Enhancement of the thermostability and hydrolytic activity of xylanase by random gene shuffling

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The thermostability of *Streptomyces lividans* xylanase B (SlxB-cat) was significantly increased by the replacement of its *N*-terminal region with the corresponding region from *Thermomonospora fusca* xylanase A (TfxA-cat) without observing a decrease in enzyme activity. In spite of the significant similarity between the amino acid sequences of the two xylanases, their thermostabilities are quite different. To facilitate an understanding of the contribution of structure to the thermostability observed, chimaeric enzymes were constructed by random gene shuffling and the thermostable chimaeric enzymes were selected for further study. A comparative study of the chimaeric and parental enzymes indicated that the *N*-terminus of TfxA-cat contributed to the observed thermostability. However, too many substitutions decreased both the thermostability and the activity of the enzyme. The mutants with the most desirable characteristics, Stx15 and Stx18, exhibited significant thermostabilities at 70 °C with optimum temperatures which were 20 °C higher than that of SlxB-cat and equal to that of TfxA-cat. The ability of these two chimaeric enzymes to produce reducing sugar from xylan was enhanced in comparison with the parental enzymes. These results suggest that these chimaeric enzymes inherit both their thermostability from TfxA-cat and their increased reactivity from SlxB-cat. Our study also demonstrates that random shuffling between a mesophilic enzyme and its thermophilic counterpart represents a facile approach for the improvement of the thermostability of a mesophilic enzyme.

Key words: chimaera, biobleaching, *Streptomyces lividans*, *Thermomonospora fusca*, xylan.

INTRODUCTION

Xylan is the most abundant hemicellulose in the cell walls of terrestrial plants and is usually associated with the cellulosic and lignin components. Xylan consists of a backbone of β-(1,4)-D-xylopyranoside residues which are commonly substituted with acetyl, arabinose and 4-*O*-methyl-glucuronose residues. A wide variety of micro-organisms is known to produce xylan-degrading enzymes. The enzymic cleavage of β-1,4-xylosidic linkages is performed by xylanases (β-1,4-xylan xylanohydrolase, EC 3.2.1.8). On the basis of amino acid similarities, xylanases are classified into the glycosyl hydrolase families 10 and 11 [1]. Their biotechnological applications are of interest to the animal-feed, food-processing, and pulp-and-paper industries. In particular, xylanase has been found to be effective in reducing chlorine dosage requirements in the Kraft pulp-bleaching process [2]. The xylanases used in biobleaching applications are usually family-11 xylanases. This is because a low-molecular-mass enzyme is desirable for fibre penetration, and a lack of cellulase activity is required to maintain pulp yield and strength. In addition, it is desirable that xylanases used for biobleaching are stable and active under alkaline conditions at high temperatures. There has been a considerable amount of research devoted to identifying new xylanases and improving the properties of wild-types of xylanases to meet the requirements of the pulp-and-paper industry.

Thermomonospora fusca xylanase A is known as one of the most thermostable xylanases, retaining 96 % of its activity even after 18 h at 75 °C [3]. Xylanase A consists of a substrate-binding domain and a catalytic domain connected by a linker region. The amino acid sequence of the catalytic domain of the enzyme shows that it belongs to family 11 and it has 72 % identity with the catalytic domain of *Streptomyces lividans* xylanase B. However, the thermostabilities of the two xylanases are quite different – the stability of *S. lividans* xylanase B decreases gradually above 37 °C [4]. Thus *T. fusca* xylanase A is a thermostable homologue of *S. lividans* xylanase B and, as such, these two enzymes are suitable for studying the mechanistic aspects of thermostability.

DNA shuffling is a powerful approach for studying structure–function relationships. However, to our knowledge, no DNA shuffling work has previously been performed on xylanases. In the present study, we chose the random fragmentation and PCR reassembly method [5,6] and DNA coding from the catalytic domains of xylanase A from *T. fusca* (TfxA-cat) and xylanase B from *S. lividans* (SlxB-cat) to construct a pool of chimaeric xylanases. Resulting chimaeric enzymes displaying thermostability were selected, and the properties of these enzymes were compared with those of the parent enzymes.

