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ORIGINAL PAPER

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Fusion of carbohydrate binding modules from *Thermotoga neapolitana* with a family 10 xylanase from *Bacillus halodurans* S7

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Abstract Xylanase A of *Thermotoga neapolitana* contains binding domains both at the N- and C-terminal ends of the catalytic domain. In the N-terminal position it contains two carbohydrate-binding modules (CBM) which belong to family 22. These CBMs bind xylan but not to cellulose. The gene encoding the mature peptide of these CBMs was fused with an alkaline active GH10 xylanase from *Bacillus halodurans* S7 and expressed in *Escherichia coli*. The $(His)_6$ tagged hybrid protein was purified by immobilized metal affinity chromatography and characterized. Xylan binding by the chimeric protein was influenced by NaCl concentration and pH of the binding medium. Binding increased with increasing salt concentration up to 200 mM. Higher extent of binding was observed under acidic conditions. The fusion of the CBM structures enhanced the hydrolytic efficiency of the xylanase against insoluble xylan, but decreased the stability of the enzyme. The optimum temperature and pH for the activity of the xylanase did not change.

Keywords Xylanase · Xylan binding domain · CBM · Chimeric gene · *Thermotoga neapolitana* · *Bacillus halodurans*

Introduction

Cellulose and xylan are the two most abundant polysaccharides in the biosphere. Degradation of these residues is largely done by microorganisms and is an important process in the global carbon cycle. Microbial degradation of these polysaccharides is achieved by a concerted action of diverse enzyme systems. These enzymes have been the focus of intense research and a vast array of polysaccharide degrading enzymes have been purified and characterized, the genes encoding the proteins are sequenced, and expressed in heterologous systems, and structures for a number of enzymes are determined (Sunna and Antranikian 1997; Kulkarni et al. 1999; Collins et al. 2005).

Most hemicellulose and cellulose degrading enzymes have modular structures comprising usually a catalytic module and one or more noncatalytic carbohydrate-binding modules (CBM) which are connected via a linker (van Solingen et al. 2001; Harhangi et al. 2003). In modular enzymes, CBMs are located either at the N- or C-terminal or at both positions, and are varying in size. CBMs are classified into families based on their amino acid sequence similarities. At the time of writing, there were 45 CBM families in the latest update of the CAZy database (http://www.afmb.cnrs-mrs.fr/CAZY/) (Coutinho and Henrissat 1999).

The biological role of CBMs appears to be to mediate a tight association between the enzyme and the substrate. This can increase the effective enzyme concentration on the substrate, thereby enhancing the hydrolytic action (Bolam et al. 1998). Thus, the fusion of CBM structures to nonmodular hydrolytic enzymes would be assumed to improve the hydrolytic efficiency of the enzyme. Previously, fusion of a family 6 cellulose binding CBM from *Clostridium stercorarium* xylA at the C-terminal of a *Bacillus halodurans* C-125 family 10 xylanase resulted in a better activity toward insoluble xylan (Mangala et al. 2003).

In case of xylanases, fusion with xylan specific CBMs has some advantages over fusion with cellulose binding CBMs, since the binding of the enzyme specifically to xylan would be expected to increase, hence resulting in a better xylan hydrolysis. On the other hand, when cellulose binding CBMs are fused with xylanases, it is possible that some of the hybrid enzyme can bind to cellulose surface even if there is no xylan within catalytic

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proximity. This can keep the enzyme away from xylan and lead to a drop in the total hydrolytic efficiency. Moreover, for the production of high quality cellulose fibers, the use of xylanases linked to cellulose binding CBMs may lead to disruption of cellulose fibers. This has been demonstrated for family 2 CBM from *Cellulomonas fimi* Cel6A (Din et al. 1991), family 2 CBM from *Cellulomonas fimi* Xyn10A (Creagh et al. 1996), and family 1 CBM from *Penicillium janthinellum* Cel7 (Pei-Ji et al. 2001). Moreover, some CBMs bind soluble xylan and enhance the enzyme hydrolytic efficiency on soluble xylan (Kittur et al. 2003). Therefore, fusion of xylan specific CBM structures to xylanases is interesting.

