A novel thermostable multidomain 1,4-β-xylanase from ‘Caldibacillus cellulovorans’ and effect of its xylan-binding domain on enzyme activity

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The nucleotide sequence of the complete xynA gene, encoding a novel multidomain xylanase XynA of ‘Caldibacillus cellulovorans’, was determined by genomic-walking PCR. The putative XynA comprises an N-terminal domain (D1), recently identified as a xylan-binding domain (XBD), homologous to non-catalytic thermostabilizing domains from other xylanases. D1 is followed by a xylanase catalytic domain (D2) homologous to family 10 glycosyl hydrolases. Downstream of this domain two cellulose-binding domains (CBD), D3 and D4, were found linked via proline-threonine (PT)-rich peptides. Both CBDs showed sequence similarity to family IIb CBDs. Upstream of xynA an incomplete open reading frame was identified, encoding a putative C-terminal CBD homologous to family IIb CBDs. Two expression plasmids encoding the N-terminal XBD plus the catalytic domain (XynAd1/2) and the xylanase catalytic domain alone (XynAd2) were constructed and the biochemical properties of the recombinant enzymes compared. The absence of the XBD resulted in a decrease in thermostability of the catalytic domain from 70°C (XynAd1/2) to 60°C (XynAd2). Substrate-specificity experiments and analysis of the main products released from xylan hydrolysis indicate that both recombinant enzymes act as endo-1,4-β-xylanases, but differ in their ability to cleave small xyloooligosaccharides.

Keywords: thermophilic bacteria, thermostabilizing domain, xynA, hemicellulose degradation

INTRODUCTION

Hemicelluloses are non-cellulosic low-molecular-mass polysaccharides that are found together with cellulose in plant tissues. Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Timell, 1967). Xylans are heterogeneous polysaccharides consisting of a main chain of 1,4-linked β-D-xylopyranosyl residues that often carry acetyl, arabinosyl and glucuronosyl substituents. The action of the main xylanolytic enzyme, β-endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is to convert polymeric xylan to xyloooligosaccharides (Biely, 1985).

Many xylanases from fungi and bacteria have been characterized and cloned (Kulkarni et al., 1999; Sunna & Antranikian, 1997). However, substantial interest has been focused on thermostable xylanases due to their potential application in the development of environmentally friendly technologies in the paper and pulp industry (Viikari, 1991; Viikari et al., 1994). Xylanases are classified into two families, 10 and 11, according to the similarity of amino acid sequences of their catalytic domain in hydrophobic cluster analyses (Henrissat, 1991). Sequence analysis of glycosyl hydrolase family 10 and 11 xylanase genes shows that they often encode multidomain structures comprising a catalytic domain associated with discrete non-catalytic domains of various functions (Gilkes et al., 1991; Tomme et al., 1995).

Abbreviations: CBD, cellulose-binding domain; PT-linker, proline-threonine linker; TSD, thermostabilizing domain; XBD, xylan-binding domain; XU, xylanase unit.

The GenBank accession number for the sequence reported in this paper is AF200304.
Family 10 xylanases of thermophilic origin often have associated duplicated family IX cellulose-binding domains (CBDs) at the C terminus of the catalytic domain and thermostabilizing domains (TSDs, typically found in tandem domains), at the N terminus (Bergquist et al., 1999; Lee et al., 1993; Morris et al., 1999; Winterhalter et al., 1995; Zverlov et al., 1996).

‘Caldibacillus cellulovorans’ is a new thermophilic spore-forming aerobic bacterium isolated from an artificial compost that is able to grow on crystalline cellulose (X. P. Huang, unpublished). Here, we report the cloning, sequencing and expression of a glycosyl hydrolase family 10β-xylanase gene (Henriissat, 1991) from ‘C. cellulovorans’ that encodes a protein with an unusual structure and which is covalently associated with a single xylan-binding domain (XBD) and two CBDs. The effect of the XBD on thermostability and substrate hydrolysis is also discussed.

METHODS

Bacterial strain and genomic DNA. Escherichia coli JM101 [Δ(lac-proAB) thi-1 supE44 F’ (traD36 proAB’ lacZΔM15)] was used as the bacterial host for all DNA cloning and expression studies. ‘Caldibacillus cellulovorans’ cells were kindly provided by Professor Hugh W. Morgan, University of Waikato, New Zealand. Genomic DNA was prepared as described previously (Morris et al., 1995). Media and other reagents are described by Croft et al. (1987).

