The type II and X cellulose-binding domains of *Pseudomonas* xylanase A potentiate catalytic activity against complex substrates by a common mechanism

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Xylanase A (Pf Xyn10A), in common with several other *Pseudomonas fluorescens* subsp. *cellulosa* polysaccharidases, consists of a Type II cellulose-binding domain (CBD), a catalytic domain (Pf Xyn10A_{CD}) and an internal domain that exhibits homology to Type X CBDs. The Type X CBD of Pf Xyn10A, expressed as a discrete entity (CBD_X) or fused to the catalytic domain (Pf Xyn10A'), bound to amorphous and bacterial microcrystalline cellulose with a K_a of $2.5 \times 10^5 \text{ M}^{-1}$. CBD_X exhibited no affinity for soluble forms of cellulose or cello-oligosaccharides, suggesting that the domain interacts with multiple cellulose chains in the insoluble forms of the polysaccharide. Pf Xyn10A' was 2–3 times more active against cellulose–hemicellulose complexes than Pf Xyn10A_{CD}; however, Pf Xyn10A' and Pf Xyn10A_{CD} exhibited the same activity against soluble substrates. CBD_X did not disrupt the structure of plant-cell-wall material or bacterial microcrystalline cellulose, and did not potentiate Pf Xyn10A_{CD}

when not covalently linked to the enzyme. There was no substantial difference in the affinity of full-length Pf Xyn10A and the enzyme's Type II CBD for cellulose. The activity of Pf Xyn10A against cellulose–hemicellulose complexes was similar to that of Pf Xyn10A', and a derivative of Pf Xyn10A in which the Type II CBD is linked to the Pf Xyn10A_{CD} via a serine-rich linker sequence [Bolam, Cireula, McQueen-Mason, Simpson, Williamson, Rixon, Boraston, Hazlewood and Gilbert (1998) *Biochem. J.* **331**, 775–781]. These data indicate that CBD_X is functional in Pf Xyn10A and that no synergy, either in ligand binding or in the potentiation of catalysis, is evident between the Type II and X CBDs of the xylanase.

Key words: cell-wall breakdown, cellulase, hemicellulose, polysaccharidase, xylan.

INTRODUCTION

Cellulases and hemicellulases expressed by aerobic micro-organisms generally have a modular structure in which the catalytic domain is linked to a cellulose-binding domain (CBD) by flexible sequences rich in hydroxy amino acids [1]. Based on primary structure homologies, there are at least ten different types of CBD [2], with Type I and Type II being the most common domain in aerobic-fungal and bacterial cellulases, respectively.

Recently the three-dimensional structures of Types I, II, III, IV and V CBDs have been determined [3–7]. These domains consist mainly of β -strands and have very little α -helical structure. The cellulose-binding region of Types I, II, III and V CBDs consists of a planar hydrophobic region on the surface of the domains, in which ligand binding is mediated by hydrophobic-stacking interactions between solvent-exposed aromatic residues and the glucosyl-pyranose rings [8,9]. Rather than binding to cello-oligosaccharides or soluble cellulose, the domains exhibit a strong preference for insoluble forms of the polysaccharide, suggesting that the binding surface interacts with multiple cellulose chains [4,10].

Several studies have shown that Types I and II CBDs potentiate the activity of cellulases and xylanases (1,4- β -D-xylan xylanhydrolase, EC 3.2.1.32) against recalcitrant substrates [11–13]. The *Cellulomonas fimi* Type II domains appear to mediate their

effects, not only by promoting substrate attachment, but also by disrupting the structure of the cellulose surface, making the microfibrils more accessible to enzyme attack [13,14]. The observation that the type III CBD of the *Clostridium cellulovorans* cellulosome integrating protein A (CipA) also potentiates cellulase activity, when not covalently attached to these enzymes, indicates that this domain may also mediate its effect, at least in part, by a disruptive mechanism [15]. In contrast, two *Pseudomonas* Type II CBDs did not appear to disrupt the structure of cotton, and only potentiated cellulase and xylanase activities when covalently attached to these enzymes [10]. These data suggest that the *Pseudomonas* Type II CBDs elevate enzyme activity simply by increasing the effective concentration of these biocatalysts on the surface of the recalcitrant substrate.

