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Expression in yeast of secreted lignin peroxidase with improved 2,4-dichlorophenol degradability by DNA shuffling

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ABSTRACT

Lignin peroxidase (LiP) from *Phanerochaete chrysosporium* was shown to mineralize a variety of recalcitrant aromatic compounds and oxidize a number of polycyclic aromatic and phenolic compounds. The major problem of the wild type LiP is that it can be inactivated by excess H_2O_2 and high concentrations of aromatic compounds. We applied a directed evolution technique coupled with a rapid colorimetric screening method to obtain mutant genes with improved H_2O_2 stability and polychlorinated phenol degradability, and they were successfully expressed as the secretive LiPs in recombinant *Saccharomyces cerevisiae*. The resulting variants showed approximately 1.6-fold improved 2,4-dichlorophenol (2,4-DCP) degradation activity and stability against H_2O_2 compared with the parent strain. The kinetic properties of the variants toward 2,4-DCP and H_2O_2 were also increased compared with the wild type for all three mutants studied. Amino acid sequence analysis indicated that the greatest number of amino acid substitutions was located near the surface or Ca^{2+} binding sites of the enzyme.

1. Introduction

Chlorinated phenols are widely used by the chemical industry as intermediate products in chemical synthesis (Balfanz and Rehm, 1991; Häggblom and Valo, 1995). They have high toxicity, are very difficult to degrade in natural conditions, and thus pose serious ecological problems as environmental pollutants (Salkinoja-Salonen et al., 1989; Sharma et al., 1997). Environmental scientists have been increasingly interested in lignin-degrading white rot fungus, mainly *Phanerochaete chrysosporium*, for applications such as the cleanup of toxic organic chemicals in soils and water. *P. chrysosporium* is capable of degrading a wide variety of organopollutants (Bumpus et al., 1985). Biodegradation and biotransformation of 2,4-dichlorophenol (2,4-DCP) by *P. chrysosporium* have been reported (Valli and Gold, 1991).

Peroxidases catalyze the peroxide-dependent oxidation of a range of inorganic and organic compounds. They are primarily intracellular enzymes with important roles in cellular processes (Everse et al., 1990). They catalyze the oxygen radical transfer in the presence of H_2O_2, resulting in one-electron oxidation of various materials including phenol, aromatic amines, etc. (Lewis and Lewis, 1993; Orten and Neuhaus, 1970). H_2O_2-dependent peroxidases related to lignolysis by *P. chrysosporium* are divided into two independent groups. The fungus secretes two types of heme-containing peroxidases, i.e., lignin peroxidase (LiP) and manganese peroxidase (MnP) (Kirk and Farrell, 1987). Both LiP and MnP are involved in lignin and xenobiotic degradation (Hammel et al., 1993). LiP isozymes can directly oxidize a variety of organic substrates (Tien, 1987). Due to its high redox potential, the preferred substrates for LiP are nonphenolic methoxy-substituted lignin subunits. LiP follows the same reaction pathway as horseradish peroxidase (HRP) and cytochrome *C* peroxidase (Ccp) in catalyzing the oxidation of substrate by H_2O_2. Some of the most important features distinguishing these enzymes from other oxidoreductases such as HRP, for example, are their very low pH optima and much higher redox potentials. The optimum pH for steady-state turnover of LiP is near 2.0 (Tien et al., 1986), lower than those of all other peroxidases.

One problem with the wild type LiP is its inactivation by excess H_2O_2 and high concentration of aromatic substrates. Some researchers have used directed evolution methods (Morawski et al., 2000; Cherry et al., 1999; Petrounia and Arnold, 2000; Ryu et al., 2008) to engineer new catalysts starting from peroxidase. Stability, activity towards non-natural substrates, and expression in heterologous hosts are the important features that should be manipulated. Directed evolution, however, requires functional expression in a host that is suitable for making and screening libraries of thousands, and preferably tens of thousands, of mutants.

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LiP is one of the few peroxidases that have not been successful in recombinant expression. LiP was expressed in recombinant *Escherichia coli*, but all the expressed peptides were associated with inclusion bodies. Heterologous expressions of LiP in yeast expression systems have not yet been attempted. We previously reported that, by using directed evolution with a rapid colorimetric screening method, we were able to screen several LiP mutants with improved functionality through yeast surface display technique (Ryu et al., 2008). In this study, the goals were to express functionally improved lignin peroxidase H2 (LiP H2), obtained by DNA shuffling for improved stability against excess H_2O_2 and degradability at high 2,4-DCP concentration, in an active soluble form using the *Saccharomyces cerevisiae* secretion system.

2. Materials and methods

2.1. Strains, vectors and chemicals

P. chrysosporium KCTC 6147 (ATCC 24725) was obtained from the Korea Research Institute of Bioscience and Biotechnology and grown as described elsewhere (Tien and Kirk, 1988). JM109, DH5α and pGEM TE easy cloning vector were purchased from Promega Co. The pGEM TE easy cloning vector was used for subcloning. Plasmid pYEX-S1 and *S. cerevisiae* strain BJ5465, generously provided by Dr. F.H. Arnold (Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, USA), were used for secretive expression of the LiP. The yeast plasmid miniprep kit was from Zymo Research (Orange, CA). All chemicals were of reagent-grade purity. 2,4-DCP, 4-antiamino pyrine, HRP, H_2O_2, ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma (St. Louis, MO). Restriction enzymes, ligase, *Taq* polymerase and dNTP were purchased from Promega. Proofreading polymerase *Pfu* was from Stratagene (La Jolla, CA). Plasmid DNA preparation, PCR purification and gel elution kit were purchased from NucleoGen (Korea). All primers for cloning were provided by Bionics (Korea). For the secretion of LiP, the sequences of oligonucleotides were EX5H2 (5′-aa**gagctc**atgccgaacctcgacaagcg-3′) and EX3H2 (5′-ataga**ggatcc**ttagttgggggacggcggc-3′).

