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Importance of C-Terminal Region for Thermostability of GH11 Xylanase from *Streptomyces lividans*

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Abstract The amino acid sequences of xylanase B (XlnB) and xylanase C (XlnC) from *Streptomyces lividans* show significant homology. However, the temperature optima and stabilities of the two enzymes are quite different. XlnB exhibits an optimum temperature of 40 °C and retains 50% of its maximum activity at 43 °C, whereas the corresponding values for XlnC are 60 and 70 °C. To analyze these properties further, as well as to study the effect of the *exchange* of homologous segments in the C-terminal region, four chimeras designated as BSC, BFC, CSB, and CFB were constructed by substituting segments from the C-terminal homologous region of XlnB gene with that of XlnC and in turn substituting XlnC gene with that of XlnB. The purified chimeric enzymes were characterized with respect to pH/temperature activity, stability, and kinetic parameters. Most of enzymatic properties of chimeras were admixtures of those of the two parents. The chimeric enzymes were optimally active at 45–55 °C and pH 7.0. Both K_m and k_{cat} values of chimeric enzymes for *p*-nitrophenyl-β-D-cellobioside were admixtures of both parental enzymes, except that the k_{cat} value of chimeric BFC (2.79 s^{-1}) was higher than that of parental XlnC (1.99 s^{-1}). Notably, *thermal* stability of chimeric BSC and BFC was increased by 25 and 13 °C separately, as compared to one of parental XlnB, whereas the thermal stability of chimeric CSB and CFB was decreased by 23 and 21 °C, respectively, as compared to another parental XlnC. These results suggest that homologous C-terminal region in *S. lividans* GH11 xylanase appears to play an important role in determining enzyme characteristics, and *exchanging* of different segments of gene in this region might significantly alter or improve the enzymatic properties such as thermal stability.

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Abbreviations

GH	glycoside hydrolase
XlnB	*Streptomyces lividans* xylanase B
XlnC	*Streptomyces lividans* xylanase C
PNP-G_2	*p*-nitrophenyl-β-D-cellobioside
RBB-xylan	4-*O*-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R

Introduction

Xylanases (EC 3.2.1.8) catalyze the hydrolysis of xylan, a major constituent of hemicellulose. On the basis of primary structure homology, the majority of xylanases have been classified into glycoside hydrolase families 10 and 11 (GH10 and GH11, respectively) [1, 2]. The enzyme has potential economical and environment-friendly applications, including paper and pulp industries and food and feed industries [3]. Xylanases can be used in the bleaching of pulp to reduce the use of toxic chlorine-containing chemicals. In addition, it is desirable that xylanases used for biobleaching are stable and active under alkaline conditions at high temperatures. Therefore, attention is focused on discovery of new xylanases or improvement of existing ones to meet the requirements of the pulp-and-paper industry.

Streptomyces lividans is a well-known *Actinomycete* that produces three xylanases: xylanases A, B, and C [4]. Xylanase A (XlnA) belongs to family GH10; xylanase B (XlnB) and xylanase C (XlnC) belong to family GH11 [4, 5]. XlnB and XlnC are homologous to the low MW/basic xylanase and show similar biochemical features. They are hydrophilic in nature and hydrolyse xylan by an endo-type mechanism to produce xylopentose, xylotetraose, and xylotriose. The gene encoding XlnB and XlnC were cloned and expressed in *S. lividans* [4]. XlnB is a 31-kDa protein comprising of an N-terminal catalytic domain and a C-terminal substrate-binding domain. XlnC is a 22-kDa protein comprising only of a catalytic domain. The amino acid sequences of the XlnB gene show significant similarity (about 68%) with those of the XlnC gene (Fig. 1a). Despite this similarity, their enzymatic properties, especially temperature activity and thermal stability, are quite different. The XlnC is contained within the XlnB N-terminal amino acids sequence, which suggests that the catalytic site is located in N-terminal domain of XlnB and XlnC. Furthermore, the catalytic amino acid, glutamic acid, was reported to be located at position 87 of N-terminal region, indicating that N-terminal domain could be catalytic [4, 5]. However, the function of the C-terminal domain has not thus been properly clarified.