Consensus and genomic-walking PCR. A consensus PCR fragment from a glycosyl hydrolase family 10 xylanase was amplified from the ‘Caldibacillus cellulovorans’ genomic DNA using the XYNF and XYNR consensus primer pair (Table 1). PCRs were performed as described by Morris et al. (1998). Linker assembly, linker library construction and genomic-walking PCR were performed according to Morris et al. (1995, 1998). The forward and reverse genomic-walking primers used in this study are shown in Table 1. When necessary, genomic-walking PCR was performed using the GC-Rich PCR System (Roche Diagnostics). PCRs were performed as recommended by the manufacturer with an annealing temperature of 55°C and an extension time of 3 min at 72°C.

DNA sequencing. DNA sequencing was carried out on an Applied Biosystems 377 DNA sequencer using Big Dye terminator chemistry. Computer analysis of sequence data were carried out with the Genetics Computer Group (GCG) software package (Devereux et al., 1984).

Construction of a xynAd1/2 recombinant plasmid. The catalytic or non-catalytic domains are numbered from the N terminus and the linkers are ignored in this nomenclature (see Fig. 1). The specific primers XYNACBD4BF and XYNACBD4BR (Table 1) were designed to allow PCR amplification of DNA encoding the first two N-terminal domains (D1 and D2) of xynA from ‘Caldibacillus cellulovorans’. The primers XYNACBD4BF and XYNACBD4BR incorporate the restriction sites of the expression plasmid pJLA602 (Schauder et al., 1987), to give the recombinant plasmid pSUN18. Xylanase-positive transformants were identified after induction of enzyme production at 42°C for 3 h on plates containing birchwood xylan (Sigma) using Congo red staining (Teather & Wood, 1982). The recombinant plasmid encoding XynAd1/2 was sequenced on both strands to confirm that there were no PCR-derived base changes in the DNA except for those introduced in the engineered restriction sites of the PCR primers (Pro→Met change at the N terminus, and Thr→Ser change at the C terminus of the XynAd1/2 peptide). Translation of XynAd1/2 was terminated using a stop codon in the multiple cloning site of pJLA602 directly adjacent to the BamHI site.

**Table 1.** Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 10 xylanase consensus primers*</td>
<td></td>
</tr>
<tr>
<td>XYNFB</td>
<td>CAT ACK TTK GTT TGG CA</td>
</tr>
<tr>
<td></td>
<td>H T F/L V W</td>
</tr>
<tr>
<td>XYNFR†</td>
<td>TGG GAY GTK GTM AAY GA</td>
</tr>
<tr>
<td></td>
<td>W D V V N</td>
</tr>
<tr>
<td>Genomic-walking primers</td>
<td></td>
</tr>
<tr>
<td>GW1F</td>
<td>GACACATTAAAACCGTGCTCAAGCC</td>
</tr>
<tr>
<td>GW1R</td>
<td>TCCCTTTTGATGGCGGCTGACGAGG</td>
</tr>
<tr>
<td>GW2F</td>
<td>GACTGCGCGCTTGGTTGCTGATAGCC</td>
</tr>
<tr>
<td>GW2R</td>
<td>CGACGACGTTTCCAGGCTGACAC</td>
</tr>
<tr>
<td>3NEWGW2F</td>
<td>CCGGAGATTTCCAGTCTG</td>
</tr>
<tr>
<td>GW6R</td>
<td>CCGAAGCCTTCTCGGGCAGCGGACGTGGTCGAGT</td>
</tr>
<tr>
<td>xynAd1/2 and xynAd2 specific primers‡</td>
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<tr>
<td>XYNACBD4BF</td>
<td>CGATTCGCGTCATGCCATGTCGCTTCCAGG</td>
</tr>
<tr>
<td>XYNACBD4BR</td>
<td>TGACGCGGCAAGCGATCGACTCCAGGCCCCACATATGC</td>
</tr>
<tr>
<td>XYNA-CBD4BF</td>
<td>GGTAGAAGCCCTGCTCCGAGAGATG</td>
</tr>
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</table>

