RESEARCH COMMUNICATION

The thermostabilizing domain, XynA, of *Caldibacillus cellulovorans* xylanase is a xylan binding domain

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We show that the N-terminal 'thermostabilizing domain' (TSD) of the xylanase, XynA, from the thermophilic bacterium *Caldibacillus cellulovorans* also acts as a xylan binding domain. Affinity electrophoresis experiments show that this TSD selectively binds soluble xylan and binds weakly to hydroxyethylcellulose. Based on this, and previously reported evidence, we propose that xylanase-associated TSDs are xylan binding domains.

Key words: cellulose binding domains, glycosyl hydrolases, multidomains, TSD.

INTRODUCTION

Over the last decade there has been considerable interest in the biotechnological applications of endoxylanases, particularly for use as pulp bleaching aids [1,2]. Consequently, a large number of bacterial and fungal recombinant xylanase genes have been isolated, sequenced and expressed. There have been at least 270 endoxylanase gene sequences reported, and all can be classified into either of two families, glycosyl hydrolase families 10 and 11 [3]. As with many polysaccharide hydrolysing enzymes, endoxylanases are observed frequently to be composed of distinct catalytic and non-catalytic domains that are often linked via flexible linker sequences. Separation of the individual domains has generally demonstrated that they can function independently of each other [4].

Xylanase-associated thermostabilizing domains (TSDs) were first defined by Winterhalter et al. [5], who identified unique N-terminal repeated domains (A1-A2) directly adjacent to the family 10 xylanase catalytic domain (B) of *Thermotoga maritima* XynA. Removal of the A1-A2 domains was found to substantially reduce the thermostability of the xylanase domain (50 % decrease at 75 °C when compared with the full-length enzyme). It was argued that the multidomain organization of XynA may be a strategy adopted to protect the protein against thermal denaturation [5]. Numerous homologues of TSDs have since been identified, mainly in association with family 10 xylanases [6]. The effect of their removal in enzyme thermostability has supported the assumption of their function as TSDs [5,7–9]. However, four observations suggest a function for TSDs other than thermostabilization. First, TSDs are not essential for thermal stability, since hyperthermophilic bacteria produce single domain xylanases that are more thermostable than TSD-associated xylanases. For example, *T. maritima* produces two family 10 xylanases [5,10]. XynB, a single domain xylanase, has been shown to display higher thermostability than its respective multidomain counterpart XynA, which has two N-terminal TSDs. Furthermore, XynB from *T. maritima* has an optimum temperature of activity more than 10 °C above that of XynA (92 °C).

Secondly, the presence of TSD has been reported in xylanases from mesophilic bacteria. In the family 10 xylanase XylC, from the mesophilic soil bacterium *Cellulomonas fimi* [11], and XynC, from the mesophilic *Bacillus* sp. BP-23 [7], removal of TSDs was associated with decrease in optimal temperature for activity and thermostability. TSDs have also been described in the family 11 xylanases, XynB and XynD, from the mesophilic bacterium *Ruminococcus flavefaciens* [12,13]. However, no studies on the effect of these domains in enzyme thermostability have been reported. There seems to be no advantage for increased thermostability in xylanases of mesophilic origin and, thus, a more general role for TSDs in conferring protection against proteolytic attack and extreme pH conditions has been postulated [11].

Thirdly, if this class of TSDs had a general role in thermostabilization, one might expect to find them associated with other proteins besides xylanases. However, so far these TSDs are restricted entirely to association with family 10 and 11 xylanases, with two exceptions. A small open-reading frame (TM0062) is found on the *T. maritima* genome directly downstream of the *xynA* gene [14]. TM0062 encodes a putative protein, consisting of two partial TSD domains with no associated catalytic domain. Also, in a single case, a TSD is found in association with a family 43 arabinofuranosidase, an enzyme also involved in xylan degradation [6].

Fourthly, we have recently characterized a novel thermostable multidomain family 10 xylanase from *Caldibacillus cellulovorans*, XynA, with the modular 'TSD-catalytic domain–PT-linker–cellulose binding domain (CBD)IIIb–PT-linker–CBDIIIb' structure (A. Sunna, M. D. Gibbs and P. L. Bergquist, unpublished work). Experiments with two expression plasmids coding for the TSD plus the catalytic domain (XynAd1/2) and the xylanase catalytic domain alone (XynAd2) indicated that, in addition to reducing the thermostability of the enzyme, removal of the TSD also affects the specific activity and processivity of the xylanase. Removal of the TSD changed the hydrolysis specificity of the enzyme against xylo-oligosaccharides, and also the pattern of the major products released from xylan hydrolysis.

In the present work, we report on the ability of the TSD of *Ca. cellulovorans* XynA to selectively bind to soluble xylan, and propose that substrate binding is the primary function of this class of domain.