Xylanases produced by the hyperthermophilic bacterium, *Thermotoga neapolitana* and the closely related species, *Thermotoga maritima* are known to be the most stable xylanases ever reported (Zverlov et al. 1996; Wassenberg et al. 1997). Some of these xylanases are comprised of CBM structures located both at the N- and C-terminal positions of the catalytic domain. At the C-terminus two family 9 CBM modules exist in tandem and are known to have a high affinity to crystalline cellulose (Boraston et al. 2001). The N-terminus structure contains in tandem two CBM structures that belong to CBM family 22 in CAZy database (http://www.afmb.cnrs-mrs.fr/CAZY/). These CBM motifs in *T. maritima* XynA have been shown to bind xylan and not to insoluble microcrystalline cellulose, and are proposed to be a novel class of CBMs (Meissner et al. 2000). The primary sequence similarity between the respective domains (A1 and A2) of *T. maritima* and *T. neapolitana* CBM structures is about 80 and 91%. Due to the relatively high sequence similarity, it is expected that N-terminal CBM modules of *T. neapolitana* would have a specific affinity to xylan. Moreover, these CBMs are not studied in detail.

In this study, fusion of the N-terminal CBMs from *T. neapolitana* xylanase A with the catalytic domain of a nonmodular family 10 alkaline active xylanase from *B. halodurans* S7, and the characterization of the chimeric xylanase is reported. The xylanase has optimal activity and reasonable stability under alkaline conditions and elevated temperature; however, it degrades insoluble xylan at a relatively low efficiency. For example, compared to the respective soluble xylans, the relative activity of the enzyme on insoluble oat spelts and beechwood xylan was only 55 and 54%, respectively (Mamo et al. 2006a). Thus, enhancing the hydrolytic efficiency of this enzyme is an advantage considering its application potential.

Materials and methods

Bacterial strains and plasmids

T. neapolitana (DSM 4359), the source for CBM encoding gene, was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). The gene encoding the glycoside hydrolase family 10 (GH10) xylanase catalytic domain was from *B. halodurans* S7, which was isolated in our laboratory (Mamo et al. 2006a). *Escherichia coli* strains NovaBlue and BL21(DE3), and the vector pET-22b(+) were purchased from Novagen (Madison, USA).

Media and growth conditions

T. neapolitana cells were grown anaerobically in *Thermotoga* medium (Medium 343) of DSMZ at 80°C. Cells of *B. halodurans* were cultivated as described previously (Mamo et al 2006a). *E. coli* cultures were grown in liquid or on solid LB media at 37 or at 30°C.

Chimera gene construction

Genomic DNA extraction was performed following the method of Sambrook et al. (1989). Plasmid DNA was extracted from cells by the standard alkaline lysis method. Plasmids and PCR products were recovered from agarose gel using Qiagen purification kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's instructions.

A three-step polymerase chain reaction (PCR) was used to construct the chimeric gene (Fig. 1). In the first step, the family 10 catalytic domain (mature peptide) encoding gene segment from *B. halodurans* S7 was amplified using primers P3 and P4 as described previously (Mamo et al. 2006b). The CBMs together with the linker regions were amplified using primers P1 and P2. Primers P1 and P4 contain, respectively, *Nco*I and *Xho*I restriction sites for cloning into the vector pET-22b(+). The two primers, P2 and P3, have an overlapping region that was used for self-priming in the second step of the overlapping PCR. Sequences of the primers are given in Table 1.

After purification of the PCR products, equimolar concentrations of the catalytic domain and CBM encoding gene fragments were used in the second step overlapping PCR. In the final PCR step, primers P1 and P4 were used to amplify the full-length chimeric gene (CBM–xylS7). The first PCR step amplification of CBM was done with 20 cycles denaturation at 95°C for 30 s, annealing for 30 s at 55°C, and 2 min extension at 72°C. The overlapping PCR step was done for ten cycles of 1 min at 95°C, 1.5 min at 50°C, and 2 min at 72°C. The third PCR step condition was similar to the first step of CBM amplification except that the extension time was 4 min. In all the PCR steps a final extension was carried out for 7 min at 72°C. All PCRs were carried out with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using a high fidelity PCR enzyme mix (MBI Fermentas INC, Opelstrasse 9, Germany).