MATERIALS AND METHODS

Preparation of TfxA-cat and SlxB-cat genes

The DNA encoding of the TfxA-cat was amplified from the plasmid pD731, which was kindly provided by Professor David B. Wilson (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY, U.S.A.). The PCR primers used were: TfA-N (5′-GTGGGATCCGCCGTGACCTCCAACGA-GACCGGGTACC-3′) and TfA-CdB (5′-GTGCTGCAG*TTA*-GCTGGTGCCCAACGTCACGTTCCAGGACCCGCTGC-TCTG-3′). TfA-N includes the *Bam*HI recognition site (underlined), which occurs just before the first codon of the

Abbreviations used: TfxA-cat, catalytic domain of *Thermomonospora fusca* xylanase A; SlxB-cat, catalytic domain of *Streptomyces lividans* xylanase B; LB, Luria–Bertani; RBB-xylan, 4-*O*-methyl-D-glucurono-D-xylan–Remazol Brilliant Blue R.

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mature enzyme. TfA-CdB includes the *C*-terminal region of the catalytic domain; the termination codon is shown in *italics* and the *Pst*I recognition site is underlined. One base was intentionally changed and is shown in **bold** type. This base was changed to remove the *Bam*HI cleavage site which exists in the original gene.

The gene encoding the SlxB-cat was amplified from the genomic DNA of *S. lividans* 66 mycelium using the following PCR primers: SlB-N (5′-GTGGGATCCGACACGGTCGT-CACGACCAACCAGGAGGGC-3′) and SlB-C(5′-GTG-CTGCAG*TTA*CCCGCCGACGTTGATGCTGGAGCTGCC-3′). SlB-N includes the *Bam*HI restriction site (underlined), which is located just before the first codon of the mature enzyme. SlB-C includes the *C*-terminal region of the catalytic domain; the termination codon is in *italics* and the *Pst*I restriction site is underlined.

Amplification of DNA was performed using a MiniCycler™ (MJ Research, Watertown, MA, U.S.A.) employing the following temperature program: 5 min at 98 °C, 25 cycles of 1 min at 98 °C, 1 min at 65 °C, 1 min at 72 °C, followed by 10 min at 72 °C. After the first 5 min denaturation step the temperature program was paused, TaKaRa LA Taq™ (1.25 units; Takara Shuzo, Shiga, Japan) was added to the reaction mixture (25 μl), and the temperature program was immediately continued. Using this method, GC-rich target genes were efficiently amplified. The PCR products were subcloned into pCR2.1 (Invitrogen) and the nucleotide sequences were confirmed. TfxA-cat and SlxB-cat genes were cleaved by *Bam*HI and *Pst*I and ligated with pQE30 (Qiagen) using the *Bam*HI and *Pst*I sites to obtain pTfxA-cat and pSlxB-cat respectively.

DNase I digestion and fragment reassembly

Approx. 6.0 μg of pTfxA-cat and pSlxB-cat were diluted to 90 μl with distilled water and 10 μl of 10 × DNase I buffer [0.5 M Tris/HCl (pH 7.4)/100 mM $MgCl_2$] was added. These plasmids were digested with DNase I (0.88 unit) at 25 °C for 15 min and the digests were electrophoresed on a 2 %-(w/v)-agarose gel. DNA fragments (< 100 bp) were extracted from the gel and recovered in 50 μl of distilled water. The resultant DNase I digests were mixed (total 6.4 μl) and 1.0 μl of 10 × PCR buffer (Takara Shuzo), 1.0 μl of 25 mM $MgCl_2$ and 1.6 μl of dNTP mixture (2.5 mM each) were added. The subsequent PCR program used was the same as described above, except that annealing was performed at 45 °C. A portion of this reaction mixture (8.0 μl) was added to 0.2 μl of 10 × PCR buffer, 0.2 μl of 25 mM $MgCl_2$ and 1.6 μl of dNTP mixture, and the PCR was repeated. This extra PCR step was necessary to complete the assembly of the DNA.