Abbreviations used: CBD, cellulose binding domain; TSD, thermostabilizing domain; XBD, xylan binding domain.

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EXPERIMENTAL

Construction of a *xynAd1*:pPROEX HTc recombinant plasmid

XynAd1, encoding the TSD of *Ca. cellulovorans* XynA, was amplified from the recombinant plasmid pSUN18 (A. Sunna, M. D. Gibbs and P. L. Bergquist, unpublished work), using the specific primers XYNAD1F (5′-CCGCATGTCGCTTC-CATGGAAAACGTCCTG-3′) and XYNAD1R (5′-TTCAG-GGAATTCTAATTCTCTTCTACCGTC-3′). XYNAD1F and XYNAD1R incorporate, respectively, *Nco*I and *Bam*HI restriction sites to allow directional in-frame ligation of the xynAd1 PCR fragment into complementary sites of the expression plasmid pPROEX HTc (Life Technologies, Melbourne, Australia), to give the recombinant plasmid pSUN31. The expression vector pPROEX HTc encodes an N-terminal His_6-tag followed by a protease cleavage site.

Production and purification of XynAd1 fusion protein

Luria-Bertani medium (600 ml), supplemented with ampicillin (100 μg/ml), was inoculated with 6 ml of an overnight culture of *Escherichia coli* strain DH5α [15] harbouring the recombinant pSUN31 plasmid. The culture was incubated at 37 °C, with shaking, until the D_{600} was 0.7. To induce the expression of the recombinant XynAd1 fusion protein, 0.6 mM isopropyl β-D-thiogalactoside was added and the growing culture was incubated for a further 4 h. After induction, the cells were collected by centrifugation (5000 ***g*** for 15 min at 4 °C), resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by passage through a French pressure cell. The resulting crude extract was centrifuged at 20000 ***g*** for 30 min at 4 °C. The final extract was used for XynAd1 purification. Purification was performed with Ni^{2+}-nitrilotriacetate ('Ni-NTA') magnetic agarose beads (Qiagen) as described by the manufacturer. Purity of the final sample was determined by SDS/PAGE in 12% acrylamide precast gels (Gradipore, Sydney, Australia) by the method of Laemmli [16]. Proteins were stained with Coomassie Brilliant Blue R-250 (Sigma). Pure samples were finally desalted using Microcon ultrafitration spin columns (cut-off 3 kDa; Millipore) and stored in 50 mM sodium phosphate buffer (pH 6.0) at 4 °C. Protein concentrations were determined using the Micro-BCA protein quantification kit (Pierce).

Affinity electrophoresis with soluble polysaccharides

Non-denaturing continuous PAGE (12.5% acrylamide gels) was performed in 1.5 M Tris/HCl buffer, pH 8.2. For ligand-containing gels, birchwood xylan (Sigma), hydroxyethylcellulose (Merck), locust bean gum galactomannan (Sigma) or laminarin (Sigma) were added to the gel mixtures at a final concentration of 0.1% (w/v) prior to polymerization. Native gels without ligand were run simultaneously under the same conditions. Electrophoresis was carried out at room temperature and 100 V. Chicken egg albumin and BSA (Sigma) were used as negative non-binding controls. Separated proteins were revealed by staining with Coomassie Blue.

RESULTS AND DISCUSSION

Affinity electrophoresis was used to determine binding specificity of the TSD of *Ca. cellulovorans* XynA against different soluble glycans. The results of the affinity electrophoresis experiments are presented in Figure 1. The TSD of XynA interacted strongly with soluble xylan (β-1,4-linkages), as shown by the severely retarded migration of TSD on the xylan gel. The TSD did not interact with the soluble glycans, laminarin (β-1,3-linkages) and