Structure–function relationships and the engineering of xylanases have attracted considerable interest [6–8]. However, previous work has concentrated on only a limited number of amino acids using site-directed mutagenesis. Such technique only determines the function of isolated amino acids and cannot investigate the nature of larger regions of protein. Rather than concentrating on one or two amino acids at a time using site-directed mutagenesis, an alternative approach is to use recombinant DNA technology to investigate the function of larger regions of protein. Recently, exchange of two or more genes for chimeric enzyme study using gene fusion and gene shuffling technology has emerged as a very powerful and effective tool in generating enzymes with maximal substitution of amino acids [9–12]. Biochemical analysis of chimeric enzymes can reveal the structure and function relationships of closely related enzymes. This approach should provide insight into the function of the C-terminal region of GH11 xylanases from *S. lividans*.

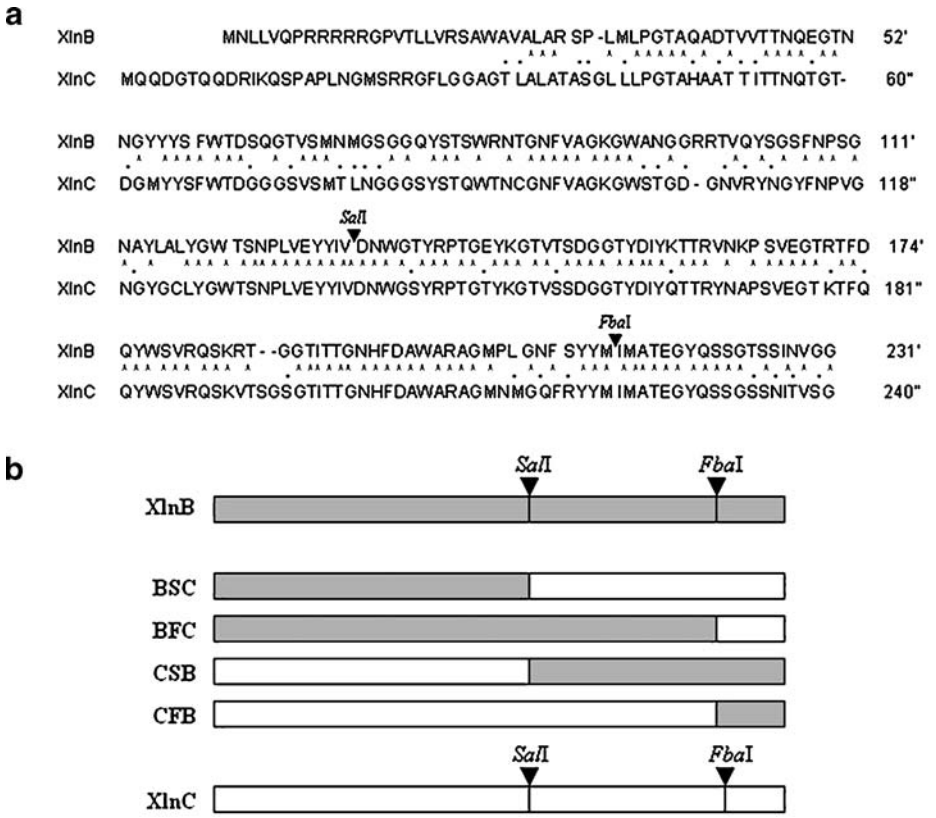

Fig. 1 **a** Homology in amino acid sequences of xylanase B catalytic domain (XlnB) and xylanase C (XlnC) from *S. lividans*. Identical and similar amino acid residues are designated by *asterisk* and *period*, respectively. Chimeric xylanases were constructed using the two restriction enzyme sites *Sal*I and *Fba*I marked by *arrowheads*. **b** Schematic representation of parental and chimeric xylanase genes. *Dark* and *white* bars represent regions derived from XlnB and XlnC, respectively. Two restriction enzymes *Sal*I and *Fba*I used for the construction of chimeric enzymes are shown with *arrowheads*