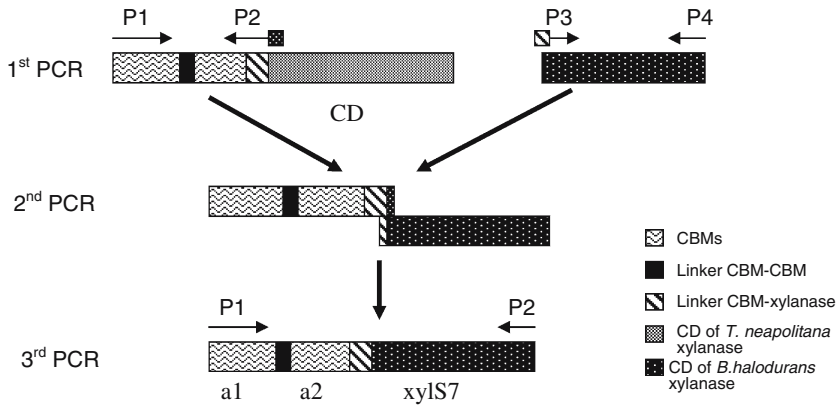


Fig. 1 Steps followed for chimeric gene construction that encodes for CBM–xylS7. *P1–P4* symbolizes the primers. *a1 and a2* stands for CBM22-1 and CBM22-2 of *T. neapolitana* whereas *xylS7* is for the *B. halodurans* S7 family 10 xylanase. *CD* stands for catalytic domain. Details are given in the text. Symbols and their representations are indicated in the figure

Table 1 Primers used in the chimera gene construction

Primer	Sequence
P1	5-CATG***CCATGG***CCACAGGAGCTCTGGGATTC-3
P2	5-TCCTTGGGGTATCTCTTCTTC CGGATTCATTCGAG-3
P3	5-GAAGAGATACCCCAAGGAGGACCACCA-3
P4	5-AGCTAC***CTCGAG***ATCAATAATTCT CCAGTAAGCAG-3

Restriction sites are in bold and italicized. The overlapping regions are underlined. In primer P2, the T in bold type is G in the *T. neapolitana* gene sequence (EMBL accession number Z46945) but mutated to avoid digestion by *Xho*I

Recombinant vector construction and sequencing

The amplified chimeric gene was purified and digested by the restriction enzymes (*Nco*I and *Xho*I) and cloned at *Nco*I–*Xho*I restriction sites in the multiple cloning site of pET-22b(+). The constructed vector contains *pel*B leader peptide for possible export of the enzyme to the periplasm. At the C-terminus, Leu-Glu-$(His)_6$ sequence was added before the stop codon for easy metal ion affinity purification of the expressed protein. The vector was transformed into *E. coli* NovaBlue cells by electroporation with a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, USA). PCR screening was done to identify clones harboring the insert containing plasmids. Insert containing plasmids were purified and sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) following the manufacturer's protocol. Sequencing reactions were electrophoresed using ABI 3100 DNA sequencer.

Protein expression and purification

The plasmids containing the chimeric gene in correct reading frame were transformed into *E. coli* BL21 (DE3). A single colony of *E. coli* BL21 (DE3) cells harboring the recombinant vector was cultured overnight, which was then used to inoculate 100 ml of LB broth containing ampicillin (100 μg/ml) and incubated at 30°C with a shaking rate of 200 rpm in an orbital shaker incubator. When the optical density (OD) of the culture at 600 nm was about 0.7, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM.

Cells were harvested after 12 h of induction and resuspended in 20 mM Tris–HCl buffer, pH 7.4. Lysozyme (0.1 mg/ml) was added and the suspension was kept at room temperature for 30 min before being subjected to freezing and thawing for two cycles and sonicating using UP 400S Sonicator (GmbH, Stahnsdorf, Germany). The cell homogenate was heated at 60°C for 30 min, cooled on ice for 1 h, and the insoluble fraction was removed by centrifugation. The enzyme was purified from the clarified supernatant using immobilized metal ion affinity chromatography (IMAC) as described previously (Mamo et al. 2006b). The native recombinant xylanase (xylS7), which was purified in the same way, was used for comparison.

Protein analysis

Protein concentration was determined following bicinchoninic acid method using bovine serum albumin as standard (Smith et al. 1985).

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels and the proteins were visualized by staining with Coomassie brilliant blue R-250.

Enzyme assay

Xylanase activity was measured based on the release of reducing sugar from xylan using the dinitrosalicylic acid (DNS) method (Miller 1959). An appropriately diluted enzyme solution was added to 1% (w/v) birchwood xylan dissolved in glycine–NaOH buffer (pH 10) and incubated at 70°C for 10 min and then the reaction was stopped by adding DNS reagent. One unit of the

xylanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent to xylose per minute under the assay conditions.

Effect of temperature and pH on the enzyme activity and stability

The effect of temperature on the enzyme activity was determined at pH 10 in glycine–NaOH buffer. A mixture of the enzyme and xylan solution was incubated for 10 min at different temperatures ranging between 40 and 75°C and the amount of reducing sugar released was measured. Thermal stability was determined by incubating the enzyme at 65°C in 50 mM glycine–NaOH buffer, pH 9. Samples were taken at different time intervals and residual activity was measured. The effect of pH on enzyme activity was determined at 70°C in a pH range of 4–12 using the standard assay conditions. The effect of pH on the enzyme stability was studied by preincubating the enzyme at 50°C in different buffers for 6 h and determining the residual activity.

