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ORIGINAL PAPER

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Comparative characterization of deletion derivatives of the modular xylanase XynA of *Thermotoga maritima*

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Abstract The modular Xylanase XynA from *Thermotoga maritima* consists of five domains (A1-A2-B-C1-C2). Two similar N-terminal domains (A1-A2-) are family 22 carbohydrate-binding modules (CBMs), followed by the catalytic domain (-B-) belonging to glycoside hydrolase family 10, and the C-terminal domains (-C1-C2), which are members of family 9 of CBMs. The gradual deletion of the non-catalytic domains resulted in deletion derivatives (XynAΔC; XynAΔA1C and XynAΔNC) with increased maximum activities (V_{max}) at 75°C, pH 6.2. Furthermore, these deletions led to a shift of the optimal NaCl concentration for xylan hydrolysis from 0.25 (XynA) to 0.5 M (XynAΔNC). In the presence of the family 22 CBMs, the catalytic domain retained more activity in the acidic range of the pH spectrum than without these domains. In addition to the deletion derivatives of XynA, the N-terminal domains A1 and A2 were produced recombinantly, purified, and investigated in binding studies. For soluble xylan preparations, linear β-1,4-glucans and mixed-linkage β-1,3-1,4-glucans, only the A2 domain mediated binding, not the A1 domain, in accordance with previous observations. The XynA deletion enzymes lacking the C domains displayed low affinity also to hydroxyethylcellulose and carboxymethylcellulose. With insoluble oat spelt xylan and birchwood xylan as the binding substrates, the highest affinity was observed with XynAΔC and the lowest affinity with XynAΔNC. Although the domain A1 did not bind to soluble xylan preparations, the insoluble oat spelt xylan-binding data suggest that this domain does play a role in substrate binding in that it improves the binding to insoluble xylans.

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Introduction

Xylans, cellulose, and lignin are closely associated with each other in the secondary cell walls of plants. Xylans are heteropolysaccharides, based on a non-branched β-1,4-glycosidically linked xylose backbone. Depending on the origin, the backbone structure is substituted to varying degrees with acetyl, L-arabinofuranosyl, glucuronyl, and 4-*O*-methylglucuronyl groups (see Coughlan and Hazlewood 1993). In addition, covalent linkages between xylan and lignin can be found. Non-substituted linear xylans are found in guar seed husk, tobacco stalks, and esparto grass (Kulkarni et al. 1999). Because of the complex structure of xylans, their complete decomposition requires the action of a variety of enzymes specific for the different linkages between the xylan building blocks (Meissner et al. 2000). Endo-1,4-β-D-xylanases (EC 3.2.1.8) hydrolyse the xylan backbone and produce mixtures of substituted and unsubstituted xylo-oligosaccharides. The xylo-oligosaccharides are degraded by β-xylosidases (EC 3.2.1.37) to release monomeric xylose residues (Decelle et al. 2004). In addition, for complete xylan degradation, accessory enzymes are needed to remove the non-xylose residues from the backbone. Endo-1,4-β-xylanases are found in a variety of microorganisms. They are classified into two glycoside hydrolase (GH) families, 10 and 11, according to the similarity of the amino acid sequences of their catalytic domains in hydrophobic cluster analyses. A variety of cellulolytic and hemicellulolytic enzymes have multi-domain structures with one (in some cases two) catalytic domain(s), and one or more non-catalytic domains (see Gilkes et al. 1991; Gilbert et al. 2002).

Thermotoga maritima is a strictly anaerobic hyperthermophilic bacterium that grows at temperatures up to 90°C. During growth on xylose or xylan as carbon