In this study, to elucidate the role of the C-terminal region, chimeric xylanases were constructed between XlnB and XlnC by substituting different segments from one enzyme in the C-terminal homologous region of other and comparing the enzyme characteristics of parental and chimeric enzymes.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Media

Escherichia coli TOP10F′ (Invitrogen, Carlsbad, CA), JM109 (Takara Shuzo, Shiga, Japan) were used as hosts for production of parental or chimeric enzymes. Plasmid pCR2.1-TOPO (Invitrogen) was used as cloning vector. pQE60 (Qiagen, Hilden, Germany) was used as cloning vectors. *E. coli* strains were grown at 37 °C in LB medium containing 50 μg/ml ampicillin or 20 μg/ml kanamycin. Clones were screened for xylanase activity on plates containing 0.5% Remazole Brilliant Blue (RBB)-xylan (Sigma, Germany) [13].

Cloning and DNA Sequencing

Amplified DNA fragments were cloned into pCR2.1-TOPO cloning and sequencing vector according to the protocol of the supplier (Invitrogen). Recombinant pCRTOPO plasmids were extracted using a QIAminiprep kit and were sequenced. Big dye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, CA, USA) was used as per the supplier's instructions to prepare sequencing samples and assayed with a DNA sequencer (Model 373A, Applied Biosystems). The sequence data were analyzed using the GENETIX program (Software Development, Tokyo, Japan).

Construction of Chimeras

Plasmid pQE60 was used as a vector. Two restriction nuclease sites, the *Sal*I and *Fba*I were used to construct four chimeric xylanase genes (Fig. 1b). For construction of chimeric gene BSC, each parental gene was first cloned into the pQE60 expression vector. To obtain the insert fragments, sites suitable for binding appropriate polymerase chain reaction (PCR) primers were located outside the required boundaries of the genes by the aid of appropriate software. The PCR products obtained using XlnC gene as the template were then digested by *Sal*I and *Hin*dIII to yield an insert having cohesive ends. Another parental XlnB gene was similarly digested by *Sal*I and *Hin*dIII. The desired DNA fragments which included the bulk of vector and PCR purified using agarose gel electrophoresis. Finally, both the digested vector and PCR fragment were ligated and transformed into *E. coli.* The other chimeric genes depicted in Fig. 2 were similarly constructed.

Expression of Chimeric Enzymes

DNA chimeras without any mutations in their amino acid sequences were subcloned into *Nco*1–*Bam*H1 sites of expression vector pQE60. Thus, the fragments were positioned under control of the strong T5 promoter of pQE60, which ensured their highest level of expression. Moreover, this was tagged six-histidine residues of pQE60 at the C-terminal ends of each DNA chimera, which were facilitated one-step purification of the translated