PCR amplification of the fragment reassembly products

PCR was carried out using 2.5 μl of the fragment reassembly products using the primers 30BU (5′-CTATGAGAGGATCG-CATCACCATCACCATCACGGATCC-3′) and 30PL (5′-CA-GGAGTCCAAGCTCAGCTAATTAAGCTTGGCTGCAG-3′), which correspond to the outer region of the *Bam*HI and *Pst*I sites of pQE30 respectively. The PCR products were digested with *Bam*HI and *Pst*I, purified by agarose-gel electrophoresis, then ligated into pQE30 between the *Bam*HI and *Pst*I sites. The recombinant plasmids were transformed into *Escherichia coli* M15[pREP4] (Qiagen) and plated on to Luria–Bertani (LB) agar containing 50 μg/ml ampicillin, 50 μg/ml kanamycin and 0.5 % 4-*O*-methyl-D-glucurono-D-xylan–Remazol Brilliant Blue R (RBB-xylan; Sigma). The colonies which were capable of acting on RBB-xylan were picked for screening.

Screening for thermostable chimaeric enzymes

The colonies of recombinant *E. coli* were transferred on to an RBB-xylan plate [0.5 % RBB-xylan and 1.5 % agar in 0.4 × McIlvaine buffer (1 × McIlvaine buffer is 0.1 M citric acid/0.2 M Na_2HPO_4), pH 5.7] using toothpicks and the plate was incubated at 60 °C for 1 h. The plate was then left overnight at room temperature. 'Successful' colonies were identified by their ability to form a distinct halo in the RBB-xylan-containing agar.

Enzyme production and purification

The recombinant *E. coli* was grown at 37 °C in 100 ml of LB medium containing ampicillin and kanamycin until an attenuance (D_{600}) of 0.7 was reached. A 100 μl portion of 1 M isopropyl 1-thio-β-D-galactoside was added and incubation was continued for a further 5 h. The cells were harvested by centrifugation at 10000 ***g*** for 15 min and resuspended in 50 mM phosphate buffer, pH 7.0 (5 ml). The cells were sonicated three times for 2 min each time in a Branson model 250D sonifier, and cell debris was removed by centrifugation. The supernatant was subjected to FPLC (Pharmacia Biotech FPLC system) using a column of HiTrap chelating resin charged with Ni^{2+} and equilibrated with 50 mM phosphate buffer, pH 7.0. After application of the enzyme, the column was washed with 50 mM phosphate buffer, pH 7.0, followed by the same phosphate buffer containing 100 mM imidazole. The enzyme was then eluted with 250 mM imidazole in the same phosphate buffer.

Assay of xylanase activity and protein determination

Soluble birchwood xylan (Fluka), prepared as described previously [7], was used in the determination of xylanase activity. Assays were performed in 0.4 × McIlvaine buffer, pH 5.7, containing 5 mg/ml soluble birchwood xylan and 0.1 mg/ml BSA. The pH value of the McIlvaine buffer was confirmed at room temperature. After incubation at 50 °C for 30 min, the reducing power released was measured by the Somogyi–Nelson method [8], using D-xylose as the standard. Thermostability was tested by heating enzyme samples (10 ± 2 μg of protein/ml) containing 1.0 mg/ml BSA and 0.4 × McIlvaine buffer, pH 5.7, at 70 °C for various periods of time, then assaying the activity as described above. K_m and k_{cat} values were determined using substrate concentrations ranging from 0.4 to 10 mg/ml xylan. Assays were performed as described above, except that the reaction mixture was incubated for 5 min at 40 °C and the production of reducing sugar was measured by using *p*-hydroxybenzoic acid hydrazide [9]. Protein concentration was measured using DC Protein Assay Reagents (Bio-Rad) with BSA as the standard.

RESULTS

Construction and screening of chimaeric xylanases

The amino acid sequence similarities of TfxA-cat and SlxB-cat are shown in Figure 1. TfxA-cat and SlxB-cat consist of 189 and 191 amino acid residues respectively, with 148 residues being identical. The recombinant enzymes exhibit the same activity as the native enzymes when soluble xylan is used as the substrate. Both *T. fusca* xylanase A and *S. lividans* xylanase B are known to be composed of a catalytic domain, a binding domain and a linker region [3,10]. It has been reported previously that the removal of the substrate-binding domain from the native enzyme has no effect on the hydrolysis of soluble xylan [3,11]. Both TfxA-cat and SlxB-cat were functionally expressed in *E. coli* and showed similar properties to those reported for the native

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          1       10        20        30        40        50
                =B1=>    ==B2==>   =A2=>     =A3=>     ==B3=
SlxB-cat  DTVVTTNQEGTNNGYYYSFWTDSQGTVSMNMGSGGQYSTSWRNTGNFVAG
TfxA-cat  --A··S·ET·YHD··F·····AP·····EL·P··N·············