Aerobic-bacterial plant-cell-wall hydrolases often contain duplicated CBD sequences [16,17]. This feature is exemplified by *Pseudomonas fluorescens* subsp. *cellulosa* plant-cell-wall hydrolases which generally consist of a catalytic domain and a Type II CBD, separated by a highly conserved small 40-residue non-catalytic domain [18–21] which shows strong sequence similarity to the C-terminal Type X CBD of *Pseudomonas* xylanase E [22]. Although the Type X CBD is functional in xylanase E (the enzyme contains no other CBDs except the Type X domain); in the *Pseudomonas* enzymes that contain multiple CBDs, it is currently unclear whether both the Type II and Type X domains retain their capacity to bind to cellulose.

Abbreviations used: CBD, cellulose-binding domain; LB, Luria broth; MUC, methylumbelliferyl- β -D-cellobioside; Pf Xyn10A, xylanase A from *Pseudomonas fluorescens* subsp. *cellulosa*.

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Although multiple sequences that exhibit similarity to CBDs are a relatively common phenomenon among bacterial plant-cell-wall hydrolases [16,21], the selection pressures that have led to their evolution are not readily apparent. In this paper we have analysed the biological significance of the Type X CBD (CBD_x) of xylanase A (Pf Xyn10A) from *P. fluorescens* subsp. *cellulosa*, which also contains an N-terminal Type II CBD, an internal Type X CBD homologue (CBD_x) and a C-terminal glycosyl hydrolase family 10 xylanase catalytic domain [21,23]. In this report we show that CBD_x binds to cellulose, with an affinity approximately six times lower than the Type II domain, and that it potentiates Pf Xyn10A activity against insoluble complex substrates by increasing enzyme substrate proximity alone, rather than through the additional mechanism of disruption of the cellulose structure. CBD_x does not appear to interact synergistically with the Type II domain in full-length Pf Xyn10A, either in ligand binding or in the potentiation of catalytic activity. The functional significance of CBD_x in Pf Xyn10A is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The *Escherichia coli* strains used in this study were BL21(DE3):pLysS (Novagen, Madison, WI, U.S.A.), JM83 [24] and XL1-Blue (Stratagene). The plasmid vectors employed in this study were pCR-Blunt (Invitrogen) and pET16b (Novagen). Plasmid pRS4, which encodes the catalytic domain of Pf Xyn10A (Pf Xyn10A_{CD}), was described previously [23]. Plasmids constructed during the course of this study are denoted as follows: pJG1, encoding the mature Pf Xyn10A; pJG2, encoding the Type X CBD homologue from Pf Xyn10A (CBD_x); pJG3, encoding a truncated version of Pf Xyn10A (Pf Xyn10A') comprising the catalytic domain and Type X CBD, but lacking the Type II CBD; pJG4, encoding a catalytically inactive mutant of Pf Xyn10A' (E246A) in which the catalytic nucleophile, Glu-246, was mutated to an alanine residue; pJG5, encoding a truncated version of Pf Xyn10A (Pf Xyn10A'') lacking the Type X CBD, in which the Type II CBD and catalytic domain of the enzyme are joined by a serine-rich linker sequence. The molecular architectures of the proteins used in this study are displayed in Figure 1. All *E. coli* strains were cultured in Luria broth (LB) supplemented with ampicillin (50 µg/ml). To produce Pf

Xyn10A_{CD}, *E. coli* XL1-Blue harbouring pRS4 was grown for 16 h at 37 °C in LB containing ampicillin. To generate the other recombinant proteins *E. coli* BL21(DE3), harbouring pLysS and the appropriate recombinant plasmids, was cultured to mid-exponential phase (*D*₅₅₀ 0.5) in LB containing ampicillin at 37 °C. Isopropylthio-β-D-galactopyranoside was then added to a final concentration of 1 mM and the cultures were incubated for a further 4 h at 30 °C.

Construction of plasmids

The plasmids pJG1, pJG2, pJG3 and pJG5 were constructed by using PCR to amplify regions of the gene (*xynA*) flanked by appropriate restriction sites, and inserting the resultant DNA into pET expression vectors. The primer pairs and the amplified regions of *xynA* are displayed in Table 1. The blunt-ended PCR products were ligated into pCR-Blunt, and selected transformants were sequenced using a 373 Applied Biosystems (Foster City, CA, U.S.A.) sequencer and the Dye Prism dideoxy terminator kit (Applied Biosystems). The *xynA* sequences were then excised from the recombinant plasmids by digestion with *Nde*I and *Bam*HI and cloned into pET16b to generate pJG1, pJG2 and pJG3. To construct pJG5, the region of *xynA* between nucleotides 78 and 538, encoding the Type II CBD of Pf Xyn10A and the upstream 45-residue-linker sequence, was amplified and the resultant PCR product cloned into pET16b which had been cut with *Nde*I and *Bam*HI to generate pJG5'. The region of *xynA* between nucleotides 779 and 1832, encoding the catalytic domain of Pf Xyn10A, was amplified with primers that incorporated *Bam*HI sites at the 5' and 3' ends of the PCR product. The resultant DNA was then cloned into the *Bam*HI site of pJG5', and recombinants in which the two regions of *xynA* were in the same orientation were designated pJG5, and retained for further use. The conditions used in all the PCR reactions were as described previously [25]. The plasmid pJG4 was constructed by subjecting pJG2 to site-directed mutagenesis using the Transformer kit supplied by ClonTech (Heidelberg, Germany). The selection and mutagenic primers used in the Transformer method were 5'-GACTGGTGAGTATTCAACCAAGTC-3' and 5'-ACGCACATCCAATGCAGTAATCTTGAT-3', respectively. The nucleotides in bold are the mutations incorporated into *xynA*.