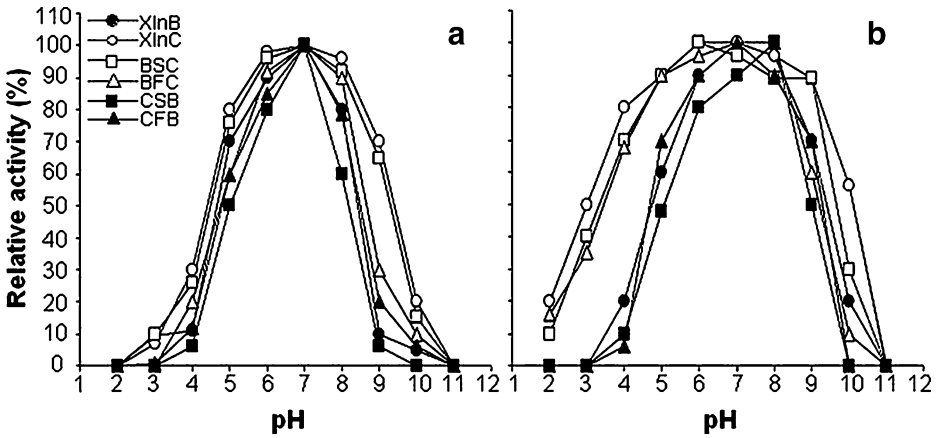

Fig. 2 pH activity (**a**) and pH stability (**b**) profiles of parental and chimeric xylanases

enzymes by affinity column chromatography. Ligation-High T4 DNA ligase (TOYOBO, Osaka, Japan) was used for DNA-vector ligations during subclonings. *E. coli* JM109 competent cells were transformed with recombinant pQE60, and positive subclones were screened out from LB-plates containing X-gal and kanamycin.

Purification of Enzymes

LB-broth (800 ml) supplemented with ampicillin (50 mg/ml) was incubated with 100 ml of freshly prepared seed culture of *E. coli* JM109 harboring recombinant pQE60. Protein production was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside to the culture media when the OD of the broth reached to 0.4–0.5 at 660 nm. Cultivation was then continued overnight. Cells were harvested by centrifugation (8,000 rpm, 10 min at 4 °C). About 2.5 g of cells (*w*/*w*) were subjected to ultrasonication by Sonifier Model 250D (Branson Sonic Power, CT, USA) to extract enzymes. The cell-free lysates were then centrifuged (12,000 rpm, 10 min at 4 °C), and each lysate was charged with 1 ml of Ni-NTA resin, which bound to the target proteins. This resin was then packed into 1-ml column and eluted with a linear gradient of 250 mM imidazole in 50 mM Na-phosphate buffer, pH 8.0 at a flow rate of 0.8 ml/min using an fast protein liquid chromatography (FPLC) system (Pharmacia, Sweden). The active fractions were combined and dialyzed against 1 l of 20 mM CHES buffer, pH 9.0 or 20 mM MES buffer, pH 6.0 for overnight at 4 °C. The dialyzed enzyme solutions were reloaded onto a Q-Sepharose or a SP-Sepharose column (8 ml) previously equilibrated with either 20 mM CHES buffer, pH 9.0 or 20 mM MES buffer pH 6.0, and the enzymes were eluted with a linear gradient of 0.5 M or 1.0 M NaCl at a flow rate of 0.5 ml/min. The homogeneity of the purified enzyme fractions was monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme Assay

Crude and purified enzymes were assayed quantitatively using 0.2% of RBB-xylan (Sigma) as a substrate. Concentrated enzyme solution was diluted with assay buffer, 50 mM McIlvaine, pH 7.5 (a mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4) to give a final concentration of 0.05 μg protein/5 μl of solution. Five microliters of diluted enzyme was applied to a pre-warmed mixture of 20 μl of 50 mM McIlvaine buffer, pH 7.5 and 25 μl of 0.4% aqueous solution of RBB-xylan and incubated at 40 °C for 15 min. The reaction was stopped by addition of 100 μl of 100% EtOH and kept at room temperature at least for 15 min. The precipitated residual substrate was collected by centrifugation at 15,000 rpm for 2 min, and the OD of the supernatant was measured at 595 nm against respective substrate blanks.

Determination of pH and Thermal Profiles

All of the following parameters were assayed twice with standard assay method as described above. For determining pH stability, purified enzyme aliquots containing 5 μg of protein were exposed to different pH levels using either 50 mM McIlvaine buffer for pH 2.0 to 8.0 or 50 mM Tris–HCl buffer, for pH 8.0 to 11.0 and incubated at 30 °C or 40 °C for 1 h. The residual activities of these treated enzymes were determined. For determinations of optimum pH, the same sets of buffers having pH values 2.0 to 8.0 and 8.0 to 11.0, respectively, were used instead of the usual pH 7.5 buffer to assay the enzyme activity. To determine optimum temperatures, 0.05 μg purified protein was incubated with the substrate

solution at different temperatures for 30 min each, and the relative enzyme activity was measured. For determination of thermal stability, 5 μg purified enzyme was incubated at its optimal pH, with 0.1% of bovine serum albumin (BSA) as stabilizer at different temperatures for an hour. The residual activity of the treated enzymes was then measured according to the standard assay method.

