Mode of action and active site of an extracellular peroxidase from *Pleurotus ostreatus*

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The properties of the haem environment of an extracellular peroxidase from *Pleurotus ostreatus* were studied by electronic absorption spectroscopy. A high-spin ferric form was predominant in the native enzyme and a high-spin ferrous form in the reduced enzyme. Cyanide was readily bound to the haem iron in the native form, thereby changing the enzyme to a low-spin cyano adduct. The electronic absorption spectra of the enzyme were similar to those of lignin peroxidase from *Phanerochaete chrysosporium*. Compound III of the enzyme was formed after the addition of an excess of H_2O_2 to the native enzyme, and thereafter spontaneously reverted to the native form. The enzyme oxidized 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)-1,3-dihydroxypropane in the presence of H_2O_2 to

produce 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)-1-oxo-3-hydroxypropane, 2,6-dimethoxyhydroquinone, 2-(2-methoxyphenoxy)-3-hydroxypropanal, 2-(2-methoxyphenoxy)-3-hydroxypropanoic acid, 2,6-dimethoxy-1,4-benzoquinone and guaiacol. A similar oxidation pattern was demonstrated with a one-electron oxidant, ammonium cerium(IV) nitrate. Free radicals were detected as intermediates of the enzyme-mediated oxidation of 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)-1,3-dihydroxypropane and acetosyringone. These results can be explained by the mechanisms involving an initial one-electron oxidation of the lignin substructure. This radical may undergo C_α - C_β cleavage, C_α -oxidation and alkyl-phenyl cleavage.

INTRODUCTION

Lignin is a complicated macromolecule primarily composed of methoxylated phenylpropane units interconnected by various stable carbon–carbon and ether linkages. The latter bonds most often connect side chains of one monomeric unit with the aromatic ring of adjacent units. Among them, the arylglycerol- β -aryl ether bond (β -O-4) is the most abundant substructure in lignin [1]. Most studies for biological degradation of lignin have focused on two enzymes from *Phanerochaete chrysosporium*: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Both are haem-containing extracellular glycoproteins [2]. Earlier papers [2–8] reported that LiP catalysed a variety of reactions involved in the degradation of lignin, such as C_α - C_β cleavage, cleavage of β -O-4 ether bonds, oxidation of C_α carbonol to C_α -oxo compounds, hydroxylation of benzylic methylene groups and styrene olefinic bonds, decarboxylation of phenylacetic acid, and opening of the aromatic ring. The second peroxidase, MnP, oxidizes Mn(II) to Mn(III), and the Mn(III) in turn oxidizes monomeric phenols [9,10], phenolic lignin dimers [11] and synthetic lignin [12], through the formation of the phenoxyl radical.

Various redox states of LiP and MnP were characterized by electronic absorption spectroscopy [10,13–15]. During the catalytic cycle, LiP and MnP have three redox states, i.e. native ferric form, compound I and compound II. In the presence of an excess of H_2O_2 , LiP and MnP are readily converted into compound III, which is not involved in the catalytic cycle of peroxidases [10,15].

Lignin peroxidases and manganese-dependent peroxidases of other white-rot fungi such as *Trametes versicolor* [16] and *Phlebia radiata* [17,18] have also been isolated and characterized. Spectral and catalytic properties of these enzymes are similar to those of *P. chrysosporium*.

It is known that lignin peroxidase and manganese-dependent peroxidase are absent in the white-rot fungus *Pleurotus ostreatus*. However, we recently reported some basic properties of another type of extracellular peroxidase and D-glucose oxidase, as a generator of H_2O_2 from *P. ostreatus*, and suggested roles for the enzymes in lignin degradation [19,20]. The *P. ostreatus* extracellular peroxidase (PoP) has similar substrate specificity to that of MnP, with respect to the finding that it cannot oxidize non-phenolic compounds. But, it is not dependent on manganese for catalytic activity in contrast to MnP. PoP is a H_2O_2 -dependent haem protein with iron protoporphyrin IX as a prosthetic group. It oxidizes a variety of organic compounds including phenolic lignin model compounds. In this paper, we report the electronic absorption spectroscopic properties of PoP and the mechanisms of the oxidation of phenolic β -O-4 lignin substructure by PoP.

MATERIALS AND METHODS

Chemicals

1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)-1,3-dihydroxypropane (see Figure 5, structure **i**) and 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)-1-oxo-3-hydroxypropane (**ii**) were prepared according to the method proposed by Hwang and Lee [21]. Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone), guaiacol (**vii**), ammonium cerium(IV) nitrate, 1-(trimethylsilyl)-imidazole, sodium cyanide, and sodium dithionite were purchased from Aldrich. All other reagents used were of the highest quality generally available.

Enzyme preparations

PoP was purified from the culture medium of *P. ostreatus* as described previously [19].

Abbreviations used: HRP, horseradish peroxidase; LiP, lignin peroxidase from *Phanerochaete chrysosporium*; MnP, manganese-dependent peroxidase from *Phanerochaete chrysosporium*; PoP, extracellular peroxidase from *Pleurotus ostreatus*; TMS, trimethylsilyl.

