Evidence that linker sequences and cellulose-binding domains enhance the activity of hemicellulases against complex substrates

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Xylanase A (XYLA) and arabinofuranosidase C (XYLC) from *Pseudomonas fluorescens* subsp. *cellulosa* are modular enzymes consisting of discrete cellulose-binding domains (CBDs) and catalytic domains joined by serine-rich linker sequences. To evaluate the role of the CBDs and interdomain regions, the capacity of full-length and truncated derivatives of the two enzymes, lacking either the linker sequences or CBDs, to hydrolyse a range of substrates, and bind to cellulose, was determined. Removal of the CBDs did not affect either the activity of XYLA or XYLC against soluble arabinoxylan. Similarly, deletion of the linker sequences did not alter the

affinity of the enzymes for cellulose or their activity against soluble substrates, even when bound to cellulose via the CBDs. Truncated derivatives of XYLA lacking either the linker sequences or the CBD were less active against xylan contained in cellulose–hemicellulose complexes, compared with the full-length xylanase. Similarly, removal of the CBD from XYLC diminished the activity of the enzyme (XYLC'') against plant-cell-wall material containing highly substituted arabinoxylan. The role of CBDs and linker sequences in the catalytic activity of hemicellulases against the plant cell wall is discussed.

INTRODUCTION

Analysis of the molecular architecture of cellulases and xylanases has shown that many of these enzymes have a modular structure in which the functional domains are spatially separated by linker sequences [1–3]. These interdomain regions vary in size from six to over 300 amino acids, and generally consist of single amino acids [4] or of tandem repeats of dipeptides [5]. In general, modular cellulases consist of discrete catalytic domains (CDs) and cellulose-binding domains (CBDs) which are connected by linker sequences [1–3]. Although the removal of CBDs from endo- β 1,4-glucanases and cellobiohydrolases does not affect the activity of the respective enzymes against soluble substrates, CBDs appear to play a pivotal role in the activity of cellulases against the more recalcitrant crystalline forms of cellulose [6–8]. Knowles et al. [9] suggested that CBDs facilitate cellulose hydrolysis by disrupting the inter- and intra-chain hydrogen bonds within the substrate and channeling individual cellulose chains into the active site of the enzyme. Recently, Din et al. demonstrated that discrete CBDs had the capacity to disrupt the structure of highly crystalline forms of cellulose [10,11].

It is now well established that numerous hemicellulases, including endo- β 1,4-xylanases (xylanases), endo- β 1,4-mannanases, an arabinofuranosidase (arabinofuranosidase C, XYLC) and an acetyl xylan esterase similarly contain discrete CBDs and CDs linked by sequences rich in hydroxy amino acids [12–15]. Ferreira et al. [14] suggested that CBDs may potentiate the catalytic activity of hemicellulases by mediating intimate contact between the enzymes and the plant cell wall, although there is currently no experimental data to support this hypothesis.

Although the majority of modular plant-cell-wall hydrolases

contain easily identifiable linker sequences, there is a paucity of information on their functional importance. Removal of the proline/threonine interdomain region from *Cellulomonas fimi* endoglucanase A affected the relative orientation of the CBD and CD, reduced the activity of the enzyme against both soluble and crystalline forms of cellulose and affected the desorption of the cellulase from Avicel [16]. Deletion of the linker sequences from *Trichoderma reesei* cellobiohydrolase I did not affect the activity of the enzyme against soluble substrates, although there was a significant reduction in its capacity to hydrolyse crystalline cellulose [17]. The removal of linker sequences from *Pseudomonas fluorescens* subsp. *cellulosa* xylanase A (XYLA) had no effect on the capacity of the enzyme to bind to cellulose or hydrolyse purified soluble and insoluble arabinoxylans, and led to the suggestion that the DNA encoding the linker sequences may provide a mechanism for the rapid evolution of modular plant cell wall hydrolases [12].

Although a number of studies have attempted to elucidate the role of CBDs in cellulases, the functional significance of this domain in xylanases and other hemicellulases remains obscure. Likewise, no unifying role has been defined for linker sequences. The purpose of the present study was to determine whether linker sequences and CBDs play an important role in the activity of hemicellulases. To achieve this objective the catalytic activity of full-length and truncated forms of XYLA and XYLC from *P. fluorescens* subsp. *cellulosa*, against plant cell wall material, was evaluated. The results show that the removal of the linker sequences does not diminish the capacity of XYLA or XYLC to attack soluble arabinoxylans when bound to cellulose via the CBD. Removal of the linker sequences or CBD from XYLA reduced the activity of the enzyme against a cellulose–

Abbreviations used: XYLA, xylanase A; XYLC, arabinofuranosidase C; CBD, cellulose-binding domain; CD, catalytic domain; IPTG, isopropyl thio- β -D-galactopyranoside; MUC, methylumbelliferyl β -D-cellobioside; MUA, methylumbelliferyl α -L-arabinofuranoside.