source, *T. maritima* is able to produce a high-molecular mass xylanase (120 kDa, XynA), a low-molecular mass xylanase (40 kDa, XynB), a β-xylosidase, an α-L-arabinofuranosidase, and an α-D-glucuronidase (Winterhalter and Liebl 1995; Winterhalter et al. 1995; Ruile et al. 1997). XynA of *T. maritima*, a 1,059 amino acid (120 kDa) xylanase with maximum activity at 92°C, pH 6.2 and 0.25 M NaCl, and high stability at temperatures up to 90°C (Winterhalter and Liebl 1995) has a modular structure with five domains in the order A1-A2-B-C1-C2. Its N-terminal signal peptide is followed by two related ~150 residue domains (A1-A2), a ~340-amino acid catalytic domain belonging to GH family 10, a short proline-rich linker sequence, and two related ~170 residue domains (C1-C2). The four non-catalytic domains belong to two different families of carbohydrate-binding modules (CBMs). Previous studies concerned with XynA and recombinantly produced deletion and fusion enzymes have helped to gain some information on the stabilizing and polysaccharide-binding functions of the CBMs of XynA (Winterhalter and Liebl 1995; Winterhalter et al. 1995; Wassenberg et al. 1997; Meissner et al. 2000). The N-terminal repeated domains (A1 and A2) are family 22 CBMs while the C-terminal domains (C1 and C2) belong to the CBM familiy 9 (Winterhalter et al. 1995; Meissner et al. 2000). Domain C2 and, based on its amino acid sequence similarity to C2, possibly also C1 represent cellulose-binding domains that confer upon XynA the ability to reversibly bind to microcrystalline cellulose (Winterhalter et al. 1995). Thermostablisation was the first described function for enzyme domains related to A1 and A2 of XynA, and several examples of family 22 CBM-containing enzymes are known where their deletion led to heat lability (Lee et al. 1993; Fontes et al. 1995; Winterhalter et al. 1995; Clarke et al. 1996; Zverlov et al. 1996; Kulkarni et al. 1999; Kim et al. 2000; Sunna et al. 2000; Araki et al. 2004). Further investigation has shown that xylan-binding seems to be their main function. It could be shown that glutathion-S-transferase (GST) hybrid proteins containing A2 of XynA (GST-A1A2 and GST-A2) interact with various soluble xylan preparations and with mixed-linkage β-1,3/β-1,4-glucans. Binding of the A1 domain to these substrates could not be demonstrated (Meissner et al. 2000).

The main goals of this study are to investigate the carbohydrate-binding properties of the A1 domain and to elucidate the influence of the CBMs of the catalytic activity and the binding capacity.

Materials and methods

Bacterial strains and plasmids

Escheerichia coli strains used for DNA manipulations were XL1-Blue (Stratagene, Heidelberg, Germany), JM83 (Yanish-Perron et al. 1985), and BL21 (DE3) (Studier and Moffat 1986). *E. coli* strains were routinely grown in Luria broth (LB, 10 g of peptone, 5 g of yeast extract, 5 g of NaCl and 1,000 ml of deionized water, pH 7.2) supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin depending on the containing plasmid. The pBluescriptII (Stratagene) and pCR4-Topo (Invitrogen, Karlsruhe, Germany) were used as a cloning vector; pBAD*myc* (Invitrogen) and pET24c/d (Novagen, Schwalbach, Germany) were used as expression vectors.

Construction of xynA deletion derivatives

The domain borders (the approximate borders are shown in Fig. 1) used as the basis for expression plasmid construction were as proposed by Winterhalter et al. (1995) and Meissner et al. (2000). Vectors for the recombinant production of XynA (pUCXyl1), XynAΔC (p74) and XynAΔNC (p24), and PCR methods the amplification of DNA fragments were described earlier (Winterhalter et al. 1995; Meissner et al. 2000). For PCR-mediated amplification, the DNA was first denatured at 95°C for 5 min. The PCR cycles were: 35 cycles of 95°C for 45 s, 55°C for 1 min, and 72°C for 1 min for amplification of A1; 35 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 1 min for amplification of A2; 35 cycles of 95°C for 45 s, 65°C for 1 min, and 72°C for

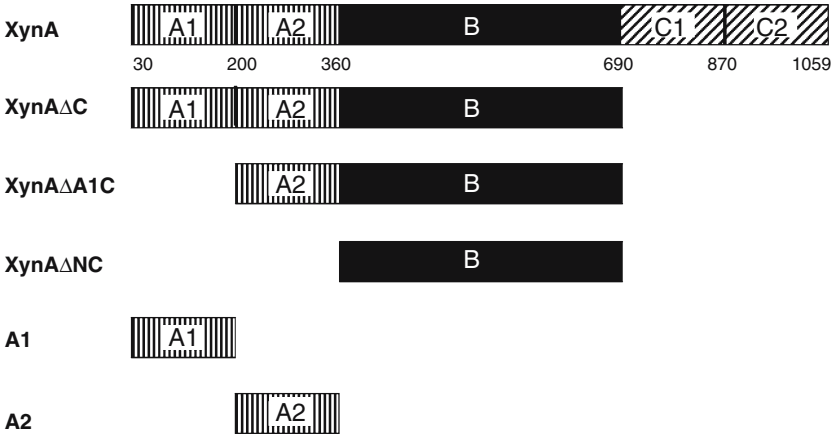

Fig. 1 Modular structure of XynA and deletion derivatives. The two similar N-terminal domains (A1-A2) of XynA are family 22 carbohydrate-binding modules (*CBMs*). The catalytic domain (*B*) belongs to glycoside hydrolase family 10. The two similar C-terminal domains (C1-C2) are members of family 9 of CBMs. The *numbers* below the XynA boxes represent the approximate borders of the XynA domains as proposed by Winterhalter et al. (1995) and Meissner et al. (2000)

3.5 min for amplification of XynAΔA1C. The final elongation for all amplifications was at 72°C for 5 min.

