Studies on a lignin-degrading enzyme of the basidiomycete *Pleurotus ostreatus* using a homologous gene expression system: Oxidation mechanism and molecular breeding of the versatile peroxidase MnP2

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Lignin is one of the most abundant natural polymers on the earth and a primary component of plant cell walls that confers strength to wood. Lignin forms a matrix surrounding hemicellulose and cellulose, that is the most abundant natural. Lignin is a variable three-dimensional network. Due to its various stable linkages and heterogeneity, lignin is rather resistant to hydrolytic attack and protects the surrounding hemicellulose and cellulose from biological attack by the most microorganisms. However, a certain group of basidiomycetes called white-rot fungi are able to completely degrade lignin to carbon dioxide and water. Thereby, these fungi gain access to the carbohydrate polymers of plant cell walls, which they use as carbon and energy sources. White-rot fungi are also able to degrade toxic aromatic pollutants, because lignin contains a variety of bonds that are commonly present in aromatic pollutants. Therefore, these filamentous wood decaying fungi have a potential application in conversion of lignocellulosic materials to fuels and chemicals, bioremediation for a broad range of environmental pollutants, and biological pulping.

A white-rot basidiomycete, *Pleurotus ostreatus*, secretes a versatile peroxidase (VP), MnP2, which has an extraordinarily wide range of substrate specificity. VP has a substrate spectrum of both of the two common fungal-peroxidase families concerned with lignin degradation, namely, lignin peroxidases (LiP) and manganese peroxidases. Most interestingly, *P. ostreatus* MnP2 oxidizes even high-molecular-weight compounds with high redox potential such as RNase A and a polymeric azo dye, Poly R-478, directly by itself without redox mediators [1]. In *Phanerochaete chrysosporium*, the best studied white-rot fungus, it was reported that Poly R-478 and RNase A were oxidized by LiP only when veratryl alcohol (VA) was present concomitantly in the reaction mixture [2,3]. The purpose of this study is to express a high level of MnP2 in a homologous system and elucidate its oxidation mechanism of high-molecular-weight substrates with high redox potential such as Poly R-478 and RNase A. At first, a molecular breeding approach to isolate P. ostreatus transformants with enhanced productivity of its MnP2 is achieved. Next, with optimization of the culture conditions, hyper-production and exclusive expression of recombinant MnP2 from P. ostreatus is accomplished. The last, MnP2 variants with desired amino acid substitution(s) are produced and their catalytic properties for various substrates are characterized.

Using a DNA-mediated transformation technique [4], a molecular breeding approach to isolate *P. ostreatus* strains with enhanced productivity of MnP2 was achieved. A recombinant *mnp2* construct under the control of *P. ostreatus sdi1* expression signals was introduced into the wild type *P. ostreatus* strain by cotransformation with a carboxin-resistant marker plasmid. A total of 32 transformants containing the recombinant *mnp2* sequence were isolated in a screening with specific amplification by PCR. Productivity of MnP2 in the recombinants was evaluated by the decolorization ability of Poly R-478 on agar plates in the absence of Mn^{2+}. Recombinant *P. ostreatus* strains with elevated MnP productivity were successfully isolated. One of the recombinants, TM2-10, was demonstrated to secret recombinant MnP2 predominantly on a synthetic medium, which was confirmed by RT-PCR and isozyme profile analysis using anion-exchange chromatography. The benzo[*a*]pyrene-removing activity by fungal treatment was analyzed using the isolated recombinant strains, which exhibited enhanced benzo[*a*]pyrene-removing activity. This is the first report to homologously express VP in the basidiomycete. It is demonstrated that *P. ostreatus* strains with enhanced productivity of MnP2 are useful as a biocatalyst, which removes high-molecular compounds and polycyclic aromatic pollutants [5].