Purification of recombinant proteins

Pf Xyn10A_{CD} was purified by anion-exchange chromatography as described previously [21]. CBD_x, Pf Xyn10A, Pf Xyn10A', E246A and Pf Xyn10A'' all contained N-terminal His₁₀ tags, and were therefore purified by nickel-ion-affinity chromatography, using a Talon[™] metal-affinity column (ClonTech). The purification protocols were as described previously [10].

Enzyme, ligand and protein assays

The activities of Pf Xyn10A and its derivatives against soluble xylan, methylumbelliferyl-β-D-cellobioside (MUC) and cellulose-xylan complexes were determined as described previously [12]. Protein concentration was determined by the method of Pace et al. [26] and the purity of protein preparations was evaluated by SDS/PAGE [27]. Affinity-gel electrophoresis was performed essentially as described by Tomme et al. [28]. Release of carbohydrate from cotton fibres was determined by the phenol/sulphuric acid method of Dubois et al. [29], and the binding of the Type X CBD and full length Pf Xyn10A to different forms of cellulose was performed as described by Bolam et al. [10].

Figure 1 Molecular architecture of Pf Xyn10A and its derivatives

The domains present in the derivatives of Pf Xyn10A are represented as follows: Type II CBD (■), Type X CBD (▨) and the catalytic domain (□). Linker sequences are depicted by a heavy bold line. The plasmid pJG4 encodes Pf Xyn10A' in which the catalytic nucleophile of the catalytic domain (Glu-246) has been mutated to an alanine. This protein is referred to as E246A.

Table 1 Primers used to amplify segments of *xynA*

Plasmid	Encoded protein	Primer pairs used to amplify regions of <i>xynA</i>	Region of <i>xynA</i> amplified
pJG1	Pf Xyn10A	5'-GGCAGATCTGCATATGGCACAACAGCAACCTTGC-3' 5'-GCGGGATCTTAACGACCACTCAATGCCTCGAC-3'	78–1832
pJG2	CBD _x	5'-GCGACACCCGGCTCGCATATGGGTAAACCAGCAATGTA-3' 5'-GCGGGATCCGGACACGGGTGTGGAACCTGCCGC-3'	536–701
pJG3	Pf Xyn10A'	5'-GCGACACCCGGCTCGCATATGGGTAAACCAGCAATGTA-3' 5'-GCGGGATCTTAACGACCACTCAATGCCTCGAC-3'	536–1832
pJG5	Pf Xyn10A''	5'-GGCAGATCTGCATATGGCACAACAGCAACCTTGC-3' 5'-GCGGGATCCCTGGACGAGCCGGGTGTACTGCTGGC-3' 5'-GCGGATCCTGCCACCGGCAACGGTTTGGCCAGCC-3' 5'-GCGGGATCTTAACGACCACTCAATGCCTCGAC-3'	78–538 779–1832

Figure 2 SDS/PAGE of purified Pf Xyn10A and its derivatives

The purified proteins were subjected to SDS/PAGE using a 10% acrylamide gel. The following proteins are shown: (A) lane 1, Pf Xyn10A; lane 2, Pf Xyn10A''; lane 3, Pf Xyn10A'; lane 4, Pf Xyn10A_{CD}; lane 5, E246A; (B) lane 1, CBD_x. Sigma low molecular mass standards (with masses, in kDa, of 66, 45, 36, 29, 24, 20 and 14) are in lanes L.