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hemicellulose complex containing xylan that was not highly substituted. Similarly, removal of the CBD from XYLC reduced the activity of the enzyme against plant-cell-wall material containing highly substituted arabinoxylan. A possible role for the linker sequences and CBDs in hemicellulases is discussed.

MATERIALS AND METHODS

Microbial strains vectors and culture conditions

The *Escherichia coli* strain employed in the present study was JM83. The vectors used were pUC18/19 [18], pCRII (Invitrogen), pNM52 [19] and pMTL22/23p [20]. The construction of plasmids that encode full-length XYLA (pRS6) and XYLC (pLE7), the CD of XYLA (pRS16) and a derivative of XYLA that contains no linker sequences (pLH4), are described elsewhere [12,13]. *E. coli* strains were cultured in Luria broth (LB) or on LB-agar at 37 °C unless otherwise stated. Ampicillin (100 µg/ml), 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (80 µg/ml) and 4-O-methyl-O-glucurono-D-xylan-Remazol Brilliant Blue R (500 µg/ml) were added to the media to select for transformants, recombinants and xylanase-expressing strains respectively. To induce the expression of genes under control of *lacO*, media were supplemented with isopropyl thio-β-D-galactopyranoside (IPTG; 100 µM).

Construction of derivatives of XYLC

The region of the XYLC gene (*xynC*) between nucleotides 1 and 413, that encodes the CBD, was amplified using the primers 5' AGCGGATAACAATTTACACAGGA 3' (Primer A) and 5' CTCGAATTCGGTGGTTGCACTGTTACAAG 3', while the region of *xynC* encoding the CBD and a small linker region of XYLC, was amplified using Primer A and the primer 5' CTCGAATTCGGAGGCCACACTGGATGCAG 3'. The two amplified DNA sequences were restricted with *EcoRI* and *HpaI*, and cloned into *EcoRI*/*SmaI*-digested pMTL22p to generate pGB1 (encodes the CBD of XYLC) and pGB2 (encodes the CBD and a small linker region of XYLC). The region of *xynC* between nucleotides 965 and 1712, encoding just the CD of XYLC, was amplified by the primer pair 5' CTCGAATTCACCCCATCA-CAAACCTGTGAG 3' and 5'CGTTGTAAAACGACGGC-CAGTGCC 3'. The amplified DNA was restricted with *EcoRI* and *SalI*, and cloned into *EcoRI*/*XhoI*-digested pMTL22p to generate pGB3. The DNA inserts in pGB1 and pGB2 were then excised on *EcoRI*/*XhoI* restriction fragments and cloned into *EcoRI*/*SalI*-digested pGB3 to generate pGB4 and pGB5 respectively. In pGB4 and pGB5, the regions of *xynC* encoding the XYLC CBD, and the XYLC CBD joined to a small linker respectively were in frame with the sequence encoding the CD of XYLC. DNA sequences were amplified by the PCR using the conditions described by Fontes et al. [21]. All amplified DNA sequences were resequenced using a PRISM™ ready reaction dye-deoxy terminator cycle sequencing kit and an Applied Biosystems ABI 373A sequenator to verify that no mutations were generated during PCR.

Purification of full-length and truncated derivatives of XYLC and XYLA

To purify full-length and truncated derivatives of XYLC, *E. coli* strains harbouring pLE7, pGB4 and pGB5 were grown in 400 ml of medium at 30 °C to mid-exponential phase. IPTG was added to each culture, and cells were incubated at 30 °C with aeration for a further 16 h. Bacterial cells were pelleted, resuspended in 5 ml of 100 mM Tris/HCl buffer, pH 7.5, and disrupted by

ultrasonication. Cell debris was removed by centrifugation at 12000 g for 30 min at 4 °C, and the supernatant, comprising the cell-free extract, was incubated with 5 ml of a 5% (w/v) suspension of Avicel in 50 mM sodium phosphate/12 mM citric acid (PC) buffer, pH 6.5, for 1 h at 4 °C. After washing the polysaccharide five times with 5 ml of ice-cold PC buffer, bound protein was eluted by incubating the cellulose with 100% (v/v) ethylene glycol. The Avicel was then removed and the protein-containing ethylene glycol solutions were dialysed three times against 300 vol. of PC buffer at 4 °C to renature the enzyme.

