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Fusion of family VI cellulose binding domains to *Bacillus halodurans* xylanase increases its catalytic activity and substrate-binding capacity to insoluble xylan

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Abstract

A tandem repeat of the family VI cellulose binding domain (CBD) from *Clostridium stercorarium* xylanase (XylA) was fused at the carboxyl-terminus of *Bacillus halodurans* xylanase (XylA). *B. halodurans* XylA is an enzyme which is active in the alkaline region of pH and lacks a CBD. The constructed chimera was expressed in *Escherichia coli*, purified to homogeneity, and then subjected to detailed characterization. The chimeric enzyme displayed pH activity and stability profiles similar to those of the parental enzyme. The optimal temperature of the chimera was observed at 60 °C and the enzyme was stable up to 50 °C. Binding studies with insoluble polysaccharides indicated that the chimera had acquired an increased affinity for oat spelt xylan and acid-swollen cellulose. The bound chimeric enzyme was desorbed from insoluble substrates with sugars and soluble polysaccharides, indicating that the CBDs also possess an affinity for soluble sugars. Overall, the chimera displayed a higher level of hydrolytic activity toward insoluble oat spelt xylan than its parental enzyme and a similar level of activity toward soluble xylan.

Keywords: Xylanase; *Clostridium stercorarium*; *Bacillus halodurans*; Cellulose binding domains

1. Introduction

The plant cell wall consists of cellulose, hemicellulose (mainly xylan) and lignin which are closely associated with each other. Xylan is a major component of the hemicellulose in the plant cell wall, and it consists of a backbone of β-1,4 linked xylopyranosyl residues which are substituted mainly with acetyl, arabinofuranosyl and glucuronosyl residues. β-Xylanase (E.C. 3.2.1.8) hydrolyzes the β-1,4-glycosidic linkages within the xylan backbone in an endo-wise manner. On the basis of amino acid sequence similarities present in their catalytic domains, xylanases are classified into two major families (glycosyl hydrolases F or 10 and G or 11) [1,2]. Some of the bacterial and fungal xylanases that exist are modular proteins consisting of a distinct cellulose binding domain (CBD) connected to a catalytic domain (CD) [1] and these are often separated by a linker segment rich in either Pro, Thr, and/or Ser residues [3]. These binding modules,

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which are found at either terminus of the enzyme as well as internally, range in size from the small fungal CBDs (36 residues) to the larger bacterial CBDs (up to 200 residues). CBDs are also found in other plant cell wall hydrolases such as β-mannanase [4], acetyl xylan esterase [5] and arabino furanosidases [6] as well as xylanases [7] and cellulases [8]. The exact biological function of these domains in vivo is unknown, however they have been shown to enhance the catalytic activity of the enzyme either by increasing the enzyme concentration around the substrate [9] or by non-covalent disruption of the polysaccharide structure of the substrate [10].

Alkalophilic xylanases are enzymes that are prized because of their ability to hydrolyze xylan under highly alkaline conditions. Thermophilic and alkalophilic xylanases are of great importance in the pulp and paper industry. They reduce the level of chlorine bleaching agent used. *Bacillus halodurans* C-125 strain is an alkalophilic aerobe isolated from soil. This organism is similar to *Bacillus circulans* except for its optimum pH for growth. Alkaline conditions were essential for both growth and xylanase production. [11]. *B. halodurans* C-125 strain produces a highly alkalophilic family 10 xylanase with a broad pH range for the display of activity. It is capable of hydrolyzing xylan at pH 12.0 and it grows at relatively high temperatures (up to 55 °C) [11]. Therefore, this enzyme is an interesting enzyme not only from a scientific perspective but also from an industrial point of view. However, due to a lack of binding domains, it shows only a limited level of activity toward insoluble xylan [11]. On the other hand, *Clostridium stercorarium* is a thermophilic xylanolytic anaerobe that produces at least three modular xylanases. For example, XylA from *C. stercorarium* is a multidomain enzyme consisting of a catalytic domain (family 11) and two family VI CBDs at its C-terminus separated by a proline rich linker. These domains have been shown to bind both cellulose and xylan [12].

The aim of this study was to investigate whether the artificial connection of the CBDs to a single domain enzyme enhances its catalytic activity towards soluble and insoluble xylan. To this end, the family VI CBDs from *C. stercorarium* [12] and the F10 catalytic domain from *B. halodurans* xylanase [11] were employed in this study.

2. Materials and methods

2.1. Construction of the chimeric gene

The strain of *B. halodurans* C-125 was obtained as *Bacillus* sp. (K. Horikoshi C-125) strain number 9153 from the Institute of Physical and Chemical Research (Wako, Japan). The *pXYN* plasmid was provided by Professor Ohmiya in Mie University (Tsu, Japan).