The vector pET24c (Novagen) was used for the construction and expression of the isolated domains A1 and A2. The following primers were used for the PCR-aided amplification of A1 and A2: A1-f, 5′-GCC CAT ATG TCT TCT CTC GAA ACA GTT CTT GCT TTG AGT-3′; A1-r, 3′-GAA CTG TCC CAA GTC CAT GAA CGA GGT TTT ATC GAA TTC CCG-5′; A2-pET-f, 5′-GCC CAT ATG GAA TCG GGC CCA AAG GTC ATC TAC GAA ACA-3′; A2-r, 3′-CTG CTG CAC TTC TAT CAC CTA TGT TGA AGG ATC GAA TTC CCG-5′. The PCR products obtained were purified from an agarose gel (Gel Extraktion Kit, Qiagen, Hilden, Germany), incubated with *Taq* DNA polymerase in the presence of 0.2 mM dATP, and cloned in the pCR4-Topo vector (Invitrogen) yielding plasmids pCR4-A1 and pCR4-A2. The vector obtained was cut with *Nde*I and *Eco*RI, the A1- and A2-encoding DNA-fragments were purified from an agarose gel and inserted into pET24c treated with the same restriction endonucleases, thus, yielding the plasmids pET24-A1 and pET24-A2. The plasmids pET24-A1 and pET24-A2 were transformed into *E. coli* BL21 for subsequent high-level expression.

For the construction and expression of XynAΔA1C, the following primers were used: A2-pBAD-f, 5′-GA TCC ATG GAA TCG GGC CCA AAG GTC ATC TAC GAA ACA-3′; GHF-r, 3′-GAG CGA ATG ACC CGC TAT CAC CGT ATC GAG CTC CCG-5′. The PCR products obtained were purified from an agarose gel (Gel Extraktion Kit, Qiagen) and ligated with pBluescriptII (Stratagen) treated with *Eco*RV, yielding the plasmid pBlue-XynAΔA1C. In order to construct an expression vector for XynAΔA1C, pBlue-XynAΔA1C was subjected to *Nco*I and *Xho*I restriction, and the XynAΔA1C fragment was ligated with pBAD*myc* (Invitrogen), placing it under control of the arabinose-inducible *araB* promoter. The resulting plasmid pBAD-XynAΔA1C was introduced into *E. coli* XL1-Blue for subsequent high-level expression. The XynA deletion derivatives consisted of the following sequence regions of XynA (numbering of residues as described by Winterhalter et al. 1995): XynAΔC, A_{31}-P_{692}; XynAΔA1C, E_{200}-A_{691}; XynAΔNC, M_{361}-P_{692}; domain A1, S_{47}-K_{199}; domain A2, E_{200}-S_{354}. Due to construction reasons, most of the deletion derivatives produced carried an additional methionine residue at the N-terminus.

Expression and purification of the recombinant proteins

For high-level expression of the recombinant proteins A1 and A2 in *E. coli* BL21(DE3)(pET24-A1) and *E. coli* BL21(DE3)(pET24-A2), the strains were grown in LB medium supplemented with 25 μg/ml kanamycin at 37°C. The strains were grown in 1.5 l batches in 5 l Erlenmeyer flasks; the expression was induced after reaching an OD (600 nm) of 0.6 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.5 mM and incubation was continued over night. The cultures were harvested (5,000×*g*, 20 min, 4°C) and washed with 10 mM BisTris pH 6.2, and lysed by threefold passage through a French Press Cell (American Instruments, Silver Springs, MD, USA). The cell lysates were heated to 75°C for 20 min, and denatured protein was removed by centrifugation (20 min 15,000×*g*). After dialysing the supernatant overnight in 5 l of 10 mM Bis-Tris pH 6.2, the protein extracts were loaded onto a Source Q 15 anion exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein was eluted with a linear NaCl gradient (0–1 M) and the purest fractions containing A1 or A2 were pooled and concentrated (Amicon Ultra columns, Millipore Corp., Bedford, MA, USA).

Production of XynA and the truncated XynA derivatives was achieved by expression in the recombinant strains *E. coli* JM83(pUCXyl1, XynA), *E. coli* JM83(p74, XynAΔC), *E. coli* JM83(p24, XynAΔNC), and *E. coli* XL1-Blue(pBAD-XynAΔA1C). The strains were grown in LB medium supplemented with 50 μg/ml ampicillin at 37°C. They were grown in 1.5 l batches in 5 l Erlenmeyer flaks. The recombinant XynA was constitutively expressed, whereas XynAΔC and XynAΔNC production was induced with 0.5 mM IPTG after reaching an OD (600 nm) of 0.6, and XynAΔA1C production was induced by the addition of 0.2% arabinose at OD 0.6. Cell harvest, lysis, heat treatment, and anion exchange chromatography was done as described above. The pooled protein fractions from the anion exchange chromatography were dialysed against 10 mM BisTris pH 6.2, 0.5 M NaCl. The protein was applied to a Phenyl-Sepharose HP hydrophobic interaction column (Amersham Pharmacia Biotech) and eluted with a linear 0.5–0 M NaCl gradient in 10 mM BisTris pH 6.2. The active fractions were pooled, concentrated, and dialysed against water to remove the remaining salt.

