Mode of action, kinetic properties and physicochemical characterization of two different domains of a bifunctional (1→4)-β-D-xylanase from *Ruminococcus flavefaciens* expressed separately in *Escherichia coli*

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Two catalytic domains, A and C, of xylanase A (XYLA) from *Ruminococcus flavefaciens* were expressed separately as truncated gene products from *lacZ* fusions in *Escherichia coli*. The fusion products, referred to respectively as XYLA-A$_1$ and XYLA-C$_2$, were purified to homogeneity by anion-exchange chromatography and chromatofocusing. XYLA-A$_1$ was isoelectric at pH 5.0 and had a molecular mass of 30 kDa, whereas XYLA-C$_2$ had a pI of 5.4 and a molecular mass of 44 kDa. The catalytic activity shown by both domains was optimal at 50 °C, but XYLA-A$_1$ was more sensitive than XYLA-C$_2$ to temperatures higher than the optimum. XYLA-A$_1$ showed a higher sensitivity to pH than XYLA-C$_2$. The enzyme activity of both domains was completely inactivated in the presence of copper or silver ions and partially inactivated by iron or zinc ions. Neither domain was active on xylo-oligosaccharides shorter than xylopentaose: the rate of degradation of longer xylo-oligosaccharides (degree of polymerization 5–10) increased as the chain length increased. Analysis of the products of hydrolysis of xylo-oligosaccharides and xylan (arabinoxylan) polysaccharide showed that the two domains differed in their modes of action: xylobiose was the shortest product of the hydrolysis. With oat spelt xylan as substrate, XYLA-A$_1$ activity was apparently restricted to regions where xylopyranosyl residues did not carry arabinofuranosyl substituents, whereas XYLA-C$_2$ was able to release hetero-oligosaccharides carrying arabinofuranosyl residues. Neither domain was able to release arabinose from oat spelt xylan.

INTRODUCTION

Xylans associated with graminaceous materials are complex polysaccharides of glycosidically linked D-xylopyranosyl, L-arabinofuranosyl and 4-*O*-methyl-D-glucopyranosyluronic acid residues (Stephen, 1983). In some xylans, acetyl, feruloyl and *p*-coumaroyl residues, which are attached through ester links, are also present (Hartley and Ford, 1989), but these residues are present only in circumstances in which the polysaccharide has not been extracted from the plant cell wall with alkali. The backbone of the plant xylans is composed entirely of 1→4-linked β-D-xylopyranosyl residues, some of which carry α-L-arabinofuranosyl residues, normally on the 3 position, and 4-*O*-methyl-α-D-glucopyranosyluronic acid on the 2 position. Recent evidence indicates that hydrolysis of the backbone of these complex molecules is effected by highly specific 1→4-β-D-xylanases (Biely et al., 1992).

Two distinct families of (1→4)-β-D-xylanases can be defined by similarities in their amino acid sequences (Gilkes et al., 1991). Group G consists mostly of relatively small xylanases (less than 240 amino acids) related to that produced by the *Bacillus pumilus xynA* gene, whereas group F apparently includes both endoxylanases and exocellulases (Gilkes et al., 1991). Although many micro-organisms are known to produce multiple xylanases (Wong et al., 1988), there have been few instances where enzymes belonging to these two different families have been shown to be synthesized by the same species. Recent work on the genetic determination of xylanases in *Streptomyces lividans* and in the

Gram-positive rumen anaerobic bacterium *Ruminococcus flavefaciens* has shown, however, that sequences encoding both types of enzyme are present in the genome of these species (Shareck et al., 1991; Zhang and Flint, 1992; Flint and Zhang, 1992). Furthermore the *R. flavefaciens xynA* gene, cloned and expressed in *Escherichia coli*, encodes a bifunctional enzyme [xylanase A (XYLA)] in which the N-terminal domain (XYLA-A) is related to family G xylanases and the C-terminal domain (XYLA-C) is related to family F xylanases (Zhang and Flint, 1992). Deletion derivatives of *xynA* have been constructed that express only domain A or only domain C as fusion products with the N-terminal residues of the *E. coli lacZ* product. The functional consequences and evolutionary significance of linking two dissimilar catalytic domains acting on the same substrate in a single polypeptide are not yet known, but it is clearly possible that such organization contributes to the ability to break down the complex and variable substrates represented by plant xylans.

Investigation of the functional properties of domains A and C of XYLA can therefore be expected to shed light on their potential interactions in the complete XYLA protein, while also providing a comparison of the catalytic action of enzymes belonging to two different xylanase families. So far very little detailed functional information is available for any bacterial xylanase describing preferences and kinetic properties with respect to well-defined pure substrates. Although it is recognized that many plant xylans are highly substituted, the range of branched oligosaccharides available as pure substrates is not yet sufficient to define the effects of substituent groups on enzyme

action. The studies described here therefore concentrate mainly on defining enzyme action on unbranched xylose oligomers. In previous studies, this approach has yielded important information on patterns of bond cleavage by xylanases from the fungi *Schizophyllum commune* (Bray and Clarke, 1992), *Crytococcus albidus* (Biely et al., 1981a,b) and *Aspergillus niger* (Meagher et al., 1988). The results obtained here show that enzymes carrying XYLA domain A (XYLA-A_1) and XYLA domain C (XYLA-C_2), produced as *lacZ* fusions and purified from *E. coli*, exhibit similar properties with regard to cation sensitivity and substrate size preference, but differ in their responses to pH and temperature, and in the pattern of bond cleavage in xylo-oligosaccharides and xylan (arabinoxylan) polysaccharides.

EXPERIMENTAL

Materials

Centriprep 10 concentrators and desalting PD-10 columns were purchased from Amicon Ltd., Stonehouse, Glos., U.K., Mono P and Mono Q columns were from Pharmacia LKB, Milton Keynes, Bucks., U.K. and SDS molecular-mass markers and the isoelectric-focusing marker kit were from Sigma Chemical Company, Poole, Dorset, U.K.