Analysis of Kinetic Parameters

Steady-state kinetic parameters were determined as described by Lawson et al. [14] using *p*-nitrophenyl β-D-cellobioside (PNP-G_2) as substrates. The substrate, dissolved to give various concentrations in 30% of 0.2 M McIlvaine buffer, pH 7.0 containing 0.1% BSA, was preincubated at 30 °C for 5 min. Then, 50 μl of enzyme solution was added to the substrate, and the amount of *p*-nitrophenol released was determined by monitoring the absorbance at 405 nm spectrophotometerically (DU-650, Beckman, Palo Alto, CA).

Xylan Hydrolysis by Chimeric Xylanases

A reaction mixture containing 150 μl of McIlvaine buffer (pH 7.0), 50 μl of 1% (*v*/*w*) BSA and 250 μl of 1% soluble birchwood xylan solution was equilibrated at 30 °C for 5 min, and reactions were initiated by the addition of 50 μl of the enzyme (the final concentrations of XlnB, XlnC, BSC, BFC, CSB, and CFB were 52, 95, 65, 150, 62, and 62 μg/ml, respectively. The amount of enzyme was adjusted by the production of reducing sugar from soluble birchwood xylan. After 0-, 10-, 20-, 30-, and 60-min incubations, the reaction was terminated by boiling for 5 min. The reducing power generated from the soluble xylan was determined by the Somogyi-Nelson method [15].

Results and Discussion

Construction of Chimeric Xylanases and Expression in *E. Coli*

XlnB and XlnC from *S. lividans* belonging to GH11 xylanases shared 68% amino acid identity (Fig. 1a). Despite the significant similarity of the deduced amino acid sequences, the enzymatic features, especially optimum temperature and thermostability, of the two xylanases were quite distinct. XlnB shows lower temperature optimas and thermal stability as compared to the one from XlnC. To analyze these properties further as well as to study the effect of the *exchange* of homologous segments in the C-terminal region, four chimeric xylanases derived from two genes were constructed.

Construction of chimeras was carried out by exchanging the DNA fragments produced by restriction enzyme digestion at *Sal*I and *Fba*I sites, which are conserved in the C-terminal homologous region of XlnB and XlnC and existed uniquely at analogous sites in the two genes (Fig. 1a). Schematic representation of the structure of the chimeric xylanase gene is shown in Fig. 1b. Chimerias BSC and CSB were constructed by homologous exchanges of XlnB and XlnC at *Sal*I site. Two additional chimeras BFC and CFB were obtained similarly by reciprocal exchange at *Fba*I site. Chimeric BSC and BFC shared 133 and 212 amino acid residues of XlnB's N-terminal region with 102 and 19 amino acid residues of XlnC's C-terminals, whereas chimeric CSB and CFB shared 138 and 221 amino acid residues of XlnC's N-terminal region with 100 and 19 amino acid residues of XlnB's C-terminals. Therefore, chimeric xylanases BSC and BFC possess the C-terminal region of XlnC,

whereas CSB and CFB possess the C-terminals of XlnB. The nucleotide sequences of the DNA chimeras were determined to confirm that they encoded the corresponding amino acid sequences of their respective parents.

Xylanase activity expressed by recombinant *E. coli* cells were initially confirmed on LB-plates containing RBB-xylan as substrate and then confirmed in crude cell-free extracts harvested from recombinant *E. coli* cells against the same substrate. All of the chimeric xylanases exhibited enzymatic activity when assayed. Enzymatic activity is a sensitive criteria for determining correct folding of engineered proteins, indicating that the protein chimeras, BSC, BFC, CSB, and CFB, were folded functionally.