Insoluble xylan preparation and binding assays

Insoluble xylan was prepared by the method of Irwin et al. (1994). The chimeric xylanase or the native enzyme was mixed with 20 mg of Avicel or insoluble oat spelts xylan in a final volume of 0.5 ml 50 mM phosphate, pH 7.0 containing 100 mM NaCl and 0.1% (w/v) bovine serum albumin (BSA). The mixtures were maintained on ice for 30 min with periodic mixing (at 5 min interval), then centrifuged at 12,000*g* for 5 min to sediment the polysaccharide and bound proteins. Xylanase activity was determined in the clear supernatant. Loss of activity in the supernatant was assumed to be due to binding to the polysaccharide.

Sequence alignment

For convenience, the first and the second CBM, from the N-terminal end of *T. neapolitana* xylanase A are named as CBM22-1 and CBM22-2, respectively (Fig. 1). The amino acid sequences of CBM22-1 and CBM22-2 were compared to other sequences available in public databases using the BLAST algorithm (Altschhul et al. 1990). CLUSTAL W program (Thompson et al. 1994) was used for multiple sequence alignment of amino acid sequences.

Results

T. neapolitana CBMs and their fusion to *B. halodurans* S7 xylanase

Similarity searches for the primary structures of A1 and A2 from *T. neapolitana* xylanase A were carried out independently against entries in public databases. The closest hits were found to be CBMs linked to GH10 xylanases and most are from thermophiles. Multiple sequence alignment was done with some of the closest sequences and conserved residues are identified (Fig. 2).

The gene fragment encoding CBMs from *T. neapolitana* xylanase A was fused with the *B. halodurans* S7 xylanase gene, and the chimeric enzyme was expressed in *E. coli* and purified to electrophoretic homogeneity. The purified chimeric and native xylanases are used in all the characterization studies. The molecular weight of the chimeric xylanase (CBM–xylS7) was estimated to be about 78 kDa in comparison to 43 kDa of the native (xylS7) (Fig. 3). The specific activity of xylS7 and CBM–xylS7 on unfractionated xylanases (i.e. containing both soluble and insoluble fractions) at pH 7 and 70°C was determined to be 200 and 183 U/mg, respectively.

