Lignin Peroxidase-Catalyzed Oxidation of Monomeric Lignin Model Substrates

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Abstract——The substrate specificity of lignin peroxidase (LiP) was investigated by determining the K_ms and V_{max}s of LiP for the four lignin model substrates of 3, 4-dimethoxybenzyl alcohol (I), 3, 4-dimethoxybenzyl glycerol (II), 3, 4, 5-trimethoxybenzyl alcohol (III), and 3, 4, 5-trimethoxybenzyl glycerol (IV). The K_ms (μM) and V_{max}s (turnover number, sec^{-1}) of LiP were determined. The K_m/V_{max} values calculated to be 4.7, 18.2, 29.9, and 76.7 for I, II, III, and IV, respectively. Among the substrates tested, I (veratryl alcohol), which is a secondary metabolite of white-rot fungi, was found to be the best substrate for LiP.

Keywords : Lignin peroxidase, lignin model substrate, *Phanerochaete chrysosporium*, specificity, veratryl alcohol

1. Introduction

Since the discovery of lignin peroxidase (LiP) in the ligninolytic culture of *Phanerochaete chrysosporium* in 1983[1,2], world wide efforts have been made to characterize the enzyme, to clone the genes encoding the enzyme, to improve the production of the enzyme, and to rationalize the mechanism of lignin degradation by white-rot fungi.

LiP is a peroxidase with an optimum of pH 3.0, having a molecular weight of 41,000–42,000 dalton[3-5]. The enzyme is a glycoprotein with an iron protoporphyrin IX as the prosthetic group. LiP catalyzes the initial one-electron oxidation of a variety of lignin model substructures in the presence of H_2O_2, yielding aryl cation radical (from non-phenolic model compounds) intermediates, which undergo $C\alpha$-$C\beta$ bond cleavage and aromatic ring opening reactions[4,6-17].

Natural lignin macromolecules are extremely complex and heterogeneous biopolymers, having no optical activity. Nevertheless, lignin is decomposed by microorganisms in nature. This is due to the multifunctional activities of LiP or to the lack of the stereospecificity of the enzyme. Actually, Kurosaka et al.[18] reported that LiP did not show any stereospecificity for threo- and *erythro*-3, 4-dimethoxybenzyl glycerol substrates, although a *threo*-form of β-0-4 substracte was oxidized by LiP in preference to the *erthyro*-

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form[19]. It is interesting to further investigate the effectiveness of the monomeric lignin model substrates for LiP in connection with the biodegradation of lignins.

Here we report that 3, 4-dimethoxy benzyl alcohol (veratryl alcohol), which is a secondary metabolite of some of the white-rot fungi, is the best substrate for LiP among the related monomeric lignin model substrates examined. The results are discussed in relation to the multifunctional activities of LiP of white-rot fungi.

2. Materials and Methods

2.1 Preparation of the lignin model substrates

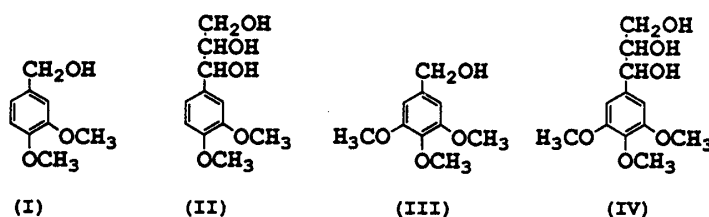The monomeric lignin model substrates (I, II, III, and IV in Fig. 1) were prepared as follows.

Fig. 1. Structures of the lignin model compounds used in this experiment. I, 3, 4-dimethoxybenzyl alcohol; II, 3, 4-dimethoxybenzyl glycerol; III, 3, 4, 5-trimethoxybenzyl alcohol; IV, 3, 4, 5-trimethoxybenzyl glycerol.

The 3, 4-dimethoxybenzyl alcohol (I) used was purified from the commercial one with TLC. 3, 4-Diomethoxybenzyl glycerol (II) was synthesized from 3, 4-dimethoxycinnamyl alcohol as previously reported[20]. 3, 4, 5-Tri-methoxybenzyl alcohol (III) was prepared by reducing the corresponding aldehyde with $NaBH_4$. 3, 4, 5-Trimethoxybenzyl glycerol (IV) was synthesized from 3, 4, 5-trimethoxycinnamyl alcohol according to the procedure for 3, 4-dimethoxybenzyl glycerol synthesis. The other chemicals used in this experiment were all analytical grade reagents.

2.2 Production and purification of LiP

LiP was produced by growing *P. chrysosporium* in shaking culture as previously described[21] and purified to electrophoretically homogeneous protein by using DEAE and GPC HPLC column chromatography successively[22].

2.3 Determination of K_m and V_{max} values

Enzymatic reactions were carried out at 30°C, in 0.1 M Na-tartrate buffer, which contained 0.15 μM LiP, 0.5 mM H_2O_2 and various concentrations of substrate according to the Tien and Kirk method[23]. The reaction was started by adding H_2O_2. The reaction rates were determined spectrophotometrically by measuring increases in the absorbance at 310 nm for 3, 4-dimethoxybenzyl alcohol and 3, 4-dimethoxybenzyl glycerol (ε_{310}=9.3×10^3

$M^{-1}cm^{-1}$) and at 290 nm for 3, 4, 5-trimethoxybenzyl alcohol and 3, 4, 5-trimethoxybenzyl glycerol ($\varepsilon_{290} = 1.09 \times 10^4\,M^{-1}cm^{-1}$). The values for K_m (μM) and V_{max} (turn over in molar activity) were determined from Lineweaver-Burk (1/v − 1/s) plots.

3. Results and Discussion

Since it is more rational to evaluate the effectiveness of the substrates (substrate specificity) with the K_m/V_{max} ratios rather than with the K_m or V_{max} values alone, we calculated the K_m/V_{max} ratios for the substrates, I, II, III, and IV. The results are shown in Table 1, which clearly indicates that I is the most effective substrate for LiP since the smallest K_m/V_{max} ratio was obtained and the ratios increased in the order of II, III, and IV : the ratio for II, III, and IV increased about 4-, 6- and 16-fold, respectively, by addition of one methoxyl group (to the substrate I), one glycol moiety to the side chain of I, and both one methoxyl group and the glycol moiety to I, respectively. Thus, there is a strong correlation between the structures of both side chain and the substituting groups of the monomeric substrates and the substrate specificity of LiP. Therefore, it is noteworthy that LiP prefers I as the substrate to the artificial lignin model substrates, which is in good harmony with the fact that the substrate I is the secondary metabolite produced by several white-rot fungi and that a new biosynthetic pathway of veratryl alcohol has been proposed[24].

Table 1. The K_m and V_{max} of LiP for four lignin model compounds.

Lignin model substrates used	K_m (μM)	V_{max}	K_m/V_{max}
I	229	48.7	4.7
II	758	41.6	18.2
III	630	21.1	29.9
IV	2,115	27.6	76.7

Since the substrate I is known to serve as the mediator, as well as the enzyme protector during the LiP-catalyzed oxidation[25,26], lignin may be oxidized indirectly by mediation of the veratryl alcohol cation radical intermediate in nature.

In view of the recent findings that the substrate I has been established as the mediator for oxidation of both lignin[17,27] and oxalic acid[28,29], the fungal peroxidase, "LiP" might have been evolved for metabolism of the secondary metabolite (I)[30]. Thus, it is postulated that these white-rot fungi successfully applied the LiP/veratryl alcohol system to decompositions of their endogenously produced oxalic acid, the exogenous lignin in wood, and even xenobiotic pollutants[31].

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