Thermal Stabilization of *Bacillus subtilis* Family-11 Xylanase by Directed Evolution*

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We used directed evolution to enhance the thermostability of glycosyl hydrolase family-11 xylanase from *Bacillus subtilis*. By combining random point mutagenesis, saturation mutagenesis, and DNA shuffling, a thermostable variant, XylT, was identified which contained three amino acid substitutions: Q7H, N8F, and S179C. The half-inactivation temperature (the midpoint of the melting curves) for the XylT variant compared with the wild-type enzyme after incubation for 10 min was elevated from 58 to 68 °C. At 60 °C the wild-type enzyme was inactivated within 5 min, but XylT retained full activity for at least 2 h. The stabilization was accompanied by evidence of thermostability; that is, an increase in the optimal reaction temperature from 55 to 65 °C and lower activity at low temperatures and higher activity at higher temperatures relative to wild type. To elucidate the mechanism of thermal stabilization, three-dimensional structures were determined for the wild-type and XylT enzymes. A cavity was identified around Gln-7/Asn-8 in wild type that was filled with bulky, hydrophobic residues in XylT. This site was not identified by previous approaches, but directed evolution identified the region as a weak point. Formation of an intermolecular disulfide bridge via Cys-179 was observed between monomers in XylT. However, the stability was essentially the same in the presence and absence of a reducing agent, indicating that the increased hydrophobicity around the Cys-179 accounted for the stability.

Xylanases (EC 3.2.1.8) catalyze the hydrolysis of xylan, a major constituent of hemicellulose. The enzyme is the focus of much attention because of its potential for use in industrial processes, including paper and pulp industries and food and feed industries (1, 2). Many efforts have been made to improve the properties of the glycosyl hydrolase family-11 (previously family G) xylanase to handle industrial tasks. In particular, thermostability is a major target of such modifications (3–7). However, the majority of these studies have been performed by site-directed mutagenesis based on high resolution three-dimensional structures, and solutions that identify functional changes are well beyond our predictive ability with these approaches (8, 9). We, therefore, used a directed evolution technique, an alternative approach to protein engineering that can complement the weaknesses of the site-directed approach (8). Our approach included a global search for weakness using random mutagenesis, an extensive search for the best-fit residue by saturation mutagenesis, and rapid fitness improvement by DNA shuffling. By using these techniques, we attempted to identify positions that confer thermostability and that have not been identified by other methods.

Directed evolution does not require three-dimensional structures but does require a quick and sensitive screening system (10). From this point of view, the high-throughput screening systems developed for this enzyme are a poor fit except for the clear zone (halo) assay (11). With this assay, xylanase-producing bacteria form a clear zone (halo) around the colonies when grown on agar plates containing xylan (or dye-coupled xylan such as Remazol Brilliant Blue to increase sensitivity). Formation of a complex between the dyes and sugars enables us to detect using the naked eye enzyme-producing colonies as a clear zone. Although these assays are simple and quick, the method is limited since the activity needs to be detected under physiological conditions. Therefore, we first attempted to establish a more flexible high-throughput xylanase activity screening system. To achieve this, we modified the dinitrosalicylic acid (DNS) method (12) to a 96-well format. We also used a commercial mild detergent, the BugBuster™ reagent (Novagen), for efficient and consistent extraction of enzymes from *Escherichia coli*. After setting up reliable screening conditions, we applied directed evolution and screened for increased thermostability. We successfully obtained a thermostable variant that retained full activity at 60 °C for more than 2 h, whereas the wild type inactivated in ～5 min.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Taq DNA polymerase was purchased from Takara Bioneer (Shiga, Japan); PfuTurbo® was purchased from Stratagene (La Jolla, CA); DpnI was purchased from New England Biolabs (Beverley, MA); CircleGrow® was purchased from BIO101 (Carlsbad, CA); BugBuster™ reagent and benzonase nuclease were purchased from Novagen (Madison, WI); the DiversiTy™ PCR random mutagenesis kit was purchased from Clontech (Mountain View, CA); oligonucleotides were purchased from Japan Bio Services (Saitama, Japan); competent *E. coli* JM109 cells were purchased from Toyobo (Osaka, Japan). Soluble birch wood xylan was kindly provided by Dr. Kazuhiro Ishikawa.