*The sequences below the primer sequences are the amino acid sequences encoded.
† Reverse complement of synthesized oligonucleotide.
‡ Engineered restriction sites are underlined.
Construction of a xynAd2 recombinant plasmid. The primers Xyna-CBD4BF and XynaCBD4BR (Table 1) were used to amplify xynAd2 encoding the xylanase catalytic domain from the recombinant plasmid pSUN18. The primers Xyna-CBD4BF and XynaCBD4BR incorporate the restriction sites NcoI and BamHI, respectively, allowing the directional in-frame ligation of xynAd2 into pJLA602 to give the recombinant plasmid pSUN24. Xylanase-positive transformants were identified as described above. The recombinant plasmid encoding XynAd2 was sequenced on both strands to confirm that there were no PCR-derived base changes in the DNA, except for those introduced in the engineered restrictions sites of the PCR primers (Asn→Met and Ile→Val change at the N terminus, and Thr→Ser change at the C terminus of the XynAd2 peptide). Translation was terminated as described for pSUN18.

Protein purification. XynAd1/2 and XynAd2 were produced from E. coli strains harboring the recombinant plasmid pSUN18 and pSUN24 plasmids, respectively, as described previously (Morris et al., 1998). XynAd1/2 was purified by anion-exchange chromatography in a similar fashion to that reported earlier (Morris et al., 1998), whilst XynAd2 was purified by anion-exchange chromatography on a HiTrapQ column (Amersham Pharmacia Biotech) pre-equilibrated with 20 mM Tris/HCl buffer, pH 8.0. More than 90% of contaminating proteins bound to the anion exchanger, whereas the recombinant XynAd2 was found in the flow-through in a highly purified form (data not shown). Both XynAd1/2 and XynAd2 were concentrated, desalted and stored in 50 mM sodium phosphate buffer (pH 6.0) at 4 °C.

Enzyme assay and protein determination. Xylanase activity was determined by the dinitrosalicylic acid method of Bernfeld (1955), using birchwood xylan as substrate. The standard assay reaction mixture consisted of 0.5% (w/v) xylan supplemented with 120 mM universal buffer (Britton & Robinson, 1931), pH 6.0, and enzyme to give a final volume of 0.1 ml. The reaction mixture was incubated at 90 °C (XynAd1/2) or 70 °C (XynAd2) for 15 min. One xylanase unit (XU) is defined as the amount of enzyme required to liberate 1 µmol xylose per minute at the assay temperature. Protein concentrations were determined using the BCA protein quantification kit (Pierce).

Effects of temperature, pH and thermostability on xylanase activity. The effects of temperature, pH and thermostability on the activity of XynAd1/2 and XynAd2 were determined as described by Sunna et al. (2000b). Universal buffer was used for determination of optimal pH for activity and accordingly, all the pH values were adjusted at 90 °C and 70 °C.

Protein electrophoresis. SDS-PAGE was performed in 4–20% precast gradient gels (Gradipore) by the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (Sigma). Low-molecular-mass marker proteins (Amersham Pharmacia Biotech) were used to determine the molecular mass of the enzymes.

Kinetic determinations. The initial rate of xylan hydrolysis using the purified enzymes was determined with birchwood and oat spelts xylan at various concentrations, under the standard assay conditions. The values of the Michaelis–Menten constant (Km) and the maximum velocity (Vmax) were estimated from linear regression of Hanes–Woolf plots.

Substrate specificity and mode of action of XynAd1/2 and XynAd2. The substrate specificity of XynAd1/2 and XynAd2 were determined by incubating the enzymes with different polymeric substrates under the standard assay conditions. Hydroxyethylcellulose was from Merck and all other substrates were from Sigma. The reaction mixtures consisted of 160 µl 0.5% (w/v) solutions of either birchwood or oat spelts xylan in 120 mM universal buffer (pH 6.0) and 40 µl purified enzyme. The mixtures were incubated at 70 °C (XynAd1/2) or 60 °C (XynAd2) for 3 h. Products from enzymic hydrolysis were extracted and desalted as described previously (Gibbs et al., 1999). The ability of the purified enzymes to hydrolyse short xylooligosaccharides was also investigated as described by Sunna et al. (2000b). Sugars liberated from xylan and xylooligosaccharide hydrolysis at 70 °C (XynAd1/2) or 60 °C (XynAd2) for 3 h were separated on silica gel plates as described previously (Sunna et al., 2000b). Reference xylooligosaccharides were obtained from Megazyme International.