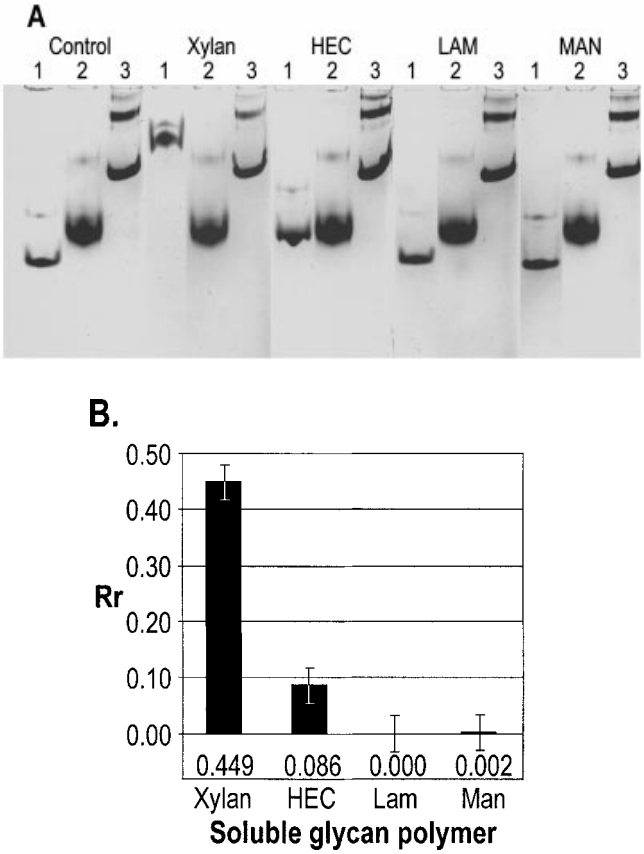

Figure 1 Affinity electrophoresis of *Ca. cellulovorans* XynAd1

(**A**) Affinity electrophoresis of XynAd1 in the absence or presence of soluble glycans (0.1%, w/v). Lanes 1, XynAd1 (10 μg); lanes 2, chicken egg albumin; lanes 3, BSA. Control, no soluble glycan; Xylan, birchwood xylan; HEC, hydroxyethylcellulose; LAM, laminarin; MAN, locust bean gum galactomannan. (**B**) Relative retardation of XynAd1. Rf (distance migrated/length of gel) values for XynAd1 were corrected, based on the Rf of chicken egg albumin standard in each gel. Rr was calculated as: (corrected Rf of XynAd1 in a native acrylamide gel) — (Rf of XynAd1 in native gels containing 0.1% soluble glycan). Error bars represent mean ($\pm$2 S.D.s) Rf of BSA in each gel. Gels were scanned and images were processed using Adobe Photoshop 3.0.

locust bean gum galactomannan (β-1,4-linkages), but weak interaction with hydroxyethylcellulose (β-1,4-linkages) was observed. Since the migration of both control proteins (chicken egg albumin and BSA) was not retarded in the presence of these substrates, we conclude that the interaction of the TSD with xylan is specific and not caused by viscosity. Similarly, affinity electrophoresis has successfully been used to determine the binding specificity of the CBDs of *Ce. fimi* CenC [17] and the xylan binding domain (XBD) of *Ce. fimi* XylD [18] against soluble polysaccharides.

A number of xylanases have TSD-associated domains. The latest update of the ProDom domain PD002546 (www.toulouse.inra.fr) recognizes 22 xylanases containing at least one TSD homologous domain; of these, 16 were found to be N-terminal to the catalytic xylanase domain (three single, 12 in tandem and one in triplicate), five were single domains C-terminal to the catalytic domain, and one had both an N-terminal and internal TSD (C-terminal to the catalytic domain). It is noteworthy that all 16 N-terminal TSD homologues were associated only with family 10 xylanases (mainly thermophilic xylanases), whereas four of the C-terminal TSD homologues