Characterization of Chimeric Xylanases

Parental and chimeric xylanases were purified by FPLC system using HisTrap chelating column and Q-Sepharose or a SP-Sepharose column. The molecular weight and purity of the purified enzymes were analyzed by SDS-PAGE. The purified enzymes were subjected to characterization.

Like other enzyme chimera [16, 17], BSC, BFC, CSB, and CFB also shared properties with their parents, XlnB and XlnC. The pH optima of XlnB and XlnC were observed as 7.0. Similarly, four chimeric BSC, BFC CSB, and CFB also showed optimal activity at pH 7.0. The pH stabilities of XlnB and XlnC were observed between pH 6.0–8.5 and 4.0–9.5, respectively, whereas BSC, BFC, CSB, and CFB displayed stability within the pH ranges of 4.5–9.0, 4.5–8.5, 6.0–8.0, and 6.0–8.5, respectively (Fig. 2). Substitution of segments in the homologous C-terminal region seems to have no influence on pH stability. In a study on chimeric β-glucosidases from *Cellvibrio gilvus* and *Agrobacterium tumefaciens*, pH activity profiles were found to be influenced by the C-terminal parts [16].

The chimeric xylanases also exhibited a significant variation in temperature optimum from their parent enzymes (Fig. 3). The chimeric enzymes were optimally active at 40–5 5°C, showing an intermediate temperature optimum between XlnB (40 °C) and XlnC (60 °C). BSC exhibited maximum activity at 55 °C and 80% of its maximum activity at 60 °C. BFC was optimally active at 50 °C and exhibited 40% of its maximum activity at 60 °C. On the other hand, CSB exhibited maximum activity at 50 °C and 20% of its maximum activity at

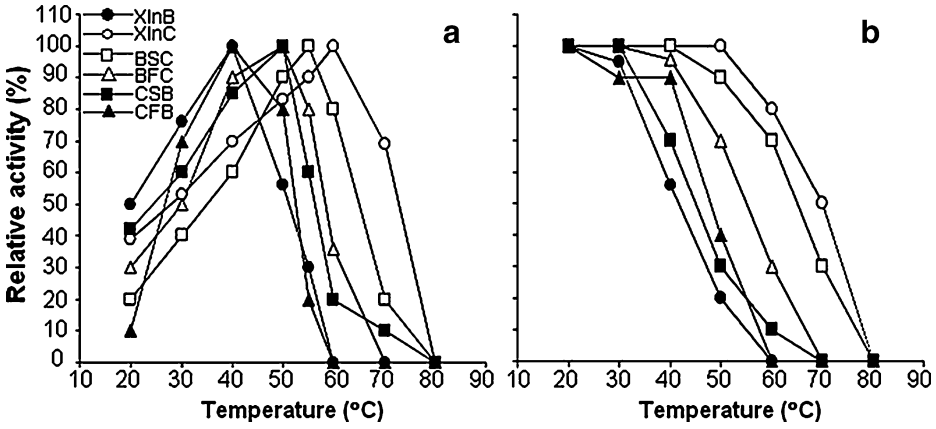

Fig. 3 Optimum temperature (**a**) and thermal stability (**b**) of parental and chimeric xylanases