Characteristics of the chimeric enzyme

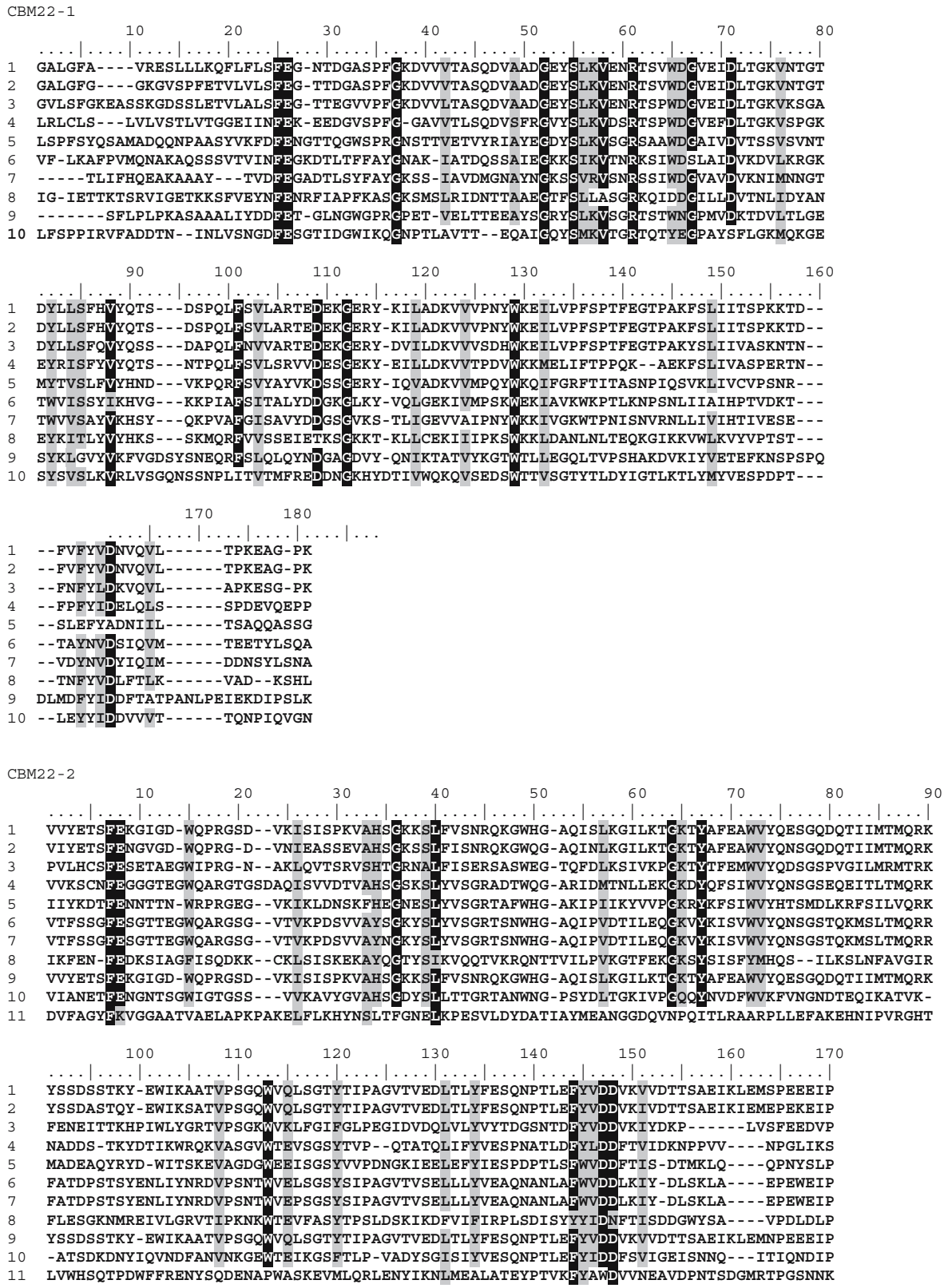
The properties of the chimeric enzyme (CBM–xylS7) were compared with that of the native xylanase (xylS7). CBM–xylS7 and xylS7 exhibited a similar temperature profile and showed optimal activity at 70°C when assayed at pH 10 (Fig. 4). However, the thermal stability of CBM–xylS7 was significantly lower than that of xylS7 (Fig. 5). The chimeric enzyme lost almost all its activity after incubation for 3 h at 65°C, pH 9, while the recombinant native *B. halodurans* xylanase retained about 60% of its original activity after similar treatment. The pH profile and stability of the chimeric and the native xylanases were similar (data not shown). Both enzymes show higher activity at pH 6–10 and are stable in a pH range of 5–10.5.

Evaluation of the xylanase binding characteristics at pH 7 showed that the hybrid protein has affinity to insoluble xylan but not to Avicel (which is 50% amorphous and 50% crystalline cellulose) (Table 2). Binding of CBM–xylS7 to insoluble xylan was influenced by

▶

Fig. 2 Amino acid sequence alignments for CBM22-1 and CBM22-2. Accession numbers are given in *brackets*. For CBM22-1, the following sequences are aligned: (1) *Thermotoga neapolitana* (Z46945), (2) *Thermotoga* sp. strain FjSS3-B.1 xynB (AAD32593), (3) *T. maritima* MSB8 xynA (AAD35155), (4) *Thermotoga* sp. strain FjSS3-B.1 xynC (AAD32594), (5) *Caldicellulosiruptor* sp. Rt69B.1 (AAB95325), (6) *C. saccharolyticus* (ZP_00884870), (7) *Caldicellulosiruptor* sp. Tok7B.1 xynA (AAD30363), (8) *Caldicellulosiruptor* sp. Rt69B.1, (AAB95326), (9) *Anaerocellum thermophilum* (CAA93627), and (10) *Clostridium thermocellum* (ZP_00504483). For CBM22-2, the aligned sequences are: (1) *T. neapolitana* (Z46945), (2) *T. maritima* MSB8 (AAD35155), (3) *Ruminococcus flavefaciens* (CAB51934), (4) *Thermotoga* sp. strain FjSS3-B.1 xynC (AAD32594), (5) *R. albus* (AAA85198), (6) *Thermoanaerobacterium saccharolyticum* (A48490), (7) *Cellulomonas fimi* (CAA90745), (8) *Caldicellulosiruptor saccharolyticus* (AAB87372), (9) *Caldicellulosiruptor* sp. Rt69B.1 (AAB95326), and (10) *Caldibacillus cellulovorans* (AAF61649)

NaCl concentration. There was no measurable binding in the absence of NaCl, and the binding increased with increasing NaCl concentration up to 200 mM (Fig. 6). Similarly, pH influenced the chimeric enzyme binding to insoluble xylan, the lower the pH the better was the binding. At pH 9, the extent of binding was only 54% of



