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Cloning and expression of a new manganese peroxidase from *Irpex lacteus* F17 and its application in decolorization of reactive black 5

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ABSTRACT

A novel gene (*imnp*) encoding manganese peroxidase (MnP) from a new and local white-rot fungus, *Irpex lacteus* F17, was cloned and sequenced. It contains a coding region of 1632 bp, eleven exons and ten introns. The deduced protein, Il-MnP, contains 333 amino acids, with a signal peptide of 26 amino acids. It shows conserved motifs that also exist in other fungal MnPs, including a Mn^{2+}-binding site, Ca^{2+}-binding sites, and eight cysteines. Based on a phylogenetic tree analysis, Il-MnP was deemed to be a new member of the short-type hybrid manganese peroxidases belonging to the class II fungal heme peroxidase subclass/subfamily A.2. Furthermore, the cDNA encoding the mature protein sequence was cloned into the expression vector pET28a and successfully expressed in *Escherichia coli* Rosetta (DE3). The recombinant protein was refolded and purified to homogeneity, and then partially characterized. Kinetic properties of the recombinant MnP differed from native MnP produced by *I. lacteus* F17, whereas the spectrum of substrates oxidized by both enzymes was similar. Other attractive features of the recombinant enzyme were its high stability at extreme pH values (from pH 3.5 to 9) and its ability to decolorize a high redox potential azo dye, reactive black 5. The results indicate that this enzyme has a promising biotechnological potential.

1. Introduction

White-rot fungi have the unique ability to efficiently degrade lignin and a broad range of diverse aromatic pollutants due to their extracellular and metal-containing oxidoreductases, mainly including heme cofactor peroxidases and laccases [1]. Ligninolytic heme peroxidases from white-rot fungi belong to the so-called plant peroxidase (non-animal peroxidase) superfamily, class II fungal peroxidase family. As already described by Lundell, the class II family is divided into three main peroxidases clusters comprising: (i) group A, the short peroxidases-encoding genes, which further diverge into three clades, including lignin peroxidase (LiP) subfamily A.1, the short manganese peroxidase (MnP) subfamily A.2, and the versatile peroxidase (VP) subfamily A.3; (ii) group B, the typical fungal Mn^{2+}-oxidizing, long MnPs, such as the MnPs from *Phanerochaete chrysosporium*; and (iii) group C, *Coprinopsis cinerea* peroxidase (CiP) [2]. All peroxidases within this family are extracellular and possess an Fe protoporphyrin IX (heme) prosthetic group.

Among fungal class II peroxidases, MnPs (EC 1.11.1.13) are more ubiquitous members in forest ecosystems [3]. With frequent evolutionary changes and diverse catalytic adaptations of MnPs, they are evolved into two types: the short-type hybrid MnPs (hMnPs, group A.2) and the long-MnPs (group B), which play a critical role in the Earth's carbon cycle [2]. The main differences between these groups are based on their diverse amino acid sequences, *e.g.*, the presence or absence of a C-terminal extension and a fifth disulfide bridge [4]. In the presence of H_2O_2, MnPs oxidize Mn^{2+} to Mn^{3+}, which is stabilized by oxalic and other organic acids secreted by white-rot fungi, forming Mn^{3+} chelates as diffusing oxidizers of phenolic compounds [5]. In addition, MnP can also oxidize non-phenolic aromatic compounds through radical intermediates, such as peroxidized lipids [6].

In general, MnPs always occur as different types of isozymes encoded by a family of genes [7,8]. Since the discovery of three MnP isozymes in *P. chrysosporium* and the isolation of their respective encoding genes [9,10,11], there have been constant reports of MnP isozymes and genes from different white-rot fungi, including *Dichomitus squalens* [12], *Ceriporiopsis subvermispora* [13,14], *Pleurotus ostreatus* [15], *Lentinula edodes* [16,17], *Physisporinus rivulosus* [18], *Irpex lacteus* [19], and *Fomitiporia mediterranea* [20]. These reports showed primary differences in the lengths of the MnP-encoding genes, the organization of introns and exons, as well as in

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their protein structures, *etc*. In addition, following the isolation of some MnP genes, their regulatory sequences have also been investigated. These regulatory elements, such as heat shock elements (HSEs) and metal response elements (MREs), mainly exist in the 5′ upstream region of the MnP genes [21].

I. lacteus F17 is an indigenous, MnP-producing white-rot basidiomycetous organism that was recently isolated by our group. This strain is able to produce MnP whether in agitated liquid cultivation or stationary solid-state cultures, however, the latter exhibited a much higher level of MnP than that of the former. In the present study, this strain was identified to be *I. lacteus* F17 by internal transcribed spacer (ITS) analysis, and its MnP-encoding sequence and 5′ regulatory sequence were isolated using inverse polymerase chain reaction (IPCR) and thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). To the best of our knowledge, this is the first case of obtaining a full-length sequence of MnP from the white-rot fungus*I. lacteus*. Although many strains of *I. lacteus* have been reported for their good capacity for degradation of organopollutants, showing a crucial role of MnP in the bioremediation process [22–25], no information about MnP-encoding genes from the fungus was disclosed.