To generate the catalytic domain of XYLC, the full-length enzyme was incubated with chymotrypsin, at a ratio of 1 unit of chymotrypsin per mg of XYLC, for 1 h at 37 °C. Following the addition of chymotrypsin/trypsin inhibitor (Bowman-Birk inhibitor), the size of truncated XYLC was determined by SDS/PAGE as described by Laemmli [22].

The purification of XYLA' encoded by pLH4 was as described by Ferreira et al. [12], while XYLA and XYLA'', encoded by pRS6 and pRS16 respectively, were purified as described by Harris et al. [23].

Enzyme and protein assays

All enzyme assays were performed at least three times, and the data presented are the means of the results obtained. The purified polysaccharides used as substrates in the present study were soluble oat-spelt arabinoxylan, prepared as described by Ghangas et al. [24], and soluble wheat arabinoxylan (Megazyme Ltd). To evaluate the role of linker sequences and CBDs in enzymes acting on cellulose/hemicellulose complexes, we utilized two further substrates. The first comprised of kraft-pulped, delignified hardwood (supplied by the Department of Paper Science, University of Manchester Institute of Science and Technology), which contained principally cellulose and hemicellulose. The second consisted of destarched cell walls from *Festuca arundinacea* grown in suspension culture; analysis of this material revealed that 47% (w/w) constituted non-starch polysaccharides of which 12.9% (w/w) was arabinose, 11.6% (w/w) xylose, 7.9% (w/w) galactose and 14.8% (w/w) glucose. Substrates were incubated with XYLA, XYLC or their truncated derivatives, and at regular time intervals samples were removed and either analysed for reducing sugar, as described by Miller ([25] xylanase assays), or arabinose (arabinofuranosidase assays), as described by Kellett et al. [13]. The concentration of the soluble substrates varied from 0.4 to 3.0 mg/ml, while the plant cell wall materials were incubated with the different enzymes at a concentration of 20 mg/ml (hardwood kraft pulp) and 3 mg/ml (*Festuca* cells). To verify that enzymes which bound to the plant cell wall material had retained catalytic activity, 10 µl of the enzyme/plant-cell-wall mixtures were removed at regular time intervals and incubated with 1 ml of PC buffer containing either 1 mM methylumbelliferyl β-D-cellobioside (MUC; XYLA assays) or 1 mM methylumbelliferyl α-L-arabinofuranoside (MUA; XYLC assays), and the release of methylumbelliferone determined as described previously [26]. Cellulose-binding assays were as described by Ferreira et al. [12]. Protein was estimated by the method of Sedmak and Grossberg [27].

RESULTS

Characterization of native and deletion mutants of XYLC

To evaluate the role of linker sequences and CBDs in *endo*-acting and side-chain-cleaving hemicellulases, full-length and truncated forms of *xynA* and *xynC*, which encode xylanase A (XYLA) and arabinofuranosidase C (XYLC) from *Ps. fluorescens* subsp.

Figure 1 Structure of XYLA and XYLC and their derivatives

The regions of XYLA and XYLC were as follows: signal peptide (▨), CBD (■), linker sequence (—) and CD (▤). The designations of the two enzymes and their encoding plasmids are defined. XYLC''' was derived by treating XYLC with chymotrypsin as described in the Materials and methods section. The position of the amino acids at the extremities of the CBDs and CDs, in the respective full-length native enzymes, is indicated. The size of the linker sequence in XYLC'' is 17 amino acids and corresponds to residues 139–155 in the full-length sequence of XYLC.