The plasmid DNA preparations, DNA electrophoresis and other basic DNA manipulations described in this paper were performed as described previously [13]. Full-length *xylA* was amplified from the genomic DNA of *B. halodurans*. The gene segment encoding the CBDs, along with the linkers, were amplified (from 236 to 512 residues) from a plasmid *pXYN* containing the full-length *C. stercorarium xylA* gene. The two domains were fused by using a three step overlapping PCR (Fig. 1) [14]. In the first and second steps, full-length *xylA* (*B. halodurans*) and the gene segment encoding the CBDs and the linker were amplified using primers 1–2 and 3–4, respectively (Table 1). Primers 1 and 4 had *Nco* I and *Sal* I sites for cloning into a *pET*-28b (+) vector, whereas primers 2 and 3 had 10 base pair overlapping regions, which were used as templates for the self-priming in the second step of the overlapping PCR. For cloning into the *Nco* I site of the *pET*-28b (+) vector, the second amino acid, Ile of *B. halodurans* was mutated to a Val. The third PCR step was self-priming and was carried out with equimolar concentrations of the two fragments obtained in the first and second PCR steps. In the third step, primers 1 and 4 were used to amplify the full-length chimeric gene (*xylA–cbd*). All three PCR steps consisted of denaturation at 98 °C for 1 min, annealing at 55 °C for 1 min, and primer extension at 72 °C for 5 min with 25 cycles. All PCR steps were carried out with a GeneAmp PCR system 9700 (Applied Biosystems, Foster city, CA) using

Table 1
Primers used in the construction of the chimeric gene

1	5′-**CCATGG**TTACACTTTTTAGAAAGCCT-3′
2	5′-CAGGAGTTGGATCAATAATTCTCCAGTAAGCAG-3′
3	5′-AATTATTGATCCAACTCCTGCCCCATCT-3′
4	5′-**GTCGAC**AGTTCCTGATTTTGAGAATACAAA-3′

Restriction enzyme sites are in bold letters. Ten base pairs of the overlapping regions are underlined.

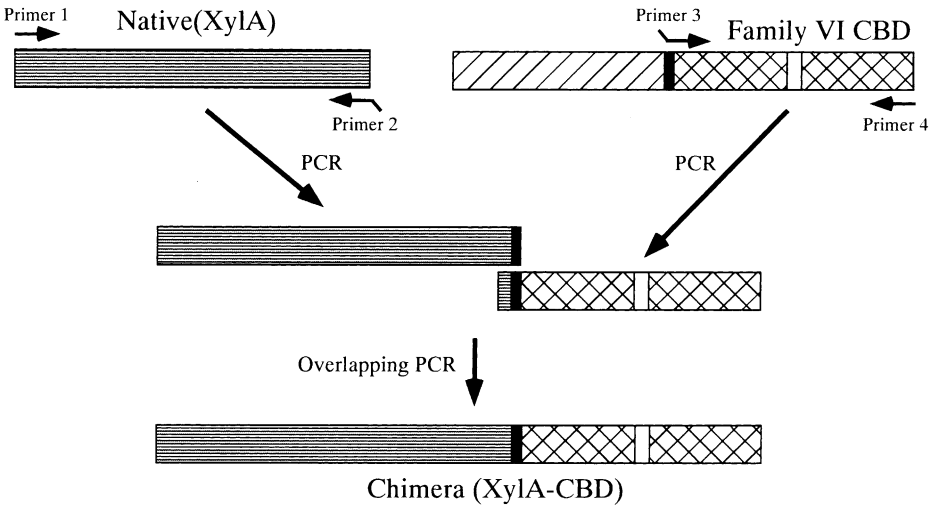

Fig. 1. Strategy for the construction of the chimera (XylA–CBD). (▤) Family 10 catalytic domain of *B. halodurans*; (▨) family 11 catalytic domain of *C. stercorarium*; (▩) family VI CBD; (■) linker.

high fidelity KOD-plus DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). Then, the chimeric gene was cloned into a pDrive vector (Qiagen) and transformed into *E. coli* EZ competent cells (Qiagen, USA). Isolated plasmids were subjected to DNA sequencing (DNA sequencer, Applied Biosystems, Foster city, CA) using a BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA) to confirm the sequence of whole insert including the fused region.

2.2. Production of the enzyme in E. coli

The *xylA–cbd* was excised from the pDrive vector by digestion with the restriction enzymes *Nco* I and *Sal* I and then ligated with the *pET*-28b (+) expression vector which had been previously digested with the same set of restriction enzymes. The host *E. coli* BL 21-Codonplus-RIL competent cells (STRATAGENE, LaJolla, CA, USA) were transformed using the pET-*xylA–cbd* plasmid. Ligation-high T4 DNA ligase (TOYOBO, Osaka, Japan) was used for the gene-vector ligations during the sub-cloning process. The enzyme was expressed as a fusion protein consisting of a tag of six histidine residues at the carboxyl-terminus. Transformants were cultivated in 1 l of Luria-Bertani (LB) medium supplemented with kanamycin (50 mg ml^{-1}) at 25 °C, with shaking, to an optical density of 0.6 at 600 nm. After the addition of isopropyl-1-thio-β-D-galactoside (IPTG) to give a final concentration of 1 mM, the culture was incubated at 25 °C, with agitation for 5 h. The cells were harvested and lyzed and the supernatant was subjected to further purification.