In this study, a detailed investigation of the phylogenetic relationships of MnP from *I. lacteus* F17 and related MnPs from class II fungal heme peroxidase subclasses was undertaken using a maximum likelihood method based on their amino acid sequence similarities. Meanwhile, the cDNA encoding the mature protein was subcloned into the expression vector pET28a and expressed in *Escherichia coli* Rosetta (DE3). The characteristics of the recombinant enzyme and its ability to decolorize the recalcitrant azo dye reactive black 5 (RB5) were also analyzed.

2. Materials and methods

2.1. Materials

The *E. coli* expression vector pET28a(+) was purchased from Invitrogen (Carlsbad, CA, USA) and the *E. coli* expression host Rosetta (DE3) was obtained from TransGen Biotech (Beijing, China). Lysozyme, glutathione (GSSG), dithiothreitol (DTT), H_2O_2, 2,6-dimethoxyphenol (DMP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phenylmethanesulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma–Aldrich (St. Louis, MO, USA). All restriction enzymes and DNA polymerases were purchased from TaKaRa (Ostu, Japan). Unless otherwise stated, all other chemicals used were obtained from Sangon (Shanghai, China) and were of analytical grade.

2.2. Strain cultures and media

I. lacteus F17 was isolated from a decayed wood chip pile in the vicinity of Hefei, China, and was stored at the China Center for Type Culture Collection (CCTCC AF 2014020). *I. lacteus* F17 was cultured on potato dextrose agar (PDA) medium (200 g/L of potato extract, 20 g/L of glucose, and 20 g/L of agar) for 7 days at 28 °C and then preserved at 4 °C.

Compound potato dextrose agar (CPDA) liquid medium (200 g/L of potato extract, 20 g/L of glucose, 3.0 g/L of KH_2PO_4, 1.5 g/L of $MgSO_4{\cdot}7H_2O$, and 0.01 g/L of VB1) was used for genomic DNA extraction, and malt yeast peptone glucose (MYPG) liquid fermentation medium (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, and 10 g/L glucose) was employed for the expression of MnP-encoding genes and total RNA extraction [16].

Table 1
Primers used in this study.

Primer	Nuleotide sequence
Df	GGHGGTGCCGATGGSTC
Dr	TCRGACTGGAGSCGCATC
Inf	GTAATGTTGTGCCTGGCGATGAA
Inr	GTTCAACTCCGTGGCACGCTCTT
Ca	ATGGCCTTCAAACACCTCATC
Cs	TTACGAGGGAGGGCTGAAAGC
Cf	ATGGCCTTCAAACACCTCATCGCT
Cr	TTACGAGGGAGGGACAGGGGCG
SpA	CCGAATGGCTCAGGTACAGTCTTGT
SpB	CGGAATGCGATACGATACCAAGATC
SpC	GGGTAATGTTGTGCCTGGCGATGAA
Sp1	TCACGGATGGCGAACAAAGAACAGC
Sp2	CACTGGAGGCTACCTGAGTACAAGA
Sp3	CGAAGGTTCATCTACCAATCGGCTA
AD1	TGWGNAGWANCASAGA
AD2	AGWGNAGWANCAWAGG
AD3	STTGNTASTNCTNTGC
AD4	WTCTGNCTWANTANCT
AD5	NTCGASTWTSGWGTT
AD6	NGTCGASWGANAWGAA
AD7	WGTGNAGWANCANAGA

2.3. MnP activity assay

MnP activity was determined spectrophotometrically based on the oxidation of Mn^{2+}–Mn^{3+} at 25 °C and 240 nm ($\epsilon = 6500\ M^{-1}\ cm^{-1}$) [26]. Assay mixtures (1 mL) contained 0.11 M sodium lactate buffer, pH 4.5, 1 mM $MnSO_4$, 0.1 mM H_2O_2, and 25 μL of sample. One unit (U) of MnP activity was defined as the oxidization of 1 μM–Mn^{2+} per minute. The enzyme activities were expressed in U/L.

2.4. Extraction of genomic DNA and total RNA

For genomic DNA extraction, mycelia were cultured in CPDA liquid medium and harvested on the fifth day, and genomic DNA was extracted as described previously [27]. For total RNA extraction, mycelia were cultured in liquid fermentation medium and harvested when the MnP activity reached its highest level, and total RNA was extracted as described previously [28].

2.5. ITS amplification and analysis

To amplify the ITS sequence of *I. lacteus* F17, the fungal primers ITS1F and ITS4 were used in a conventional polymerase chain reaction (PCR) [29]. The products were examined by gel electrophoresis in 1% agarose gels, stained with ethidium bromide, and then sequenced.

Subsequently, the ITS sequence of *I. lacteus* F17 was aligned with those of other fungi by BLAST analysis in GenBank. From the results, some fungal ITS sequences were selected for alignment with that of *I. lacteus* F17 using Clustal W. The alignment was then input into MEGA 6.0 to construct a phylogenetic tree based on the neighbor-joining method.

2.6. Cloning of a fragment of the imnp gene

A fragment of *I. lacteus* F17 *imnp* was obtained by degenerate PCR. Degenerate primers Df and Dr (Table 1) were designed based on two amino acid sequences that are highly conserved in the ten MnP amino acid sequences registered in GenBank. Using the genomic DNA of *I. lacteus* F17 as template, degenerate PCR was performed with Taq DNA polymerase (Sangon). The PCR products were examined by gel electrophoresis in 1% agarose gels, stained with ethidium bromide, and subsequently sequenced.