RESULTS AND DISCUSSION

Analysis of consensus and genomic-walking PCR nucleotide sequence

A single 160 bp PCR fragment was amplified from ‘Caldibacillus cellulovorans’ genomic DNA with the XYNFB–XYNFR consensus PCR primer pair (Table 1). The nucleotide sequence of this fragment revealed homology to bacterial xylanases belonging to the glycosyl hydrolase family 10 (Henrisat, 1991). The amplification of the DNA fragments upstream and downstream of the xynA consensus fragment region was achieved using genomic-walking primers (see Table 1) and genomic-walking PCR. The nucleotide sequence data were combined to generate a 3237 bp sequence. A GC-rich specific PCR was required to successfully amplify the C-terminal portion of xynA due to the relatively high G + C content (65 mol% mean) of the DNA encoding the PT-linkers and type IIIb CBDs compared to the G + C content (44 mol% mean) of the DNA encoding the catalytic domain (see Fig. 1b). Overall, ‘Caldibacillus cellulovorans’ has a mol % G + C content of 62.4 (X. P. Huang, unpublished). These sequence data have been submitted to the GenBank database under accession number AF200304.

Analysis revealed the presence of two ORFs, ORF1 and xynA. The partial ORF1 consists of a sequence encoding a putative C-terminal type IIIb CBD (1–279 bp). Downstream of the ORF1 terminator codon TAA, a 104 bp intergenic region was identified (280–383 bp), containing a 14 bp palindromic repeat sequence, which may serve as a transcription-terminator signal. The second ORF, xynA (384–3149 bp), encodes a multidomain β-xylanase, XynA, with a putative size of 921 amino acids. The initiation codon ATG was preceded at a spacing of 7 bp by a potential ribosome-binding sequence (GAG-GA).

Analysis of the deduced amino acid sequences

Fig. 1(a) shows a diagrammatic representation of the elements encoded by the two ORFs of the 3237 bp sequence. Similarity searches were carried out between the deduced amino acid sequences of ORF1 and XynA against entries in the GenBank and SWISS-PROT databases. The incomplete nucleotide sequence of ORF1 encodes a partial C-terminal CBD homologous to family
IIIb CBDs (Fig. 1a), as classified by Tormo et al. (1996). It showed highest homology (% identity) to a partial ORF encoding a C-terminal family IIIb CBD upstream of ‘Caldicellulosiruptor’ ManA and to the C-terminal IIIb CBD (ManAd4) from ManA of the same organism (99%; Sunna et al., 2000b). ORF1 exhibited only moderate sequence identity to the internal CBD of Caldicellulosiruptor sp. Rt69B.1 multidomain XynC (55%; Morris et al., 1999), a partial ORF encoding a CBD directly upstream of Bacillus lautus CelA (53%; Hansen et al., 1992), and the C-terminal CBD from Clostridium stercorarium CelY (52%; Bronnenmeier et al., 1997).

Analysis of XynA revealed a multidomain structure. The N-terminal sequence has a putative signal peptide sequence with a predicted cleavage site between position 33 (Ala) and position 34 (Glu). Removal of the signal peptide yields a mature protein with a predicted molecular mass of 98–99 kDa. The mature protein has a XBD, XynAd1, at its N terminus (483–965 bp, Fig. 1a). XynAd1 shows only low sequence identity (34%) to the N-terminal TSD of Clostridium thermocellum XynC (Hayashi et al., 1997) and the internal TSD of Caldicellulosiruptor sp. Rt69B.1 XynC (Morris et al., 1999). XynAd1 is followed by a second domain, XynAd2 (966–1946 bp, Fig. 1a), homologous to glycosyl hydrolase family 10 β-1,4-xylanases (Henrissat, 1991). The xylanase domain shares 51% sequence identity with the xylanase catalytic domains of Caldicellulosiruptor saccharolyticus XynE (V. S. J. Te'o, unpublished), Caldicellulosiruptor sp. Rt69B.1 XynC (Morris et al., 1999) and Thermotoga maritima XynA (Winterhalter et al., 1995). The XynAd2 catalytic domain is linked to a family IIIb CBD, XynAd3 (2076–2519 bp, Fig. 1a), through a 43 amino acid PT-linker. This CBD shares greatest sequence identity (97%) with the internal IIIb CBD (ManAd2) of ‘Caldicellulosiruptor’ ManA, but only 53% sequence identity to the C-terminal IIIb CBD (ManAd4) of the same enzyme (Sunna et al., 2000b). Furthermore, XynAd3 exhibited only 43% sequence identity with the internal CBD of Caldicellulosiruptor sp. Rt69B.1 XynC (Morris et al., 1999) and the C-terminal CBD of Bacillus subtilis endoglucanase EgI8 (MacKay et al., 1986). XynAd3 is linked via a 59 amino acid PT-linker to a second family IIIb CBD, XynAd4 (2697–3149 bp, Fig. 1a), at the C terminus of the protein that is almost identical (93% sequence identity) to the partial C-terminal CBD of ORF1, but shows only 51% sequence identity to the internal XynAd3 CBD. In addition, XynAd4 exhibited 93% sequence identity to the partial ORF encoding a C-terminal family IIIb CBD upstream of ‘Caldicellulosiruptor’ ManA, and 95% sequence identity to the C-terminal IIIB CBD (ManAd4) from ManA of the same organism (Sunna et al., 2000b). XynAd4 was homologous to the internal CBD of Caldicellulosiruptor sp. Rt69B.1 XynC (55% sequence identity; Morris et al., 1999), the C-terminal CBD from Clostridium stercorarium CelY (53% sequence identity; Bronnenmeier et al., 1997) and the partial ORF encoding a CBD directly upstream of B. lautus CelA (52% sequence identity; Hansen et al., 1992).