cellulosa, respectively [4,13], were inserted into *E. coli* expression vectors. The molecular architecture of the various enzymes used in this study are displayed in Figure 1. The construction of genes encoding full-length XYLA and two derivatives of the enzyme consisting of the CD (XYLA''), and the CD joined directly to the CBD (XYLA'), and the subsequent purification of the respective enzymes were as described by Ferreira et al. [12]. In the present study, full-length and two truncated derivatives of XYLC were purified by cellulose affinity chromatography. The truncated enzyme, XYLC', contained no linker sequences, while in XYLC'' the CBD and CD were joined by a small serine-rich region. The purified enzyme preparations, shown in Figure 2, contained single polypeptides, as judged by SDS/PAGE, with M_r values 59 000 (XYLC), 44 000 (XYLC') and 42 000 (XYLC''). The sizes of the three forms of XYLC were similar to their predicted M_r values, based on deduced primary sequence data [13]. To generate XYLC''', full-length XYLC was treated with chymotrypsin, which decreased the size of the enzyme from 59 000 to 33 000 (Figure 2). The capacity of the 33 kDa polypeptide to release arabinose from arabinoxylan, but not to bind to cellulose (Table 1), confirmed its identity as the CD of XYLC.

To determine whether the removal of linker sequences and CBDs affected the capacity of XYLC derivatives to fold correctly, the catalytic activity of full-length and truncated derivatives of the enzyme, and their affinity for cellulose, were assessed. The data, presented in Table 1, showed that removal of either the CBD (XYLC''') or linker sequences (XYLC' and XYLC'') from XYLC did not influence the catalytic activities of the resultant

enzymes. Although removal of the CBD from XYLC abolished cellulose binding, truncated hemicellulases in which the linker sequences had been deleted but the CBD retained, exhibited the same affinity for cellulose as the full-length enzyme. These data indicate that removal of either the CBD or linker sequences from XYLC does not affect the ability of the enzyme to fold correctly.

Catalytic properties of hemicellulases bound to cellulose

To evaluate the influence of linker sequences on the catalytic activity of enzymes that are anchored to cellulose via the CBD, full-length and linker-less derivatives of XYLA and XYLC were bound to Avicel, and the activities of the enzymes against soluble arabinoxylans determined. The data, presented in Table 2, show that when XYLA, XYLC and their respective derivatives were bound to cellulose, there was no substantial difference in either the k_{cat} or K_m values of the full-length enzymes and their corresponding linker-less derivatives. It would appear, therefore, that linkers do not play an important role in the activity of the two hemicellulases against soluble substrates when bound to cellulose.

Activity of hemicellulases against plant-cell-wall material

To assess the role of linker sequences and CBDs on the catalytic activity of enzymes which hydrolyse arabinoxylans that are an integral component of cellulose/hemicellulose complexes, the

Figure 2 SDS/PAGE of full-length and truncated forms of XYLC
(A) Purified XYLC' (lane 1), XYLC'' (lane 2) and XYLC (lane 3) were subjected to SDS/PAGE using a 10% polyacrylamide gel. (B) depicts SDS/PAGE of purified XYLC (lane 1) and XYLC''' (lane 2). XYLC''' was generated by treating XYLC with chymotrypsin as described in the Materials and methods section. The M_r values ($\times 10^{-3}$) of the purified polypeptides are shown to the left of each panel.

Table 1 Catalytic properties of full-length and truncated derivatives of XYLC

The enzyme species are defined in Figure 1. The kinetic parameters were determined as described in the Materials and methods section using wheat arabinoxylan as the substrate. The k_{cat} values are defined as the molecules of substrate cleaved/s per molecule of enzyme.

Enzyme	K_m (mg/ml)	k_{cat}	Avicel (mg) that binds 50% of enzyme activity*
XYLC	1.269 ± 0.246	3.32 ± 0.167	0.83
XYLC'	1.160 ± 0.160	3.10 ± 0.221	0.71
XYLC''	1.402 ± 0.368	3.17 ± 0.178	0.76
XYLC'''	1.326 ± 0.134	3.27 ± 0.213	NB†

* Various quantities of Avicel were mixed with 0.026 units of the four enzymes. After 1 h incubation on ice, the cellulose was pelleted by centrifugation and the supernatant was assayed for unbound enzyme.
† NB, no enzyme bound to the Avicel.

Table 2 Catalytic activity of cellulose-bound full-length and truncated derivatives of XYLA and XYLC

The enzyme species, which are defined in Figure 1, were bound to Avicel as described in the Materials and methods section. The kinetic constants were determined using soluble wheat and oat-spelt arabinoxylan as the substrates for XYLC and XYLA respectively.

Enzyme	K_m (mg/ml)	k_{cat} *	Bound enzyme (%)
XYLA	0.967 ± 0.05	557 ± 5.7	98
XYLA'	1.034 ± 0.07	534 ± 7.9	99
XYLC	1.143 ± 0.143	0.91 ± 0.16	100
XYLC'	1.076 ± 0.195	0.93 ± 0.21	98
XYLC''	1.016 ± 0.208	0.81 ± 0.15	99

* k_{cat} is defined as molecules product/molecule of enzyme/s.