2.3. Purification of the chimera

The expressed chimera (XylA–CBD) was purified by metal chelate affinity chromatography followed by ion-exchange chromatography using an AKTA System (Pharmacia LKB Biotechnology Inc.). The crude extract was allowed to bind with a Ni-NTA agarose slurry by mixing on ice. The resin was packed into a column (HR 5/5, Pharmacia) and the elution was performed with a linear gradient of 0–250 mM imidazole in sodium phosphate buffer (25 mM, pH 8.0) at a flow rate of 0.5 ml min^{-1}. The active fractions were dialyzed overnight at 4 °C against 5 mM phosphate buffer (pH 7.0) and then applied onto a HiLoad Q-sepharose column (HR 16/10, Pharmacia), which was previously equilibrated with 25 mM phosphate buffer (pH 7.0). The protein was eluted with a linear gradient of 0–500 mM NaCl at flow rate of 0.5 ml min^{-1}. The active fractions were pooled and dialyzed overnight at 4 °C against 5 mM phosphate buffer (pH 7.0). The purity of the eluted protein

was assessed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) [15]. Proteins were stained with Coomassie brilliant blue R-250 and a protein ladder (Invitrogen, USA) was used as a molecular weight standard. The native enzyme xylanase (XylA) was purified as described previously [16].

2.4. Xylanase assay

Xylanase activity was determined by incubating with soluble birch wood xylan at 50 °C for 15 min in 100 mM phosphate buffer (pH 7.0). Released reducing sugars were determined by using the Nelson–Somogyi method [17,18]. One unit of enzyme activity is defined as the amount of enzyme that produces 1 mmol of reducing sugar as a xylose standard per minute under the above conditions. Enzyme assay was carried out in duplicate.

2.5. Effect of pH on enzyme activity and stability

To determine the effect of pH, 50 mM concentrations of the following buffers were used: sodium citrate (pH 2.1–4.1), sodium acetate (pH 3.7–5.8), 2-[*N*-morpholino]ethanesulfonic acid (MES) (pH 5.1–7.2), 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 6.2–8.2), phosphate (pH 6.2–8.2), 2-[*N*-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (pH 6.5–8.6), 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) (pH 8.2–10.3) and 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 9.4–11.5). For determinations of pH stability, the enzyme was pre-incubated in the various buffers described above at 50 °C for 30 min followed by the determination of residual activity using the standard assay.

2.6. Effect of temperature on enzyme activity and stability

Temperature optima of the chimeric and native enzymes were determined using the standard assay in 100 mM phosphate buffer, pH 7.0, at temperatures ranging from 0 to 100 °C. Thermal stability was determined by incubating the enzyme for 30 min at different temperatures. After cooling the samples on ice for 10 min, residual activity was determined using the standard assay.

2.7. Adsorption assay

Phosphoric acid-swollen cellulose (ASC) was prepared by phosphoric acid treatment of Avicel and insoluble xylan from oat spelt xylan as reported previously [19,20]. Binding assays were carried out in 500 ml of sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 mg of insoluble xylan (or acid-swollen cellulose or Avicel) (Sigma, USA) and an appropriate amount (0.04 μg) of enzyme, unless otherwise stated. The mixture was incubated on ice for 30 min with continuous stirring and the polysaccharides were sedimented by centrifuging at 12,000 rpm for 5 min. The amount of enzyme bound to the insoluble polysaccharides was determined by measuring the protein concentration in the supernatant. Protein concentrations were determined by using the Bradford method with bovine serum albumin as a standard [21]. Assay was carried out in duplicate.

2.8. Desorption assay

Desorption assays were performed as follows: the enzyme and acid-swollen cellulose were incubated on ice in the presence of 100 mM phosphate buffer (pH 7.0) for 30 min with continuous stirring. After centrifugation, the precipitate (enzyme–ASC complex) was incubated on ice for 30 min in the same buffer containing 5% soluble saccharides such as maltose, glucose, xylose or polysaccharides such as birch wood xylan, barley β-glucan and carboxy methyl cellulose (CMC) (low viscosity grade) (Sigma, USA). The precipitate (enzyme–ASC complex) was washed with 100 mM phosphate buffer (pH 7.0). After centrifugation, the residual activity and protein concentration in the supernatant were determined. The ratio of the xylanase activity released from the enzyme–ASC complex by washing to the total amount of xylanase bound to the ASC was expressed as a relative desorption (%).