Recently, we have identified two further putative family IIIb CBDs in a gene sequence that codes for a multi-domain cellulase in ‘Caldicellulosiruptor’ (A. Sunna, unpublished). Interestingly, all family IIIb CBDs...
identified so far in the modular glycosyl hydrolases from \textit{Caldibacillus cellulovorans} can be grouped into two distinct groups. Type 1 family IIIb CBDs are all C-terminal, preceded by a PT-linker region and end with a stop codon. This subclass shares more than 93\% sequence identity at the amino acid level. Type 2 family IIIb CBDs are all internal and flanked by two PT-linker regions. Type 2 IIIb CBDs share more than 97\% amino acid sequence identity. Type 1 and 2 family IIIb CBDs exhibit only around 50\% sequence identity.

**Comparison with other family 10 glycosyl hydrolases**

The N-terminal region of \textit{Caldibacillus cellulovorans} XynA is homologous to regions found in several thermostable xylanases. These regions have been described as TSDs as their removal has been associated with loss of thermostability of the adjacent catalytic domain (Fontes \textit{et al}., 1995). The molecular architecture of thermophilic family 10 multidomain xylanases is usually based on the domain arrangement TSD–TSD–catalytic domain–CBDIX–CBDIX (Bergquist \textit{et al}., 1999). This arrangement is shared by XynB and XynC from \textit{Thermotoga} FjSS3B.1 (Reeves \textit{et al}., 2000), XynA from \textit{Thermotoga maritima} (Winterhalter \textit{et al}., 1995), XynA from \textit{Thermotoga} neapolitana (Zverlov \textit{et al}., 1996), and XynA from \textit{Thermoanaerobacterium saccharolyticum} B6A-RI (Lee \textit{et al}., 1993). In the case of XynB from \textit{Caldicellulosiruptor} sp. Rt69B.1 (Morris \textit{et al}., 1999), three TSDs are found associated with the catalytic domain at the N terminus, while at the C terminus the catalytic domain is associated with two type IX CBDs. The modular XBD–catalytic domain–PT-linker–CBDIIIb–PT-linker–CBDIIIb structure of XynA from \textit{Caldibacillus cellulovorans} is unique when compared to other thermophilic family 10 xylanases. The only xylanases from this family associated with type IIIb CBDs are XynC from \textit{Caldicellulosiruptor} sp. Rt69B.1 (Morris \textit{et al}., 1999) and CelB from \textit{Caldicellulosiruptor} saccharolyticus (Saul \textit{et al}., 1990). It should be noted that \textit{Caldibacillus} is not closely related to \textit{Caldicellulosiruptor}, a low-percentage G+C anae-robe. A selection of xylanases with domain structures related to \textit{Caldibacillus cellulovorans} XynA is shown in Fig. 2.