Gene fusions

Construction of the *lacZ* fusions X4530 and X4723 was described previously (Zhang and Flint, 1992). The xylanase polypeptide made from X4530 is predicted to consist of the first 16 amino acids of the *E. coli lacZ* product (not including the initial formylmethionine), plus an additional proline residue, followed by residues 23–266, inclusive, of the *R. flavefaciens* XYLA product (comprising the last five residues of the signal peptide, all 221 residues of domain A and the first 18 residues of domain B) followed by three further *lacZ*-derived residues, giving a total of 264 amino acids. The xylanase polypeptide made from X4723 is predicted to consist of the same 16 amino acids of the *E. coli lacZ* product, plus an additional histidine residue, followed by residues 576–954 of the *R. flavefaciens* XYLA product (comprising the last 47 amino acids of domain B plus all 332 amino acids of domain C) giving a total of 396 amino acids.

Growth of bacteria and extraction of enzymes

A culture (5 litres) of *E. coli* DH5α carrying X4530 DNA encoding XYLA-A was produced using Luria broth with ampicillin (50 μg/ml) at 37 °C. After 15 h growth, the cells were recovered by centrifugation at 6000 *g* and 4 °C for 15 min, resuspended in 80 ml of 0.05 M sodium phosphate buffer, pH 6.5, and disrupted by sonication (MSE Soniprep): 10 × 1 min treatments. Cell sonicates were centrifuged at 12000 *g*. Protein in the supernatant was recovered by sequential precipitation at 1 °C (at 30%, 50% and 80% satn.) with $(NH_4)_2SO_4$ and collected by centrifugation (75000 *g*; 20 min). Each pellet was resuspended in 0.1 M phosphate buffer, pH 6.8, and assayed for protein and xylanase activity.

In the case of *E. coli* DH5α/X4723 carrying DNA encoding XYLA-C, the extraction procedure was the same except that 10 ml of a 10% (w/v) solution of streptomycin sulphate was added after the sonication treatment. The solution was left stirring overnight at 4 °C and centrifuged at 25000 *g* at the same temperature for 45 min. The supernatant was treated with $(NH_4)_2SO_4$ as described above.

Enzyme purification

Domain A (XYLA-A)

The redissolved material, precipitated between 30 and 50%-satd. $(NH_4)_2SO_4$ (10 ml), was desalted by molecular-sieve chromatography on a PD-10 column (Amicon), concentrated (Centriprep 10), equilibrated with 20 mM Tris/HCl buffer, pH 7.5, 10 mM with respect to NaCl, and applied to a Mono Q anion-exchange column (HR5/5) previously equilibrated with the same buffer. The column was washed at a flow rate of 0.3 ml/min with a linear gradient of NaCl (10–300 mM) in the equilibration buffer (total volume 30 ml). Fractions of 1 ml were collected. Fractions containing xylanase activity were pooled where appropriate, concentrated and equilibrated in 25 mM Bistris, pH 6.3, using a Centriprep 10. The enzyme in the concentrate was separated by chromatofocusing on a Mono P column, previously equilibrated in the same buffer, and eluted at a flow rate of 0.5 ml/min with Polybuffer 74 (Pharmacia) which had been diluted 1:10 and adjusted to pH 4.0. Fractions containing xylanase were pooled, concentrated and equilibrated in 25 mM Bistris, pH 5.8, using a Centriprep 10 concentrator before being applied to an ion-exchange column (Mono P) equilibrated in the same buffer. The elution was effected at 0.5 ml/min with Polybuffer 74, diluted 1:10 and adjusted to pH 4.5. Fractions of 1 ml were collected. Fractions containing xylanase were pooled, equilibrated in 25 mM Bistris, pH 6.3, by using a Centriprep 10 concentrator and applied to a Mono P column which had been equilibrated with the same buffer; the column was washed at a flow rate of 0.5 ml/min with Polybuffer 74 previously diluted 1:10 and adjusted to pH 5.0.

Domain C (XYLA-C)

The redissolved material, precipitated between 30 and 50%-satd. $(NH_4)_2SO_4$ (30 ml), was desalted on a PD-10 column, concentrated and equilibrated using a Centriprep 10 concentrator with 20 mM Tris/HCl buffer, pH 7.5, 10 mM with respect to NaCl. The concentrate (15 ml) was applied to an anion-exchange column (DEAE-Sepharose CL-6B) (27.5 cm × 1.5 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.5, 10 mM with respect to NaCl. The column was eluted with a linear gradient of NaCl (10–500 mM) in the same buffer at a flow rate of 15 ml/h. Fractions of 5 ml were collected. Selected fractions were pooled, desalted, concentrated (2.6 ml) and equilibrated in 25 mM Bistris buffer, pH 6.7, using a Centriprep 10 concentrator. The concentrate was applied to a Mono P column (HR5/5) equilibrated with the same buffer and eluted with Polybuffer 74 which had been diluted 1:10 and adjusted to pH 5.0. The flow rate of the eluting buffer was 0.5 ml/min and 1 ml fractions were collected.

Enzyme assays

Activity on arabinoxylan

Assay mixtures (1 ml) containing 0.5% (w/v) of the suspension of oat spelt (Sigma) arabinoxylan (in 250 mM NaOH/acetic acid, pH 5.0) were incubated for 15 min at 50 °C in 0.1 M Mes buffer, pH 6.2. Reducing sugars were determined colorimetrically at 520 nm using the Nelson–Somogyi method (Nelson, 1952) and expressed as xylose equivalents. One unit of activity (xylanase) is defined as the amount of enzyme releasing 1 μmol of reducing sugars/min under the conditions described.