*Mutagenesis—* Plasmid pBSx2, which carries a thermolabile variant (a G510A nucleotide substitution occurs which causes a Met-169 to Ile amino acid substitution) of *Bacillus subtilis* xylanase (13), was cloned into pTDT-tac (14).

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2. The abbreviations used are: DNS, dinitrosalicylic acid; DTT, dithiothreitol; XylT, thermostable variant xylanase of *B. subtilis*.
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TABLE 1
Oligonucleotide sequences used for site-saturation mutagenesis

The target amino acid position was coded by degenerated codon NNK (sense strand, upper sequence) and MNN (antisense strand, lower sequence), where N = A, G, C, T, or K = G or T, and M = A or C.

| Target sites | Oligonucleotide sequences |
|--------------|--------------------------|
| 7 and 8      | 5′−CCATGCTAGCACTAGCTGNNKTKGGGCTGATGGGGGCGGTATAAGT−3′ |
| 5′−ACTAATTTGACCCCGATCACAACMNCCCAAGTAATATGCCTTTGGC−3′ |
| 169          | 5′−CATATTTGACCACTTTCGTCGCMGNNACTGTGAAGCGCCTACTGT−3′ |
| 5′−GGCCAGAAGGATATCAGAAATGAGTGAGNGTCTCTACGTTAAGA−3′ |
| 179          | 5′−TCTTACACACACTGTGTAACGAMNNTCCACTCCCATGTATCACTTGTGCC−3′ |

Error-prone PCR was carried out using a Clontech Diversify™ PCR random mutagenesis kit. A mutation condition, which should generate an error frequency of ~4–5 base substitutions per 1000 bases per gene copy, was used. The reaction mixture contained 40 mM Tricine-KOH (pH 8.0), 16 mM KCl, 3.5 mM MgCl₂, 3.75 mM DTT, 0.64 mM MnSO₄, 40 μM dGTP, 1× Diversify™ dNTP mix, 10 μM concentrations of each flanking primers, 1 ng of pBSx2, and 5 units of Taq polymerase in a total volume of 50 μL. The solution was heated at 94 °C for 30 s followed by 25 cycles of incubation at 94 °C for 30 s and 68 °C for 1 min. Products were purified and cloned back into the pTD-tac vector. The plasmid library thus obtained was used to transform competent E. coli JM1109.

Site-saturation mutagenesis was carried out using a pair of oligonucleotide primers. The sequences of oligonucleotides are listed in Table 1. The target amino acid position was coded by NNK (sense strand) and MNN (antisense strand), where N = A, G, C, or T, K = G or T, and M = A or C. The reaction was carried out following the Stratagene QuikChange™ site-directed mutagenesis protocol (15). Briefly, a pair of primers was mixed in 1× Pfu Turbo buffer, 50 ng of pBSx2, 10 μM concentrations of each primer, 0.2 mM concentrations of each dNTP, and 2.5 units of Pfu Turbo® DNA polymerase in a total volume of 50 μL. The solution was heated for 30 s at 95 °C followed by 20 rounds of incubation at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min. After cooling down the mixture to 4 °C, 10 units of DpnI was added to the products and incubated at 37 °C for 2 h to digest the template pBSx2. The mixture was used to transform competent E. coli JM1109.

DNA shuffling was carried out using the SteEP method (16). The reaction contained ~0.2 μg each of parent plasmid DNA, 1× Taq buffer, 0.2 mM concentrations each of dNTP, 0.15 μM concentrations of each flanking primer, and 2.5 units of Taq polymerase in a total volume of 50 μL. The mixture was heated at 95 °C for 5 min and then subjected to 80 rounds of thermal cycling at 94 °C for 30 s and 55 °C for 15 s in a Stratagene Robocycler. The product was purified and cloned back into the pTD-tac vector. The plasmid library thus obtained was used to transform competent E. coli JM1109.

