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Escherichia coli expression and *in vitro* activation of a unique ligninolytic peroxidase that has a catalytic tyrosine residue

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ABSTRACT

Heterologous expression of *Trametes cervina* lignin peroxidase (LiP), the only basidiomycete peroxidase that has a catalytic tyrosine, was investigated. The mature LiP cDNA was cloned into the pET vector and used to transform *Escherichia coli*. Recombinant LiP protein accumulated in inclusion bodies as an inactive form. Refolding conditions for its *in vitro* activation—including incorporation of heme and structural Ca^{2+} ions, and formation of disulfide bridges—were optimized taking as a starting point those reported for other plant and fungal peroxidases. The absorption spectrum of the refolded enzyme was identical to that of wild LiP from *T. cervina* suggesting that it was properly folded. The enzyme was able to oxidize 1,4-dimethoxybenzene and ferrocytochrome *c* confirming its high redox potential and ability to oxidize large substrates. However, during oxidation of veratryl alcohol (VA), the physiological LiP substrate, an unexpected initial lag period was observed. Possible modification of the enzyme was investigated by incubating it with H_2O_2 and VA (for 30 min before dialysis). The pretreated enzyme showed normal kinetics traces for VA oxidation, without the initial lag previously observed. Steady-state kinetics of the pretreated LiP were almost the same as the recombinant enzyme before the pretreatment. Moreover, the catalytic constant (k_{cat}) for VA oxidation was comparable to that of wild LiP from *T. cervina*, although the Michaelis–Menten constant (K_m) was 8-fold higher. The present heterologous expression system provides a valuable tool to investigate structure–function relationships, and autocatalytic activation of the unique *T. cervina* LiP.

Lignin in wood and other lignocellulosic materials is the most abundant renewable aromatic polymer, and one of the most recalcitrant biomaterial on the earth [1,2]. Lignin peroxidase (LiP[1]; EC: 1.11.1.14), first described in the white-rot basidiomycete *Phanerochaete chrysosporium* [3–5], is an extracellular heme peroxidase involved in the oxidative depolymerization of lignin by white-rot fungi. Different aspects of LiP molecular structure provide to this enzymes the high redox potential and ability to oxidize bulky substrates, required for lignin biodegradation [6,7]. These unique properties of LiP have been shown by oxidation of 1,4-dimethoxybenzene [8] and ferrocytochrome *c* [9], as well as by its action on polymeric lignin and different model compounds [10,11]. 1,4-Dimethoxybenzene is hardly oxidized by other peroxidases because of its high redox potential ($E_{1/2}$ 1.34 V), and ferrocytochrome *c* is too large to penetrate into the heme cavity, as is also the case with the large lignin macromolecule. The catalytic properties of LiP and other ligninolytic enzymes are of interest for applications in paper pulp bleaching and bioethanol production from woody biomass [12].

Several kinetic, crystallographic and spectroscopic studies including chemical modification and site-directed mutagenesis of LiP from *P. chrysosporium* have demonstrated that a tryptophan residue located at the surface of the protein (Trp171) is the oxidation site for high redox-potential aromatic substrates [13–17] (Fig. 1A). This implies the existence of a long-range electron transfer (LRET) pathway from this exposed Trp171 to the heme cofactor in the peroxide activated enzyme. Studies of versatile peroxidases (VP) from *Pleurotus eryngii* and *Pleurotus ostreatus*, which possess catalytic properties of LiP including veratryl alcohol (VA) oxidation, have shown that one of the VP substrates oxidation sites is also a tryptophan residue at the same location of *P. chrysosporium* LiP Trp171 [18–20]. In fact, the tryptophan residue corresponding to the above Trp171 is conserved in all genes encoding LiP and VP

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[1] *Abbreviations used:* ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSH, oxidized glutathione; HRP, horseradish peroxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; k_{cat}, catalytic constant; K_m, Michaelis–Menten constant; LiP, lignin peroxidase; LiP*, recombinant LiP; p-LiP*, pretreated LiP*; LRET, long-range electron transfer; MnP, manganese peroxidase; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VA, veratryl alcohol.

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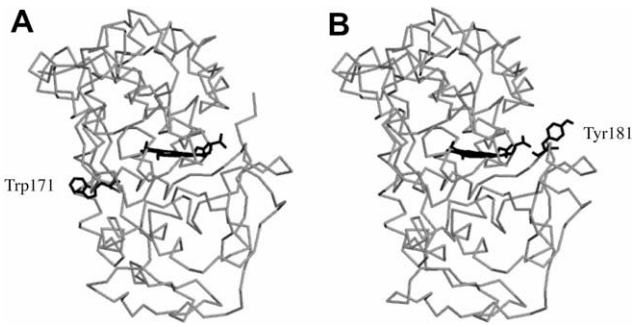

Fig. 1. General scheme of the *P. chrysosporium* (A) and *T. cervina* LiP (B) molecular structures showing the protein backbone, the heme cofactor (in the center), and the aromatic residues involved in catalysis being Trp171 and Tyr181, respectively. PDB entry 1LLP is shown in (A), while a theoretical structure obtained by homology modeling [27] is shown in (B). Homology modeling was performed with the Molecular Operating Environment (MOE) program (Chemical Computing Group), using 1LLP structure as template.

proteins [21,22]. Thus, this residue is considered as essential for the LiP-type catalytic mechanism including the LRET pathway from high redox-potential substrates to heme.