Protein analytical methods, enzyme assays and binding studies

Determination of protein concentrations and SDS-PAGE were carried out as described before (Liebl et al. 1992). Commercial protein assay kits (Micro BCA Protein Assay Reagent Kit of Pierce, Rockford, USA or the microassay of the Protein Assay Kit of Bio-Rad, München, Germany) were used to measure low-protein concentrations in xylan-binding studies. Xylanase activity was determined as described before (Winterhalter et al. 1995).

Native affinity polyacrylamide gel electrophoresis (NAPAGE) analysis for binding studies with soluble subtrates was done as described by Meissner et al. (2000). Binding assays with insoluble substrates were performed with 0.5 ml 1% (w/v) insoluble polysaccharide substrate in binding buffer A (10 mM BisTris pH 6.2, 0.5 M NaCl) incubated with 0.1 nmol enzyme. After incubation at 20°C with slow rotation for various periods (0.5, 1, 1.5, 2, 2.5, and 3 h), the substrate was pelleted by centrifugation

(1 min, 13,000×*g* in a table-top centrifuge) and the supernatants were tested for xylanase activity.

Results and discussion

Expression in Escherichia coli and purification of the XynA deletion derivatives and the individual domains A1 and A2

Separation of the domains of modular glycoside hydrolases from each other has demonstrated that they often function independent of each other (see Gilkes et al. 1991; Tomme et al. 1995; Winterhalter et al. 1995; Warren 1996; Meissner et al. 2000). In order to elucidate the influence of the non-catalytic domains on the activity and stability of XynA, the non-catalytic domains were gradually removed with recombinant DNA methods to yield deletion constructs XynA*Δ*C (lacking domains C1 and C2), XynA*Δ*A1C (lacking A1, C1 and C2), and XynA*Δ*NC (lacking A1, A2, C1, and C2) (Fig. 1). XynA and the deletion constructs XynA*Δ*C and XynA*Δ*NC were isolated from *E. coli* strains carrying the plasmids pUCXyl1, p74 and p24, respectively (Winterhalter et al. 1995). For production of XynA*Δ*A1C, a *xynA* deletion gene was constructed using specific primers matching the borders of the region coding for A2 and B (residues E_{200} to A_{691} of XynA). The *xynA* gene segment obtained by PCR amplification was cloned in the pBAD*myc* vector to yield pBAD-XynA*Δ*A1C as described in 'Materials and methods'. Recombinant XynA was constitutively expressed after transformation of pUCXyl1 in *E. coli* JM83. XynA*Δ*C and XynA*Δ*NC were expressed via IPTG-induction after transformation of p74 and p24, respectively, into *E. coli* JM83. XynA*Δ*A1C was produced in *E. coli* XL1-blue containing pBAD-XynA*Δ*A1C after induction with L-arabinose. The deletion constructs were purified by heat treatment, anion exchange, and hydrophobic interaction chromatography. The level of enzyme purity reached was estimated to be higher than 95%. SDS-PAGE analysis revealed protein bands with sizes of about 120 kDa (XynA), 75 kDa (XynA*Δ*C), 57 kDa (XynA*Δ*A1C), and 39 kDa (XynA*Δ*NC) (data not shown), which corresponds well to the expected sizes. All four XynA derivatives displayed xylanase activity.

By the analysis of the GST hybrid proteins GST-A1 and GST-A2, Meissner et al. (2000) found that the A2 module but not A1 was a functional XBD. However, in GST-A1, the GST part of the fusion protein may have had a negative influence on the function of the A1 domain. Therefore, the plasmids pET24-A1 and pET24-A2 were constructed for overexpression of the individual domains A1 and A2, respectively, and transformed into *E. coli* BL21. Crude cell extracts were prepared and analysed by SDS-PAGE, which revealed overexpressed protein bands with sizes of about 15 and 17 kDa for the pET24-A1 and pET24-A2-bearing strains, respectively, which corresponds well to the sizes expected for the single A1 and A2 domains. The A1 and A2 domains, which under optimized induction conditions were expressed at levels of about 15% (A1) and 7% (A2) of soluble cellular proteins, were purified by heat treatment and anion exchange chromatography on Source 15 Q as described in 'Materials and methods'. The level of protein purity reached was estimated to be higher than 95%.