Library Screening—E. coli JM109 cells harboring wild-type or mutant xylanase genes were grown overnight in LB medium (1.6% Tryptone, 1.0% yeast extract, and 0.5% NaCl) containing 100 μg/ml ampicillin at 37 °C. The culture was then diluted 1/1000 in a fresh LB medium (1 liter) containing 100 μg/ml ampicillin and 1 mM isopropyl-β-d-thiogalactopyranoside and agitated at 37 °C for 12 h. Cells were harvested by centrifugation (5000 × g, 10 min, 4 °C) and suspended in 25 ml of BugBuster™ containing 25 μL of benzozone nucleace. Proteins were extracted by agitation at room temperature for 20 min. Cell debris was removed by centrifugation (20,000 × g, 20 min, 4 °C), and supernatant was applied to a Resource S® (Amersham Biosciences) column (6.4 mm inner diameter × 3 cm) pre-equilibrated with 20 mM sodium acetate buffer (pH 5.5). The column was rinsed with 20 ml of the buffer, and proteins were eluted with a linear gradient of NaCl from 0 to 0.2 M. Active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). Then proteins were applied to a TSK gel CM-SPW (Tosoh, Tokyo, Japan) column (7.5 mm inner diameter × 7.5 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was rinsed with 20 ml of the buffer, and proteins were eluted with a linear gradient of NaCl from 0 to 0.4 M. Active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). The concentration of purified protein was determined using the extinction coefficient (ε280 = 4.08) as reported previously (17).

Thermal Inactivation—Half-lives of thermal inactivation of wild-type and Xylst enzymes were determined by incubating the enzymes (0.1 mg/ml) at 60 or 65 °C in 20 mM sodium acetate buffer (pH 5.5) containing 0.2 mM NaCl and 1 mM DTT. At various time intervals, 10-μL aliquots were removed and diluted into 90 μL of assay solution containing 0.1 M Mes-NaOH (pH 5.7) and 0.1% (w/v) soluble xylan for the measurement of residual activity at 37 °C. Inactivation profiles of wild-type and Xylst enzymes were plotted by incubating the enzymes (0.1 mg/ml) at various temperatures (from 30 to 85 °C) for 10 min followed by determining the residual activity at 37 °C using 10 μL of enzymes in 90 μL of assay solution containing 0.1 M Mes-NaOH (pH 5.7) and 0.1% (w/v) soluble xylan.

X-ray Analysis—The initial screening of the crystallization conditions was carried out according to the sparse-matrix sampling method.
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(18) using Crystal Screen (Hampton Research, Aliso Viejo, CA) and Wizard and Cryo Screens (DeCODE Genetics, Reykjavik, Iceland). Crystallization was performed using the hanging-drop vapor diffusion method (19) at 20 °C using a 24-well VDX plate (Hampton Research). Before crystallization, wild-type xylanase and Xylst were concentrated to 17.7 and 17.4 mg/ml, respectively. The size of the droplet, which consisted of equal volumes of protein and reservoir solution, was 1 μl. The initial conditions under which crystals appeared were refined by varying the pH of the buffer and the concentration of the precipitant.

Diffraction data of the wild-type xylanase crystal were collected at beamline BL6A and the Xylst crystal were collected on NW12 of the Photon Factory, KEK, Tsukuba, Japan at a wavelength of 1.000 Å. Before data collection, the crystal was in the crystallization solution containing 20% glycerol then mounted on a nylon fiber loop and immersed in liquid nitrogen for freezing. The diffraction data were processed using HKL2000 (20) and the CCP4 program suite (21).

Structures of wild-type xylanase and Xylst were determined by a molecular replacement method with the program MOLREP (22) in the CCP4 package using the structure of Bacillus circulans xylanase (PDB codes 1XNB) as a search model. CNS (23) and REFMAC5 (24) were used for structural refinement. A randomly chosen 5.0% of the data were used to calculate the free R factor (25).

RESULTS AND DISCUSSION

High-throughput Screening System for Xylanase Thermostability—Because Miller (12) reported the use of DNS reagent to determine the concentration of reducing sugars released, the DNS method has routinely been used in determination of glycosidase activities. In this study we modified the DNS method by downsampling all the reaction components to fit the 96-well format. In addition, for consistent extraction of cytosolic proteins from E. coli we modified the DNS method by downscaling all the reaction components to fit the 96-well format. In addition, for consistent extraction of cytosolic proteins from E. coli with high efficiency and to minimize well-to-well or plate-to-plate fluctuations, we used the BugBuster™ protein-extracting reagent. Although the reagent is usually used in a 1× concentration, we identified that a 0.2× concentration was the best. Increasing the concentration of the reagent had an adverse effect; the extraction efficiency of the enzyme did not increase, whereas the solution became more viscous. Because pipetting viscous solution is highly problematic, it was at least in our case not advantageous to use the regular 1× concentration.

