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Structure-based protein engineering of bacterial β-xylosidase to increase the production yield of xylobiose from xylose

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ABSTRACT

Xylobiose consists of two molecules of xylose and has been highly recognized as a food supplement because it possesses high prebiotic functions. β-xylosidase exhibits enzymatic activity to hydrolyze xylobiose, and the enzyme can also catalyze the reverse reaction in the presence of high concentrations of xylose. Previously, β-xylosidase from *Bacillus pumilus* IPO (BpXynB), belonging to GH family 43, was employed to produce xylobiose from xylose. To improve the enzymatic efficiency, this study determined the high-resolution structure of BpXynB in a complex with xylobiose and engineered BpXynB based on the structures. The structure of BpXynB deciphered the residues involved in the recognition of the xylobiose. A site-directed mutation at the residue for xylobiose recognition increased the yield of xylobiose by 20% compared to a similar activity of the wild type enzyme. The complex structure of the mutant enzyme and xylobiose provided the structural basis for a higher yield of the engineered protein. This engineered enzyme would enable a higher economic production of xylobiose, and a similar engineering strategy could be applied within the same family of enzymes.

1. Introduction

Plant cell walls are a complex mixture consisting mainly of cellulose, hemicellulose, and lignin [1]. Xylan is the major non-cellulosic polysaccharide of hemicellulose and constitutes ~30% of the total cell wall dry mass [2]. The backbone of xylan is made of a linear polymer of xyloses linked by β-1,4-glycosidic bonds, and its degradation requires a series of enzymatic actions. β-1,4-xylosidases belonging to the GH43 family have been found in diverse bacteria. These enzymes catalyze the release of xylose units from the non-reducing end of short xylooligosaccharides. The crystal structure of β-1,4-xylosidase from the thermophilic bacteria *Geobacillus stearothermophilus* T-6 (GsXynB3) was determined [3]. The structure revealed that each protomer consists of two domains with a tight dimeric assembly.

Xylooligosaccharides are low-calorie and are used as a soluble dietary fiber because they cannot be broken down by enzymes in the human digestive system [1,4]. Xylobiose (X2) has been utilized as a higher value food supplement, as it has the highest prebiotic ability among xylooligosacharides and is better than that of other non-digestible oligosaccharides for the proliferation of *Bifidobacteria* in the human gut [4–7]. While β-1,4-xylosidases can hydrolyze X2, the enzyme can also catalyze the production of an X2 molecule from two xylose molecules in the presence of high concentrations of xylose. In the latest study, the X2 reaction yield using β-1,4-xylosidase from *Bacillus pumilus* IPO (BpXynB) was 17% in a solution containing 90% (w/v) xylose [8].

To enhance the X2 production yield with BpXynB, we employed a structure-based protein engineering approach. We determined the crystal structures of BpXynB at high resolutions, elucidating the substrate-binding pocket and substrate interacting residues. Based on this structural information, BpXynB was engineered to make

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more interactions with the second xylose unit at the entrance of the substrate-binding pocket.

2. Materials and methods

2.1. Protein expression and purification

We used the plasmid for expression of the wild type BpXynB inserted in a pET21a vector (Novagen, USA), which was described previously [8]. The procedure of protein expression and purification was included in the Supplementary materials.

2.2. Site-directed mutagenesis

To construct the expression vector for the mutant BpXynB proteins (E186Q, F503Y, and E186Q/F503Y), site-directed mutagenesis was performed with *n*Pfu-Special polymerase chain reaction (PCR) premix (Enzynomics, Republic of Korea) and Dpn1 restriction enzyme using the plasmid for the wild type or E186Q mutant enzyme.