Catalytic properties of the XynA derivatives

XynA and the deletion derivatives XynA*Δ*C had an optimum NaCl concentration at 0.25 M, which is the NaCl optimum reported previously for XynA (Winterhalter and Liebl 1995). Deletion of one (A1) or both (A1 and A2) family 22 CBMs in addition to the C-terminal CBMs led to an increased NaCl optimum of 0.4 M for XynA*Δ*A1C and 0.5 M for XynA*Δ*NC (Fig. 2). Thus, by deletion of all non-catalytic domains from XynA, its NaCl optimum approached the value reported for the naturally CBM-less 40 kDa xylanase XynB (Winterhalter and Liebl 1995) of *T. maritima* (NaCl optimum 0.5 M; XynB and the catalytic domain of XynA share about 39% amino acid sequence identity).

The deletion of the non-catalytic domains had no dramatic influence on the optimal pH, but with the N-terminal family 22 CBMs, the catalytic domain retained more activity in the acidic range of the pH spectrum than the catalytic domain alone (Fig. 2). A similar broadening of the activity profile in the acidic range was

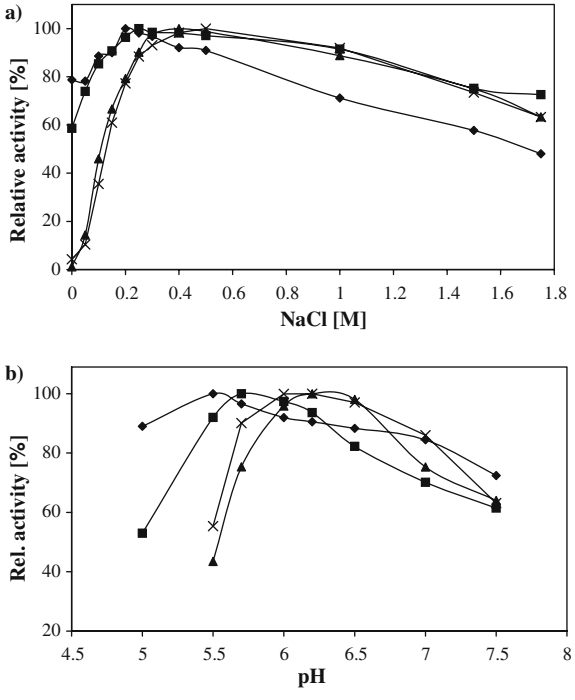

Fig. 2 Effect of NaCl concentration (a) and pH (b) on xylanase activity. All measurements were carried out at 75°C. Symbols used: XynA (*rhomboids*), XynA*Δ*C (*squares*), XynA*Δ*A1C (*triangles*), and XynA*Δ*NC (*crosses*)

also found in experiments with other members of family 22 CBMs (Devillard et al. 2003; Kittur et al. 2003; Ali et al. 2005).

Interestingly, the deletion derivatives differed significantly in their catalytic efficiencies for hydrolysis of oat spelt xylan. The maximum initial velocity at 75°C measured with the catalytic domain devoid of all non-catalytic domains (XynAΔNC) was 9.6-fold higher than for XynA. The other deletion derivatives showed a twofold (XynAΔC) or sixfold (XynAΔA1C) higher V_{max} than XynA (Table 1). The kinetic data for oat spelt xylan hydrolysis were also measured at 65°C. The V_{max} for the catalytic domain (XynAΔNC) was 6.5-fold higher than for XynA whereas the other truncated enzymes had twofold (XynAΔC) and threefold (XynAΔA1C) higher V_{max} values. Also, it was observed that the K_M was higher for the truncated XynA derivatives without the family 22 carbohydrate-binding domains, which was more pronounced at 75°C than at 65°C.

Taken together, the kinetic data for oat spelt xylan hydrolysis by the deletion derivatives (Table 1) demonstrate that the gradual deletion of the CBMs increased the enzymes' maximum activity and simultaneously moderately increased the K_M for the substrate of hydrolysis. The generally accepted concept about CBM function is to promote a close interaction between the enzymes and substrates and thereby to assist in substrate hydrolysis in different ways (Millward-Sadler et al. 1994; Black et al. 1996, 1997; Warren 1996; Boraston et al. 1999, 2003; Charnock et al. 2000; Meissner et al. 2000; Notenboom et al. 2001, 2002; Sunna et al. 2001; Scharpf et al. 2002). Many cellulases and xylanases contain CBMs, which increase the hydrolytic activity of the catalytic domain, or lead to a decrease of the activity after deletion of the CBMs (Linder and Teeri 1997; Srisodusk et al. 1997; Bolam et al. 1998; Charnock et al. 2000; Ali et al. 2001; Chhabra and Kelly 2002; Mangala et al. 2003). Also, CBMs can increase the activity against cellulose-xylan-complexes (Black et al. 1996; Gill et al. 1999). In general, it is believed that CBMs increase the catalytic activity against insoluble substrates, but family 2b, 6, 13, and 22 of CBMs have affinity against soluble substrates. Recently, a family 2b CBM from *Streptomyces thermoviolaceus* STX-II was fused to the carboxy-terminus of the single-domain GH 10 xylanase XynB from *T. maritima*, yielding a chimeric xylanase with increased activity against soluble xylan (Kittur et al. 2003).

