Preliminary

Molecular Cloning of Genes Encoding Manganese Peroxidase (MnP) from *Pleurotus ostreatus*

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Introduction

Lignin is a structurally complex aromatic biopolymer and one of the major components of woody plants. White-rot basidiomycetes are primarily responsible for the initiation of the decomposition of lignin in wood. Two groups of heme proteins, manganese peroxidases (MnP) and lignin peroxidases (LiP) are considered to be responsible for fungal depolymerization of lignin[1]. However, mechanism of lignin degradation is not completely elucidated. The aim of present study is to elucidate the mechanism of ligninolytic system in white-rot fungus *Pleurotus ostreatus*. *P. ostreatus* presents a particularly interesting system, because it lacks LiP activity while it expresses MnP activity in the culture media[2]. For the isolation of MnP encoding genes, we used Polymerase Chain Reaction (PCR) with synthesized primers according to conserved amino-acid sequence region among the peroxidases[3].

Materials and Methods

Isolation of mnp *gene fragments using Polymerase Chain Reaction (PCR)*

Genomic DNA of *P. ostreatus* strain *Ishizduchi Shimeji* (IS1) was extracted by the means of Yelton *et al.*[4] Messenger RNA was extracted from six-day old *P. ostreatus* mycelium cultured with 200 mM Manganese at 30°C. Using these nucleotides as template, genome PCR and RT-PCR were carried out. PCR primers were designed on the basis of the *Phanerochaete chrysosporium* MnP1[5] amino acid sequences surrounding active site residues, distal histidine, proximal histidine, and H-bonded aspartate. All PCR reactions were performed in 100 μl mixtures with the following buffer : 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, and 2.5 U of Taq polymerase. Approximately 100 ng of DNA template was subjected to initial denaturation (2 min, 94°C) followed by

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45 cycles of denaturation (30 sec, 94°C), annealing (1 min, 46°C), and extention (1 min, 72°C). A final 3-min extention at 72°C was also included. In the case of RT-PCR, reverse transcription was performed at 42°C for 1 h using total RNA (1 mg) and Reverse Transcriptase (2.5 U) before PCR. PCR products were ligated into pGEM-T vector (Promega). After cloned in *E. coli* cells, the DNA was sequenced by the dideoxy chain-termination method using ABI 373A DNA sequencer.

Construction of genomic DNA library

For the construction of genomic DNA library, high molecular weight genomic DNA was isolated from *P. ostreatus* mycelium. The isolated DNA was partially digested with *Sau*3AI to give main fragments in the size range of about 18–23 kb. The DNA fragments were ligated to *Bam*HI-cleaved phage vector lambda DASHII (STRATAGENE) and packaged into phage particles in vitro using a commercial kit.

Screening of the genomic DNA library

The *P. ostreatus* genomic DNA library was screened by plaque hybridization with the DIG-labelled probe (Boehringer Mannheim). Hybridization was conducted at 65°C for 15 h. Exposure was performed at 28°C with X-ray films.

Results and Discussion

At first, *P. ostreatus* genomic DNA was probed with *P. chrysosporium mnp-1*. But no positive signal was observed. It is conceivable that *P. ostreatus* has no highly homologous DNA sequence to *P. chrysosporium mnp-1* for the detection by genomic southern hybridization. Then, genomic PCR was performed, and 20 ml aliquots were electrophoresed on agarose gels. Clearly six amplified bands were recovered and cloned into pGEM-T vector. The DNAs were sequenced and putative amino-acid sequences were compared with several peroxidase sequences. One of these fragments, POP-1 (924 bp), showed high homology to the *P. chrysosporium* LiP, and MnP. Secondly, RT-PCR was performed and amplified bands were recovered as well. After subsequent cloning and sequencing of the RT-PCR products, two highly homologous distinct cDNA fragments, POP-2A and POP-2B, were obtained. The deduced amino-acid sequence from POP-2B fragment are 57.9% identical and 85.2% similar to *P. chrysosporium* MnP1 protein. This result strongly suggest that these fragments are portion of genes encoding MnP of *P. ostreatus*.

P. ostreatus genomic DNA library was constructed and screened with POP-1 and POP-2 as probes. Approximately 15,000 plaques of the library were screened by plaque hybridaization. And strongly hybridized 27 clones were isolated and subjected to southern analysis of the insert DNA. As the result of this analysis, it was showed that at least 20 distinct clones were obtained. In addition, it was suggested that the MnP isozymes of *P. ostreatus*

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POP2B     1  IRLTFHDAIGFSPKLSRQGKFGGGGADGSLMVHTAIEAAFNANNGIDDIV
             *********··*  ·*   **   ·*******···  ···*··*·******* *
P.cMnP   62  IRLTFHDAIAIS-RL--QGPKAGGGADGSMLLFPTVEPNFSANNGIDDSV

POP2B    51  EVQRPFAIKHK-VSFGDFIQFAGAVGVSNCAADPRLEFLAGRSNHSIASP
             ·    **  **·  ·*  ·*··******··***···*********·*··**·
P.cMnP  109  NNLIPFMQKHNTISAADLVQFAGAVALSNCPGAPRLEFLAGRPNKTIAAV

POP2B   100  DLLVPEPSDSVDAILARMGDAPPIR
             * *·*** ***· **·*··**
P.cMnP  159  DGLIPEPQDSVTKILQRFEDAGGFT
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Fig. 1. Comparison of deduced amino-acid sequence of POP2B with that of *P. chrysosporium* MnP1 protein. Asterisks and dots indicate identical and similar amino-acid residues, respectively. Only the corresponding region within the 357 amino-acid residures of MnP1 was shown.

are also encoded by a family of closely related genes as in the case of *P. chrysosporium*[6]. To clone and analysis all of genes encoding MnP from *P. ostreatus*, it is needed to determine nucleotide sequences of clones obtained here. The authors are grateful to Dr. Tohru Komano, Kyoto University, for his courtesy in DNA-sequence analysis.

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