To test the validity of our screening system, we performed site-saturation mutagenesis at amino acid position 169. The residue is fully buried in the molecule, and the “solutions” that confer thermostability may be limited; thus, some hydrophobic residues will be accommodated. We previously found that the thermal stability of the enzyme is greatly reduced by altering the wild-type residue Met to Ile.3 We, therefore, used the thermolabile M169I variant and employed saturation mutagenesis to test if our screening was sensitive enough to solve the “problem” correctly.

Eight clones were randomly picked from the saturation mutagenesis library to check for the randomness of the library. DNA sequencing revealed that the site was occupied with 2× Asp (GAT), 1× Phe (TTT), Trp (TGG), Lys (AAG), Ala (GCT), Val (GTG), and Ile (ATT), ensuring the randomness of the library. We then screened for activity and thermostability of 360 randomly picked clones. Approximately half of the clones retained the activity (Fig. 1a). We next screened 164 active clones for thermostability by incubation at 55 °C for 5 min (Fig. 1b). Fourteen thermostable clones that showed essentially the same stability were picked and subjected to DNA sequencing. As a result, the position was occupied with Met (5× ATG) or Leu (2× TTG, 2× CTG, and 5× CTT). Taking the different types of codons in Leu-169 variants into account, our screening system is accurate and sensitive enough to identify thermostable variants of xylanase.

Directed Evolution—The thermolabile M169I variant was used as a starting enzyme to enhance thermostability by directed evolution. To avoid sacrificing activity at low temperature, which is often seen in naturally thermophilic enzymes (26), simultaneous screening for activity at 25°C and thermostability at elevated temperatures was performed.

At first, error-prone PCR was carried out for the entire sequence of the xylanase gene. A total of 744 clones were screened for thermostability by incubation at 55 °C for 5 min. Five thermostable variants, 2D6, 4C3, 4A6, 4D12, and 6B5, were identified, and their DNA sequences were determined (Table 2). Interestingly, some mutations were localized at three sites. Mutations at 169 were identified in 4A6 (I169V), 4D12 (I169L), and 6B5 (I169M), mutations at residues 7 and 8 were identified in 4C3 (Q7H) and 4A6 (N8Y), and mutations at residues 151/152 were identified in 4A6 (A152T) and 6B5 (N151S). To identify true-positive amino acid substitutions, we then conducted recombination experiments of those variants by DNA shuffling (15).

Two StEP libraries were created to recombine variants 2D6/4C3 and 4A6/4D12/6B5. As for the 2D6/4C3 StEP library, 168 clones were screened for thermostability by incubation at 65 °C for 5 min. Two thermostable variants that surpassed the thermostability of parents

3 K. Miyazaki and M. Takenouchi, unpublished result.
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TABLE 2  Thermostable variants of xylanase obtained from the library prepared by error-prone PCR

| Variants | Mutations |
|----------|-----------|
| 2D6      | S179C (535A→T) |
| 4C3      | Q7H (21A→C), N159S (476A→G) |
| 4A6      | N8Y (22A→T), A152T (454G→A), I169V (505A→G) |
| 4D12     | I169L (505A→T), 54t→312a→ct, 363t→cc |
| 6B5      | N114K (342C→A), N151S (452A→G), I169M (507A→G) |

were identified. DNA sequencing showed that both variants contained the same mutations, Q7H (21A→C) and S179C (535A→T). As for the 4A6/4D12/6B5 STEP library, 192 clones were screened for thermostability by incubation at 60 °C for 5 min. Two thermostable variants that surpassed the thermostability of parents were identified. DNA sequencing revealed that one variant contained N8Y (22A→T), N151S (452A→G), and I169M (507A→G), and the other contained N8Y (22A→T) and I169M (507A→G). Because the thermostability was nearly the same for the two variants, the latter variant was used for further study. Although the sites 151 and 152 appeared to be a weak point of the molecule, they did not contribute to enhancing stability in the context of other mutations, N8Y and I169M. The mutations might be false positive mutations, and DNA shuffling did not pick such non-adaptive mutations. There were three amino acid substitutions both in 6B5 and 4A6, and the rest of the mutations (N114K and I169M in 6B5; N8Y and I169V in 4A6) might play a more important role in thermostabilization.