Biophysical properties of cellulose and plant cell walls

Evaluation of microfibril release from cotton by the Type X CBD was as described previously [10]. The capacity of CBD_x to influence the extensibility of plant cell walls was determined by measuring the rate at which the plant cell wall is extended when subjected to a constant force (Creep assay) as described previously [10], and the influence of the protein on the tensile strength of filter paper was assessed using an Instron 91-51-17M extensometer (Instron, Boston, MA, U.S.A.), as described by Bolam et al. [10].

Figure 3 The capacity of Pf Xyn10A' and Pf Xyn10A_{CD} to bind to Avicel

Pf Xyn10A' and Pf Xyn10A_{CD} were incubated with Avicel and the capacity of the proteins to bind to the polysaccharide was evaluated by SDS/PAGE using a 10% acrylamide gel. The following samples are shown: (A) lane 1, Pf Xyn10A'; lane 2, material from the Pf Xyn10A' sample that did not bind to Avicel; lanes 3 and 4, respectively, penultimate and last washes of Avicel previously incubated with Pf Xyn10A'; lane 5, material recovered from Avicel pellet incubated with Pf Xyn10A'. (B) shows the following samples: lane 1, Pf Xyn10A_{CD}; lane 2, material in Pf Xyn10A_{CD} sample that did not bind to Avicel; lanes 3 and 4, respectively, penultimate and last washes of Avicel previously incubated with Pf Xyn10A_{CD}; lane 5, material recovered from Avicel pellet incubated with Pf Xyn10A_{CD}. Lanes L contain Sigma low molecular mass standards (with masses, in kDa, of 66, 45, 36, 29, 24, 20 and 14).

NMR spectroscopy

NMR spectroscopy of CBD_x was performed as described previously [10].

RESULTS

The internal domain of Pf Xyn10A is a functional CBD

To establish whether the internal CBD_x homologue of PfXyn10A is functional, we purified Pf Xyn10A', which lacks the N-terminal Type II CBD (Figures 1 and 2), and the catalytic domain of the enzyme (Pf Xyn10A_{CD}), and incubated the proteins with cellulose. The data (Figure 3) showed that Pf Xyn10A' bound to Avicel, whereas Pf Xyn10A_{CD} did not interact with the insoluble matrix. These results demonstrate that the internal domain of Pf Xyn10A is a functional Type X CBD.

Cellulose-binding properties of CBD_x

The affinities of Pf Xyn10A' and CBD_x, expressed as a discrete domain, for different forms of cellulose were analysed by binding-isotherm measurements using the model described previously [30]. A typical example of the data obtained is displayed in Figure 4. The results, which seemed to fit a one-site binding model, were used to calculate the affinity constant for binding to cellulose. The data (Table 2) showed that Pf Xyn10A' and CBD_x exhibited similar affinities for acid-swollen cellulose and bacterial microcrystalline cellulose, which were approximately six-fold

Table 2 The affinity of Pf Xyn10A and its derivatives for crystalline and amorphous cellulose

Protein	Ligand affinity (K_d ; M^{-1})	
	Bacterial microcrystalline cellulose	Acid-swollen cellulose
Pf Xyn10A	1.82×10^6	1.72×10^6
Pf Xyn10A'	2.74×10^5	2.63×10^5
Pf Xyn10A''	1.70×10^6	1.56×10^6
CBD _x	2.54×10^5	2.32×10^5

lower than the affinity of the Pf Xyn10A Type II CBD for the corresponding ligands [10].

To investigate whether CBD_x exhibits any affinity for soluble cellulose derivatives, Pf Xyn10A' was subjected to non-denaturing gel electrophoresis in the presence and absence of hydroxyethyl-cellulose. There was no apparent retardation in the electrophoretic mobility of the enzyme when the soluble cellulose was added to the matrix (results not shown). In addition, there

Figure 4 Binding isotherms of Pf Xyn10A, Pf Xyn10A', Pf Xyn10A'' and CBD_x incubated with Avicel

Adsorption measurements of Pf Xyn10A (A), Pf Xyn10A'' (B), Pf Xyn10A' (C) and CBD_x (D) were performed at 30 °C in 50 mM sodium phosphate buffer pH 7.0 as described previously [10].