Activity on xylo-oligosaccharides

Hydrolysis of xylo-oligosaccharides ranging from X_3 to X_{10} (Puls et al., 1988) were determined as follows: reaction mixtures

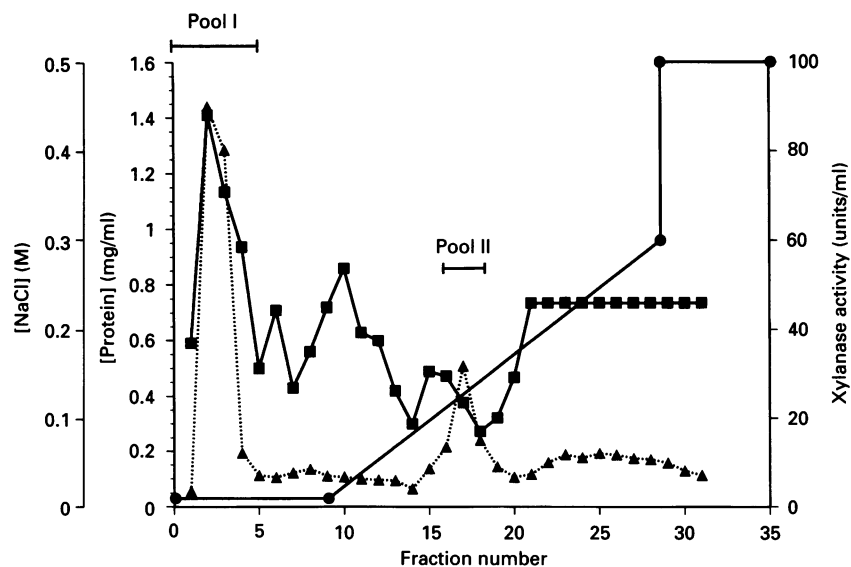**Figure 1   Partial purification of domain A (XYLA-A) on Mono Q anion exchanger**

A 10 ml sample of 30–50%-satd. $(NH_4)_2SO_4$ fraction (see the Experimental section), desalted, concentrated and equilibrated with 20 mM Tris/HCl buffer, pH 7.5, 10 mM with respect to NaCl, was applied to the Mono Q column (HR5/5), previously equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl (10–300 mM) in 20 mM Tris/HCl buffer, pH 7.5. Fractions of 1 ml were collected and assayed for protein (■) and xylanase (▲). ●, NaCl gradient. For further details see the Experimental section. Fractions 2–5 (pool I) and 15–19 (pool II) were combined.
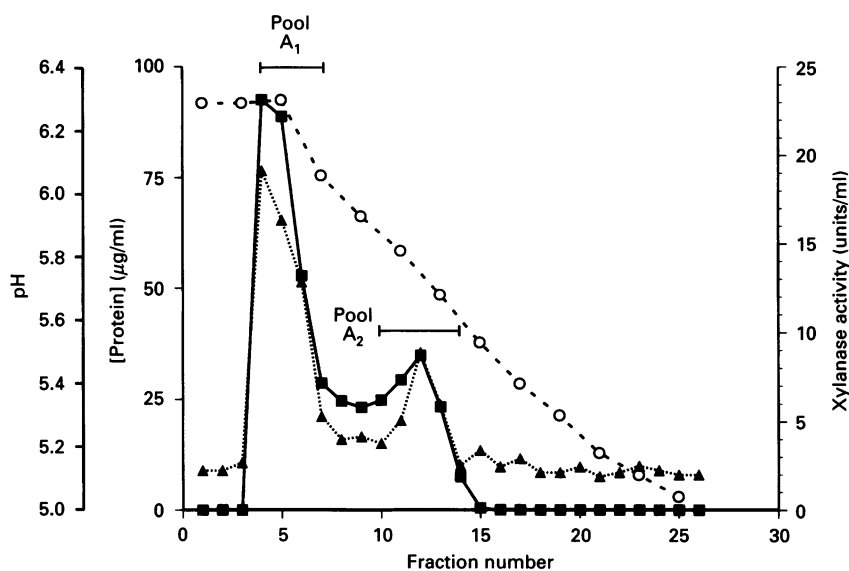

Figure 2   Further purification of domain A (XYLA-A) on Mono P chromatofocusing gel

Pool I (Figure 1) was concentrated, equilibrated with 25 mM Bistris buffer, pH 6.3, and applied to a column of Mono P chromatofocusing gel (HR5/5), previously equilibrated with the same buffer. The column was eluted at a flow rate of 0.5 ml/min with Polybuffer 74 (Pharmacia), diluted 1:10 and adjusted to pH 4.0. Fractions of 1 ml were collected and assayed for protein (■) and xylanase (▲). ○, pH. Fractions 4–8 (pool A_1) and 10–14 (pool A_2) were combined. For further details see the Experimental section.

(0.25 ml) containing 0.8 mg/ml xylo-oligosaccharide in 0.025 M Mes buffer, pH 6.2, and 0.047 μg of protein (Redinbaugh and Turley, 1986) of domain A or 0.063 μg of domain C were incubated at 50 °C. Samples were removed at intervals up to 5 h and the reaction was terminated by immersing the tubes in a boiling-water bath for 8 min. Analyses of the reaction mixtures were performed with a Dionex h.p.l.c. system (model DX-300) equipped with a pulsed amperometric detector (PAD), a pump for post-column addition of NaOH, and a Spectraphysics Chromjet integrator. The PAD had electrode potentials set at $E_1 = +0.05$ V, $E_2 = +0.6$ V and $E_3 = +0.6$ V, and these were applied in durations of 300, 120 and 60 ms. Samples were injected on to a Carbopac PA1 anion-exchange column (4 mm × 250 mm) previously equilibrated with a solution made up by mixing the three solutions, (I) 1 M sodium acetate/0.1 mM NaOH, (II) water and (III) 1 M NaOH, in the ratio of 5:7:20

(by vol.). The column was then eluted with a gradient constructed by adding solutions I, II and III in the ratio of 21:59:20 (by vol.) over 20 min. The post-column base (0.5 M NaOH) was added at 0.3 ml/min. Fucose was used as internal standard.

Hydrolysis of arabinoxylan by XYLA-A and XYLA-C

Reaction mixtures (1.1 ml) containing 0.5% (w/v) of the suspension of arabinoxylan (in 250 mM acetate buffer, pH 5.0) and 0.5 μg of XYLA-A_1 or 1.1 μg of XYLA-C_2 were incubated at 50 °C in 0.025 M Mes buffer, pH 6.2. Portions of volume 0.11 ml were taken at different time intervals, immersed in a boiling-water bath for 8 min and centrifuged for 5 min at 10000 g. Analysis of the products of hydrolysis was performed by h.p.l.c. as described above.

Protein determination

Protein was determined by the bicinchoninic acid method described by Redinbaugh and Turley (1986).

Gel electrophoresis

SDS/PAGE was by the method of Laemmli (1970), gels being stained with Coomassie Brilliant Blue R-250. Low- and high-molecular-mass calibration mixtures (Sigma) were used as standards.