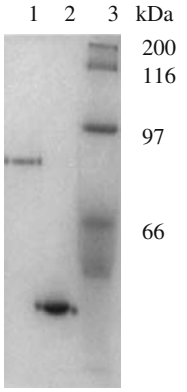


Fig. 3 SDS-PAGE of the purified proteins. *Lane 1*, CBM–xylS7, *lane 2*, xylS7, and *lane 3*, protein molecular weight markers

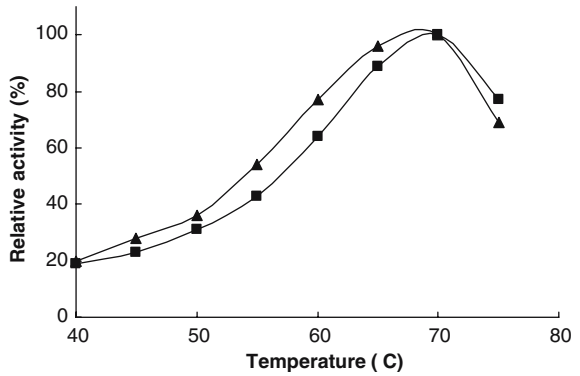


Fig. 4 Temperature profiles of xylS7 (*filled triangle*) and CBM–xylS7 (*filled square*) at pH 10, 50 mM glycine–NaOH buffer

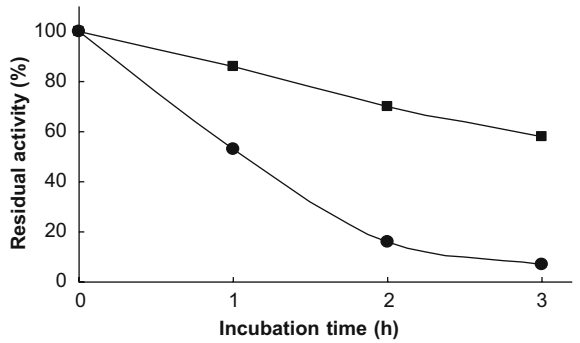


Fig. 5 Residual activity of xylS7 (*filled square*) and CBM–xylS7 (*filled circle*) during incubation at 65°C in 50 mM glycine–NaOH buffer, pH 9. Experimental details are given in the text

the maximum adsorption observed at pH 4 (Fig. 7). Binding studies were not done below pH 4 due to its effect on the stability of the enzyme.

The efficiency of *B. halodurans* S7 GH10 xylanase to hydrolyze insoluble xylan was improved by the CBM fusion as shown in Fig. 8. Hydrolysis was performed at pH 7 and 9, respectively. Better degradation was achieved at pH 7. Although the hydrolysis was lower at pH 9, it was still slightly higher than that obtained by

Table 2 Binding of native (xylS7) and chimeric (CBM–xylS7) xylanases to Avicel and insoluble xylan

Polysaccharide	xylS7 bound (%)	CBM–xylS7 bound (%)
Avicel	1	1
Insoluble xylan	1	62

About 5 units of the enzymes were added to the mixtures containing 20 mg of polysaccharide, 100 mM NaCl, and 0.1% BSA. After centrifugation, unbound activity was determined from the supernatant and used to calculate the % of enzyme bound to the polysaccharide

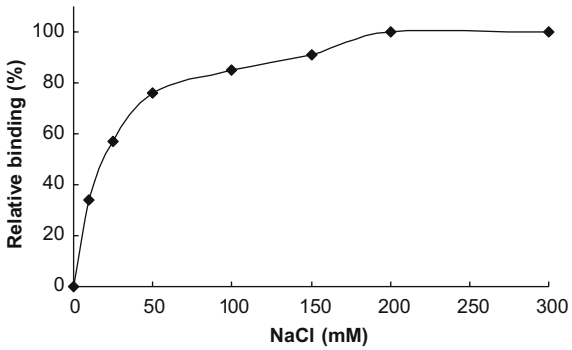


Fig. 6 Effect of NaCl concentration on CBM–xylS7 binding to insoluble xylan. The study was made in aqueous solutions containing different concentrations of NaCl, 0.1% BSA and 10 mg of xylan and 1 U of enzyme

xylS7. Soluble xylan degradation was nearly the same for both CBM–xylS7 and xylS7 (data not shown).