                60        70        80        90       100
          =>      ====A5===> ====B5===>    ==B6b=> B6a>  co
SlxB-cat  KGWANGGRRTVQYSGSFNPSGNAYLALYGWTSNPLVEYYIVDNWGTYRPT
TfxA-cat  ····T·····T··A·········T·····R·········ES·······

                110       120       130       140       150
          rd  =B9=>   ===B8===>  thumb    ==B7==>     =A6>
SlxB-cat  GEYKGTVTSDGGTYDIYKTTRVNKPSVEGTRTFDQYWSVRQSKRTGGTIT
TfxA-cat  ·T·M····T···········Y·A··I·················S····

                160       170       180       190
            ---helix---       ===B4==>  ===A4==>
SlxB-cat  TGNHFDAWARAGMPLGNFSYYMIMATEGYQSSGSSSINVGG-
TfxA-cat  A·········H··H··THD-·············NVTL·TS
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Figure 1 Amino acid sequence comparison of SlxB-cat and TfxA-cat

The SlxB-cat and TfxA-cat genes were amplified by PCR and the amino acid sequences shown were deduced from the nucleotide sequences. The amino acid numbering corresponds to SlxB-cat. The secondary-structure elements (arrows indicate β-strands and dotted lines indicate helices) refer to xylanase XYNII from *T. reesei* [19]. For TfxA-cat, only the amino acids which differ from SlxB-cat are shown.

enzymes [3,4]. Using the random-gene-shuffling technique, the DNA fragments encoding the catalytic domains TfxA-cat and SlxB-cat were used in the construction of a pool of chimaeric xylanase genes. Equimolar mixtures of DNase I digests of TfxA-cat and SlxB-cat genes were reassembled by PCR without primers. The resultant shuffled genes were digested with *Bam*HI and *Pst*I, and inserted into the expression vector pQE30, then transformed into *E. coli* by electroporation. For the selection of a chimaeric enzyme with higher thermostability than SlxB-cat, recombinant *E. coli* colonies produced by the random-gene-shuffling method were placed on to RBB-xylan plates and incubated at 60 °C for 1 h. Theoretically, colonies capable of acting on RBB-xylan after this treatment possessed either the TfxA-cat gene or were thermostable mutants, because these incubation conditions inactivated SlxB-cat. In cases where the recombinant enzyme is not stable at 60 °C, only a small halo, due to residual activity, is produced. A small halo may also indicate low specific activity, even if the enzyme is relatively thermostable at 60 °C. Using this screening method, 18 colonies were selected from a total of 48 colonies as thermostable mutants relative to SlxB-cat.

Sequence analyses of the chimaeric xylanases

The amino acid sequences of the chimaeric xylanases were determined and are shown in Figure 2(A), along with the halo sizes after 1 h incubation at 60 °C. The sequences indicate that DNA shuffling of the TfxA-cat and SlxB-cat genes was successful. From the 18 thermostable colonies, only 15 different chimaeric genes were obtained, since three colonies were found to share identical sequences and also another two colonies were found to

