, to influence the activity of a heterologous cellulase (catalytic domain of EGE from *C. thermocellum*) against cellulosic substrates were evaluated. Results described in this paper show clearly that the CBDs from both the xylanase and cellulase increased the activity of the cellulase against insoluble substrates. This is in sharp contrast with a previous study [35] that indicated that xylanase CBDs did not enhance cellulase activity against insoluble substrates. In the study by Poole et al. [35], the amount of cellulase added to assays containing insoluble cellulose was too small to elicit a significant release of reducing sugar, obscuring any possible influence that the CBD was having on enzyme activity against crystalline cellulose. Thus, from results presented here, we propose that Type II CBDs, irrespective of their enzymic or microbial origin, have the capacity to increase the activity of cellulases against insoluble substrates.

Results presented here indicated that the two *Pseudomonas* CBDs do not cause a significant disruption of the structure of either plant cell walls or crystalline cellulose. However, this interpretation must be viewed with some caution as it could be argued that the biophysical measurements would detect only an extensive reduction in the structural integrity of these macromolecules, such as the weakening of the forces that hold individual cellulose microfibrils or cellulose microfibrils and hemicellulose chains together. It has been suggested that CBDs cause only subtle changes to microfibril structure, such as the solvation of single cellulose chains at the surface of cellulose crystals [36], which would probably not cause a substantial change in the integrity of cellulose microfibrils or whole plant cell walls. However, the observation that no potentiation of xylanase or cellulase activity against cellulose/hemicellulose complexes and crystalline cellulose occurred when the isolated *Pseudomonas* CBDs were mixed with either xylanase or cellulase catalytic domains supports the view that the two binding domains are not increasing enzyme activity by disrupting the structure of recalcitrant substrates and making the glycosidic bonds more accessible to enzyme attack. We therefore propose that the *Pseudomonas* CBDs, by bringing cellulases and xylanases into intimate and prolonged contact with the plant cell wall, increase the effective concentration of the enzymes on the substrate

surface and thus facilitate more efficient glycosidic bond cleavage. This is in contrast with studies by Din et al. [5,6] that showed that the *C. fimi* Type II CBD of CenA disrupted the structure of cotton (crystalline cellulose), which contributed to the capacity of the CBD to potentiate cellulase activity when covalently linked to the catalytic domain or when present as a discrete entity. The structural basis for the different modes of action of the Type II *Pseudomonas* and *Cellulomonas* CBDs is currently unclear.

In addition to the different modes of action of the *Pseudomonas* and *Cellulomonas* CBDs described in this report, there is increasing evidence that CBDs from different protein families influence cellulase activity in different ways. Thus Pages et al. [37] showed that a Type III CBD increased the activity of a *Clostridium* cellulse against ASC, when covalently attached to the cellulase or as a discrete entity, whereas the potentiation of cellulase activity afforded by the CBD against BMCC was much less. In contrast, Coutinho et al. [30] and Tomme et al. [29] showed that *C. fimi* Type II CBDs do not increase enzyme activity against amorphous cellulose, but only against crystalline substrates. Results presented here showed that *Pseudomonas* Type II CBDs can increase cellulase activity against both crystalline and amorphous cellulose. It therefore seems, that although CBDs that bind to crystalline cellulose increase the activity of plant cell wall hydrolases against insoluble substrates, the mechanism by which these domains elicit their effects is not universal.

The observation that some CBDs and the plant proteins termed expansins disrupt the structure of cellulose suggested that the two protein species might have a common mechanism of action. However, results presented in this paper showed that the two *Pseudomonas* CBDs and expansins have very different effects on both the plant cell wall and crystalline cellulose. Expansins are thought to be able to break hydrogen bonds between cellulose microfibrils and matrix hemicelluloses within the plant cell wall, and between cellulose microfibrils in paper; their effect is manifested by a decrease in the rigidity of the plant cell wall [31]. The *Pseudomonas* CBDs are clearly unable to mediate these effects. It could be argued that the disruptive effects of the *Cellulomonas* Type II CBDs on cotton fibres (microfibril release, roughening of surface fibres and capacity to potentiate cellulase activity when present as a discrete entity) points to a common mechanism of action with expansins. However, unpublished work indicates that the Type II CBD of *C. fimi* CenA does not mediate a loss in plant cell wall rigidity (R. A. J. Warren, personal communication), providing further evidence for the view that CBDs and expansins have very different effects on plant cell wall structure.

In conclusion, this paper has shown that Type II CBDs of different enzymic and microbial origins display similar discrimination between insoluble cellulose and soluble cello-oligosaccharides. It is also apparent that a Type II CBD derived from a xylanase is able to potentiate cellulase activity against insoluble substrates. However, the differences in the capacities of the CBDs from *Pseudomonas* and *Cellulomonas* to disrupt the structure of crystalline cellulose, and to potentiate cellulase activity when present as discrete entities, suggest that Type II CBDs do not have a common mechanism of action. Some CBDs enhance hydrolytic activity by increasing the effective concentration of the enzymes on the substrate surface, whereas others additionally mediate their effect by disrupting crystalline cellulose structure, thereby improving substrate accessibility.

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