Figure 5 NMR spectra of CBD_x in the presence of increasing amounts of cellohexaose

CBD_x (0.8 mM) was titrated with increasing molar equivalents (approx.) of cellohexaose. The vertical lines mark the initial positions for peaks associated with protons on Trp-42 (W42), Trp-44 (W44), Cys-25 (C25) and the δ [$\text{I51}(\delta)$] and γ [$\text{I51}(\gamma)$] carbons of Ile-51.

was no significant difference in the binding of CBD_x to bacterial microcrystalline cellulose in the presence of cellohexaose, and the oligosaccharide did not elute the domain from insoluble cellulose, even at saturating sugar concentrations (results not shown). Analysis of the NMR spectrum of CBD_x , titrated with increasing concentrations of cellohexaose, showed that there was a small downfield shift in two of the Trp N^{H} signals, and a similar shift in an Ile signal (Figure 5). The affinity constant for the binding was estimated from a plot of the change in chemical shift of the γ carbon of Ile-51 against ligand concentration using the method described by Johnson et al. [6]. The results (Figure 6) showed that the K_a value for cellohexaose was approximately $1.1 \times 10^2 \text{ M}^{-1}$. It is apparent, therefore, that the affinity of the CBD for cellohexaose was approx. 2500 times as low as for insoluble cellulose. These results show that CBD_x , like many other CBD families [4,10], binds much more tightly to insoluble cellulose than to soluble forms of the polysaccharide or cello-oligosaccharides, suggesting that the domain interacts with more than one cellulose chain within the crystalline lattice.

To establish whether Type II and Type X CBDs act in synergy to bind to cellulose in full length Pf Xyn10A, the wild-type Pf Xyn10A was purified (Figure 2) and its affinity for different forms of cellulose was evaluated. The data displayed in Table 2 showed that native Pf Xyn10A and the Type II CBD of the xylanase exhibited very similar affinities for cellulose, indicating that there is no synergy in ligand binding between the two CBDs in Pf Xyn10A.

Does CBD_x potentiate catalytic activity?

To establish whether CBD_x potentiates xylanase activity against cellulose-xylan complexes, Pf Xyn10A, Pf Xyn10A', Pf Xyn10A'' and Pf Xyn10A_{CD} were incubated with the insoluble cellulose-xylan complex, soluble xylan and MUC, and the capacity of the three enzymes to hydrolyse these substrates was assessed. When the four enzymes were incubated with the cellulose-xylan complex, more than 99% of Pf Xyn10A_{CD} was not associated with the insoluble polysaccharide complex during the experiment, while > 90% of Pf Xyn10A, Pf Xyn10A' and Pf Xyn10A'' bound to the cellulose-xylan complex, and there was no substantial decrease in the activity of the enzymes during the course of the experiment (results not shown). The activities of the four enzymes against the three substrates are displayed in Table 3 and Figure 7. All the enzymes exhibited similar activities against soluble xylan or MUC; however, Pf Xyn10A, Pf Xyn10A' and Pf Xyn10A'' were 2.5 times more active against the xylan-cellulose complex than Pf Xyn10A_{CD}. These data indicate that CBD_x potentiates xylanase activity against insoluble cellulose-xylan complexes, but not against soluble substrates that contain no cellulose.

Does CBD_x disrupt plant cell wall structure?

To investigate whether CBD_x disrupts the structure of the plant cell wall and crystalline cellulose, the domain was incubated with

Figure 6 Plot of change in the chemical shift of the NMR spectrum against cellobiose concentration

The change in chemical shift was for the protons attached to the γ carbon of Ile-51. The data in this figure are derived from the NMR spectra shown in Figure 5.

Table 3 Activity of Pf Xyn10A and its derivatives with different substrates

The activities of Pf Xyn10A, Pf Xyn10A' and Pf Xyn10A'' are relative to the catalytic activities of Pf Xyn10A_{CD} with each of the three substrates.

Enzyme	Relative enzyme activity		
	MUC	Soluble xylan	Cellulose–xylan complex
Pf Xyn10A _{CD}	1.00	1.00	1.00
Pf Xyn10A	0.95	1.07	2.60
Pf Xyn10A'	0.98	1.04	2.55
Pf Xyn10A''	1.02	1.02	2.47

plant cell walls derived from cucumber hypocotyls and with Whatman 3MM filter paper strips, and its effect on the integrity of these macromolecules was monitored by the Creep and the Instron assays. CBD_x did not affect the extensibility of plant cell walls when exposed to a constant force (Creep assay), or decrease the load required to break paper strips in the Instron assay (results not shown). To further investigate whether CBD_x can disrupt crystalline cellulose, the domain was incubated with cotton fibres and the release of microfibrils was monitored. No release of small particles from the cotton was observed (data not shown). The Creep, Instron and small-particle-release assays indicated that CBD_x does not cause a substantial disruption of cellulose or the plant cell wall.