60 °C, whereas CFB showed the temperature optima of 40 °C with no activity at 60 °C. With regard to *thermal* stability, XlnB shows complete activity up to 20 °C, retains about 55% of its maximum activity at 40 °C, and inactivates completely at 60 °C. On the other hand, XlnC is stable up to 55 °C, and, even at 65 °C, it retains 60% of its maximum activity. *Thermal* stability experiments revealed that BSC was completely active up to 45 °C, retained about 65% of its maximum activity at 65 °C, and was completely inactivated at 80 °C. BFC was stable up to 40 °C, retained about 70% of its maximum activity at 50°C, and was completely inactivated at 70°C. CSB was completely active up to 30 °C and, at 40 °C, 70% of its maximum activity was retained. CFB was least the stable among the four chimeras. It was stable up to 25 °C and retained only 20% of its maximum activity at 55 °C. The temperatures at which 50% loss of the enzyme activities occurred were 43, 70, 68, 56, 47, and 49 °C for XlnB, XlnC, BSC, BFC, CSB, and CFB enzymes, respectively. Thus, *thermal* stability of chimeric BSC and BFC was increased by 25 and 13 °C separately as compared to one of parental XlnB, whereas the thermal stability of chimeric CSB and CFB was decreased by 23 and 21 °C, respectively, as compared to another parental XlnC. To summarize, the thermal stability variations based on the exchanged fragments, the 13–25°C enhancement in thermal stability was obtained when 8–43% of the XlnB C-terminal were replaced by the corresponding XlnC regions, whereas the 21–23 °C decrease in thermal stability was observed after 8–43% of the XlnC C-terminal were replaced with the relevant XlnB fragments. Therefore, the thermal stability was changed distinctly by substituting different C-terminal segments of XlnC gene with that of XlnB. It is noteworthy that the ranges of enhancement or decrease in thermal stability of chimeras were dependent on the length of exchanged fragments of C-terminal region from XlnB or XlnC. *Replacement* of larger fragment of XlnB C-terminal region with that of XlnC increased the thermal stability of chimera significantly, whereas exchanging small part improved the thermal stability slightly. Thermal stability was also found to be in the order of BSC>BFC>CFB>CSB. These data suggested that the amino acid residues corresponding to C-terminal fragment of XlnC DNA were partly responsible for inducing thermal stability in chimeric BSC and BFC.

Thermal stability may be influenced by only a few amino acid substitutions [18, 19]. In general, protein stability increases with the insertion into an α-helix of helix-forming amino acids (alanine, glutamic acid, etc.) and decreases with the insertion of helix-breaking amino acids (proline, glycine, etc.). The secondary structures of the parental and chimeric enzymes were predicted by Robson's method [20]. There were similar numbers of helix-breaking but more helix-forming amino acid residues in the α-helix regions of XlnC, chimeric BSC, and BFC than XlnB, chimeric CSB, and CFB (data not shown), suggesting that it could be one of the factors influencing the thermal stability of chimeras. Hydrophobic interaction inside the protein molecule is another important factor in stabilizing protein structure. Hydrophobic cluster analysis [21, 22] of native and chimeric enzymes revealed that the amino acids of C-terminal region substitution from XlnB to XlnC significantly increased the hydrophobic properties of the chimeric BSC and BFC, especially BSC. These substitutions might be important for thermal stability of *S. lividans* GH11 xylanase (data not shown).

In chimeric isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophile*, and a mesophile, *Bacillus subtilis*, the stability of each chimeric enzyme was approximately proportional to the content of the amino acid sequence from the *T. thermophile* enzyme [23]. Similarly, Singh and Hayashi [16] have constructed chimeric β-glucosidases from *Cellvibrio gilvus* and *Agrobacterium tumefaciens* and concluded that the C-terminal is important for the stability. In this study, our results also indicate that C-termial region is important for enzyme stability of *S. lividans* GH11 xylanases, and exchanging a segment in this region significantly alter or improve the thermal stability of chimeras.

Table 1 Kinetic parameters of parental and chimeric xylanases for the substrate of *p*-nitrophenyl-β-D-cellobioside.

