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Structure-based protein engineering for thermostable and alkaliphilic enhancement of endo-β-1,4-xylanase for applications in pulp bleaching

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ABSTRACT

In the pulp bleaching industry, enzymes with robust activity at high pH and temperatures are desirable for facilitating the pre-bleaching process with simplified processing and minimal use of chlorinated compounds. To engineer an enzyme for this purpose, we determined the crystal structure of the Xyn12.2 xylanase, a xylan-hydrolyzing enzyme derived from the termite gut symbiont metagenome, as the basis for structure-based protein engineering to improve Xyn12.2 stability in high heat and alkaline conditions. Engineered cysteine pairs that generated exterior disulfide bonds increased the k_{cat} of Xyn12.2 variants and melting temperature at all tested conditions. These improvements led to up to 4.2-fold increases in catalytic efficiency at pH 9.0, 50 °C for 1 h and up to 3-fold increases at 60 °C. The most effective variants, XynTT and XynTTTE, exhibited 2–3-fold increases in bagasse hydrolysis at pH 9.0 and 60 °C compared to the wild-type enzyme. Overall, engineering arginines and phenylalanines for increased pK_a and hydrogen bonding improved enzyme catalytic efficiency at high stringency conditions. These modifications were the keys to enhancing thermostability and alkaliphilicity in our enzyme variants, with XynTT and XynTTTE being especially promising for their application to the pulp and paper industry.

1. Introduction

Growing concerns regarding the environmental impact of chemical wastes from the paper industry, particularly chlorinated compounds from the bleaching process, have driven development of alternative pulping processes that can reduce or eliminate the requirement for these chlorine compounds. This awareness has led to new technologies in enzyme-based processes, with endo-β-1,4-xylanase for pre-bleaching being the most widely used (Bajpai, 1999). This application of xylanase reduces the use of chlorine compounds for removal of leftover lignin after the chemical pulping process, thereby increasing pulp brightness without concomitant cellulose degradation and decreased pulp strength (Bajpai et al., 2006). Since wood pulp is in a strong KOH alkaline solution after chemical pulping, xylanases would ideally work effectively under such alkaline conditions with relatively high thermal stability.

Unfortunately, most xylanases from microbial sources and commercially available xylanases function in slightly acidic or neutral conditions. Xylanases from *B. agaradhaerens* and *Bacillus* sp. 41M-1 have been reported to function in alkaline conditions but their activity was observed to decline rapidly (Nakamura et al., 1993; Sabini et al., 1999). Such enzymes require pH modification with acid prior to their use, introducing additional steps and chemical use. Researchers have therefore turned to the engineering of xylanases to generate enzymes with the requisite characteristics. Many strategies, including directed evolution and site-directed mutagenesis combined with structure-based design, have been explored to improve thermal stability and activity of acidic and neutral xylanases in industrial conditions. Approaches for improving alkaliphilicity, and thereby enhancing stability, of xylanase include increasing the polar surface with arginine and formation of H-bonds and salt bridges. Replacement of surface serines and threonines of *Trichoderma reesei* xylanase II with arginines slightly elevated the optimal pH from 6.5 to 7 (Turunen et al., 2002). Several charged residues were also introduced in the catalytic cleft of the xylanase Xyl1 from *Streptomyces sp.* S38; an E139 K mutation successfully shifted the optimal pH from 6 to 7.5, likely due to interaction between the engineered lysine and the substrate (De Lemos Esteves et al., 2005).

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Introduction of arginines to the alkaliphilic *Bacillus sp.* 41M-1 xylanase on the surface and to reinforce a salt bridge managed to shift its optimal pH from 8.5 to 9.5, but unfortunately resulted in a dramatic decrease in activity without any obvious improvement in thermal stability (Umemoto et al., 2009). Enhancing such thermal stability can be achieved via disulfide bond formation. It has been reported that a disulfide bond-containing protein is approximately 2 kcal mol^{-1} more stable at 25 °C, has a higher T_m (> 14 °C), and greater enthalpic change for protein denaturation than its non-crosslinked variant (Betz, 1993). Increasing hydrophobic interaction has also been attempted, as seen with *Streptomyces* sp. S38 xylanase where interaction of an engineered tyrosine (T11Y) with a downstream tyrosine (Y16) improved thermophilicity and stability of the enzyme. The mutant's optimal temperature for activity was raised from 60 to 70 °C, and it was characterized by increased T_m (9 °C) and a six-fold enhancement in stability at 57 °C (Georis et al., 2000).

We applied a combination of these enzyme engineering concepts to Xyn12.2, an alkaline endo-β-1,4-xylanase collected from the microbial comm y residing in hindguts of the wood-feeding higher termite *Microcerotermes* sp. in Thailand. Xyn12.2 is a potentially useful enzyme for pulp bleaching as it has shown enzyme activity in a broad pH and temperature range (Nimchua et al., 2012). In this study, we employed structure-based protein engineering and site-directed mutagenesis towards Xyn12.2 modification, and report improvement of Xyn12.2 activity, alkaliphilicity and thermostability. Performance of the engineered xylanases was demonstrated on hydrolysis of beechwood xylan and various biomasses. Increased alkaliphilicity and thermostability in alkaline conditions of these xylanase variants have strong potential for application in pulp bleaching as well as catalyzing biomass hydrolysis for other purposes.