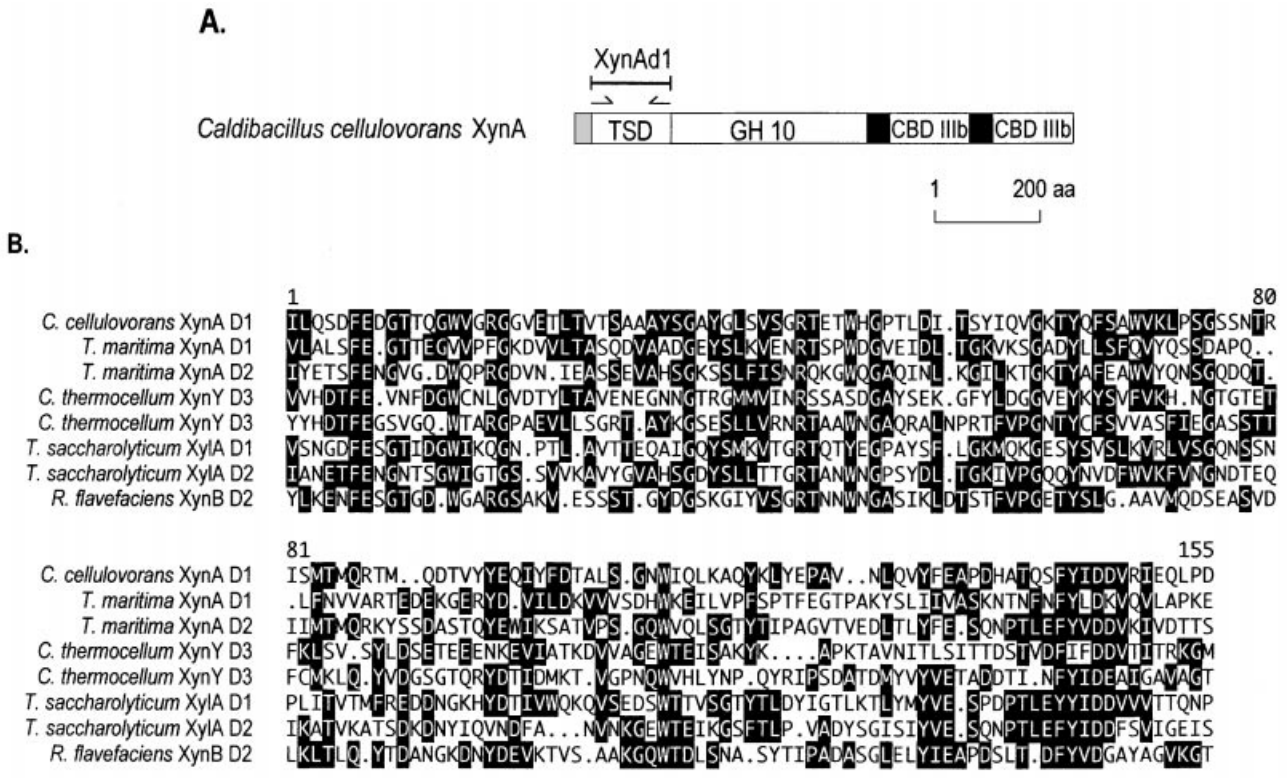

Figure 2 Domain structure of *Ca. cellulovorans* XynA and amino acid sequence alignment of XynAd1

(**A**) Domain structure of multidomain XynA from *Ca. cellulovorans*. GH10, glycosyl hydrolase family 10 xylanase domain; CBD IIIb, family IIIb CBD. The signal peptide is indicated with a grey box, and the interdomain linker peptides are indicated with black boxes. Arrows indicates the position of primers used to amplify the TSD domain. (**B**) Amino acid alignment of a selection of TSD homologous domain sequences of several xylanases. Relative position of domains (D) with respect to the N-terminus of the mature protein are indicated on the left. *Ca. cellulovorans* XynA (A. Sunna, M. D. Gibbs and P. L. Bergquist, unpublished work); *T. maritima* XynA [5]; *Clostridium thermocellum* XynY [8]; *Thermoanaerobacterium saccharolyticum* XylA [9]; *Ruminococcus flavefaciens* XynB [12].

were associated with family 11 mesophilic xylanases. An amino acid alignment of a selection of TSD homologous domain sequences of several xylanases is presented in Figure 2.

The classification of these domains as TSDs is based on the observation that removal of the domain substantially reduced the thermostability of its associated xylanase domain [5,7–9]. A similar decrease in thermostability of a catalytic domain upon removal of an associated glycan-binding domain has been observed for the family 9 endoglucanases, CelZ, from *Clostridium stercorarium* [19] and E4 from *Thermomonospora fusca* [20]. Both of these enzymes comprise a family-9-endoglucanase domain joined with limited flexibility to a family IIIc CBD. The crystal structure of the *Thermomonospora fusca* E4 catalytic domain and IIIc CBD has been solved [21]. The catalytic domain can be seen to interact with the CBD in two loop regions, so that the catalytic cleft is aligned with the substrate binding face of the CBD in such a way that a cellulose strand could bind along both domains [20,21]. It has been proposed that the interaction of the loop regions contributes to the thermostability of the enzyme, resulting in a loss of functional independence of the individual domains [20]. Furthermore, this close association between the catalytic domain and CBD has been shown to alter the processivity of the catalytic domain by greatly enhancing its ability to degrade crystalline cellulose substrates and by conferring an exo-acting processivity to the enzyme [19,20].

Recently, we have shown that the TSD of *Ca. cellulovorans* XynA also changes the catalytic specificity of its associated xylanase domain (A. Sunna, M. D. Gibbs and P. L. Bergquist, unpublished work). In the present report we provide further evidence that this TSD has a more complex function than previously assumed. Accordingly, we propose that xylan binding is the primary role for this class of xylanase-associated domain, and that TSDs act in a way analogous to CBDs. Furthermore, we suggest that the thermostabilizing function of this class of domains is a result of a lack of discrete linker peptides separating TSDs from the adjacent catalytic domain.

It appears that this new class of XBD, which is unrelated to the three classes of XBDs described so far [18,22–24], may have evolved in close association with family 10 xylanase domains in a manner similar to that observed for *Clostridium stercorarium* CelZ [19] and *Thermomonospora fusca* E4 [20], and, as a consequence, the combination of a XBD directly proceeding a family 10 xylanase domain should be considered, in terms of optimal function, as a single inseparable unit.

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