3. Results and discussion

3.1. Construction of the chimeric enzyme

In order to investigate whether the addition of binding domains increased the catalytic activity of the

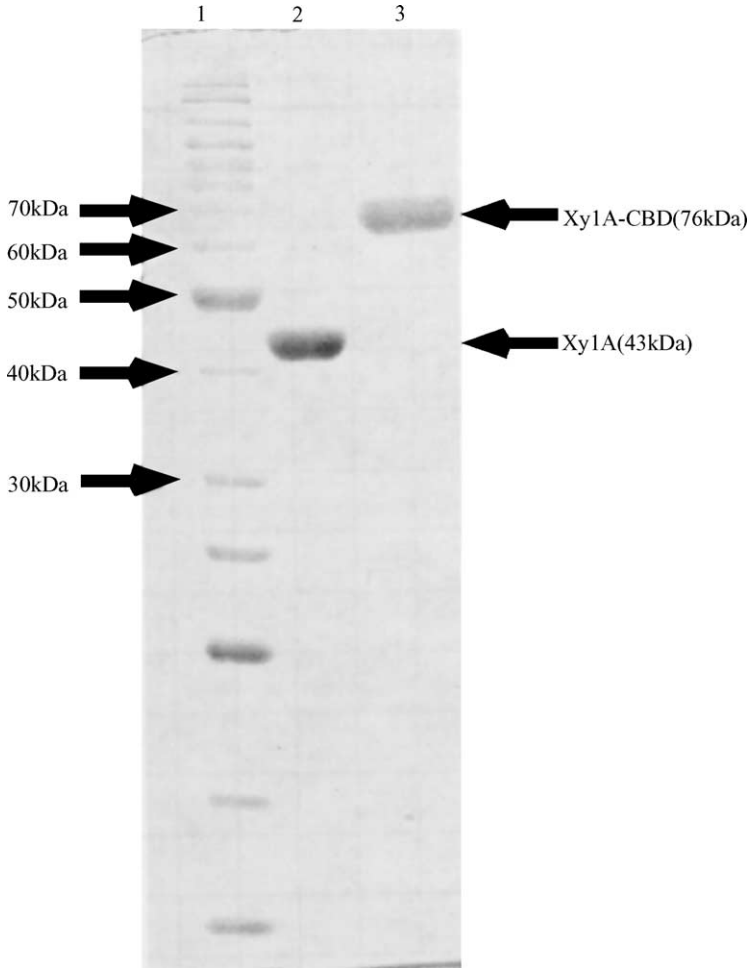

Fig. 2. SDS-PAGE analysis of purified XylA and XylA–CBD. Lane 1: 10 kDa protein marker; lane 2: purified XylA; lane 3: purified XylA–CBD.

parental enzyme towards both soluble and insoluble xylan, two CBDs of *C. stercorarium* XylA were fused to the C-terminus of *B. halodurans* XylA. Native *B. halodurans* XylA is a single domain enzyme without a CBD and it belongs to family 10 of the glycosyl hydrolases. In contrast, *C. stercorarium* XylA is a modular enzyme consisting of a family 11 catalytic domain and two family VI CBDs at the C-terminus (Fig. 1) with a proline rich linker separating the catalytic domain from the CBDs. The chimeric enzyme constructed in this study consisted of a family 10 catalytic domain, a proline rich linker, and two family VI CBDs at its C-terminus.

3.2. Purification of the chimeric enzyme

The chimeric enzyme was purified to homogeneity by using Ni-NTA and Q-sepharose column chromatography steps and the purity was confirmed by the presence of a single band by SDS-PAGE (Fig. 2). Native *B. halodurans* XylA has a molecular weight (M_W) of approximately 43 kDa, whereas the chimera displayed a M_W of about 76 kDa, indicating the presence of the CBDs. These values are in close agreement with the M_W calculated using the ExPASy ProtoParam tool of 43,564 and 76,176 for the native *B. halodurans* XylA and the chimeric enzyme, respectively.