	XlnB	XlnC	BSC	BFC	CSB	CFB
K_m (mM)	0.68	2.89	1.05	2.06	1.97	1.69
k_{cat} (s^{-1})	0.93	1.99	0.89	2.79	1.03	1.02
k_{cat}/K_m (s^{-1}/mM)	1.37	0.69	0.84	1.35	0.52	0.60

Kinetic Properties and Xylan Hydrolysis of Parental and Chimeric Enzymes

Kinetic parameters were investigated for chimeric BSC, BFC, CSB, and CFB along with parental XlnB and XlnC using *p*-nitrophenyl-β-D-cellobioside (PNP-G_2) as the substrates (Table 1). The K_m values of chimeric BSC, BFC, CSB, and CFB for PNP-G_2 were calculated to be 1.05, 2.06, 1.97, and 1.69 mM, respectively. These are moderate values relative to 0.68 and 2.89 mM measured for XlnB and XlnC, respectively, suggesting that the chimera may inherit particle their characteristics from each parental enzyme. Similarly, the k_{cat} values of chimeric BSC, CSB, and CFB acting on PNP-G_2 were 0.89, 1.03, and 1.02 s^{-1}, respectively. They also lie between 0.99 s^{-1} and 1.99 s^{-1} measured for the parental XlnB and XlnC. But the k_{cat} value of chimeric BFC (2.79 s^{-1}) was higher than that of parental XlnC (1.99 s^{-1}). The k_{cat}/K_m values of chimeric BSC and BFC for PNP-G_2 were in between those of both parental enzymes. However, the k_{cat}/K_m values of chimeric CSB and CFB were slightly lower than that of parental XlnB. Overall, the above results indicated that chimeric enzymes retained an efficient active site, as shown by the fact that their affinity and catalytical activity for PNP-G_2 were not significantly changed compared with the native enzymes from which they were derived.

The activities of the chimeras toward soluble birchwood xylan were determined (Fig. 4). All enzymes acted on soluble xylan, but no differences in the hydrolysis rates between the parents and chimeras were observed.

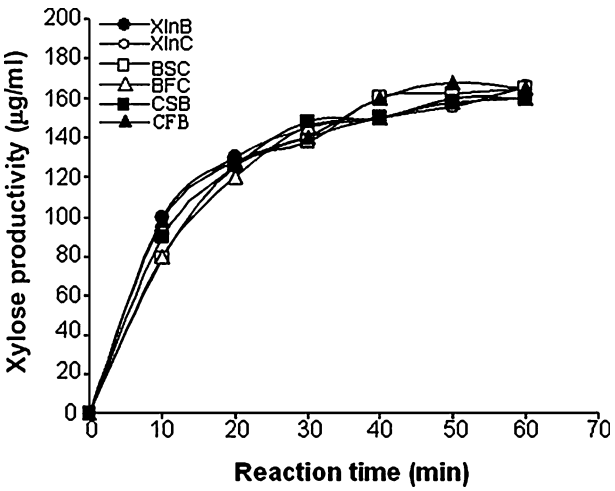

Fig. 4 Xylan hydrolysis by the chimeric enzymes. Soluble birchwood xylan was incubated at 30 °C with enzyme concentrations of 52, 95, 65, 150, 62, and 62 μg/ml for XlnB, XlnC, BSC, BFC, CSB, and CFB, respectively. After 0-, 10-, 20-, 30- and 60-min incubations, the reducing power generated from the soluble xylan was determined by the Somogyi [16]

Conclusions

Genetic construction of chimeric enzymes from two functionally related proteins sharing extensive sequence similarity is expected not only to provide valuable information on the structure–function relationship of the parent proteins but also to prepare enzymes with improved properties. Enzymatic activities are one of the sensitive criteria for judging the correct folding of engineered proteins. Our results demonstrate that different combinations of homologous C-terminal regions of XlnB and XlnC from *S. lividans* resulted in the formation of enzymatically active chimeric species. The C-terminal region in the XlnB and XlnC genes plays an important role in determining enzyme characteristics, especially thermal stability. Exchanging of different segments of gene in this region might significantly alter thermal stability. Chimeric xylanases with improved enzymatic properties can be prepared in a convenient and effective way by manipulating this region.

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