Isoelectric focusing was carried out in polyacrylamide gels (4.1% acrylamide, 0.12% bis-acrylamide) containing 4% (v/v) ampholine (Pharmacia), pH range 3.5–10.0. Gels were stained with Coomassie Brilliant Blue. Low- (Sigma) and high- (Bio-Rad) pI calibration mixtures were used as standards.

Where appropriate, enzyme activity was detected in situ by overlaying isoelectric focusing gels with 0.5% (w/v) xylan suspension in 0.1 M Mes buffer, pH 6.2, containing 1% (w/v) agar. After incubation at 37 °C for 15 min, the gel containing xylan was immersed for 30 min in a solution of Congo Red (1 mg/ml): bands showing substrate degradation were revealed by rinsing the gel with 1 M NaCl.

RESULTS AND DISCUSSION

Enzyme purification

The two catalytic domains of XYLA are expressed separately, as truncated gene products, in the E. coli clones X4530 and X4723. These clones provided the starting material for purification of the two domains (see the Experimental section).

XYLA-A

A typical elution profile of partially purified enzyme [30–50% satd. (NH$_4$)$_2$SO$_4$ fraction] from an anion-exchange (Mono Q) column is shown in Figure 1. Xylanase activity appeared as a major peak (fractions 2–4; pool I, Figure 1), which was not retained on the column (see the Experimental section), and a minor peak which was eluted with buffer containing 0.12 M NaCl. Pool I was concentrated and the enzyme purified further by chromatofocusing on a Mono P column using a pH gradient of 6.3–4.0 (results not shown). A single peak of xylanase activity was eluted when the pH of the eluent ranged from 5.8 to 4.5, and this was purified further by repeated chromatofocusing on a Mono P column using first a pH gradient covering the pH range 5.8–4.5 (results not shown) then a pH gradient over the range 6.3–5.0 (Figure 2). Two xylanase components, designated A$_1$ and

A$_2$, were separated. On the basis of analysis by SDS/PAGE and using a zymogram technique, components A$_1$ and A$_2$ appeared to be homogeneous. A$_1$ had an apparent molecular mass of 30 kDa and was isoelectric at pH 5.0 (Figures 3a and 3b); A$_2$ had an isoelectric point of pH 4.7 and a molecular mass of 32 kDa (not shown). Specific activities on xylan of 320 and 275 units/mg of protein were obtained for A$_1$ and A$_2$ components respectively. Only component A$_1$ (hereafter designated XYL-A$_1$) was chosen for further characterization. A summary of the purification of XYL-A$_1$ is given in Table 1.

XYLA-C

Initial fractionation of the enzyme concentrate [30–50%-satd. (NH$_4$)$_2$SO$_4$ fraction] was by anion-exchange chromatography on a column of DEAE-Sepharose CL-6B (see the Experimental section). Three components active on xylan (results not shown) were obtained. The first peak to be eluted was purified further by
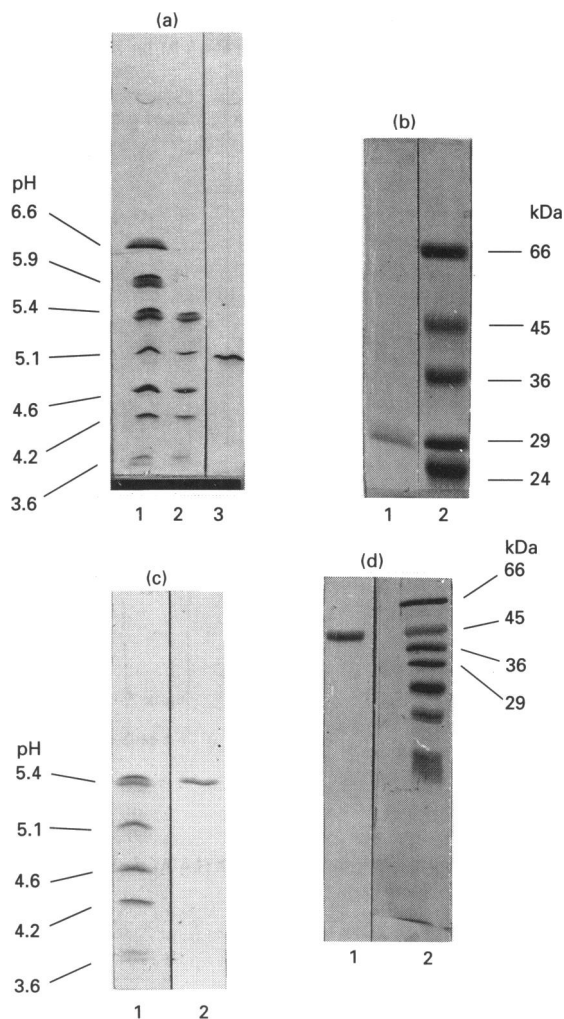

Figure 3   SDS/PAGE and isoelectric focusing on polyacrylamide gels of domains XYLA-A and XYLA-C isolated and purified from cultures of E. coli recombinants

(a) Isoelectric focusing: lanes 1 and 2, standards; lane 3, component A$_1$ from Figure 2; (b) SDS/PAGE: lane 2, standards; lane 1, component A$_1$ from Figure 2; (c) isoelectric focusing: lane 1, standards; lane 2, component C$_2$ from Figure 4; (d) SDS/PAGE: lane 2, standards; lane 1, component C$_2$ from Figure 4.