Figure 3 Release of xylose from a cellulose-hemicellulose complex by full-length and truncated forms of XYLA
Precisely 4.2 units (defined using oat-spelt arabinoxylan as the substrate) of XYLA (□), XYLA' (■) or XYLA'' (○) were incubated with 2% (w/v) kraft pulped hardwood in 20 ml of PC buffer at 37 °C. At regular time intervals, aliquots were removed and reducing sugar released was determined as described in the Materials and methods section.

Figure 4 Activity of XYLC and XYLC''' against *Festuca* cell walls
Festuca cell walls (0.3 %) were incubated with precisely 1.5 units of XYLC''' (□) or XYLC (■) respectively, and the release of arabinose was determined by HPLC analysis.

capacity of full-length and truncated derivatives of XYLA and XYLC to hydrolyse glycosidic bonds in plant cell wall-derived material was evaluated. The data are presented in Figures 3 and 4. When full-length XYLA, XYLA' (lacking the linker sequences) and XYLA'' (comprising the CD of the enzyme) were incubated with the cellulose-hemicellulose complex (hardwood kraft pulp), the full-length enzyme hydrolysed glycosidic bonds within this macromolecule at a rate 2-fold higher than the two truncated derivatives of the xylanase (Figure 3). It could be argued that the

Table 3 Activity of XYLA, XYLC and their respective derivatives when associated with the cellulose–hemicellulose complex

Enzyme	Time (min) ...	Enzyme activity retained (%) [*]			Bound enzyme (%) [†]
		0‡	60	180	
XYLA	100	100	96	93	98
XYLA'	100	100	110	115	100
XYLA''	100	100	100	102	952
XYLC	100	100	74	ND§	92
XYLC'''	100	100	94	ND	1

^{*} During the experiments displayed in Figures 3 and 4, samples from the reaction mixtures were removed at regular intervals and assayed for either xylanase activity, using MUC as the substrate, or arabinofuranosidase activity, using MUA as the substrate. Enzyme activity is presented as a percentage of the activity detected at the start of the experiment.

[†] The percentage of enzyme activity not associated with cellulose–hemicellulose complex was detected by pelleting the cellulose and assaying the supernatant for XYLC and XYLA activity, after 60 and 180 min incubations respectively.

[‡] The values are minutes after the start of the experiment.

[§] Abbreviation: ND, not determined.

difference in the activities of XYLA and XYLA' is the result of denaturation of the latter enzyme when it is bound to the plant cell wall. Alternatively, it is possible that the full-length enzyme exhibits a higher affinity for the cellulose component of the complex compared with XYLA'. To investigate these two possibilities, XYLA, XYLA' and XYLA'' were incubated with the cellulose–hemicellulose complex, and their capacity to hydrolyse MUC determined at regular intervals. The proportion of each enzyme associated with the cellulose/hemicellulose complex was also evaluated. The data (Table 3) showed that, throughout the incubation period, both XYLA and XYLA' remained bound to the cellulose–hemicellulose complex and displayed similar catalytic activities against MUC. These results confirm that XYLA derivatives possessing a CBD bind tightly to the complex substrate and show that neither enzyme is preferentially denatured when bound. Thus the data described indicate that both linker sequences and the CBD enhance the capacity of XYLA to attack xylans located in cellulose/hemicellulose complexes.

Analysis of the activity of the arabinofuranosidase against *Festuca* cell walls, which contain highly substituted arabinoxylan, revealed that XYLC released arabinose at a rate 3-fold higher than XYLC''' (Figure 4). These data support the view that CBDs facilitate the activity of *endo*- and side-chain-cleaving hemicellulases against cellulose/hemicellulose complexes.

