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Engineering endoglucanase II from *Trichoderma reesei* to improve the catalytic efficiency at a higher pH optimum

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ABSTRACT

The catalytic efficiency and pH optimum of *Trichoderma reesei endo*-β-1,4-glucanase II were improved by protein engineering. We subjected residue 342 to saturation mutagenesis, and further changed the enzyme by random mutagenesis and two rounds of DNA shuffling. Enzyme variants were purified and characterized. Variant N342V exhibited an optimal activity at pH 5.8, corresponding to a basic shift of 1 pH unit compared with the wild-type enzyme, and had improved catalytic efficiency (1.5-fold of k_{cat}/K_m) for the main substrates at pH 6.2. Variants N342R and N39R/L218H/W276R/N342T both had a pH optimum of 6.2 and the latter had improved catalytic efficiency (1.4-fold of k_{cat}/K_m) at pH 6.2. Variants L218H, Q139R/N342T and Q139R/L218H/W276R/N342T all had more than 4.5-fold higher activity in reactions compared with the wild-type at pH 7.0. The relationship between the structures and the activities of the variants were analyzed by modeling the structures of the endoglucanase II variants. More stable helixes and changed electrostatic interactions between the catalytic residues and substrates may explain the higher activities and higher pH optima of the variants.

1. Introduction

Since the first report on alkaline cellulase by Horikoshi (Horikoshi et al., 1984), alkaline cellulases from bacteria and fungi have been successfully used in laundry detergents. These cellulases also have potential applications in the textile, pulp and paper industries (Schülein, 1997; Anish et al., 2007). However, the yield of bacterial cellulases is normally low (Knowles et al., 1987). Fungal cellulases are more promising from a commercial perspective because they can be produced in rather high yields (Barbesgaard et al., 1984).

However, cellulases derived from fungi have their highest activities at acidic pH. When the pH value moves above 6, their activities are greatly reduced and this limits the application of fungal cellulases under neutral or alkaline conditions. Therefore, one of the research goals for fungal cellulases is to increase the catalytic efficiency of these enzymes at higher pH (Becker et al., 2001; Boer and Koivula, 2003; Le-Nours et al., 2003; Wohlfahrt et al., 2003; Wang et al., 2005).

Trichoderma reesei is the most widely studied and used cellulolytic fungi. It produces at least six genetically different cellulases: two cellobiohydrolases (CBH I and II; EC 3.2.1.91) and four endoglucanases (EG I, II, III, V; EC 3.2.1.4). Endoglucanase II (Cel5A) is one of the most abundant endoglucanases and has the highest activity at pH 4.6–4.8. Cel5A accounts for most of the endoglucanase activity produced by *T. reesei*. Lack of Cel5A production reduces the endoglucanase activity in the culture supernatant by as much as 55% (Suominen et al., 1993). It was the most effective at removing the color from denim and can be used for the degradation of β-glucan in feed (Miettinen-Oinonen and Suominen, 2002). The three-dimensional structure of Cel5A has not yet been determined.

In the last decade, directed evolution has become a key technology for protein engineering, especially for proteins whose 3D structures are not resolved. Directed evolution has been successfully used to modify several proteins (Tao and Cornish, 2002; Höcker, 2005). Previous work in our laboratory has demonstrated that a single substitution of Asn342 by Thr in Cel5A causes a pH optimum shift from 4.8 to 5.4 (Wang et al., 2005). In this report, we describe the successful engineering of *T. reesei* endoglucanase Cel5A, to a higher pH optimum and a higher catalytic efficiency. We used gene site saturation mutagenesis to randomize residues that were identified in our previous study (Asn342). Then the selected clones were further modified through random mutagenesis and two rounds of DNA shuffling. The structural basis of these changes in activity is discussed based upon homology modeling.

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2. Materials and methods

2.1. Strains, plasmids and media

Escherichia coli DH5α (supE44, ΔlacU169 (φ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was used as the host strain for recombinant DNA manipulations. *S. cerevisiae* H158 [GPY55-15B (MATα leu2-3 leu2-112. ura3-52 trp1-289 his4-519 prb1 cir+)] was used as a host, and the plasmid pAJ401 with URA3 gene was used as the expression vector (from VTT Biotechnology, Finland). Plasmid pUC19-cel5A containing cel5A cDNA from *T. reesei* (from this laboratory) was used as the PCR template. *E. coli* was grown in LB medium with ampicillin (0.1 mg ml^{-1}) as selection for transformants. Yeast cells were cultivated aerobically at 30 °C in synthetic medium (SDC medium: 6.7 g l^{-1} yeast nitrogen base without amino acids [Difco laboratories, MI] with the appropriate additions of 20 g l^{-1} glucose and the necessary amino acids).

2.2. Directed evolution of the enzyme

Error-prone PCR was performed using the GeneMorph Random Mutagenesis Kit (Stratagene, USA) following the manufacturer's instructions for 40 cycles. Two primers, P1 (5′-GAAGTTCG*GAATTC*GCA-3′) with an added EcoRI site and P2 (5′-TGTGGAA*CTCGAG*GAGCGGT-3′) with an added XhoI site were designed. To achieve low mutation frequencies 100 ng of pUC19-cel5A-(wt) was used as the template, while high mutation frequencies were produced using 1 ng of pUC19-cel5A-(wt) as the template. The reaction was performed using the Tgradient PCR system (Biometra, Germany), which included 1 cycle at 94 °C (2 min), 20 cycles at 94 °C (1 min), 50 °C (1 min), 72 °C (2 min) and 1 cycle at 72 °C (10 min).