Table 1 Summary of the purification of the xylanase activity of domain XYLA-A₁

Enzyme	Volume (ml)	Protein (mg)	Xylanase activity (units)	Specific activity (units/mg)	Purification factor
Sonicated fraction	80	1304	1927	1.5	1.0
30–50%-satd. $(NH_4)_2SO_4$ fraction	10	170	2170	13	8.7
Pool I (Figure 1)	20	8.88	1090	123	82
Pool A₁ (Figure 2)	5	0.71	227	320	213
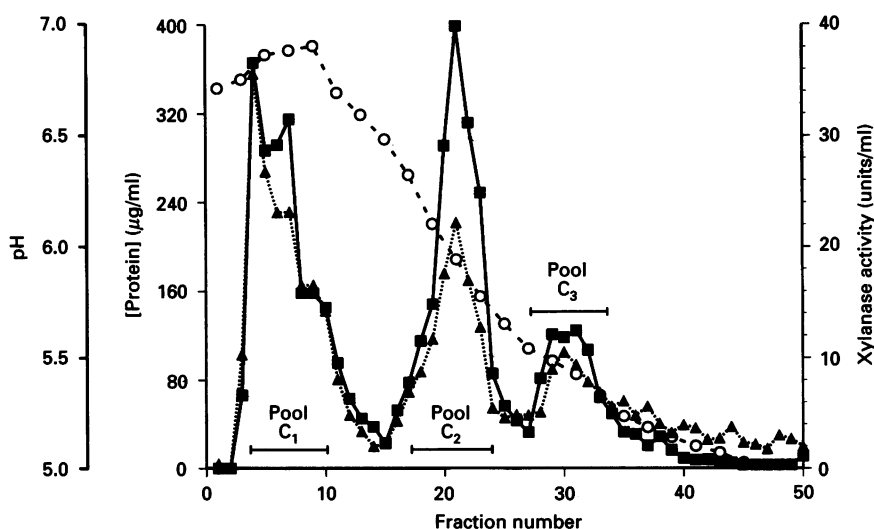
Figure 4 Purification of domain C (XYLA-C) on Mono P chromatofocusing medium

The first peak of xylanase activity eluted from a column of DEAE-Sepharose CL-6B (see the Experimental section) was desalted, concentrated, equilibrated in 25 mM Bistris buffer, pH 6.7, and applied to a Mono P chromatofocusing column (HR5/5) equilibrated with the same buffer. The column was eluted at 0.5 ml/min. Fractions of 1 ml were collected and assayed for protein (■) and xylanase activity (▲). ○, pH. Fractions 2–10 (pool C_1), 16–25 (pool C_2) and 29–35 (pool C_3) were combined. For further details see the Experimental section.

chromatofocusing (Mono P column) using a pH gradient 6.7–5.0 (Figure 4) resulting in three peaks of xylanase activity (C_1, C_2, C_3 in Figure 4). Component C_1, which was not adsorbed on the column, was shown by isoelectric focusing to be associated with a multiplicity of polypeptides (results not shown). However, components C_2 and C_3, which were eluted at pH 5.8 and 5.4 respectively, appeared to be homogeneous when analysed by isoelectric focusing. The two purified components exhibited different isoelectric pH values: 5.4 in the case of C_2 and 5.1 in the case of C_3, but there was no apparent difference in their molecular mass, which was 44 kDa (results not shown). SDS/PAGE and isoelectric focusing of component C_2 are shown in Figures 3(c) and 3(d). Only component C_2 (hereafter designated XYLA-C_2) was chosen for further characterization. A summary of the purification of this component is presented in Table 2.

Physicochemical characterization of purified enzymes

The two component catalytic domains of XYLA from *R. flavefaciens* were shown here to differ in their physicochemical properties. Both enzymes exhibited maximum activity at 50 °C, but XYLA-A₁ was considerably more thermolabile than XYLA-C_2 at higher temperatures. Thus at 55 °C the half-lives of XYLA-A₁ and XYLA-C_2 were approx. 20 and 50 min respectively.

XYLA-C_2 operated over a wider pH range than XYLA-A₁ (Figure 5).

Both XYLA-A₁ and XYLA-C_2 were completely inactivated in the presence of 1 mM solutions of copper or silver ions. Iron or zinc ions were also inhibitory: XYLA-A₁ activity decreased by 30% and 50% respectively in the presence of iron and zinc ions whereas the activity of XYLA-C_2 was reduced by 22% and 37%. Calcium and magnesium ions did not affect XYLA-A₁ but appeared to enhance slightly the activity of XYLA-C_2.

Functional characterization of purified enzymes

Action on xylo-oligosaccharides

Both enzymes appeared to act as typical endoxylanases on xylo-oligosaccharides and resembled the xylanases of *Aspergillus niger* (Conrad and Nöthen, 1984), *Cryptococcus albidus* (Biely and Vršanská, 1988) and *Schizophyllum commune* (Bray and Clarke, 1992) in that their activity increased as the degree of polymerization (d.p.) of the xylo-oligosaccharides increased. Neither XYLA-A₁ nor XYLA-C_2 could hydrolyse oligomers of d.p. 4 and lower, and in this respect they differed from the xylanases of *A. niger* (Conrad and Nöthen, 1984), *C. albidus* (Biely and Vršanská, 1988) and *S. commune* (Bray and Clarke, 1992), which could hydrolyse xylotriose and xylotetraose.

**Table 2 Summary of the purification of the xylanase activity of domain XYLA-C₂**

Enzyme	Volume (ml)	Protein (mg)	Xylanase activity (units)	Specific activity (units/mg)	Purification factor
Sonicated fraction	138	1955	51060	26	1.0
30–50%-satd. (NH₄)₂SO₄ fraction	30	747	35670	48	1.8
DEAE-Sepharose CL-6B (Pool A)	122	40	2950	74	2.8
Pool C₂ (Figure 4)	6.5	1.44	374	260	10.0
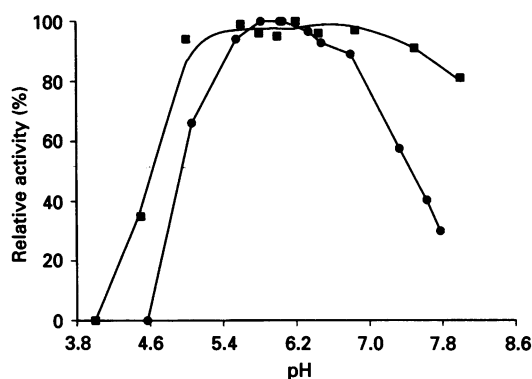
Figure 5 Effect of pH on the xylanase activity of domains XYLA-A₁ and XYLA-C₂

Enzyme (20 μl) was incubated for 15 min at 37 °C in a reaction mixture (1 ml) made up of enzyme solution and McIlvaine buffer of various pH values and then assayed for xylanase activity. ●, XYLA-A₁; ■, XYLA-C₂.