**Purification and properties of the recombinant XynAd1/2 and XynAd2**

XynAd1/2 and XynAd2 were purified to electrophoretic homogeneity with a respective final specific activity of 177 XU mg$^{-1}$ and 14 XU mg$^{-1}$. The apparent molecular mass of XynAd1/2 and XynAd2 was estimated to be 57 and 38 kDa (data not shown), respectively, which is consistent with the respective 56 and 37 kDa deduced from their translated amino acid sequences. The recombinant XynAd1/2 enzyme was active between 40 and 100 $^\circ$C, with an optimal temperature for activity...
at 90 °C (15 min assay, Fig. 3), which is comparable to that reported for thermostable xylanases from other hyperthermophilic bacteria (Sunna et al., 1997). XynAd2 was active between 40 and 85 °C and displayed an optimal temperature for activity of only 70 °C (15 min assay, Fig. 3). At 95 and 100 °C, 60 and 15% of the initial activity of XynAd1/2 was detected, respectively, whilst XynAd2 displayed only 12% of its initial activity at 85 °C. The optimal pH for activity of the XynAd1/2 (at 90 °C) and XynAd2 (at 70 °C) enzymes was 6.0 (data not shown). Both enzymes displayed 40% of initial activity when assayed at pH 8.0, whilst no activity was observed at either pH 4.0 or 9.0.

**Influence of XBD on enzyme activity**

Thermostability of the purified XynAd1/2 enzyme in the absence of substrate was studied at 70 and 80 °C while purified XynAd2 was studied at 60 and 70 °C (data not shown). XynAd1/2 was almost completely stable at 70 °C (85% residual activity) for the complete assay period of 240 min, whilst at this temperature the half-life of XynAd2 was 25 min. XynAd2 was completely stable at 60 °C for the complete assay period of 120 min. The half-life of the XynAd1/2 enzyme at 80 °C was 35 min, whilst XynAd2 was completely inactivated in less than 5 min.

The lack of the XBD in XynAd2 resulted in a decrease in the optimum temperature for activity to 70 °C, which is closer to the 65 °C growth temperature of *Caldibacillus cellulovorans* (X. P. Huang, unpublished). In addition, XynAd2 was less thermostable in the absence of substrate when compared to XynAd1/2. The fact that no other major changes in the biochemical properties of the two enzymes were observed indicates that the deleted region does thermostabilize the catalytic domain of XynA. Removal of the TSD region has been reported to result in both a decrease in optimum temperature for activity and a dramatic reduction in thermostability of the thermophilic XynY from *Clostridium thermocellum* (Fontes et al., 1995) and XynA from *Thermotoga maritima* (Winterhalter et al., 1995). A reduction in thermostability in XynA family 10 xylanase from *Thermoanaerobacterium saccharolyticum* B6A-RJ has been associated to the removal of its N-terminal TSDs (Lee et al., 1993). However, in this case the decrease in thermostability could be ascribed to deletion of a portion of the xylanase catalytic domain, rather than to the removal of the TSDs. TSDs are not a prerequisite for thermal stability as several hyperthermophilic bacteria produce single-domain family 10 xylanases that in many cases are more thermostable than their respective multidomain counterpart containing TSDs. For example the single-domain family 10 XynB from *Thermotoga maritima* has an optimum temperature for activity of 105 °C compared to 92 °C for the family 10 multidomain (tandem N-terminal TSDs) XynA (Winterhalter et al., 1995; Winterhalter & Liebl, 1995).

TSDs have mainly been found in association with thermophilic family 10 xylanases from bacteria and no TSD has yet been reported in either family 10 or 11 fungal xylanases. However, TSD domains have been reported in the XynB and XynD family 11 xylanases from the mesophilic bacterium *Ruminococcus flavefaciens* (Flint et al., 1993; Zhang et al., 1994) and the XylC family 10 xylanase from *Cellulomonas fimi* (Clarke et al., 1996). XylC was most active at 60 °C and displayed 50% residual activity at 70 °C (Clarke et al., 1996), unlike the other xylanases characterized from *Cellulomonas fimi* (Khanna & Gauri, 1993), which are active at 40–45 °C. Recently, the multidomain XynC from the mesophilic *Bacillus* sp. BP-23 was reported to have the same TSD–TSD–catalytic domain–CBDIX–CBDIX commonly found in thermophilic xylanases (Blanco et al., 1999). Removal of the tandem N-terminal TSD from XynC resulted in a 10 °C decrease (from 45 to 35 °C) in the optimal temperature for activity of the xylanase, as well as a decrease in its thermostability. There seems to be no obvious reason for the presence of TSD in enzymes from mesophilic bacteria and a more general role for TSDs in conferring protection against proteolytic attack and extremes of pH has been postulated (Clarke et al., 1996; Fontes et al., 1995). Recently, we have shown that the XBD of *Caldibacillus cellulovorans*, which is homologous to TSDs associated with glycosyl hydrolase family 10 xylanases, has the ability to selectively bind to xylan. Furthermore, it was suggested that the thermostabilizing function of this class of domain is a result of a lack of discrete linker peptides separating the XBD (or their homologous TSDs) from the adjacent catalytic domain (Sunna et al., 2000a).