Next, we employed site-saturation mutagenesis (27) to optimize the local sequence at positions 7, 8, and 179. Positions 7 and 8 were regarded as a single site, and we performed two mutagenesis experiments. For positions 7/8, a total of 744 clones were screened for thermostability by incubation at 55 °C for 5 min. Two thermostable variants were identified that contained mutations Q7H (CAT)/N8F (TTT) and Q7H (CAT)/N8F (TTT) (GTG), respectively. The former variant showed slightly higher stability. As for position 179, 192 clones were screened, and 9 thermostable variants were identified. Of these, seven variants contained the same mutations S179C (all in the TGT codon). The rest of the variants, S179Y (TAT) and S179M (ATG), showed a slightly lower stability.

Individually optimized sequences (Q7H, N8F, and S179C) were then recombined by DNA shuffling (16). As a result of the screening of 192 clones for thermostability by incubation at 65 °C for 5 min, 3 thermostable variants were obtained, all of which contained amino acid substitutions Q7H, N8F, and S179C (one variant contained an additional synonymous mutation, t66a). Based on the triple amino acid substitution variant, site-directed mutagenesis was performed to convert Ile169 to Met, to obtain a variant Xylst.

*Thermostability of Purified Enzymes*—To determine thermostability, we purified the wild-type and Xylst enzymes. Because the Xylst contained a single Cys residue at a surface of the molecule, we checked the possibility of dimer formation through intramolecular disulfide bridges. Proteins (wild type and Xylst) were incubated in the presence and absence of DTT (1 mM) and subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, Xylst formed in part a dimer in the absence of DTT, indicating that the enzyme is prone to forming a dimer upon prolonged incubation. To simplify the inactivation experiments, we added DTT thereafter to both Xylst and wild type.

First, the enzymes were heated at various temperatures for 10 min, and residual activities were determined. As shown in Fig. 3, half-inactivation temperatures (the midpoint of the melting curves) shifted ~10 °C upward for Xylst (wild type, 58 °C; Xylst, 68 °C). At 60 °C, the wild-type enzyme was inactivated, whereas Xylst retained full activity. At 65 °C, the wild-type enzyme completely lost activity, but Xylst retained activity. A slight increase in activity was observed for the Xylst enzyme especially at high temperature around 60 °C. This might be caused by heat activation of the enzyme via structural rearrangement as reported previously (28).

Next, half-lives of the enzymes upon heating were determined. As observed in the previous experiment (Fig. 3), the stability of the two enzymes was widely different, and thus, the half-life of the enzymes was not determined under the same temperature. At 60 °C, the wild-type enzyme was inactivated within 10 min (half-life, ~5 min), whereas Xylst was fully active for at least 2 h (Fig. 4A). At 65 °C, the wild-type enzyme was inactivated rapidly, and the half-life could not be determined (Fig. 4B). The half-life of Xylst was ~20 min at 65 °C.

*Temperature Dependence of the Activity*—The specific activities of wild-type and Xylst were determined over a range of temperatures from 20 to 85 °C. As shown in Fig. 5, stabilization of Xylst was accompanied by an increase in the optimal temperature of ~10 °C. The activity of Xylst was slightly lower than that of wild type below 55 °C but higher at elevated temperatures, indicating that the Xylst acquired a therophilic nature after evolution. In our previous studies of temperature adapta-
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TABLE 3
Crystallographic data and statistics of X-ray data collections and refinement of wild-type xylanase and Xylst
Numbers in parentheses represent values for the highest resolution shell.