Saturation mutagenesis at site N342 was performed with the overlap extension PCR method using three pairs of primers (P1 and P4, P3 and P2, and P1 and P2) (Urban et al., 1997). We had described the detailed process in another paper (Qin et al., 2008b).

DNA shuffling was performed using the method of Zhao and Arnold (1997) with some modifications. Mutant genes were amplified by standard PCR using 1 μmol l^{-1} primers P1 and P2. The resulting PCR product (40 μg) was digested with 0.03 Units of DNase I (TAKARA, Japan) for 15 min at 15 °C. Fragments (2 μg) that were 100–300 bp in length were selected as the assembly model and subjected to a 50-μl PCR reassembly reaction without primers. The reaction conditions were: 1 cycle at 94 °C (3 min), 50 cycles at 94 °C (1 min), 50 °C (1 min), 72 °C (1 min + 3 s) and 1 cycle at 72 °C (10 min). The PCR products were then amplified for another 25 cycles of standard PCR with P1 and P2.

The mutated cel5A DNA fragments were purified and cloned into the expression vector pAJ401 between the EcoRI and XhoI sites. The vector was transferred into *S. cerevisiae* H158 competent cells that were spread onto SDC-Ura plates for screening. Screening under alkali conditions and measuring the enzyme activity were performed as previously described (Wang et al., 2005).

The recombinant plasmids corresponding to the selected mutant Cel5A were transformed into *E. coli* DH5α and amplified. The DNA sequences were analyzed using an automated sequencing system (Shanghai Genecore Biotechnologies, China).

2.3. Purification of the recombinant Cel5A variants from S. cerevisiae H158

Purification of the recombinant Cel5A variants from *S. cerevisiae* H158 was performed as previously described (Qin et al., 2008a).

2.4. Activity assays

Carboxymethycellulose-Na (CMC-Na, low viscosity, Sigma), Avicel, ball-milled cellulose and phosphoric acid swollen cellulose (PASC) were hydrolyzed with purified wild-type Cel5A and the different variants. The concentration of CMC-Na, Avicel, ball-milled cellulose, and PASC was 5 g l^{-1}. The hydrolysis reaction lasted for 20 h at 50 °C. All assays were performed in a reaction volume of 1 ml in triplicate in 50 mmol l^{-1} acetate buffer (under pH 6.0), or in 50 mmol l^{-1} phosphate buffer (above pH 6.0). The liberated glucose and cellobiose were assayed with an HPLC system (LC-10AD VP, Shimadzu) with an HPX-87H Aminex column (Bio-Rad) and a RID-10A refractive index detector (Medve et al., 1998).

2.5. pH optimum

The optimal pH for the purified Cel5A variants was determined by measuring the amount of reducing sugar liberated from 0.5% of CMC-Na in 50 mmol l^{-1} acetate and phosphate buffer, pH 4.0–7.0, using the dinitrosalicylic acid reagent method (Bailey and Nevalainen, 1981). CMC-Na was hydrolyzed by diluted purified recombinant Cel5A variants (0.2 mg ml^{-1}) for 30 min at 50 °C.

2.6. Kinetics

The kinetic parameters of the purified wild-type and mutant Cel5A were determined at 50 °C in 50 mmol l^{-1} acetate buffer (pH 4.8), and in 50 mmol l^{-1} phosphate buffer (pH 6.2), and the initial reaction rates were assayed with a 3-min incubation. The concentrations of CMC-Na ranged from 0.05 g l^{-1} to 20 g l^{-1}. The amount of reducing sugar that was liberated was measured using the dinitrosalicylic acid reagent method described by Bailey and Nevalainen (1981). Kinetic data were analyzed using Lineweaver–Burk plots to determine the V_{max} and K_m.

2.7. Molecular modeling and analysis

The endoglucanase Cel5A from *Thermoascus aurantiacus* (PDB code: 1h1nB) was used as a homologous enzyme to generate the model structure with SWISS-MODEL.

3. Results

3.1. The process of directed evolution

Fig. 1 shows a schematic summary of the directed evolution of Cel5A expressed in *S. cerevisiae*.

3.2. Saturation mutagenesis at the N342 site and characterization of the best variants

Reardon's program (Rui et al., 2004) indicated that 292 colonies had to be screened to ensure a 0.99 probability that all 64 possible outcomes were isolated. We screened 2000 clones from the saturation mutagenesis library for Cel5A mutants and obtained all 19 mutants besides the wild-type.

As shown in Fig. 2, variant N342V had a large shift in pH optimum from 4.8 to 5.8 just as another paper had reported. However, we did not report the k_{cat}/K_m of those variants and just compared their activity in their culture broth (Qin et al., 2008b). Now we purified the proteins of variants and obtained the results. At pH 4.8, the K_m and k_{cat}/K_m of variant N342V was almost the same as the wild-type. At pH 6.2, N342V had improved catalytic efficiency (1.5-fold of k_{cat}/K_m) compared to the wild-type enzyme (Table 2). Substituting Ile or Leu at position 342 resulted in a similar shift in pH