To investigate whether CBD_x could be influencing the activity of Pf Xyn10A against the cellulose–xylan complex by increasing substrate accessibility, Pf Xyn10A_{CD} was incubated with the

Figure 7 Hydrolysis of a cellulose–xylan complex by Pf Xyn10A, Pf Xyn10A', Pf Xyn10A'' and Pf Xyn10A_{CD}

Pf Xyn10A (◆), Pf Xyn10A' (▲), Pf Xyn10A'' (▼) and Pf Xyn10A_{CD} (■) were incubated with cellulose–xylan complex derived from kraft pulp. The release of reducing sugar was determined as described in the Materials and methods section.

Table 4 The activity of Pf Xyn10A_{CD} with the cellulose–xylan complex in the presence of CBD_x and E246A

^a CBD refers to either CBD_x or E246A, which were added to the enzyme reactions containing Pf Xyn10A_{CD}. ^b Catalytic activity is relative to the activity of Pf Xyn10A_{CD} with the cellulose–xylan complex in the absence of CBD_x or E246A. When tested at a concentration equivalent to that in the 10:1 ratio, CBD_x and E246A had no measurable activity in the absence of Pf Xyn10A_{CD}.

Ratio of CBD ^a :Pf Xyn10A _{CD}	Relative catalytic activity ^b	
	Pf Xyn10A _{CD} + CBD _x	Pf Xyn10A _{CD} + E246A
0.5:1.0	1.04	1.03
1:1	0.98	0.97
2:1	1.09	1.06
5:1	1.04	1.05
10:1	1.02	1.03

polysaccharide matrix in the presence of increasing concentrations of CBD_x or E246A. The data, presented in Table 4, showed that the CBD did not potentiate the activity of Pf

Xyn10A_{CD}. These data suggest that CBD_x does not mediate its positive effect on Pf Xyn10A catalytic activity by disrupting the structure of the cellulose component of the target substrates.

DISCUSSION

Results presented in this study show that CBD_x binds to different forms of insoluble cellulose, although its affinity for these ligands is approximately six-fold lower than that of the Type II CBD from the same enzyme. These results support the view that, in *Pseudomonas* enzymes containing Types II and X CBDs, both domains retain the capacity to bind to insoluble cellulose. In contrast, CBD_x did not interact with soluble cellulose and exhibited very weak affinity for cello-oligosaccharides. This high degree of ligand specificity for insoluble cellulose also occurs in Types I, II and III CBDs [4,5,10], but not in Type IV CBDs [6,28]. Those domains that exhibit a preference for insoluble cellulose contain a planar hydrophobic face, rich in aromatic residues, that constitutes the ligand-binding region of these proteins. In view of the preference of CBD_x for insoluble cellulose, we suggest that the ligand-binding region of this domain will also contain a planar surface of exposed aromatic residues. Precisely why these CBDs exhibit a preference for insoluble cellulose is, currently, unclear, although it is likely that the ligand specificity of these domains is related to their capacity to bind to multiple cellulose chains. It is interesting to note that the molecular architecture of Pf Xyn10A is common to other *Pseudomonas* plant-cell-wall hydrolases, with three cellulases, a cellodextrinase and a mannanase all consisting of a Type II CBD, Type X CBD and a catalytic domain [18–21]. Based on the results presented in this report, we suggest that in all these enzymes both the Type II and Type X CBDs retain their capacity to bind to cellulose.

The affinity of full-length Pf Xyn10A for cellulose is very similar to the affinity of the enzyme's Type II CBD, suggesting that no significant synergy is observed between the two CBDs of the xylanase. Linder et al. [31] showed that extensive synergy occurred between two Type I CBDs (similar cellulose affinity to CBD_x) when they were covalently linked to form a single polypeptide. In contrast, tandem repeats of the relatively weak Type IV CBD from *C. fimi* endo- β -1,4-glucanase C (CenC) did not exhibit higher affinity for cellulose compared with the CBDs expressed as discrete entities. It would appear, therefore, that multiple CBDs in single enzymes do not cause a substantial increase in affinity for cellulose, and we suggest that the evolutionary rationale for having both Type II and Type X CBDs in several *Pseudomonas* plant-cell-wall hydrolases is not to increase the enzymes' affinity for cellulose.