| Data collection | Wild type | Xylst |
|-----------------|-----------|-------|
| Crystal | P2_1/2,2 | R32 |
| Space group | a = 130.4, b = 186.2, c = 380.0 | a = b = 78.2, c = 372.4 |
| Unit-cell parameters (Å) | | |
| Vcell (Å^3Da^-1) | 2.3 (Z = 20) | 2.7 (Z = 36) |
| Beam line | PF BL6A | PF NW12 |
| Wavelength (Å) | 1.0000 | 1.0000 |
| Resolution (Å) | 1.4 | 1.9 |
| Rmerge^a | 0.044 (0.301) | 0.062 (0.221) |
| Observed reflections | 646,077 | 370,222 |
| Independent reflections | 178,637 | 57,701 |
| Completeness (%) | 97.6 (87.3) | 100.0 (100.0) |
| Multiplicity | 3.7 (2.1) | 10.5 (10.6) |
| (i/σ(i)) | 13.7 (2.5) | 9.4 (3.6) |

| Refinements | | |
| Resolution range (Å) | 20–1.4 | 20–1.9 |
| R-factor | 0.197 (0.246) | 0.1987 (0.226) |
| Free R-factor | 0.217 (0.261) | 0.223 (0.284) |
| Residues | 925 (185 × 5) | 370 (185 × 2) |

| Number of non-hydrogen atoms | Protein | 7,275 | 2,902 |
| Water | 247 | 154 |
| Other molecules | 88 | 11 |
| Root mean square (bond length, angle) | 0.016, 1.70° | 0.009, 1.11° |

^a Rmerge = ΣΣ(If(h)) – I(h))/ΣΣ(If(h)), where I(h) is the mean intensity of a set of equivalent reflections.

R-factor = ΣΣFobs(h) – Fcalc(h))/ΣΣFobs(h), where Fobs and Fcalc are the observed and calculated structure factors, respectively.

FIGURE 4. Thermal inactivation profiles of the purified wild-type (open circle) and Xylst (filled circle) xylanases. Enzymes (0.1 mg/ml) were incubated at 60 °C (A) or at 65 °C (B) in 20 mM sodium acetate buffer (pH 5.5) containing 0.2 mM NaCl and 1 mM DTT. At various time intervals, 10-μl aliquots were removed and mixed in 90 μl of assay solution containing 0.1 mM Mes-NaOH (pH 5.7) and 0.1% (w/v) soluble xylan for the measurement of residual activity at 37 °C.

FIGURE 5. Temperature dependence of activities of wild-type (open circle) and Xylst (filled circle) xylanases. Enzymes (0.1 mg/ml, 10 μl) were mixed in 90 μl of assay solution containing 0.1 mM Mes-NaOH (pH 5.7) and 0.1% (w/v) soluble xylan for the measurement of residual activity at 37 °C for 30 min.

tion of psychrophilic subtilisin S41 to a high temperature (27, 29), we obtained a variant whose activity shifted upward over a wide range of temperature.

Crystal Structure Analysis and Possible Explanation for Thermostability—To elucidate the effect of mutation on thermal stability and activity by comparing wild-type xylanase and Xylst on the basis of structural information, we determined their crystal structures. Crystals of wild-type xylanase were grown from 0.1 M imidazole buffer (pH 7.7–7.9), 1.0–1.1 M potassium/sodium tartrate. Xylst was crystallized in a different form under conditions of 0.1 M Mes buffer (pH 5.9–6.1), 1.1–1.2 M ammonium sulfate, 10% dioxane, and 25 mM DTT. In the wild-type crystal, five molecules exist independently in the asymmetric unit of the cell, and two molecules are contained in the asymmetric unit of the Xylst crystal. Their structures were determined by the molecular replacement method and refined against the 20–1.4 Å intensity data for wild-type xylanase and 20–1.9 Å for Xylst. The crystallographic R-factor and free R-factor of wild-type xylanase were 0.197 and 0.217, respectively. The crystallographic R-factor and free R-factor of Xylst were 0.244 and 0.269, respectively. Table 3 summarizes the crystallographic data, statistics of x-ray data collections, and the result of the structural refinement of wild-type xylanase and Xylst.

The root mean square deviations of all atoms between pairs within five molecules of wild-type xylanase crystal existing in the asymmetric unit range from 0.17 to 0.80 Å, showing no large perturbations incurred by packing in the crystal. Exceptional large deviations (~3.0 Å) occur only on turn regions around residue 120, which is located at the edge of the central groove, protruding from the molecule and exhibiting poor electron density relative to the rest of molecule. Each of the five molecules of B. subtilis xylanase determined in this study can be superposed onto B. circulans xylanase, with the root mean square displacements ranging from 0.42 to 0.73 Å, indicating that their overall three-dimensional structures are practically identical.