Except in the case of X_6, the rate of attack of the different xylo-oligosaccharides by XYLA-A₁ (Figure 6a) was higher than that shown by XYLA-C₂ (Figure 6b). For both enzymes, the rate of degradation decreased as the chain length decreased. This decrease was particularly notable with xylo-oligosaccharide substrates of d.p. 6 and 5 in the case of XYLA-A₁ and d.p. 5 in the case of XYLA-C₂.

Further differences between the two enzymes became apparent on analysis of the products of hydrolysis of xylo-oligosaccharides, d.p. 5 and 6. As Figures 7(a) and 7(b) show, X_5 was hydrolysed at a low rate to X_3 and X_2 by both enzymes. However, whereas XYLA-A₁ preferentially cleaved the glycosidic bond in X_6 to release two molecules of X_3 (Figure 7c), XYLA-C₂, acting on the same substrate, liberated X_2, X_3 and X_4 at approximately the same rate (Figure 7d). These results demonstrate that XYLA-A₁ was highly specific for the central link of xylohexaose whereas XYLA-C₂ does not distinguish significantly between linkages that, when attacked, result in the release of xylobiose and xylotriose. The release of xylobiose in this situation could result from the cleavage of the penultimate glycosidic linkage at either the non-reducing end or the reducing end of the molecule. Xylo-oligosaccharides in which the reducing moiety has been converted into an alditol have been used elsewhere to differentiate the nature of the attack (Bray and Clarke, 1992). However, as it has been shown in studies involving endoglucanases that modification of the reducing glycosyl residue of cello-oligosaccharides induces an apparent change in the mode of action of the enzymes (Bhat et al., 1990), it is probably necessary to use radioactively labelled
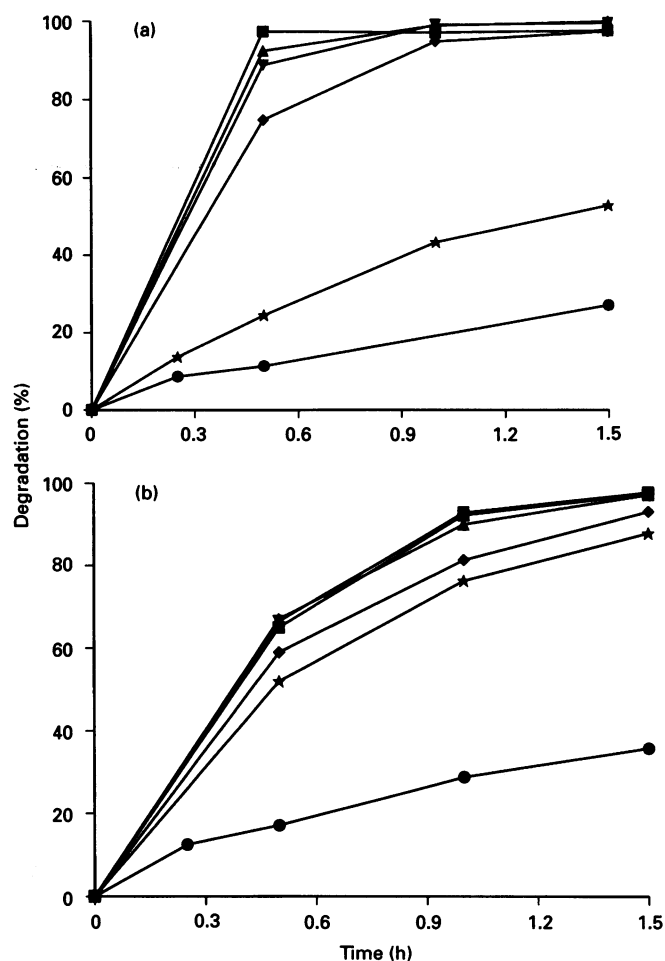
Figure 6 Percentage hydrolysis of xylo-oligosaccharides by XYLA-A₁ and XYLA-C₂ as a function of time

Details of the assay are given in the Experimental section. The abbreviations are defined in the text. (a) Hydrolysis by XYLA-A₁; (b) hydrolysis by XYLA-C₂. Key to symbols: ■, X_{10}; ▲, X_9; ▼, X_8; ◆, X_7; ★, X_6; ●, X_5.

xylo-oligosaccharides (Biely et al., 1981a,b) to provide definitive information on the mode of action of the endoxylanases.

Additional mechanistic information was obtained using xylo-oligosaccharides of d.p. 7–10. In these cases, the analysis of the products of hydrolysis was complicated by the fact that the initial breakdown products were susceptible to further degradation. The final products of hydrolysis of these substrates were X_2, X_3 and X_4. However, the relative proportions of these sugars released
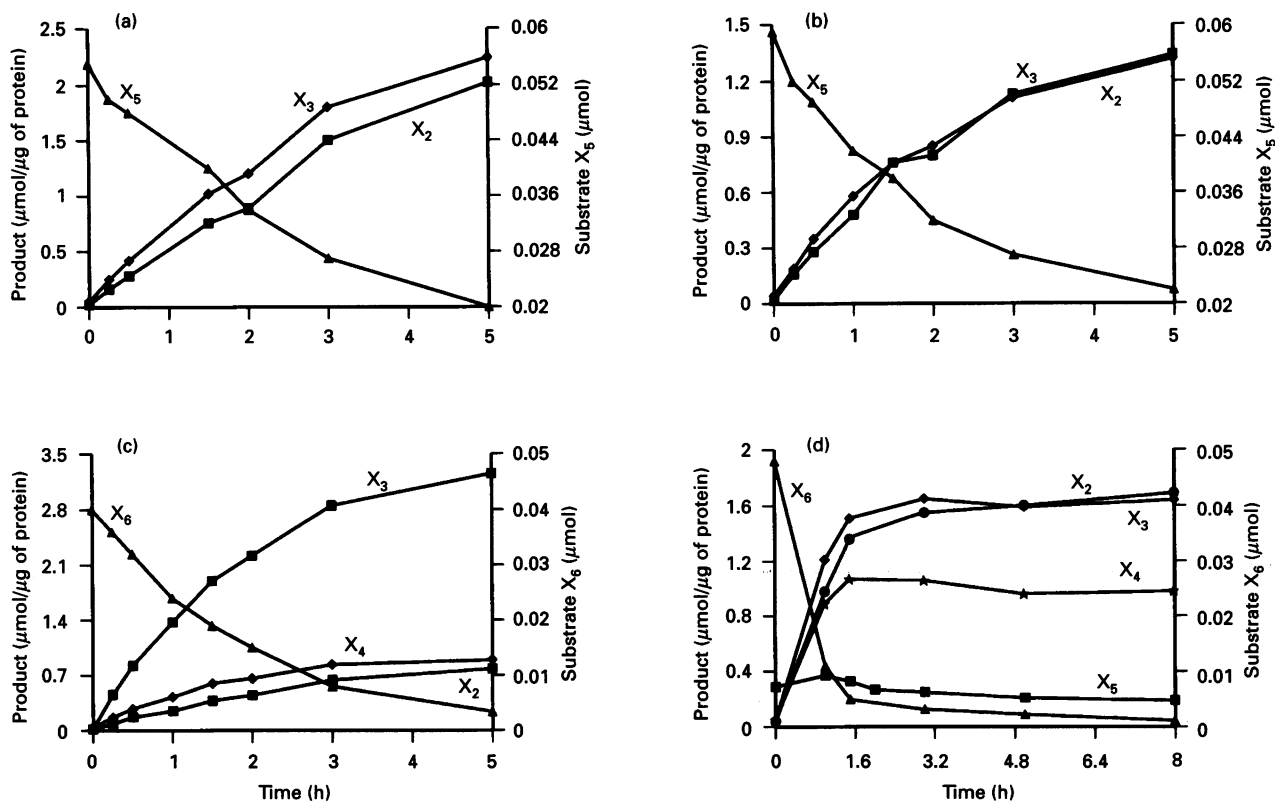
Figure 7   Products of the hydrolysis of xylo-oligosaccharides by XYLA-A₁ and XYLA-C₂ as a function of time