2. Materials and methods

2.1. Protein expression and purification

Escherichia coli Rosetta (DE3) harboring various His-tagged Xyn12.2 genes was grown in LB medium supplemented with 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol at 37 °C overnight. These overnight cultures were then used for recombinant protein expression in 6 L of antibiotic-supplemented ZYM-5052 medium (Studier, 2005) at 30 °C for 30 h with shaking at 200 rpm. Cells were harvested by centrifugation at 8600 × *g*, and recombinant proteins extracted and purified. Briefly, recombinant proteins were prepared by first resuspending the cells in lysis buffer (100 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 8.0) for lysis at 1500 psi using a French pressure cell press (Thermo Dynamics Ltd). Lysates were clarified by centrifugation at 17,400 × *g* twice. Supernatants were purified by Ni^{2+}-NTA affinity chromatography using the Hiprep Q FF 16/10 column (GE Healthcare, Uppsala, Sweden) equilibrated with lysis buffer. After loading, columns were subsequently washed with lysis buffer. The His-tagged proteins were eluted using an imidazole gradient of 20–200 mM in 100 mM sodium phosphate buffer, 500 mM NaCl, pH 8.0. Fractions with xylanase activity were pooled, buffer-exchanged with 20 mM sodium phosphate buffer, 100 mM NaCl, 10% glycerol, pH 7.4, to remove excess imidazole and then concentrated using Amicon Ultra-15 Centrifugal Filter Units with a 10-kDa cut-off (Millipore, Darmstadt, Germany). Protein purity was assessed by 12% SDS-PAGE and its concentration by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

2.2. Protein crystallization and structure determination

Protein crystallization was set up using a microbatch technique (Chayen et al., 1992; Chitnumsub et al., 2004; D'Arcy et al., 1996). Proteins were initially screened in various crystallizing solutions for optimal crystal growth. X-ray diffraction data were collected at 100 K at a wavelength of 1.54 Å using the nonious FR591 X-ray diffractometer coupled with a CCD detector (BIOTEC) or at a wavelength of 1 Å using the ADSC Quantum-315r CCD detector on beamline 13B1 (National Synchrotron Radiation Research Center, Taiwan, ROC.). Data were processed using the HKL-2000 software package (Otwinowski and Minor, 1997). Structural phases were solved by molecular replacement using PHASER (McCoy et al., 2007) in the CCP4 suite with the coordinates of a *Bacillus subtilis* xylanase (PDB code 1IGO; 30% identity) as the template. Structure refinement and adjustment were performed using REFMAC5 (Murshudov et al., 1997; Otwinowski and Minor, 1997) and COOT (Emsley and Cowtan, 2004; Emsley et al., 2010). The structures were validated in PROCHECK (Laskowski et al., 1993).

2.3. Design and construction of Xyn12.2 variants

Mutations were designed based on the crystal structure of Xyn12.2 and generated through site-directed mutagenesis by PCR. Briefly, 50 μL PCR reactions containing 10 ng plasmid DNA template, 0.2 μM forward and reverse primers (Table S1), 0.2 mM of each dNTP, and 1 U of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) were run at one cycle of 1 min at 94 °C, 18 cycles of 30 s at 94 °C, 30 s at 55 °C and 7 min at 68 °C, then 10 min at 68 °C. Amplified products were digested with 20 U of *Dpn*I at 37 °C for 1 h. The constructs were confirmed by nucleotide sequence analysis (Macrogen, Seoul, Korea).

2.4. Xylanase activity assay

Xylanase activity was determined based on the amount of liberated reducing sugar using the 3,5-dinitrosaliccylic acid (DNS) method (Miller, 1959). To determine steady-state catalytic activity of the various xylanases, reaction assays were carried out in triplicate by incubating 320 μL of 1% (w/v) beechwood xylan (Sigma-Aldrich, St. Louis, MO, USA), pH 8.0, and 20 μL purified xylanase at 50 °C for 10 min. Reactions were terminated by addition of 680 μL of DNS along with heating for 10 min at 100 °C. Reducing sugar concentrations were calculated from the final A_{540}, after subtracting the background signal of beechwood xylan alone, based on a D-xylose calibration curve. Steady-state catalytic rates were determined over 10 min with an appropriate amount of enzyme, while rates of enzyme-catalyzed hydrolysis for reducing sugar production were examined at higher enzyme concentrations and incubation times of up to 1 h.

2.5. Lignocellulosic biomass hydrolysis

Lignocellulosic substrates, namely bagasse, corn cob and rice straw, were pretreated with 5% (w/v) NaOH (at a liquor ratio of 3:1) at 80 °C for 90 min. The pretreated biomass was thoroughly washed with distilled water to remove excess alkali. All the pretreated lignocellulosic substrates were dried at 70 °C for 24 h before grinding into the final material used in the study. Biomass hydrolysis was performed in a reaction containing 10 mg lignocellulosic substrate in 900 μL of 100 mM sodium phosphate buffer at pH 7.0, 8.0 or 9.0. One hundred microliters of varying amounts of enzyme (100, 200, 400 or 800 units (U); 1U = 1 μmole of product min^{-1}) was added and reactions were incubated at 50 °C or 60 °C at pH 8.0 or pH 9.0 for 24 h. Amounts of reducing sugar liberated from lignocellulosic substrates were determined using the DNS method.

2.6. Differential scanning fluorimetry (DSF)

Differential scanning fluorimetry with Sypro Orange dye-based readout (Sigma-Aldrich, St. Louis, MO, USA) was conducted to measure xylanase thermodynamic stability (Niesen et al., 2007). Reaction mixtures containing 20 μM of purified enzyme, Sypro Orange and 60 mM sodium phosphate buffer at pH 8.0, 9.0 or 10.0 in the presence or

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