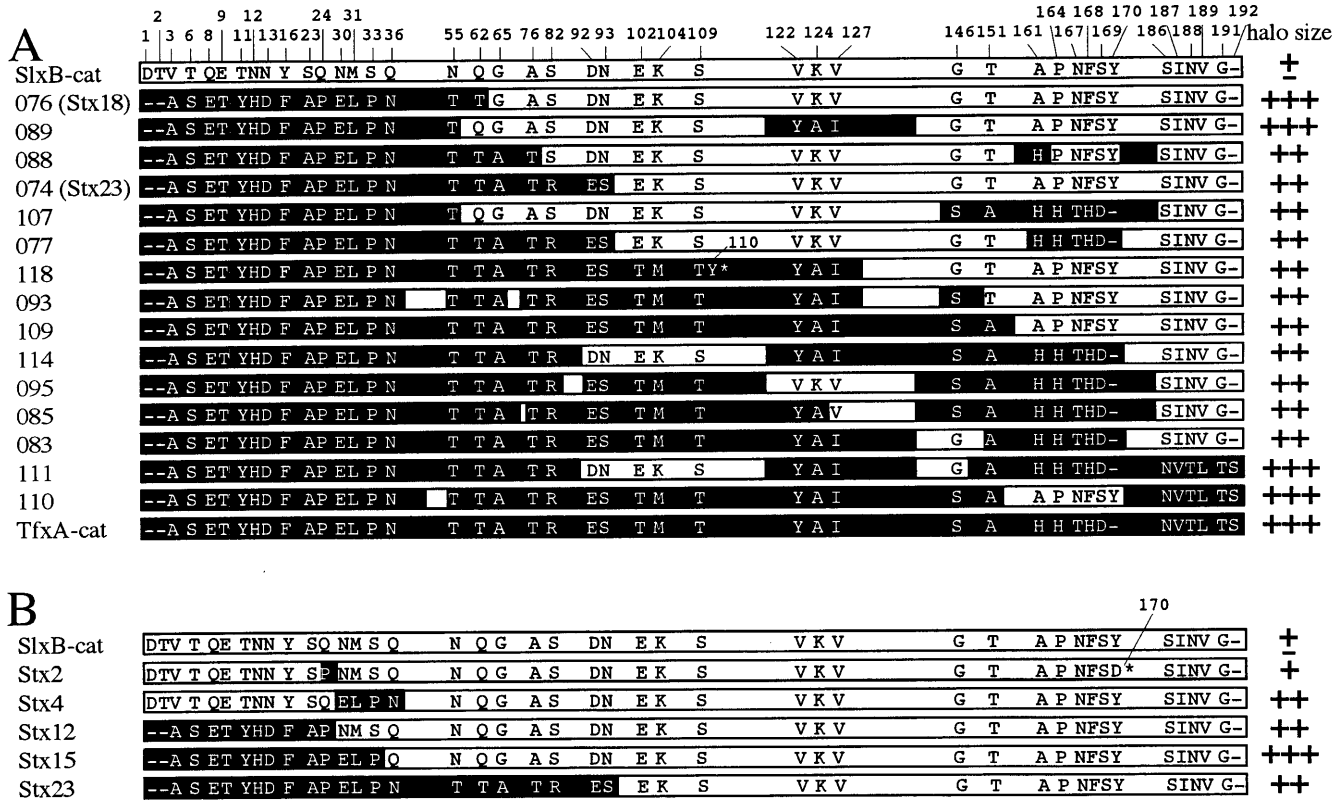

Figure 2 Schematic diagrams of parental and chimaeric enzymes and their residual activities on RBB-xylan plates

(**A**) DNA shuffling was carried out with an equimolar mixture of the DNase I digests of TfxA-cat and SlxB-cat genes. The colonies of recombinant *E. coli* were transferred on to an RBB-xylan plate. The plate was incubated at 60 °C for 1 h and then left overnight at room temperature. Colonies that were capable of acting on RBB-xylan after this treatment were selected. (**B**) DNA shuffling and the following screening methods were the same as described above except that SlxB-cat and Stx23 genes were used for the construction of a pool of chimaeric xylanase genes and were mixed in the ratio 3:1. The halo size is indicated as: $\pm$, <1 mm; $+$, 1–3 mm; $++$, 3–5 mm; $+++$, >5 mm. The 43 amino acids different between TfxA-cat and SlxB-cat are shown in the schematic diagrams. An asterisk (*) indicates a mutational change (D110Y and Y170D).

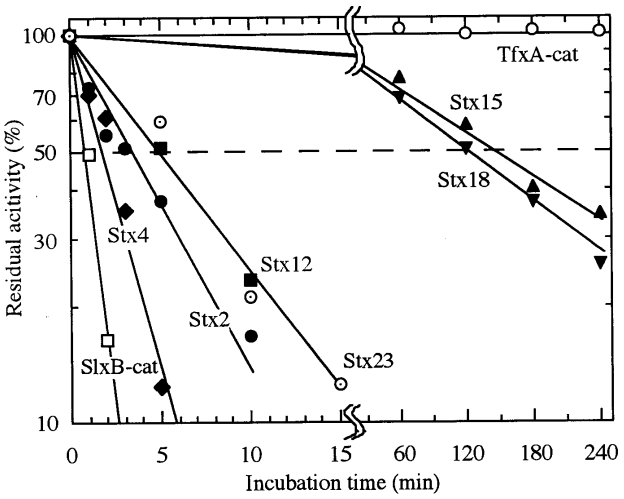

Figure 3 Heat-inactivation time courses of the parental and chimaeric enzymes

The parental and chimaeric enzymes were heated at 70 °C in 0.4 × McIlvaine buffer, pH 5.7, containing 1.0 mg/ml BSA. The residual activity was measured at 50 °C with 5 mg/ml soluble xylan in 0.4 × McIlvaine buffer, pH 5.7.

share the same sequence. In chimaera 118 a one-point mutation (D110Y) occurred, resulting in the amino acid change of Asp^{110} to Tyr.