In the case of the XynA deletion enzymes studied here, deletion of the CBMs, including the xylan-binding CBMs at the amino-terminus, was not detrimental for the specific activity on the commercial oat spelt xylan used in our standard assay. On the contrary, the fewer CBMs were left attached to the catalytic domain, the higher was the maximum initial cleavage velocity. This seems to suggest that at least in the context of the in vitro assay system used here, the non-catalytic CBMs may represent sterical hindrances for efficient hydrolysis by the central catalytic domain of XynA. To this end, the determination of the three-dimensional structure of XynA could provide additional information, but crystallization attempts were not successful so far. However, since it would not make much sense that CBMs hinder an extracellular enzyme's activity on its natural substrate, we prefer to assume that the CBMs of XynA play an overall supportive role for plant cell wall breakdown by *Thermotoga* cells. At this point, it can only be speculated that the N-terminal xylan-binding CBMs A1 and A2 and the combination of xylan- and cellulose-binding modules in one large enzyme such as XynA may be important for the hydrolysis of complex mixed-polymer substrates as found in natural habitats with decaying plant or algae cell material.

Binding studies with soluble substrates by native affinity polyacrylamide gel electrophoresis and determination of the binding substrate specifity

XynA, the deletion derivatives, and the isolated CBMs A1 and A2 were investigated by NAPAGE. The NAPAGE is based on the retarded mobility of a protein with polysaccharide-binding properties during native polyacrylamide gel electrophoresis if a soluble binding substrate is included in the separation gel (Meissner et al. 2000). The purified deletion derivatives of XynA and the isolated family 22 CBMs (xylan-binding domains, XBDs) A1 and A2 were separated on 15% non-SDS polyacrylamide gels containing soluble polysaccharides as described in 'Materials and methods'. When compared with a reference gel without polysaccharide, the mobility of the XynAΔNC (except with DMSO-xylan) was not affected by the various polysaccharides tested (Table 2). A significant retardation of the other deletion derivatives and A2 was found to occur in the presence of oat spelt xylan, DMSO-xylan, β-glucan, carboxymethyl cellulose, hydroxyethyl cellulose, and lichenan. Various other polysaccharides had no effect on the mobility of the deletion derivatives, with the exception of the isolated A2 domain (Table 2). It is interesting to note that not all of the xylans tested were suitable

Table 1 Kinetic parameters of hydrolysis of soluble oat spelt xylan by XynA and XynA deletion derivatives

All determinations were done at least in duplicate, using the standard assay with Bis-Tris buffer pH 6.2 in the presence of 0.25 M sodium chloride

Enzyme	65°C		75°C	
	V_{max} (U/mg)	K_M (%)	V_{max} (U/mg)	K_M (%)
XynA	162 ± 11	0.18 ± 0.02	284 ± 17	0.13 ± 0.02
XynAΔC	303 ± 16	0.33 ± 0.06	588 ± 35	0.18 ± 0.03
XynAΔA1C	496 ± 16	0.28 ± 0.01	1,746 ± 11	0.20 ± 0.01
XynAΔNC	1,051 ± 6	0.28 ± 0.02	2,720 ± 74	0.36 ± 0.01

Table 2 Interaction of XynA deletion derivatives and the isolated A1 and A2 domains with soluble polysaccharides, as determined by NAPAGE

Polysaccharide	(c)	XynA	XynA⊿C	XynA⊿A1C	XynA⊿CN	A2	A1
Oat spelt xylan	0.450%	+	+	+	–	+	–
Beechwood xylan	0.375%	–	–	–	–	+	–
DMSO-Xylan beech	0.375%	+	+	+	(–)	+	–
Birchwood xylan	0.750%	–	–	–	–	+	–
Wheat straw xylan	0.250%	–	–	–	–	+	–
Algal xylan	0.375%	+	+	+	–	+	–
β-Glucan	0.188%	+	+	+	–	+	–
Carboxymethyl cellulose	0.750%	+	+	+	–	+	–
Hyrdoxyethyl cellulose	0.750%	+	+	+	–	+	–
Lichenan	0.375%	+	+	+	–	+	–
Apple pectin	0.75%	–	–	–	–	–	–
Amylose	0.375%	–	–	–	–	+	–

binding substrates in the NAPAGE test. The reason for this phenomenon is not clear, but may be related to structural differences within the polysaccharide, i.e. differences in xylan backbone substitution or to differences due to different methods of xylan preparation. The latter possibility is strengthened by the fact that beechwood xylan and DMSO-xylan from beechwood gave contrary results.