2.2. Fungal growth conditions and purification of native fungal LiP

P. chrysosporium was cultivated following the previously reported methods (Fenn and Kirk, 1979; Häggblom and Valo, 1995) with slight modifications. Cultures of *P. chrysosporium* were maintained on supplemented malt agar slants. The spore production in the slants usually required 2–5 days of growth at 30 °C. Liquid mycelial culture was performed in nitrogen-limited or -sufficient media without agitation. Shallow stationary mycelial cultures (20 ml) of *P. chrysosporium* were grown in rubber-stoppered, 250-ml Erlenmeyer flasks at 30 °C under 100% oxygen. They were flushed with oxygen at the time of inoculation and again on day 3. The extracellular fluid was separated from the fungal mycelia by centrifugation at 5000 rpm for 30 min. $(NH_4)_2SO_4$ (25% saturation) was added to the supernatant at 4 °C and mixed for 30 min to precipitate impurities, which were centrifuged out (12,000 × *g*, 15 min). $(NH_4)_2SO_4$ was then added to the supernatant at 75% saturation and mixed for 3 h at 4 °C. The precipitated LiP was recovered by centrifugation (12,000 × *g*, 15 min) and resolubilized in a sodium acetate buffer (50 mM, pH 6.0). The resuspended solution was concentrated about 20-fold using an Amicon concentrator (Amicon Co., Beverly, USA) with an Amicon YM10 membrane. The concentrates were dialyzed overnight against 10 mM sodium acetate (pH 6.0) and centrifuged at 5000 rpm for 30 min to remove mucilaginous materials.

2.3. Cloning of LiP H2 from P. chrysosporium

The total RNAs of *P. chrysosporium* were purified as described by Chomczynski and Sacchi (1987). The LiP H2's mRNA was isolated and purified following the commercial technical manual of the PolyATtract™ mRNA isolation system (Promega Co.). In order to confirm, prepared RNAs were run on 1% agarose formaldehyde RNA gel. Reverse transcription (RT) from fungal mRNAs was performed according to Sambrook et al. (1989). The mature region of the LiP gene was amplified by PCR using *Pfu* DNA polymerase that had 3′–5′ proofreading activity. Reaction conditions were as follows (Guojun et al., 1998): 5 ng LiPH2 cDNA, 10 pM each of oligonucleotides EX5H2 and EX3H2, 250 μM of dNTP, 5 μl of 10 × *Pfu* DNA polymerase buffer, and 2.5 units of cloned *Pfu* polymerase in a final volume of 50 μl. PCR conditions were as follows: initial denaturation at 95 °C for 4 min followed by 30 cycles of denaturation (30 s, 95 °C), annealing (45 s, 57 °C) and extension (4 min, 72 °C). The final extension was performed for 10 min at 72 °C. The PCR product was cloned into the pGEM TE cloning vector. The ligation mixture was transformed into *E. coli* DH5α cells. The transformants were incubated for 18 h, and the clones were selected on LB medium supplemented with ampicillin (50 mg/ml). LiP-encoding plasmid DNA was isolated and used for the secretive expression of yeast. The DNA sequence was confirmed by the Basic Science Institute in Kwangju, Korea.

2.4. Construction of recombinant LiP

In the secretive expression system, the plasmid pGEM TE LiP H2 was digested with SacI and BamHI. The digestion products were gel-purified and the LiP fragments were ligated into the similarly digested vector pYEX-S1. Ligation mixtures were transformed into *E. coli* DH5α cells by the heat shock method. The transformants were incubated for 18 h. The clones were selected on LB medium supplemented with ampicillin (100 mg/ml). Colonies were directly harvested from LB plates and mixed, and the plasmid DNA was isolated. This DNA was used for transformation into protease-deficient *S. cerevisiae* BJ5465. Yeast transformation was carried out with a modified LiAc method as described elsewhere (Gietz et al., 1995), followed by plating on YNB-selective medium (0.67% yeast nitrogen base without amino acids, 20 mg/ml of leucine, histidine, and tryptophan, and 40 mg/ml adenine) and incubation at 30 °C for 48–60 h to recover transformants.

2.5. Mutagenesis and recombination by DNA shuffling

DNA shuffling was performed by the slightly modified Stemmer method (Stemmer, 1994). The entire procedures of LiP mutant library construction, DNA fragmentation and shuffling, and PCR condition were described in the previous report (Ryu et al., 2008). Small aliquots of the amplified products were run on an agarose gel to determine the yield and quality of amplification. For the secretion system, the PCR products were purified by gel elution and digested with SacI and BamHI. The digestion products were gel-purified and the reassembled LiP fragments were ligated into the similarly digested vector pYEX-S1–LiP. These DNA plasmids were used for transformation into protease-deficient *S. cerevisiae* BJ5465.

2.6. Screening of LiP mutant libraries and LiP activity assay

The first screening was the picking of rapidly grown colonies from the selective YNB medium. The second screening employed a colorimetric screening assay (Ryu and Lee, 2002). For the secretion system, single colonies were picked from the plates containing