In a previous study we showed that the Type II CBD of Pf Xyn10A potentiated the activity of the enzyme against complex substrates [12]. Results presented in this study showed that CBD_x increased the activity of Pf Xyn10A against an insoluble cellulose–xylan complex, but not against soluble xylan or MUC. These results indicate that both the Type II and Type X CBDs in Pf Xyn10A play an important role in the activity of the enzyme against plant cell walls. These observations raise the possibility that the Type II and Type X CBDs of Pf Xyn10A act synergistically to potentiate the activity of the enzyme against the cellulose–xylan complex. However, as Pf Xyn10A, Pf Xyn10A' and Pf Xyn10A'' exhibited similar activities against cellulose–xylan complexes, there is no evidence to suggest that multiple CBDs are more effective in potentiating the activity of Pf Xyn10A against complex substrates than single CBDs, regardless of whether they are Type II or Type X.

Previously we have shown that *Pseudomonas* Type II CBDs do

not affect the structural integrity of either cellulose or plant cell walls, and thus do not potentiate cellulase and xylanase activity by increasing the accessibility of the substrate [10]. The results in the present report also showed that CBD_x did not disrupt the structure of either cellulose or the plant cell wall. It could be argued that the biophysical measurements used here only detect substantial changes in the structure of these macromolecules, and if CBD_x caused only subtle changes in the structure of cellulose and/or the plant cell wall, they would not be detected by the Creep and Instron assays. Indeed, it has been suggested [14] that *Cellulomonas* Type II CBDs only cause very small changes to the structure of crystalline cellulose, such as the solvation of single cellulose chains at the surface of the microfibrils, and it is possible that these effects on the structure of these macromolecules would not be detected by the biophysical measurements used in the present study. However, if CBD_x was potentiating xylanase activity by increasing the accessibility of the substrate via a disruptive mechanism, then the domain should be capable of increasing the activity of Pf Xyn10A_{CD}, especially at high CBD_x:Pf Xyn10A_{CD} ratios, even when it is not covalently attached to the enzyme. The results presented in this study showed that CBD_x, when it was not covalently attached to Pf Xyn10A_{CD}, did not influence the capacity of the enzyme to attack the insoluble cellulose–xylan complex. This behaviour argues against the possibility that the domain is potentiating xylanase activity by disrupting the structure of the substrate and thus making the target bonds more accessible to enzyme attack. We propose, therefore, that CBD_x, by bringing Pf Xyn10A into prolonged contact with the plant cell wall, increases the effective concentration of the enzyme on the surface of the substrate, thereby facilitating an increase in glycosidic-bond cleavage.

To conclude, the results presented in this report show that the Type X CBD homologue in Pf Xyn10A binds to cellulose and, like many other types of CBD, exhibits a strong preference for insoluble forms of the polysaccharide. CBD_x enhances the activity of Pf Xyn10A by increasing the enzyme concentration on the surface of the substrate, rather than improving the accessibility of the target glycosidic bonds by disrupting the structure of the plant cell wall. No synergy was observed, either in ligand binding or in the potentiation of catalytic activity, between the Type II and Type X CBDs in Pf Xyn10A. It is difficult, therefore, to understand the selection pressure(s) that have led to the evolution of several *Pseudomonas* plant-cell-wall hydrolases which contain two distinct CBDs that are both functional. It could be argued that these enzymes recently acquired a module consisting of a Type II and Type X CBD, and that there has not been sufficient time for mutations to accumulate that result in loss of function of either of these domains. We believe that this is very unlikely as the linker sequences between the Type II and Type X CBDs in the *Pseudomonas* cellulases, xylanases and mannanases are different in sequence and length, suggesting either: (i) that several different recombination events led to the evolution of modules comprising Type II and Type X CBDs, or (ii) that there was a single module consisting of the Type II and Type X domains, which has acquired numerous mutations in the linker regions. We propose that although both domains bind to similar cellulose molecules, the two CBDs can bind to different forms of cellulose within the context of the plant cell wall. For example, maybe the Type II CBD binds to cellulose that is H-bonded with xylans, whereas the Type X domain binds preferentially to cellulose microfibrils that interact with xyloglucans. Future work will be directed towards investigating whether the different types of CBD exhibit preference for cellulose molecules that interact with specific hemicellulose molecules within the plant cell wall.