Discussion

A wide variety of organisms that degrade plant biomass produce modular enzymes containing a catalytic domain and one or more CBM structures. Several GH10 xylanases are modular in organization and their CBM structures bind cellulose (Feng et al. 2000; Gibbs et al. 2000; Notenboom et al. 2001; Ito et al. 2003) or xylan (Sun et al. 1998; Fernandes et al. 1999). Usually, xylan binding CBMs appear at the N-terminal position of the catalytic domain. However, C-terminus xylan binding modules have also been reported (Sakaguchi et al. 2004). Members of the genus *Thermotoga* produce xylanases some of which contain N-terminal CBMs. *T. maritima* has two N-terminal CBMs named as A1 and A2 (Meissner et al. 2000). A homologous structure is found in the sequence of xylanase A of *T. neapolitana*. CBM22-1 and CBM22-2 share about 26% similarity. However, they share considerable similarities on conserved residues (Fig. 2). A detailed study on the affinity of A1 and A2 of *T. maritima* xylanase shows that A2 is the one that binds xylan (Meissner et al. 2000), whereas the function of A1 still remains unknown.

The CBMs are known to share some similarities: (1) low percentage of charged amino acids; (2) two Cys

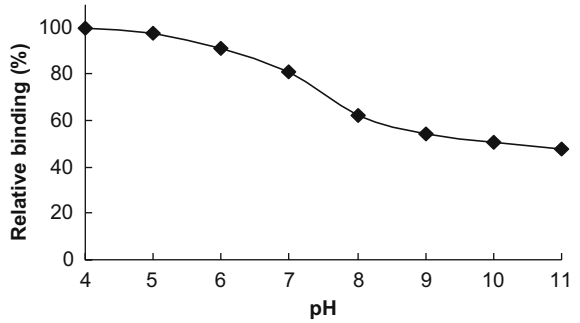


Fig. 7 Effect of pH on CBM–xylS7 binding to insoluble xylan. The buffers used are sodium acetate (pH 4 and 5), phosphate (pH 6–8), and glycine–NaOH (pH 9–11)

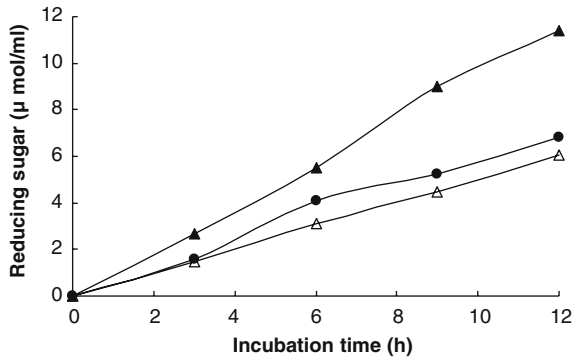


Fig. 8 Hydrolysis of insoluble oat spelts xylan by 0.1 μM of CBM–xylS7 in 50 mM phosphate buffer, pH 7 (*filled triangle*) and in 50 mM glycine NaOH–buffer, at pH 9 (*filled circle*), and by 0.1 μM of XylS7 at pH 9 (*open triangle*) and 30°C

residues present close to the N- and C-termini, respectively; and (3) highly conserved tryptophan, glycine, and asparagine residues (Tomme et al. 1995; Gibbs et al. 2000). However, as seen from the sequence in Fig. 2, the *T. neapolitana* CBMs have a significant percentage composition of charged residues. Charged residues comprise about 24 and 29% of total residues in CBM1 and CBM2, respectively. Cys residues are totally absent in the modules. They have conserved Trp, Gly, Phe, Glu, Asp, Val, and Ser but not Asn.

Most xylanases reported from alkaliphiles including *B. halodurans* do not have xylan binding domains (Mamo et al. 2006b). Although there could be other alternative explanations for why xylan binding domains are not frequent in xylanases from alkaliphiles, a possible reason might be related to xylan solubility. Xylan dissolves better under alkaline condition; and hence the presence of insoluble xylan binding CBMs might not offer a competitive advantage. Moreover, most soda lakes are hyperproductive (Melack and Kilham 1974; Grant et al. 1990) and hence there exists plenty of substrate that may diminish the advantage of having CBM structures.

The hybrid enzyme, CBM–xylS7, exhibits a similar activity profile with respect to temperature as the xylS7. Previously, the fusion of tandem CBM6 structures to the C-terminal region of a similar xylanase from another *B. halodurans* strain resulted in a downward shift in the optimal temperature by 10°C (Mangala et al. 2003). The transfer of the N-terminal xylan binding domain to the C-terminus region of the same xylanase (XynX) of *Clostridium thermocellum* has also decreased the optimum temperature of the xylanase from 65 to 30°C (Shin et al. 2002). On the other hand, Kittur et al. (2003) reported a chimeric xylanase having an optimum temperature similar to that of the native enzyme.