(a) and (c) Hydrolysis by XYLA-A₁; (b) and (d) hydrolysis by XYLA-C₂. For further details see the Experimental section.

Table 3   Final products of the action of XYLA-A₁ and XYLA-C₂ on xylo-oligosaccharides of d.p. 7–10

The products of the reaction were assayed after 5 h incubation of the xylo-oligosaccharides with the enzymes as detailed in the Experimental section.

	Domain A			Domain C		
Substrate	X_2 (μmol)	X_3 (μmol)	X_4 (μmol)	X_2 (μmol)	X_3 (μmol)	X_4 (μmol)
X_7	0.9	2.5	1.8	1.0	1.4	1.1
X_8	2.7	4.0	1.8	1.5	1.6	1.2
X_9	2.2	3.9	1.6	2.0	2.2	1.5
X_{10}	1.6	3.3	1.5	0.7	0.9	0.9

by the two domains differed significantly, showing different modes of action. XYLA-A₁ generated about twice as much X_3 as X_2 or X_4: XYLA-C₂, in contrast, produced the three sugars in approximately equal amounts (Table 3).

Action on arabinoxylan

Differences in the mode of action of the two domains were also obvious using an arabinoxylan polysaccharide from oat spelts. With this substrate, further limitations are placed on enzyme action by the arabinosyl substituents that are glycosidically linked to the 3 position (or less frequently the 2 position) of the main chain of 1→4-linked xylosyl residues. The arabinoxylan of

oat spelts was found to contain arabinose and xylose in the ratio of 1:10, but the distribution of the substituents is certainly not uniform (Kormelink et al., 1992; Vliegenthart et al., 1992). Therefore the substrate could be presumed to contain large sections of molecule in which the xylosyl residues are not substituted.

The products released by XYLA-A₁ (Figure 8a) acting on the arabinoxylan were X_2, X_3, X_4 and longer xylo-oligosaccharides of d.p. 8, 9, 10 and 11. However, in the case of XYLA-C₂ (Figure 8c), it was possible to identify X_2, X_3 and X_4 in the hydrolysate, but the oligomers of higher d.p. that were generated showed retention times that differed slightly from those of pure unbranched xylo-oligosaccharides of d.p. 8, 9, 10 and 11. When the products of XYLA-C₂ hydrolysis were incubated with purified α-L-arabinofuranosidase from *Aspergillus awamori* (Wood et al., 1992), they were shown now to contain, in addition to those xylo-oligosaccharides previously identified, X_9 and X_{10} as well as traces of X_5, X_6 and X_7: the hydrolysate also contained arabinose (Figure 8d). A similar treatment of the hydrolysate obtained with XYLA-A₁ (Figure 8b) did not result in any noticeable change in the products present but the appearance of higher amounts of X_5 and detectable amounts of X_6 and X_7 were noted. Once again free arabinose was detected.