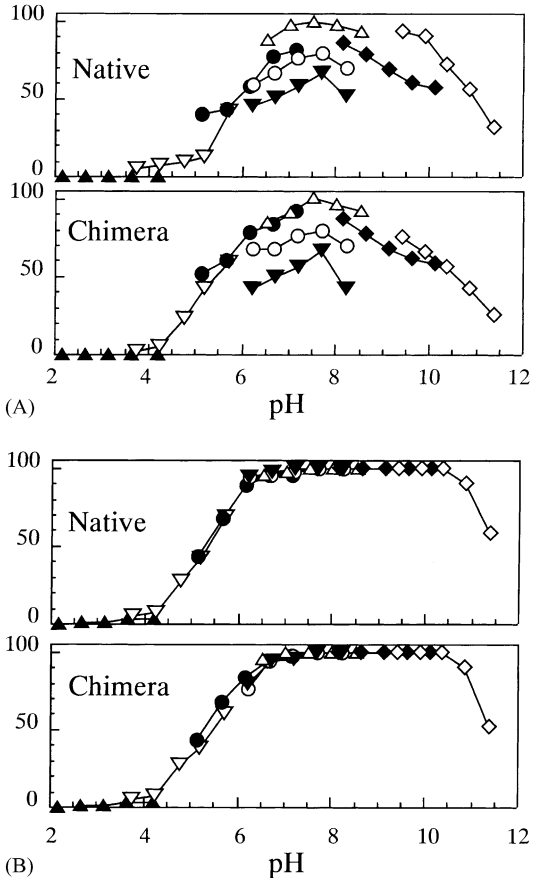

Fig. 3. Effect of pH on xylanase activity (A) and stability (B) of the native and chimeric enzymes. The pH was adjusted with 50 mM of the following buffers: sodium citrate (▲, pH 2.1–4.1), sodium acetate (▽, pH 3.7–5.8), MES (●, pH 5.1–7.2), MOPS (○, pH 6.2–8.2), phosphate (▼, pH 6.2–8.2), HEPES (△, pH 6.5–8.6), CHES (◆, pH 8.2–10.3) and CAPS (◇, pH 9.4–11.5).

3.3. Effects of pH and temperature on the activity and stability of the chimeric enzyme

The chimeric enzyme displayed an optimum pH of 8.0 and had a broad pH range from pH 6–10.0 (Fig. 3A). It was stable between pH 6–10.0 at 50 °C and retained 50% of its original activity even at pH 11.5 (Fig. 3B). Temperature optimum (Fig. 4A) and thermal stability (Fig. 4B) measurements demonstrated that maximal activity occurred at 60 °C and that the enzyme was stable up to 50 °C. Apart from the temperature optimum, the chimeric enzyme had the same characteristics as the parental enzyme. The

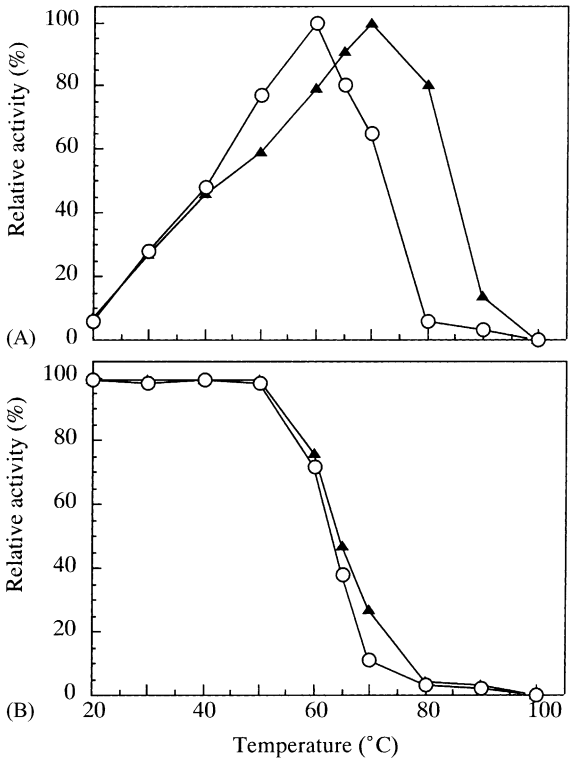

Fig. 4. Effect of temperature on enzyme activity (A) and stability (B). XylA (▲) and XylA–CBD (○).

optimum temperature for *B. halodurans* XylA is 70 °C [11] and this was shifted to 60 °C in the case of the chimeric enzyme. The reason for such a shift in the optimal temperature is not clear.

3.4. Binding studies

Qualitative binding experiments showed that the chimeric enzyme was able to bind to insoluble oat spelt xylan as well as phosphoric acid-swollen cellulose, whereas the native enzyme, which is devoid of CBDs, did not show any significant adsorption to either of these polysaccharides (Fig. 5). This result indicates that the affinity of the chimeric enzyme towards insoluble polysaccharides is due to the presence of the CBDs and not the catalytic domain of *B. halodurans* XylA. As reported previously [12,22,23], binding is highly dependent on buffer concentration and the presence of cations such as Na^+ and Ca^{2+}. Almost no binding was observed in the presence of 5 mM

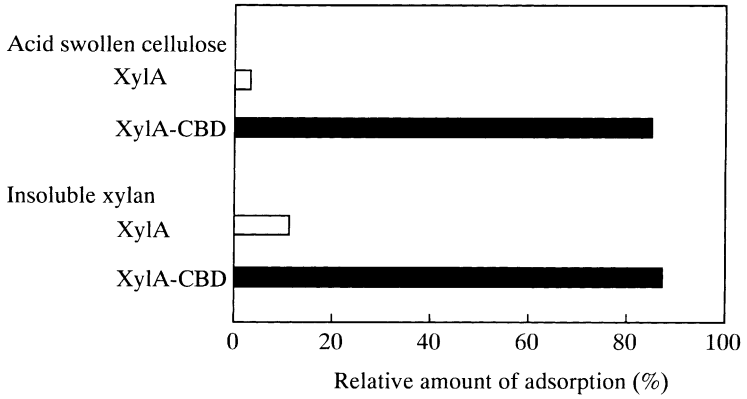

Fig. 5. Adsorption of XylA and XylA–CBD to insoluble polysaccharides.