REFERENCES

- 1 Gilbert, H. J. and Hazlewood, G. P. (1993) *J. Gen. Microbiol.* **139**, 187–194
- 2 Tomme, P., Warren, R. A. J. and Gilkes, N. R. (1995) *Adv. Microbiol. Physiol.* **37**, 1–81
- 3 Kraulis, P. J., Clore, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J. and Gronenborn, A. M. (1989) *Biochemistry* **28**, 7241–7257
- 4 Xu, G.-Y., Ong, E., Gilkes, N. R., Kilburn, D. G., Muhandiram, D. R., Harris-Brandts, M., Carver, J. P., Kay, L. E. and Harvey, T. S. (1995) *Biochemistry* **34**, 6993–7009
- 5 Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y. and Steitz, T. A. (1996) *EMBO J.* **15**, 5739–5751
- 6 Johnson, P. E., Tomme, P., Joshi, M. D. and McIntosh, L. P. (1996) *Biochemistry* **35**, 13895–13906
- 7 Brun, E., Moriaud, F., Gans, P., Blackledge, M. J., Barras, F. and Marion, D. (1997) *Biochemistry* **36**, 16074–16086
- 8 Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T. A., Knowles, J. K. C. and Teeri, T. T. (1992) *Proteins* **14**, 475–482
- 9 Nagy, T., Simpson, P., Williamson, M. P., Hazlewood, G. P., Gilbert, H. J. and Orosz, L. (1998) *FEBS Lett.* **429**, 312–316
- 10 Bolam, D. N., Ciruela, A., McQueen-Mason, S., Simpson, P., Williamson, M. P., Rixon, J. E., Boraston, A., Hazlewood, G. P. and Gilbert, H. J. (1998) *Biochem. J.* **331**, 775–781
- 11 Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. T. and Claeysens, M. (1988) *Eur. J. Biochem.* **170**, 575–581
- 12 Black, G. W., Rixon, J. E., Clarke, J. H., Hazlewood, G. P., Theodorou, M. K., Morris, P. and Gilbert, H. J. (1996) *Biochem. J.* **319**, 515–520
- 13 Din, N., Gilkes, N. R., Tekant, B., Miller, Jr., R. C. and Warren, R. A. J. (1991) *Bio/Technology* **9**, 1096–1099
- 14 Din, N., Damude, H. G., Gilkes, N. R., Miller, Jr., R. C. and Warren, R. A. J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11383–11387
- 15 Pages, S., Gal, L., Belaich, A., Gaudin, C., Tardif, C. and Belaich, J.-P. (1997) *J. Bacteriol.* **179**, 2810–2816
- 16 Gibbs, M. D., Saul, D. J., Luthi, E. and Bergquist, P. L. (1992) *Appl. Environ. Microbiol.* **58**, 3864–3867
- 17 Coutinho, J. B., Moser, B., Kilburn, D. G., Warren, R. A. J. and Miller, Jr., R. C. (1991) *Mol. Microbiol.* **6**, 1221–1233
- 18 Hall, J. and Gilbert, H. J. (1988) *Mol. Gen. Genet.* **213**, 112–117
- 19 Gilbert, H. J., Hall, J., Hazlewood, G. P. and Ferreira, L. M. A. (1990) *Mol. Microbiol.* **4**, 759–767
- 20 Ferreira, L. M. A., Hazlewood, G. P., Barker, P. J. and Gilbert, H. J. (1991) *Biochem. J.* **279**, 739–749
- 21 Hall, J., Hazlewood, G. P., Huskisson, N. S., Durrant, A. J. and Gilbert, H. J. (1989) *Mol. Microbiol.* **3**, 1211–1219
- 22 Millward-Sadler, S. J., Davidson, K., Hazlewood, G. P., Black, G. W., Gilbert, H. J. and Clarke, J. H. (1995) *Biochem. J.* **312**, 39–48
- 23 Ferreira, L. M. A., Durrant, A. J., Hall, J., Hazlewood, G. P. and Gilbert, H. J. (1990) *Biochem. J.* **269**, 261–264
- 24 Norrander, J., Kempt, T. and Messing, J. (1983) *Gene* **26**, 1816–1822
- 25 Bolam, D. N., Hughes, N., Virden, R., Lakey, J. H., Hazlewood, G. P., Henrissat, B., Braithwaite, K. L. and Gilbert, H. J. (1996) *Biochemistry* **35**, 16195–16204
- 26 Pace, N. C., Vadjos, F., Fee, L., Grimsley, G. and Gray, J. (1995) *Protein Sci.* **4**, 2411–2423
- 27 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 28 Tomme, P., Creagh, A. L., Kilburn, D. G. and Haynes, C. A. (1996) *Biochemistry* **35**, 13885–13894
- 29 Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Biochem.* **28**, 350–359
- 30 Gilkes, N. R., Jervis, E., Henrissat, B., Tekent, B., Miller, Jr., R. C., Warren, R. A. J. and Kilburn, D. G. (1992) *J. Biol. Chem.* **267**, 6743–6749
- 31 Linder, M., Salovuori, I., Ruohonen, L. and Teeri, T. T. (1996) *J. Biol. Chem.* **271**, 21268–21272

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