Thus XYLA-A₁ appears to act on the polysaccharide chain in such a way that only oligosaccharides containing residues that are unsubstituted are released. The high-molecular-mass oligomers generated by XYLA-C₂, in contrast, appeared to be branched oligomers of arabinosyl and xylosyl residues. Thus the action of XYLA-A₁ appears essentially to be confined to long sequences of unsubstituted xylose residues in the arabinoxylan
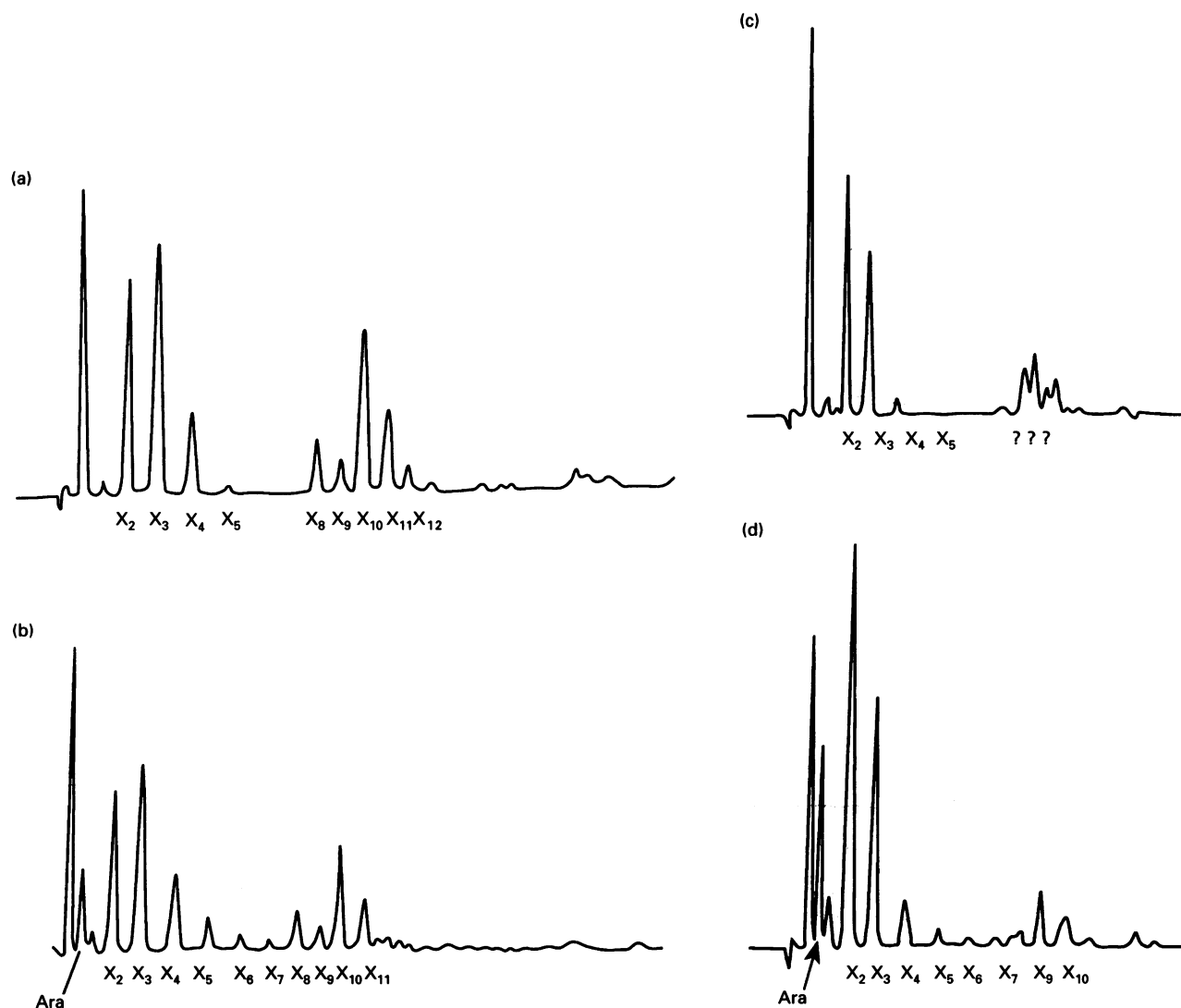
Figure 8   Products of the hydrolysis of the arabinoxylan from oat spelts by domains XYLA-A₁ and XYLA-C₂

Details of the procedure used are given in the Experimental section. (a) Hydrolysis of xylan by XYLA-A₁; (b) further hydrolysis of the hydrolysate (a) by α-L-arabinofuranosidase from *Aspergillus awamori*; (c) hydrolysis of xylan by XYLA-C₂; (d) further hydrolysis of the hydrolysate (c) by α-L-arabinofuranosidase.

molecule whereas XYLA-C$_2$ is less restricted in its action. One possible consequence of the presence of domain C in the same polypeptide as domain A is the creation of an enzyme that is less likely to be blocked by arabinosyl substituents. It is notable that some endoxylanases of bacteria and fungi appear to be able to release arabinose as well as cleave the xylosyl backbone of the molecule (Reilly, 1981). However, neither XYLA-A$_1$ nor XYLA-C$_2$ possessed this property.

Evolutionary considerations

It has been demonstrated that certain cellulase and xylanase polypeptides produced by bacteria and fungi carry separate functional domains (van Tilbeurgh et al., 1986; Gilkes et al., 1988; Tomme et al., 1988; Ferreira et al., 1990; Kellet et al., 1990). In most cases, one domain was reported to be catalytic and the other was involved in binding. However, in four cases, bifunctional enzymes with two different catalytic domains have been demonstrated. These are an endo/exoglucanase and an

endoglucanase/mannanase from *Caldocellum saccharolyticum* (Saul et al., 1990; Gibbs et al., 1992) and the xylanases XYLA and XYLD of *R. flavefaciens* (Zhang and Flint, 1992; Flint et al., 1993). 'Bifunctional' enzymes concerned with xylan degradation have also been reported in *Butyrivibrio fibrisolvens* (Utt et al., 1991) and *Bacteroides ovatus* (Whitehead and Hespell, 1990), but in these cases it was not established whether separate catalytic domains are involved.

R. flavefaciens 17 is known to possess at least four xylanase genes (Flint et al., 1989; Flint and Zhang, 1992). Sequencing studies on two further xylanase genes, in addition to *xynA*, have shown that they also encode multidomain enzymes with N-terminal domains closely resembling that of XYLA (Flint and Zhang, 1992; Flint et al., 1993). The multiplicity of sequences encoding family-G-related xylanases in the *R. flavefaciens* genome is most likely to have been the result of gene duplication events during evolution.

Further evolutionary questions are raised by the multi-functional organization of the XYLA enzyme. As xylanases so

far reported from most other bacterial species carry single catalytic domains (Gilkes et al., 1991; Henrissat, 1992), it seems clear that domains A and C must originally have been expressed from separate genes as separate enzymes. Close linkage of these genes may have been the result of selective pressure for co-regulated expression from a polycistronic mRNA, followed by fusion into a single reading frame which, in this case, appears to have entailed considerable repetition of the sequences linking the two domains (Zhang and Flint, 1992). This type of evolution towards bifunctionality has been inferred previously in other instances, for example the enzymes concerned with tryptophan synthesis in bacteria (Crawford, 1975). The selective advantage conferred by such bifunctional organization in polysaccharidases has yet to be fully explained, but the evidence presented here demonstrates significant functional differences between the two catalytic domains such that the composite enzyme can be expected to exhibit a wider range of bond cleavage than either of the domains acting independently. This type of organization represents a potentially important mechanism for generating enzymes with novel catalytic properties required for the efficient breakdown of the complex and variable structures found in plant hemicelluloses.

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