phosphate buffer and the adsorption reached a plateau at 100 mM phosphate buffer (Fig. 6). It is likely that this is due to the decrease in the ionic repulsion between the negatively charged xylan substituents such as 4-*O*-methylglucuronic acid and the enzyme in the presence of high concentrations of the buffer. However, the effect of ions on binding to PASC, which is non-ionic, is not explained by the above hypothesis. When aqueous solutions of sodium and calcium chloride were employed in the adsorption studies, maximal adsorption was observed at approximately 100 mM NaCl and 25 mM $CaCl_2$, respectively (Fig. 6). The reason for this increase in binding could be due to efficient neutralization of the intramolecular repulsion of negative charges on the protein surface, possibly near the active site or could be due to the conformational changes induced by positive divalent cations. An ion-dependent adsorption of CBDs has been proposed previously [24].

Determination of relative adsorption values for the chimeric enzyme showed higher relative adsorption values for ASC (87%) than toward Avicel (64%), suggesting that the chimera has a higher affinity for amorphous cellulose than it does for crystalline cellulose.

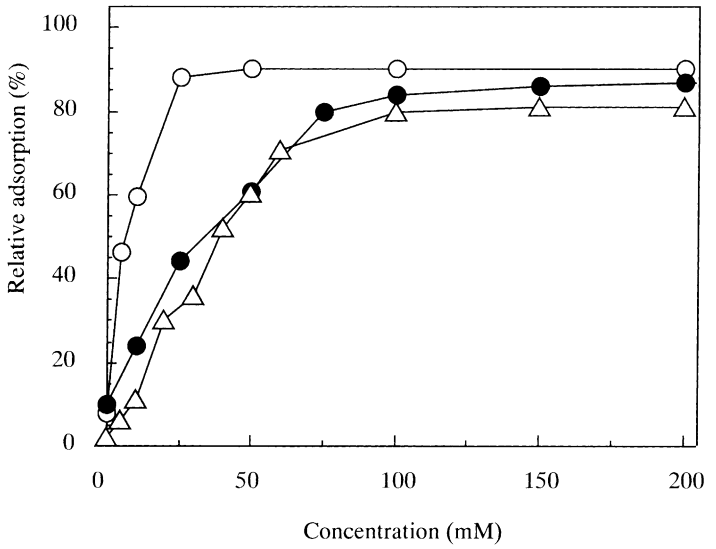

Fig. 6. Effect of $CaCl_2$, NaCl and phosphate buffer on binding of XylA–CBD to insoluble oat spelt xylan. (○) $CaCl_2$; (●) NaCl; (△) phosphate buffer.

ASC is amorphous or para crystalline in nature [25], whereas Avicel is composed of various microcrystallites embedded within an amorphous matrix and its crystallinity is estimated to be approximately 50% [26]. This suggests that the CBDs bind preferentially to cellulose chains with a relatively high degree of conformational freedom (e.g. those found in amorphous cellulose). Previous studies have shown that both *C. fimi* endoglucanase C (Cen C) [27] and *C. stercorarium* XylA [12] have an affinity for amorphous cellulose but not for crystalline cellulose.

3.5. Affinity of the chimera toward soluble saccharides

It has been shown previously that the CBDs of *C. stercorarium* XylA display an affinity for soluble saccharides [22]. In order to test whether such an affinity is retained in the chimeric enzyme, a desorption assay measuring the ability of the saccharides to release bound enzyme from the ASC–enzyme complex was conducted. Of the various saccharides tested, 5% cellobiose was able to completely desorb the chimeric enzyme from insoluble substrates, whereas maltose, xylose and glucose were less effective than cellobiose (Table 2). Birch wood xylan and barley β-glucan were also effective in releasing the enzyme from insoluble polysaccharides, but to a lesser extent. CMC, an anionic cellulose derivative, however, had no effect on insoluble polysaccharide–chimera interactions. No significant enzyme inhibition by these soluble saccharides was observed during the assay. These results indicate that the CBDs in the chimeric enzyme retained their affinity for small saccharides.