Two independent Xylst molecules exist in the asymmetric unit to form an intermolecular dimer by disulfide bond at the mutated residue, Cys-179. They are related by a non-crystallographic two-fold axis as shown in Fig. 6. Only a limited number of surface residues are involved in the interaction between each monomer, suggesting that the three-dimensional structure of Xylst is not affected by intermolecular dimerization in the crystal. The root mean square deviations between wild-type xylanase and Xylst range from 0.38 to 0.8 Å. Furthermore, structural differences at three mutated positions (His-7, Phe-8, and Cys-179) are about the same as those of other positions except for around amino acid position 120, significantly showing that amino acid substitution introduced to Xylst induces no large changes to its three-dimensional structure.
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Fig. 6 shows the location of the mutations. In crystals, the enzyme formed a dimer connecting via the newly introduced Cys-179. Because the wild-type enzyme did not form the dimer in crystal, the mutation must have promoted intermolecular disulfide-bond formation under a crystallization condition. The Cys-179 is surrounded by hydrophobic Val-57 and Ala-59 (Fig. 7). In our site-saturation mutagenesis experiment at residue 179, we identified not only the Cys variant but also the Tyr variant as the second most stable enzymes. Taking the hydrophobic environment around the residue and hydrophobic nature of Cys residue into account (30), the enzyme may have been stabilized by increasing hydrophobic contact between monomers. In fact, the addition of a reducing agent did not greatly alter the stability. Fig. 8 is a close view of the mutation site at residues 7/8 of the Xylst. A closer view around the Cys-179 of the Xylst viewed from the direction of Fig. 6 rotated by 90°.

Amino Acid Sequence Comparison to Natural and Engineered Thermostable Xylanases—We next made amino acid sequence comparisons with a naturally thermophilic xylanase from Bacillus D3 (33). The Bacillus D3 xylanase shares 73% identity with the B. subtilis xylanase, but the difference in thermostability is distinct. The B. subtilis enzyme is not stable at temperatures above 60 °C, whereas Bacillus D3 xylanase is stable even at 75 °C. Position 7 in B. subtilis xylanase (Gln-7) is identical to that of the Bacillus D3 enzyme, which was changed to His after evolution. Residue 8 is Asn in the B. subtilis enzyme, whereas the corresponding residue is Tyr in the Bacillus D3 enzyme. Although the position was changed to Phe in Xylst, we also identified the Tyr variant in the error-prone PCR library (Table 2). As for position 179, the B. subtilis enzyme has Ser, and the corresponding residue is Tyr in Bacillus D3. In our site-saturation mutagenesis experiment, we identified the Tyr variant as well as the Cys variant. This observation agrees with our structural studies, in which a major contribution to thermostability was concluded to be the result of increased hydrophobicity between monomers.

We next compared these findings with the results of site-directed mutagenesis studies of glycosyl hydrolase family-11 xylanases (3–6). Among the three sites, only the Ser-179 was targeted by site-directed mutagenesis (3). In their study, Wakarchuk et al. (3) altered the Ser of B. circulans xylanase to Cys in an attempt to introduce an intermolecular disulfide bridge. The variant, in fact, was prone to forming a dimer at 77% efficiency. Similar to their result, the S179C variant of B. subtilis xylanase also formed a dimer in solution (Fig. 2) and in crystals (Fig. 8). However, the difference in thermostability with or without the reducing agent was nominal. In addition, screening of the site-saturation mutagenesis library identified not only the Cys variant but also the Tyr and Met variants as having near-equivalent stability. Therefore, disulfide bridges may not account for the thermostability, but the hydrophobic nature of the side chain may be critical.

In this study we used directed evolution and evolved the enzyme stability without using the three-dimensional structure. Error-prone PCR quickly identified residues 7/8 as a weak point, and subsequent saturation mutagenesis further improved the stability. By sharp contrast, no studies based on a targeted design have identified the region as a weak point of the molecule even with high resolution crystal structure analysis. Directed evolution is, thus, advantageously used in protein engineering even when the high resolution crystal structure is available.

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