**Substrate specificity and hydrolysis pattern**

The action of purified XynAd1/2 and XynAd2 enzymes was tested against different polymeric substrates (data not shown). The recombinant enzymes were active against different xylans (β-1,4-linkages), including beechwood, birchwood, larchwood and oat spelts xylan. Of these, oat spelts xylan gave the highest reducing sugar values. Both enzymes were inactive against barley β-glucan (β-1,4/β-1,3-linkages), lichenan (β-1,4/β-1,3-..
linkages), laminarin (β-1,3-linkages), soluble starch (α-1,4/α-1,6-linkages), pullulan (α-1,6-linkages), dextran (α-1,6/α-1,3-linkages) and galactomannan (β-1,4/α-1,6-linkages). In addition, no reducing sugars were released upon incubation of the purified xylanases with carboxymethyl- or hydroxyethylcellulose (β-1,4-linkages).

The apparent $K_m$ and $V_{\text{max}}$ values calculated for the purified XynAd1/2 xylanase in the presence of birchwood xylan were 1.8 mg ml$^{-1}$ and 256 XU mg$^{-1}$, respectively, while in the presence of oat spelts xylan the apparent $K_m$ and $V_{\text{max}}$ values were 27 mg ml$^{-1}$ and 333 XU mg$^{-1}$, respectively. In the presence of birchwood xylan, XynAd2 displayed $K_m$ and $V_{\text{max}}$ values of 1.0 mg ml$^{-1}$ and 15.7 XU mg$^{-1}$, respectively. When oat spelts xylan was used as substrate, the $K_m$ and $V_{\text{max}}$ values calculated for XynAd2 were 2.1 mg ml$^{-1}$ and 21.3 XU mg$^{-1}$, respectively. The kinetic parameters of the two recombinant enzymes are within the range estimated from kinetic data reported for other xylanases (Sunna & Antranikian, 1997).

The action of purified XynAd1/2 (70 °C) and XynAd2 (60 °C) xylanases on partially soluble birchwood and oat spelts xylan and xylooligosaccharides was analysed qualitatively by thin-layer chromatography. Equivalent units of each enzyme (40 mXU) were used for each assay. After 3 h incubation, the major products liberated from both xylans by XynAd1/2 were xylobiose, xylotriose and xylotetraose (data not shown). The action of XynAd2 on these two substrates liberated mainly xylotriose and xylotetraose (data not shown). The ability of the purified enzymes to hydrolyse short xylooligosaccharides was also investigated (Fig. 4). Equivalent units of each enzyme (1 mXU) were used for each assay. After 3 h incubation at 70 °C, XynAd1/2 was unable to hydrolyse xylobiose, xylotriose or xylotetraose (Fig. 4a). There was minimal release of xylobiose, xylotriose and xylotetraose when xylopentaose was treated with XynAd1/2. It incompletely hydrolysed xylohexaose, releasing xylobiose, xylotriose, xylotetraose and xylopentaose. Assays using five times excess (5 mXU) enzyme did not improve the efficiency of xylooligomer hydrolysis by XynAd1/2. XynAd2 was unable to hydrolyse xylobiose and xylotriose but in contrast to XynAd1/2, it almost completely hydrolysed xylotetraose to mainly xylotriose and xylobiose (Fig. 4b). Xylotriose and xylohexaose were completely hydrolysed by XynAd2 to mainly xylotriose and xylobiose as end products, accompanied by lesser amounts of xylotetraose. Thus, based on their substrate specificity and xylan hydrolysis pattern, both purified recombinant enzymes act as endo-1,4-β-xylanases. The inability of XynAd1/2 to efficiently hydrolyse xylooligomers, and the efficient release of xylobiose, xylotriose and xylotetraose from xylan, may indicate the requirement of this enzyme for side group substitutions (i.e. acetyl, arabinosyl or glucuronosyl groups) in the xylan backbone or the inability of the active site to accommodate oligomers of equal or less than six xylose residues. In either case the substrate cleavage pattern of the xylanase is changed by the presence of its XBD.

This is the first report on a XBD domain with a dual function of conferring thermostability and also affecting the substrate cleavage pattern of a xylanase.

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xylanase gene,


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