DISCUSSION

The aim of the present study was to investigate the role of the linker sequences and CBDs of an *endo*-acting and side-chain-cleaving hemicellulase, on their capacity to attack components of, or polysaccharides released from, the plant cell wall. To achieve this objective, full-length and truncated derivatives of *Ps. fluorescens* XYLA and XYLC, lacking either the linker sequences or CBDs, were constructed, and the catalytic and cellulose-binding properties of the resultant enzymes were assessed. The data revealed that removal of the linker sequences from XYLC did not affect the catalytic activity of the enzyme against purified arabinoxylan or its ability to bind to cellulose, and that the CD and CBD of XYLC do not need to be separated by linker sequences to fold correctly. These results are consistent with those obtained in previous studies which demonstrated that linker-less derivatives of *Ps. fluorescens* XYLA and *T. reesei* cellobiohydrolase I retained the ability to hydrolyse soluble

Figure 5 Proposed model for the hydrolysis of soluble arabinoxylan by a CBD-containing hemicellulase that is bound to cellulose

The hemicellulase, which consists of a CBD (□), linker sequence (—) and CD (●), is attached to cellulose (▨) via the CBD. In (A) the linker sequence enables the CD to access multiple chains of soluble arabinoxylan (—). In (B) the cellulose, which is in close association with the CD, restricts the amount of soluble arabinoxylan available to the enzyme through steric hindrance.

substrates, and exhibited similar affinities for cellulose when compared with their respective full-length enzymes [12,17]. In contrast, removal of the linker sequences from *C. fimi* endoglucanase A reduced the activity of the enzyme against both soluble and highly crystalline substrates and affected its desorption from cellulose. This decrease in activity was thought to be a consequence of steric hindrance due to the reorientation of the CD and close proximity of the CBD [16]. Thus it is apparent that linker sequences do not have a common role in enzyme function and that the effect of removing these regions on both the activity of the CDs against soluble substrates, and the affinity of the CBDs for cellulose, depends on the overall tertiary structure of the enzyme.

In a previous study we showed the catalytic activity of XYLA against purified oat-spelt xylan was unaffected by removal of either the CBD or linker sequences, and the capacity to bind to cellulose was retained in the XYLA derivative lacking the linker sequences [12]. However, the natural substrates for hemicellulases are complex, comprising both cellulose and hemicellulose. While linker sequences and CBDs may facilitate the activity of hemicellulases against the plant cell wall, the use of purified substrates is unlikely to reveal their true role in enzyme action. When anchored to the plant cell wall via the CBD–cellulose interaction, hemicellulases have the potential to hydrolyse two types of substrate: arabinoxylan molecules, which have been released from, or hemicellulose, which is still an integral component of, the plant cell wall. It could be argued that steric hindrance by insoluble cellulose would reduce the activity of hemicellulases against soluble arabinoxylan and that linker sequences may provide the CD with the necessary flexibility to facilitate full access to the soluble hemicellulose (Figure 5). Although several studies have shown that linker sequences have a flexible structure [16–17,28–31], the data described here refute the model proposed in Figure 5 and show that linker sequences do not enhance the activities of either XYLA or XYLC against soluble substrates when bound to cellulose.

The results of this study indicate that both the CBD and linker sequences of XYLA enhance the activity of the enzyme against cellulose–xylan complexes. Similarly, the CBD of XYLC clearly increases the activity of the enzyme against a complex of cellulose and highly substituted arabinoxylan. We have previously suggested that CBDs in hemicellulases promote intimate contact with the plant cell wall, thus increasing the efficiency of the

Figure 6 Proposed model for the hydrolysis of plant cell wall xylan by a modular hemicellulase

The modular hemicellulase, whose structure is defined in Figure 5, is attached to the plant cell wall (□) via the CBD. In (A) the linker sequence provides the CD with the necessary flexibility to attack plant cell wall hemicellulose at multiple locations. In (B) the absence of linker sequences restricts the amount of substrate available to the CD when the enzyme is attached via the CBD to cellulose.

enzyme through prolonged association with its recalcitrant substrate [14]. The observation that removal of the CBD diminished the activity of the enzyme against the cellulose–hemicellulose complex provides evidence to support our hypothesis. Interestingly, the removal of the linker sequences from XYLA also decreased the activity of the enzyme against the same complex substrate. A possible explanation for this observation is that, in the hydrolysis of integral xylan, the CBD restricts substrate availability by anchoring the enzyme to a fixed location within the macromolecule. Linker sequences by conferring flexibility on the structure of the enzyme may increase the number of glycosidic bonds available to the CD (Figure 6).

To conclude, the present results demonstrate, for the first time, the importance of CBDs and linker sequences in the action of both *endo*-acting and side-chain-cleaving hemicellulases. Although these domains are not important when these enzymes are attacking soluble substrates, they clearly enhance the activity of hemicellulases against complex substrates consisting of cellulose and hemicellulose.

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