All of the selected chimaeric enzymes possessed *N*-terminal regions derived from TfxA-cat, indicating that this segment of TfxA-cat was essential for demonstrating activity under the screening conditions. Although chimaera 076 produced the largest halo, it possessed the smallest number of amino acid substitutions relative to SlxB-cat. Amino acid substitutions in the region between the 50th and 100th amino acid residues appear to affect the size of the halo on the RBB-xylan plate. For example, the difference between chimaeras 076 and 074 is due to five additional amino acid residue substitutions from TfxA-cat in chimaera 076, which are located in this region. The halo size produced by chimaera 074 was smaller than that of chimaera 076. By contrast, comparing the sequences of the chimaeric enzymes indicates that the various substitutions occurring between the 100th and the 170th amino acid residues had little effect on the halo size. These observations suggest that amino acid substitutions occurring in the *N*-terminal region are essential for activity at high temperature; however, substitutions in other regions have only minor or negative effects on the formation of haloes under the screening conditions employed.

To obtain additional chimaeric enzymes whose *N*-termini were partially replaced by the corresponding region of TfxA-cat, we constructed a pool of chimaeric genes between SlxB-cat and chimaera 074. DNase I digests of the SlxB-cat and chimaera 074 genes were mixed in a 3:1 ratio to decrease the chance of chimaera 074 reconstructing itself or producing mutants with only minor changes. Four colonies were selected from a total of 290 colonies using the same screening method described above, and the amino acid sequences of the four colonies were determined (Figure 2B). These chimaeric enzymes, namely Stx2, Stx4, Stx12 and Stx15, had one, four, 12 and 15 amino acid substitutions relative to SlxB-cat respectively. Stx2 also had another substitution, Y170D ($Tyr^{170} \rightarrow Asp$), resulting from a point mutation occurring in the PCR step. These four different enzymes were selected for further study, along with chimaeras 076 (Stx18) and 074 (Stx23).

Table 1 Kinetic constants for parental and chimaeric enzymes

Xylanase activities were determined at 40 °C using soluble xylan in 0.4 × McIlvaine buffer, pH 5.7. The concentration of soluble xylan employed ranged from 0.4 to 10 mg/ml.

Enzyme	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m
SlxB-cat	1.0	75	75
Stx2	1.9	67	35
Stx4	1.6	86	54
Stx12	1.4	88	63
Stx15	1.2	77	64
Stx18	1.1	77	70
Stx23	1.4	41	29
TfxA-cat	1.8	85	47

Thermostability

TfxA-cat, SlxB-cat and the six chimaeric enzymes were purified to homogeneity by SDS/PAGE (results not shown) using a nickel-chelating FPLC column. The residual activities of these enzymes after heating at 70 °C are shown in Figure 3. TfxA-cat was stable at 70 °C, even after incubation for 240 min, in contrast with SlxB-cat, which lost 50 % of its activity after only 1 min. All of the six selected chimaeric enzymes were more stable than SlxB-cat. Stx2 exhibited 50 % of its initial activity after 3 min incubation at 70 °C, indicating that having only two amino acid substitutions relative to SlxB-cat increased the half-life of the enzyme three-fold. Stx4, having four amino acid substitutions relative to SlxB-cat, also displayed an increase in half-life under these conditions. Significant improvements in thermostability were achieved by Stx15 and Stx18, as they displayed half-lives of about 120 min. Stx12 and Stx23 also showed an improvement in thermostability, but the half-lives of these mutants were far less than those of Stx15 and Stx18.