Upon optimization of the culture conditions, hyper overproduction of *P. ostreatus* MnP2 was accomplished. Genetically modified *P. ostreatus* strains with the recombinant *mnp2* sequence under the control of *sdi1* expression signals, were subjected to agitated culture using medium supplemented with wheat bran or its hot-water extract. The best result, whereby 7300 U/l of MnP was produced by a recombinant strain TM2-18, indicated that more than 30-fold overproduction of the recombinant MnP2 compared to the above result was achieved. On the other hand, no MnP activity was detected for the wild type strain under the same conditions. Accumulation of the recombinant, but not endogenous, *mnp2* transcripts was demonstrated in reverse-transcription PCR experiments. These results indicated that the

recombinant MnP2 was exclusively expressed by the recombinant strain. Purified recombinant MnP2 showed identical properties to native MnP2 in electrophoresis, spectroscopic and kinetic analyses, including determination of K_m and V_{max} values for Mn^{2+}, H_2O_2 and VA. Moreover, the recombinant MnP2 directly oxidized a high-molecular-weight substrate RNase A in the absence of redox mediators, as does native MnP2. The homologous expression system developed here reflects the post-transcriptional modifications, secretion and stability in the physiological condition during the enzyme production process in *P. ostreatus*. In this context, the system is suitable for analyzing not only the structure-function relationship of the enzyme, but also post-transcriptional regulations in the protein expression pathway of *P. ostreatus*. In addition, the overproduction system will also provide a plat form for exclusive production of mutant or variant peroxidases with desired properties in basidiomycete, because the production of MnP2 in this system is the best results compared to a similar recombinant gene expression system of ligninolytic peroxidases in basidiomycete has been reported [6].

To elucidate the oxidation mechanism of high-molecular-weight substrates by MnP2, a series of mutant enzymes were produced using the homologous gene expression system and their reactivity were characterized. W170A lost drastically the oxidation activity for VA, Poly R-478 and RNase A, while kinetic properties for Mn^{2+} and H_2O_2 were substantially unchanged. These results suggested that, as well as VA, the high-molecular-weight substrates are directly oxidized by MnP2 at W170. Moreover, in the mutants Q266F and V166/168L, amino acid substitution(s) around W170 resulted in a decreased oxidizing activity only for the high-molecular-weight substrates. These results, along with the 3D-modeling of the mutants, suggested that the mutations caused a steric hindrance for access of the polymeric substrates to W170. Another mutant R263N contained a newly generated *N*-glycosylation site and showed higher molecular mass in SDS-PAGE analysis. Interestingly, R263N exhibited an increased reactivity with VA and high-molecular-weight substrates but not with Mn^{2+} and H_2O_2. It was demonstrated that stability at pH 3.0 was increased and higher reactivity at wider pH range compared to wild type MnP2, when Poly R-478 was used as a substrate. On the contrary, with respect to Mn^{2+}- and VA-oxidizing activity, neither the stability at pH 3.0 nor the profile of oxidizing activity at the corresponding pH range was changed by the mutation. Furthermore, the K_m values for Mn^{2+} and VA were almost identical between R263N and wild type MnP2, indicating that recognition of Mn^{2+} and VA were not affected by the mutation. Taking all the observations into account, it is suggested that, in R263N, mode of interaction between the enzyme and the polymeric substrate is slightly changed from that of wild type MnP2, most provably due to the additional *N*-glycosylation. Direct evidence for the possible function of the additional *N*-glycan remains unclear. However, it is expected that, with higher catalytic activity and wider pH stability, R263N can be used for various bio-processes, for example, as an immobilized enzyme in bioreactors for the decomposition of recalcitrant environmental pollutants. It was demonstrated that the homologous expression system provides analysis tools for the effects of post-translational modification on the reactivity of the expressed enzyme. It is noteworthy that the results obtained with R263N could not be gained by using heterologous expression systems. This is the first study on the direct oxidation mechanism for high-molecular-weight substrates by a fungal peroxidase [7].

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