CBDs are classified into three classes based on their affinity for polysaccharide and sugar ligands: 1 (or type A), insoluble polysaccharide or "surface binders", 2 (or type B), soluble polysaccharide or "chain binders" and 3 (or type C), "small sugar binders" [28]. Type A CBDs have planar surfaces with a strip of aromatic amino acids which are coplanar and parallel to the protein surface. All type A CBDs bind planar surfaces such as crystalline cellulose. Examples of type A CBDs are found in members of families I, IIa, IIIa, V, X, XII and XV. In contrast, type B domains have a groove with aromatic residues located at the edges such a topology is suitable for interaction with soluble carbohydrate chains. Families IIb, IV and XVI CBDs represent type B CBDs. The final class, type C, includes members of families VI, IX, XIII, XVII and XXII. The binding pocket present in type C CBDs is similar to that found in type B except for the depth of the groove: it is deeper than those seen in the type B CBDs. The presence of a binding cleft in the CBD (family 4) from *C. fimi* Cen C provides a structural explanation for the selectivity of the CBD for amorphous cellulose and soluble oligosaccharides, and the lack of binding affinity toward crystalline cellulose [27,29]. It is likely that the family VI CBDs from *C. stercorarium* XylA have a deeper binding cleft, similar to *C. fimi*, since they show an affinity for amorphous cellulose and other soluble polysaccharides.

Table 2
Desorption of XylA–CBD from the ASC–enzyme complex by washing with saccharide solutions (5%)

Saccharide	Relative desorption (%)
Cellobiose	98
Birchwood xylan	59
Xylose	53
Glucose	49
Maltose	31
β-Glucan	13
CMC	3

3.6. Effect of CBDs on the catalytic activity of B. halodurans XylA

No significant difference in the hydrolytic activity was observed between the native enzyme and the chimeric enzyme when they were incubated with soluble birch wood xylan (Fig. 7A), suggesting that the CBDs did not play a role in the hydrolysis of the soluble xylan. On the other hand, when insoluble oat spelt xylan was used, the hydrolytic activity of the chimeric enzyme was greater than that of the native enzyme (Fig. 7B). The molar activities of the native XylA and XylA–CBD (chimera) were 7.5 and 35 U μmol^{-1}, respectively toward insoluble oat spelt xylan. These results indicate that the presence of CBDs improves the catalytic activity of the native xylanase toward insoluble xylan by concentrating the catalytic domain on the surface of the substrate, allowing the enzyme to interact more closely with the substrate. This result appears to be consistent with that observed previously for a chimeric enzyme

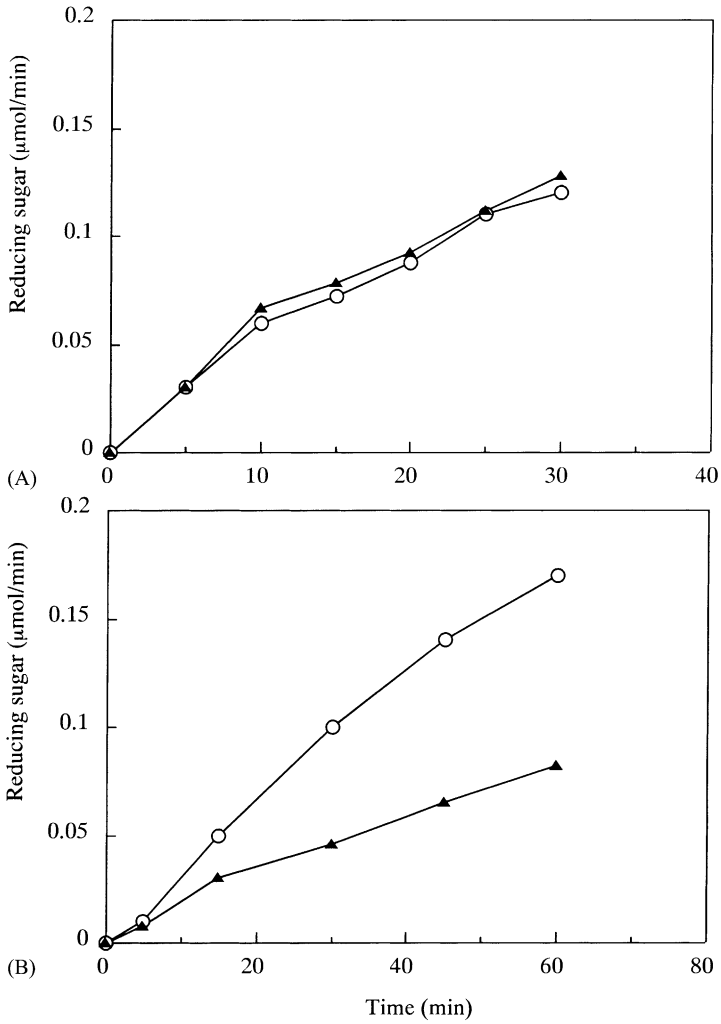

Fig. 7. Hydrolytic activity of XylA (▲) and XylA–CBD (○) toward soluble birchwood xylan (A) and insoluble oat spelt xylan (B). Polysaccharides were incubated with purified XylA and XylA–CBD in 100 mM sodium phosphate buffer at 50 °C (for soluble xylan) and 30 °C (for insoluble xylan). At the indicated time intervals, aliquots were withdrawn and the amount of reducing sugar was determined by the Nelson–Somogyi method (amount of enzyme used for the assay: 1.1×10^{-13} mol for the soluble and 1.8×10^{-10} mol for the insoluble xylan assay). The experiments were carried out in duplicate.

constructed between *Ruminococcus albus* endoglucanase IV and the CBDs of *C. stercorarium* XylA [30].