Reducing-sugar productivity from soluble xylan

Kinetic constants for the parental and chimaeric enzymes are shown in Table 1. The k_{cat}/K_m values obtained for the Stx4, Stx12, Stx15 and Stx18 were intermediate between those of SlxB-cat and TfxA-cat, indicating that these mutants were as functional as the parent enzymes in performing the hydrolysis of xylan. In contrast, k_{cat}/K_m values for Stx2 and Stx23 were lower than the values for the parent enzymes. Both the K_m and k_{cat} values for Stx2 were inferior to the values obtained for the parent enzymes. The K_m value for Stx23 was not significantly different from that of the parent enzymes, but the k_{cat} value was about half that of the parent values.

The xylan-hydrolyzing activities of the chimaeric enzymes were determined at various temperatures and the results compared with those of the parent enzymes (Figure 4). Improvement in the thermostability of Stx4, Stx12, Stx15 and Stx18 was paralleled by an increase in the production of reducing sugar at elevated temperatures. Stx4 and Stx12 were most active at 65 °C, 10 °C higher than the optimal temperature of SlxB-cat. It is noteworthy that Stx4 was significantly more active at 65 °C than SlxB-cat, and this difference is due to only four amino acid

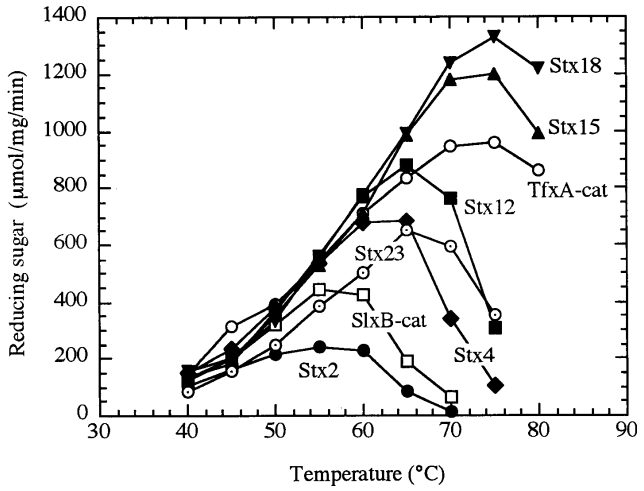

Figure 4 Reducing-sugar productivity of parental and chimaeric enzymes

Enzyme reactions were performed in 0.4 × McIlvaine buffer, pH 5.7, containing 0.1 mg/ml BSA. The data plotted are averages for three independent experiments.

substitutions. Stx15 and Stx18 exhibited maximal activity at 75 °C, which was 20 °C higher than that displayed by SlxB-cat and was equal to that of TfxA-cat. Remarkably, Stx18 produced 1.4 times more reducing sugar than TfxA-cat at 75 °C, although SlxB-cat was inactivated at this temperature. For these four chimaeric enzymes (Stx4, Stx12, Stx15 and Stx18), the enhanced thermostability contributed to an increase in reducing-sugar production at elevated temperatures. Stx23 also exhibited a rise in the observed optimum temperature (65 °C); however, it was less active than the above four chimaeric enzymes when compared at 65 °C. Stx2, which was more stable than SlxB-cat at 70 °C, did not demonstrate an increase in the optimum temperature and showed poor reducing-sugar productivity compared with SlxB-cat. These results indicate that even if a mutant displays good residual activity after heating, it is not necessarily enzymically functional at that temperature.

DISCUSSION

Two thermostable family-11 xylanases were crystallized and structural contributions to their stabilities were predicted. The crystallographic structure of *Bacillus* D3 xylanase revealed that surface aromatic residues form hydrophobic clusters between molecules [12]. These intermolecular hydrophobic contacts induce aggregation at high protein concentrations and may contribute to the thermostability of the xylanase. The crystal structure of the thermostable xylanase from *Thermomyces lanuginosus*, a thermophilic fungus, has been also described [13]. Its thermostability was ascribed to an extra disulphide bridge and ion-pair interactions throughout the protein.

Attempts to improve the thermostability of family-11 xylanases have previously been reported [14,15], but have been only partly successful. These results showed that the increase in residual activity observed after heating does not always contribute to activity at high temperatures. Although the mutants were thermostable compared with the wild-type, they were less active at low temperatures. It is common for significant conformational changes to occur in enzymes during catalysis [16]. The conformational changes occurring in xylanase from *Trichoderma reesei* were studied on the basis of crystal structure [17,18]. Structural changes led to the opening and closing of the active site, and this probably plays a role in the function of the enzyme. Therefore amino acid substitution which enhances the thermostability of an enzyme may often change its molecular structure, causing a deterioration in the enzyme activity observed.