The NAPAGE analysis of the purified isolated A1 domain revealed that this CBM had no affinity to soluble substrates (Table 2), which is in accordance with an earlier analysis of a GST-A1 fusion protein (Meissner et al. 2000). The reason for the negative results in the NAPAGE analysis with isolated A1 could lie in its amino acid sequence. The comparison of 55 known family 22 CBMs showed that only 22 of them contain five critical residues, Arg 25, Trp 53, Tyr 103, Tyr 136, and Glu 138, which appear to be important for the capacity to bind to xylan (Xie et al. 2001). While A2 posses these five residues, the A1 domain has only two of them.

It must be stressed that the binding effects shown in Table 2 are merely qualitative results and that (1) very weak interactions between protein and polysaccharide may remain unnoticed, and (2) relatively non-specific interactions between an enzyme and the polysaccharide polymerized into the gel could occur. This seems to be the case in some instances for the isolated module A2, where apparent interaction with all polysaccharides except pectin was found in the qualitative NAPAGE experiment, even with amylose, a starch-derived linear α-glucan with no similarity to xylan. However, subsequent determination of dissociation constants showed that the binding of A2 to oat spelt xylan was very specific with an affinity about tenfold higher than to carboxymethyl- or hydroxyethyl-cellulose (see below, Table 3) while the qualitative NAPAGE result was '+' for all of these polysaccharides.

In order to obtain a more quantitative measure for the binding effects observed in qualitative NAPAGE, we determined apparent dissociation constants for the binding of XynA, the four truncated XynA derivatives and A2 to oat spelt xylan, birchwood xylan, *β*-glucan, carboxymethyl cellulose, hydroxyethyl cellulose, and lichenan. To this end, NAPAGE of the purified proteins was carried out in the presence of increasing concentrations of polysaccharides in the separating gels. Among the chosen soluble binding substrates, the A2 domain was found to have the highest affinity for oat spelt xylan with an apparent dissociation constant of 31 μg/ml (Table 3). The K_d of A2 for *β*-glucan was determined to be about 54 μg/ml, which confirms the K_d value of about 55 μg/ml from previous work using a chimeric GST-A2 fusion protein (Meissner et al. 2000). It can be concluded that in this former study with GST-A2, the GST part had had no influence on the affinity of A2 to the substrates tested. The apparent dissociation constant measured with the A2 domain for lichenan (108 μg/ml) was in the same range as the value for *β*-glucan (54 μg/ml), while much larger K_d values were found for carboxymethyl cellulose (551 μg/ml) and hydroxyethyl cellulose (693 μg/ml) (Table 3). Thus, the binding affinity of the isolated binding module A2 was dramatically higher towards natural substrates of XynA than towards the substituted cellulose derivatives [XynA hydrolyses oat spelt xylan (100% relative activity) as well as the *β*-1,3/*β*-1,4-mixed-linkage polysaccharides *β*-glucan (53%) and lichenan (15%), while XynA does not cleave carboxymethyl cellulose (0%) (Winterhalter and Liebl 1995)].

Although A2 seems to have a high affinity to *β*-glucan and lichenan (Table 3), the precise contribution of the A domains to the binding of XynA to *β*-1,3/*β*-1,4-mixed-linkage polysaccharides cannot be resolved with the experiments shown here because the C2 domain of XynA also binds to *β*-glucan, whereas no affinity of C2

Table 3 Apparent dissociation constants K_d (μg/ml) of binding of XynA deletion derivatives and A2 to soluble polysaccharides, as determined by NAPAGE in the presence of increasing polysaccharide concentrations

Polysaccharide	Dissociation constant (μg/ml)			
	XynA	XynA⊿C	XynA⊿A1C	A2
Oat spelt xylan	1,142	1,205	1,321	31
β-Glucan	84	83	85	54
Carboxymethyl cellulose	514	892	778	551
Hyrdoxyethyl cellulose	542	1,177	629	693
Lichenan	64	120	137	108

for oat spelt and birchwood xylan could be demonstrated (K. Meissner and Liebl, unpublished data). With lichenan, carboxymethyl, and hydroxyethyl cellulose as the binding substrates, XynA displayed the highest affinity whereas the isolated A2 module showed lower affinity to these substrates. The higher affinity of XynA is caused by the C domains, while deletion enzymes without the C domains had a lower affinity (Table 3). The dissociation constants for binding to soluble oat spelt xylan or β-glucan did not change significantly after deletion of the C domains, neither in the presence nor absence of A1. This indicates that the A2 domain alone but neither the C domains nor A1 mediated binding to these substrates.

The question arises as to why the single A2 domain was found to have a higher affinity to oat spelt xylan than XynA or the deletion derivatives XynAΔC and XynAΔA1C, all of which also contain the A2 binding module. A possible, albeit speculative, explanation may be steric hindrance by the catalytic domain. In this context, it is noteworthy that no linker sequence was detected between domain A2 and the catalytic domain of XynA, while there is a short proline-rich linker between the catalytic domain and the C domains (Winterhalter et al. 1995).