The thermal stability of the CBM–xylS7 was lower than that of the native xylanase. A similar result is reported when CBM4 was fused with feruloyl acetyl esterase domain of *Clostridium stercorarium* xynZ (Kataeva et al. 2001). In other reports, fusion of CBM structures with catalytic domains did not significantly affect the thermal stability (Kittur et al. 2003; Mangala et al. 2003). On the other hand, Karita et al. (1996) reported fusion of CBM structure that resulted in a better thermal stability of *Ruminococcus albus* endoglucanase. The N-terminal CBM structures (most often xylan binding domains) have been referred to as thermostabilizing domains because their deletion normally leads to loss of stability (Winterhalter et al. 1995; Zverlov et al. 1996; Shin et al. 2002). However, this primary role of these modules has been challenged (Meissner et al. 2000). So far, these domains are reported only from carbohydrate active enzymes. If the primary role of these structures were thermostabilization, they would have also been found in other proteins that are produced to perform their biological role at elevated temperature. In another scenario, if these domains are thermostabilizers, why do they exist in a wide variety of cellulases and hemicellulases which are evolved to catalyze reactions under mesophilic conditions, such as in plants (Henrissat and Davies 2000)? Recent studies show that these structures are not thermostabilizing domains (Dias et al. 2004), rather they might be primarily evolved for substrate binding but somehow impart thermostabilizing effect to catalytic domains (Meissner et al. 2000).

The binding of CBMs to the substrate is influenced by the ionic strength of the binding medium. Binding improves with increasing buffer strength (Sun et al. 1998; Mangala et al. 2003), increasing concentration of $CaCl_2$ (Hachem et al. 2000; Mangala et al. 2003) and NaCl (Mangala et al. 2003; Meissner et al. 2000). Similarly, the binding of CBM–xylS7 to xylan was improved with increasing NaCl concentration (Fig. 6). There is no clear explanation as to why ionic strength improves the CBM binding to the substrate, however a few plausible explanations have been proposed. According to the first proposal the better binding at increased ionic strength is due to a decrease in ionic repulsion between negatively charged residues in CBMs and negatively charged xylan (Hachem et al. 2000), probably by charge neutralization that may facilitate CBM–substrate interaction. The second hypothesis is based on possible CBM conformational change that may be induced by ion adsorption

and facilitate substrate binding (Sakka et al. 1996; Sun et al. 1998). A third possible hypothesis could be that the ions remove water from the protein and sugar surface and improve binding. All CBMs use aromatic and often polar residues for interactions with their target substrate. Binding of CBMs to the substrates displays a face with aromatic residues, most often tyrosine and tryptophan residues (Notenboom et al. 2001; Simpson et al. 2002; Hilden and Johansson 2004). An increase in the ionic concentration may remove water from surface vicinity of polymer sugars and the aromatic rings of tyrosine and tryptophan residues and facilitate the stacking interaction. Previously, Boraston et al. (2001) reported the profound influence of osmotic conditions on CBM 4 binding to laminarin.

Binding of the chimeric xylanase, CBM–xylS7 was better in acidic range and decreased towards the alkaline pH as shown in Fig. 7. At pH 10, only 50% of the binding achieved at pH 4 was maintained. Previously, Meissner et al. (2000) reported that the binding rate of the N-terminal CBM from *T. maritima* xylanase is not affected by the pH of the binding medium. The CBM, in particular A1 (CBM22-1), has a low theoretical p*I* (4.64) and might have a negatively charged surface; hence the decrease in pH may decrease the repulsion between the negatively charged polypeptide and xylan. The other possibility is pH induced conformational change of the CBM, which at low pH favors binding.

The fusion of CBM structure improved the insoluble xylan hydrolysis efficiency of the *B. halodurans* xylanase. Better hydrolysis was achieved at pH 7 than at pH 9, which was probably due to the better binding of CBM to insoluble xylan at pH 7. However, the fusion did not improve degradation of soluble xylan. Unfractionated xylan contains insoluble and soluble components. Thus, the chimeric xylanase which degrades insoluble xylan better than the native xylanase is expected to hydrolyze unfractionated xylan better than the native xylanase. This is clear from the specific activities of the xylS7 and CBM–xylS7. The molecular weight of CBM–xylS7 is about 1.7 times of xylS7 molecular weight. Therefore, there are more catalytic sites per gram xylS7 than in a gram of CBM–xylS7. However, the specific activities of these xylanases are fairly close to each other and this may show a better hydrolysis of unfractionated xylan by the chimeric xylanase.

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