2.3. Crystallization and data collection

Single crystals of BpXynB were obtained by the hanging-drop vapor diffusion method using a precipitant solution containing 0.05 M HEPES (pH 8.0), 15% (w/v) polyethylene glycol (PEG) 3350, and 1% (w/v) tryptone. Equal volumes (1 μl) of the protein solution (7 mg/ml) and the reservoir solution were mixed and equilibrated against 500 μl reservoir solution at 14 °C. Single crystals of BpXynB-E186Q (12 mg/ml) and BpXynB-E186Q/F503Y (10 mg/ml) were obtained with a precipitant consisting of 0.05 M HEPES (pH 7.5), 20% (w/v) PEG 3350, 1% (w/v) tryptone, and 30 mM X2, which was obtained from TS Corporation Co., LTD (Incheon, Republic of Korea). Crystals of BpXynB, BpXynB-E186Q/X2, and BpXynB-E186Q/F503Y/X2 appeared after 1 day and were cryoprotected in precipitant solutions supplemented with 25% (v/v) glycerol, 25% (v/v) 2-methyl-2,4-pentanediol (MPD), and 30% (v/v) MPD, respectively, and flash-cooled in a liquid nitrogen stream at −173 °C. X-ray diffraction data sets were collected on beamline 5C and 7A at Pohang Accelerator Laboratory (PAL) (Pohang, Republic of Korea).

2.4. Structural determination and refinement

X-ray diffraction data were processed using HKL2000 software [9]. The structure of BpXynB was determined by the molecular replacement method with MOLREP in the CCP4 package [10] using the structure of β-1,4-xylosidase from *Bacillus subtilis* (PDB code: 1YIF; New York SGX Research Center for Structural Genomics, unpublished data) as a search model. The final structure of BpXynB was refined with PHENIX software suite [11]. The structures of BpXynB-E186Q and BpXynB-E186Q/F503Y were determined by the molecular replacement method using the wild type structure of BpXynB as a search model. X-ray diffraction and refinement statistics are presented in Table 1.

2.5. Multi-angle light scattering analysis

High-performance liquid chromatography (TSK-gel-G3000SWXL column, Tosoh Corporation, Japan) equipped with a multi-angle light scattering (MALS) instrument (Wyatt DAWN Heleos II (18 angles), USA) was employed. The protein sample (4 mg/ml) was applied to the column in a buffer containing 20 mM HEPES (pH 7.0), and 150 mM NaCl. Data analyses were performed with ASTRA 6 software (WYATT, USA).

2.6. Enzyme kinetic assays

The protein 5 μM in 100 mM sodium phosphate buffer (pH 6.5) was prepared as the enzyme solution, and 4-nitrophenyl β-D-xylopyranoside (pNPX) (Tokyo Chemical Industry, Japan) in DDW was prepared as the substrate solutions (0.05 mM, 0.5 mM, 1 mM, and 1.5 mM). Enzyme reactions were initiated by the addition of 50 μl of the substrate solution into 150 μl of the enzyme solution at room temperature. The amount of the chromogenic reaction

Table 1
X-ray diffraction and refinement statistics.

	wild type BpXynB	BpXynB-E186Q	BpXynB-E186Q/F503Y
Data collection			
Beam line	PAL 5C	PAL 7A	PAL 5C
Wavelength (Å)	0.99990	0.97934	0.97940
Space group	*C*2	*P*2	*C*2
Cell dimensions			
a, *b*, *c* (Å)	115.3, 105.2, 105.8	103.6, 105.2, 113.9	116.1, 103.1, 104.0
β(°)	122.7	111.0	122.7
Resolution (Å)	20.0–1.73 (1.76–1.73)	20.0–2.00 (2.03–2.00)	20.0–1.78 (1.81–1.78)
Rmerge	0.080 (0.294)	0.071 (0.334)	0.085 (0.466)
$I/\sigma I$	17.23 (2.63)	16.64 (2.84)	13.86 (1.97)
Completeness (%)	97.5 (92.1)	94.6 (88.7)	96.1 (88.8)
Redundancy	4.7 (3.0)	4.4 (2.8)	4.7 (2.6)
Refinement			
Resolution (Å)	47.2–1.73	30.7–2.00	35.6–1.78
No. reflections	98865	136082	83428
R_{work}/R_{free}	0.155/0.188	0.168/0.214	0.166/0.210
No. of Total atoms	9627	18426	9219
Wilson B-factor (Å)	16.9	22.2	16.7
R.M.S deviations			
Bond lengths (Å)	0.007	0.004	0.007
Bond angles (°)	0.875	0.713	0.916
Ramachandran plot			
Favored (%)	95.2	94.5	95.2
Allowed (%)	4.6	5.0	4.5
Outliers (%)	0.2	0.5	0.3
PDB ID	5ZQJ	5ZQX	5ZQS