Binding of the XynA deletion derivatives to insoluble polysaccharides

Previous studies have shown that the N-terminal CBMs had no affinity for insoluble microcristalline cellulose, but various xylan preparations were identified as binding substrates for GST-A2 and GST-A1A2 fusion proteins, which led to the conclusion that at least A2 was a xylan-binding domain (Meissner et al. 2000). A function for A1 could not be defined. Therefore, we decided to test the possible function of the A domains in binding to insoluble xylan components.

For this purpose, the ability of the XynA derivatives to bind insoluble Xylan was studied by incubating 0.1 nmol of the different deletion enzymes with 0.5 ml 1% insoluble oat spelt or birchwood xylan in binding buffer and determining the residual xylanase activity in the supernantant. After incubation for 3 h with insoluble oat spelt xylan, 12.4% (XynAΔC), 27% (XynAΔA1C), 46% (XynA), and 54.5% (XynAΔNC) of the initial activity was in the supernantant (Fig. 3). In an analogous experiment with insoluble birchwood xylan, the relative activity in the supernantant showed a similar pattern. The lowest activity was measured for XynAΔC with 33%, followed by XynAΔA1C with 65.1%, XynA with 69.4%, and XynAΔNC with 86.1% (Fig. 3). In general, binding to insoluble oat spelt xylan was more complete than to birchwood xylan, and the catalytic domain alone showed the weakest binding, which was expected because no CBMs are present. A possible explanation for the weaker apparent binding of full-length XynA than the C-terminal deletion XynAΔC to insoluble xylan may be steric hindrance by the C-terminal CBMs.

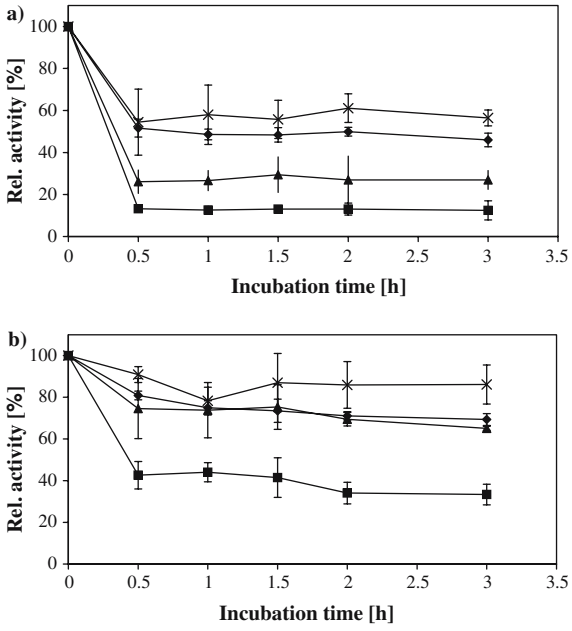

Fig. 3 Binding of truncated XynA derivatives to insoluble, washed oat spelt xylan (a) and birchwood xylan (b). Qualitative binding assays for insoluble substrates were done with 0.5 ml 1% (w/v) insoluble substrate in binding buffer A (10 mM BisTris pH 6.2, 0.5 M NaCl) incubated with 0.1 nmol enzyme. Symbols used: XynA (*rhomboids*), XynAΔC (*squares*), XynAΔA1C (*triangles*), and XynAΔNC (*crosses*)

Highly interestingly, the binding to insoluble xylans was more efficient if both A1 and A2 were present (XynAΔC) than only with the A2 domain (XynAΔA1C), which in particular was evident with the substrate insoluble oat spelt xylan (Fig. 3a). This result represents the first indication for a function of the CBM A1, i.e. in cooperation with the xylan-binding domain A2 this domain may assist the binding of XynA to insoluble xylan. In this respect, *T. maritima* XynA seems to differ from Xyn10B from *Clostridium thermocellum*, where the N-terminal CBM22-1 did not increase the affinity but rather negatively influenced the binding of Xyn10B derivatives to insoluble xylan (Dias et al. 2004). Noteworthy, however, CBM22-1 of Xyn10B contains none of the five residues (see Xie et al. 2001) mentioned above which are thought to be important for xylan binding.

In conclusion, considering the domain structure of XynA containing duplicated cellulose- and xylan-binding domains and the results of binding experiments with soluble and insoluble plant cell wall polysaccharides reported here, it can be speculated that enzymes like XynA are highly specialized for complex mixed-polysaccharide substrates and may actually be particularly useful at certain xylan/cellulose-substructures of such substrates. Clearly, more work is needed to come to a better understanding of the enzymatic breakdown of such complex mixed-polysaccharide substrates like lignocellulosic material.

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