Recently, a novel type of LiP has been found in the white-rot basidiomycete *Trametes cervina*. *T. cervina* LiP can oxidize 1,4-dimethoxybenzene and ferrocytochrome *c* indicating that it possesses high redox potential and the ability to oxidize bulky substrates like other LiP and VP [23]. However, a tryptophan residue homologous to the catalytic tryptophan of LiP and VP is absent from the *T. cervina* LiP sequence [24]. By contrast, its sequence contains one tyrosine residue (Tyr181), while no tyrosines are present in the sequences of other LiP and VP [21]. It has been reported that tyrosine can act as a redox active residue, like tryptophan, in different enzymes [25] and a tyrosyl radical has been detected in a VP variant, whose catalytic tryptophan had been substituted by tyrosine [26].

The theoretical structure obtained by homology modeling of the *T. cervina* enzyme has shown that Tyr181 is exposed at the protein surface being a candidate substrate oxidation site (Fig. 1B), in agreement with results from chemical modification studies [27]. The catalytic mechanism of *T. cervina* LiP, most probably involving a Tyr181 radical, is quite interesting but it has not been investigated so far because of the very low yield of wild enzyme obtained from *T. cervina* cultures. Thus, developing an expression system to obtain recombinant LiP (LiP*) of *T. cervina* was a requisite for further detailed mechanistic studies including site-directed mutagenesis of this unique peroxidase. In the present study, a heterologous system for *T. cervina* LiP* expression in *Escherichia coli* was constructed. Since LiP* protein was located in inclusion bodies, as other recombinant plant and fungal peroxidases expressed in *E. coli* [28–31], *in vitro* refolding was optimized for activation of *T. cervina* LiP*. Finally, the physiochemical and catalytic properties of the purified LiP* were determined, and some interesting results were observed during VA oxidation.

Materials and methods

Materials

Dithiothreitol (DTT), lysozyme, oxidized glutathione (GSSG), hemin, 1,4-dimethoxybenzene, VA, and ferricytochrome *c* were purchased from Sigma–Aldrich. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Calbiochem, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and DNase I were from Boehringer Mannhein. Restriction enzymes were from New England Biolabs. All other chemicals were from Merk. *E. coli* expression vector pET23a(+) and BL21(DE3)pLysS strain were from Merck.

Amplification of cDNA encoding the mature T. cervina LiP

The mature LiP sequence was deduced from multiple alignment of the *T. cervina* LiP predicted amino-acid sequence (GenBank Accession No.: AB191466) with those of *Pl. eryngii* VPL2 (AF007224) and *P. chrysosporium* LiPH8 (M27401) where the beginning of mature protein had been localized. Two oligonucleotides were synthesized corresponding to the N-terminal and C-terminal sequences of the mature *T. cervina* LiP: oligonucleotide *lip-S* (5′-CCAT **ATG** GTG AGC TGC GGT GGC GGC CGG-3′) corresponded to the first seven residues preceded by a NdeI restriction site, and oligonucleotide *lip-A* (5′-GGGA TCC **TTA** CCC GAG AAC GGG GGC AAC-3′) was reverse and complementary to the last seven codons with a BamHI restriction site following the termination codon.

The mature LiP cDNA (*mlip*) was amplified by polymerase chain reaction (PCR) with *Taq* polymerase, using the cDNA of *T. cervina* LiP gene (AB191466) as template, and the *lip-S* and *lip-A* as primers. PCR temperature and time were programmed as follows; 94 °C for 2 min, followed by 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 90 s in 30 cycles, with a final 5 min extension at 72 °C. PCR products were separated electrophoretically using 1.0% agarose gel, and stained with ethidium bromide. The DNA fragment corresponding to amplified *mlip* was excised from the gel and extracted using Geneclean kit (Funakoshi). Purified *mlip* was cloned into pGEM T-easy vector (Promega). Correct DNA sequence and reading frame were confirmed by sequencing using a BigDye terminator v3.1 equipment (Applied Biosystems).

Vector construction and mlip expression in E. coli

The *mlip* cDNA was excised with NdeI and BamHI from pGEM T-easy vector, and inserted under T7 promoter into the multi-cloning region of pET23a vector previously digested with the same enzymes. The resulting construct (named pET-*mlip*) was used to transform *E. coli* BL21(DE3)pLysS.

Escherichia coli cells harboring pET-*mlip* were grown in Terrific Broth containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) shaken (250 rpm) at 37 °C until an OD_{500} of 1.0 was attained. The cultures were induced by addition of 1.0 mM IPTG, and cultivated for another 4 h. Bacterial pellets harvested by centrifugation were stored at −20 °C until processed as described below.

Extraction and solubilization of the inclusion bodies

Fifteen grams of bacterial pellet (wet weight) were suspended in 200 mL of 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM DTT, and 0.1 mg/mL DNase. Cells were disrupted by sonication, and subsequent centrifugation (30 min at 16,000*g*) allowed the collection of inclusion bodies containing the LiP* polypeptide. Inclusion bodies were washed twice with 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM DTT, and solubilized in the same buffer, containing 8 M urea, at room temperature. Protein concentration was determined by the Bradford method [32] using bovine serum albumin as standard, and then adjusted to 2 mg/mL using the same urea buffer. Finally the solution was sonicated for 1 min × 3 times at 20,000 Hz frequency.

Optimization of the in vitro refolding of T. cervina LiP*

The refolding mixture was prepared by 10-fold dilution of the above solution with refolding solution containing urea, $CaCl_2$,