4. Conclusions

Employing protein engineering, an alkaline xylanase with a CBD has been successfully constructed without destroying the catalytic profile of the native enzyme. We have shown that the addition of binding domains enhances the substrate-binding capacity and catalytic activity of the native enzyme toward insoluble xylan.

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References

[1] N.R. Gilkes, B. Henrissat, D.G. Kilburn, R.C. Miller Jr, R.A.J. Warren, Microbiol. Rev. 55 (1991) 303.
[2] B. Henrissat, A. Bairoch, Biochem. J. 293 (1993) 781.
[3] P. Tomme, R.A.J. Warren, N.R. Gilkes, Adv. Microbiol. Physiol. 37 (1995) 1.
[4] H. Stalbrand, A. Saloheimo, J. Vehmaanpera, B. Henrissat, M. Penttila, Appl. Environ. Microbiol. 61 (1995) 1090.
[5] L.M.A. Ferreira, T.M. Wood, G. Williamson, C. Faulds, G.P. Hazlewood, G.W. Black, H.J. Gilbert, Biochem. J. 294 (1993) 349.
[6] G.W. Black, J.E. Rixon, J.H. Clarke, G.P. Hazlewood, M.K. Theodorou, P. Morris, H.J. Gilbert, Biochem. J. 319 (1996) 515.
[7] A. Blanco, P. Diaz, J. Zueco, P. Parascandola, F.I.J. Pastor, Microbiology 145 (1999) 2163.
[8] P. Beguin, J.P. Aubert, FEMS Microbiol. Rev. 13 (1994) 25.
[9] N. Din, N.R. Gilkes, B. Tekant, R.C. Miller Jr, R.A.J. Warren, D.G. Kilburn, Biol. Technol. 9 (1991) 1096.
[10] B. Nidetzky, W. Steiner, M. Hayn, M. Claeyssens, Biochem. J. 298 (1994) 705.
[11] H. Honda, T. Kudo, Y. Ikura, K. Horikoshi, Can. J. Microbiol. 31 (1985) 538.
[12] K. Sakka, G. Takada, S. Karita, K. Ohmiya, Ann. N.Y. Acad. Sci. 782 (1996) 241.
[13] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
[14] K. Goyal, Y.K. Kim, M. Kitaoka, K. Hayashi, J. Mol. Catal. B: Enzym. 646 (2001) 1.
[15] U.K. Laemmli, Nature 227 (1970) 680.
[16] M. Nishimoto, Y. Honda, M. Kitaoka, K. Hayashi, J. Biosci. Bioeng. 93 (2002) 428.
[17] N. Nelson, J. Biol. Chem. 153 (1944) 375.
[18] M. Somogyi, J. Biol. Chem. 195 (1952) 19.
[19] J.B. Coutinho, N.R. Gilkes, R.A.J. Warren, D.G. Kilburn, R.C. Miller Jr, Mol. Microbiol. 6 (1992) 1243.
[20] D. Irwin, E.D. Jung, D.B. Wilson, Appl. Environ. Microbiol. 60 (1994) 763.
[21] M. Bradford, Anal. Biochem. 72 (1976) 248.
[22] J.L. Sun, K. Sakka, S. Karita, T. Kimura, K. Ohmiya, J. Ferment. Bioeng. 85 (1998) 63.
[23] E. Ong, N.R. Gilkes, R.A.J. Warren, R.C. Miller Jr, D.G. Kilburn, Biol. Technol. 7 (1989) 604.
[24] M.A. Hachem, E.N. Karlsson, E.B. Roxa, S. Ragothama, P.J. Simpson, H.J. Gilbert, M.P. Williamson, O. Holst, Biochem. J. 345 (2000) 53.
[25] H. Ooshima, M. Sakata, Y. Harano, Biotechnol. Bioeng. 25 (1983) 3103.
[26] T.M. Wood, Methods Enzymol. 160 (1988) 19.
[27] P. Tomme, A.L. Creagh, D.G. Kilburn, C.A. Haynes, Biochemistry 35 (1996) 13885.
[28] A.B. Boraston, B.W. McLean, J.M. Kormos, M. Alam, N.R. Gilkes, C.A. Haynes, P. Tomme, D.G. Kilburn, R.A.J. Warren, in: H.J. Gilbert, G.J. Davies, B. Henrissat, B. Svensson (Eds.), Recent Advances in Carbohydrate Bioengineering, 1999, p. 202.
[29] P.E. Johnson, M.D. Joshi, P. Tomme, D.G. Kilburn, L.P. McIntosh, Biochemistry 35 (1996) 14381.
[30] S. Karita, K. Sakka, K. Ohmiya, J. Ferment. Bioeng. 81 (1996) 553.