To facilitate an understanding of the contribution of structure to the thermostability, we constructed the chimaeric enzymes of TfxA-cat and SlxB-cat by random gene shuffling. Chimaeric and parental enzymes used in this study were purified to homogeneity on SDS/PAGE and the activity that is seen is directly comparable with that of the parental enzyme. As shown in Table 1, the k_{cat} values for the purified chimaeric enzymes were close to that of the parental enzymes, except for Stx23, indicating that these mutants were as functional as the parental enzymes.

In the present study we revealed that the *N*-terminus of TfxA-cat contributes to thermostability. Stx2, which has two amino acid substitutions, Q24P ($Gln^{24} \rightarrow Pro$) and Y170D, showed an enhancement in thermostability at 70 °C. Of the two amino acid substitutions, Q24P must be responsible for the enhancement of the thermostability, because it was found in all thermostable chimaeric enzymes in the present study, except for Stx4 (Figure 2). Pro^{24} is thought to be located between two β-strands, B2 and A2 [13,19], and seems likely to contribute to structural stability by reducing the entropy of the unfolded state [20]. Stx4, which consists of four amino acid substitutions relative to SlxB-cat, also displayed an enhancement in thermostability. Pro^{33}, which is located in the loop between β-strands A2 and A3 [13,19], is also thought to participate in the stabilization of the enzyme in a similar manner to Pro^{24}. Enhancement of the stabilities for Stx2 and Stx4 were, however, relatively small compared with the enhancements observed in chimaeras Stx15 and Stx18, indicating that proline substitution is only a minor factor in the stabilization of the enzyme.

Significant improvement in thermostability was achieved in Stx15 and Stx18. Stx15 has 13 amino acid substitutions and two eliminations, and can be regarded as a double mutant of chimaeras Stx12 and Stx4 (Figure 2). However, the half-life of Stx15 at 70 °C was much longer than those of Stx12 and Stx4 (Figure 3), indicating that there are other, additional, major stabilizing factors present between the substituted residues in Stx12 and Stx4. Of the 13 residues, 11, namely Ala^{3}, Ser^{6}, Glu^{8}, Thr^{9}, Tyr^{11}, His^{12}, Asp^{13}, Phe^{16}, Glu^{30}, Leu^{31} and Pro^{33}, are predicted to be closely located around the *N*-terminus. Therefore it is likely that these residues are involved in the structural stability of the *N*-terminal region, resulting in a significant increase in the half-life of enzymes containing these residues. The *N*-terminal sequence of *Cellulomonas fimi* xylanase D, another thermostable enzyme from xylanase family 11, is similar to that of TfxA-cat [21]. This also lends support to the importance of this region to thermostability. However, further analysis is required to determine the exact contributions of the *N*-terminal residues to the structural stabilization of xylanases.

For soluble xylan, the K_m value for SlxB-cat was 1.0 mg/ml, which is far less than that observed for TfxA-cat (1.8 mg/ml), although the k_{cat} values for SlxB-cat and TfxA-cat were similar (75 and 85 s^{-1}; Table 1). Stx15 and Stx18 displayed K_m values of 1.1 and 1.2 mg/ml respectively and k_{cat} values of 77 s^{-1} (Table 1). These results indicate that the catalytic capacities of Stx15 and Stx18 are similar to those observed for SlxB-cat. Therefore it can be said that Stx15 and Stx18 inherit their thermostability from TfxA-cat and their catalytic efficiency from SlxB-cat, resulting in an observed increase in reducing-sugar productivity at high temperatures.

Our results suggest that random shuffling between a mesophilic and a thermophilic enzyme is an effective approach for combining

the activity and the stability from each of the parental enzymes. In general, the evolutionary-design approach produces an increase in the thermostability of a mesophilic enzyme far more readily than a rational approach [22]. In addition, the use of naturally occurring sequences increases the functional sequence diversity of the chimaeric library, allowing rapid searching of a large sequence space [23]. When a mesophilic and a thermophilic homologue are available as the starting genetic material, DNA shuffling of these enzymes is thought to be a facile approach for increasing the thermostability of the mesophilic enzyme without cost to its activity.

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