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Electronic absorption spectroscopy

Electronic absorption spectra were obtained using a Shimadzu Model UV-265 spectrophotometer (Japan) at room temperature, with 1.7 μM enzyme in 20 mM sodium phosphate buffer (pH 6.0). Compound III of PoP was made by the addition of excess H_2O_2 to the native PoP. A cyanide complex of PoP was made by the addition of a concentrated stock solution of sodium cyanide to give a final ligand concentration of 30 mM. The ferrous form of the enzyme was prepared by the addition of fresh sodium dithionite under rigorously anaerobic conditions as described previously [22,23].

EPR spectroscopy

Reaction mixtures contained 4 mM substrate, 0.2 μM PoP and 0.4 mM H_2O_2 in 0.2 ml of 0.1 M sodium acetate buffer (pH 4.0). Reactions were started with the addition of H_2O_2 at room temperature in EPR sampling tubes. After 1 min, the reaction mixtures were frozen with liquid nitrogen and immediately used for EPR measurements. For the detection of free radicals, EPR measurements were performed at 255 K with a Bruker ESP 300S EPR system (Germany) operating at X-band and 100 kHz field modulation.

Enzyme reactions and product analysis

The oxidation of model compound was carried out at 30 °C for 30 min under air in 5 ml of 20 mM sodium acetate buffer (pH 4.0), containing 10 μg of PoP and 1 mM substrate. The reaction was run with five sequential additions of 0.5 mM H_2O_2 at intervals of 5 min. The reaction mixture was then acidified with 6 M HCl and extracted three times with an equal volume of ethyl acetate. The combined organic phase was dried over Na_2SO_4 , and the solvent was evaporated by purging with nitrogen. The residue was analysed by GC/MS directly or after trimethylsilyl (TMS) derivatization. For the TMS derivatization, the residue was dissolved in pyridine and the solution was incubated for 2 h at room temperature in the presence of 1-(trimethylsilyl)-imidazole. GC/MS analysis was performed at 70 eV on a VG-Quattro mass spectrometer (U.K.) combined with a Hewlett-Packard 5890 Series II gas chromatography system (U.S.A.), using a Supelco

SPB-1 capillary column (U.S.A.). The injector temperature was 240 °C, and the column temperature was 120 °C for 2 min at the beginning; it was then increased to 280 °C at a rate of 5 °C/min and maintained at 280 °C for 3 min.

RESULTS AND DISCUSSION

Electronic absorption spectra of PoP

The electronic absorption spectra of ferric, liganded and ferrous forms together with compound III of PoP are shown in Figure 1 and the absorption maxima of various peroxidases in various oxidation states are summarized in Table 1. The native enzyme

Table 1 Electronic absorption spectral maxima of PoP and other peroxidases

Figures in columns 2 and 5 are the absorption maxima of newly generated bands.

Systems	Absorption maxima (nm)			References
	Soret	α	β	
Ferric, high-spin				
PoP (pH 6.0)	402	511	636	This work
LiP (pH 4.5)	407	500	632	[13]
MnP (pH 4.5)	406	502	632	[14]
HRP (pH 6.0)	403	500	641	[24]
Compound III				
PoP (pH 6.0)	414	528	557	This work
LiP (pH 6.0)	419	543	578	[15]
MnP (pH 4.5)	417	545	579	[10]
HRP (pH 6.0)	418	546	583	[22]
Ferric, low-spin				
CN-PoP (pH 6.0)	360	420	536	This work
CN-LiP (pH 4.5)	360	423	540	[13]
CN-MnP (pH 4.5)	421	546		[14]
CN-HRP (pH 6.0)	422		580	[24]
Ferrous, high-spin				
PoP (pH 6.0)	432	558		This work
LiP (pH 4.5)	435	556		[13]
MnP (pH 4.5)	433	554		[14]
HRP (pH 6.0)	437	556		[24]

Figure 1 Electronic absorption spectra of native PoP and other forms

(A) Cyano adduct (low-spin ferric) of PoP, (B) reduced form (ferrous) of PoP, and (C) compound III of PoP. The spectra were recorded at room temperature using native PoP (1.7 μM in 20 mM sodium phosphate buffer, pH 6.0) (broken lines) and each form of PoP (solid lines) prepared by the methods described in the text.

Figure 2 Spontaneous reversion of compound III of PoP to the native form

(A) Spectral changes accompanying the spontaneous reversion of compound III to native PoP. Compound III was prepared by the addition of 30 equivalents of H_2O_2 to PoP (1.7 μM in 20 mM sodium phosphate buffer, pH 6.0). Absorption spectra were taken at 5 min intervals. (B) First-order plots for the reversion of compound III to the native form when 51 (■), 170 (▲) and 850 (●) μM of H_2O_2 were used to make compound III. A_{∞} is the absorbance at 402 nm of native PoP and A_t is the absorbance at 402 nm at each point of time.

reveals detectable absorption bands at 402, 511 and 636 nm, indicating typical features of a high-spin ferric haem system. Its absorption maxima are similar to those of high-spin haem peroxidases with histidine as the fifth ligand [13,14,24]. The Soret maximum (402 nm) of PoP is within 1 nm of that of the high-spin pentacoordinate of horseradish peroxidase (HRP) and 5 nm of that of the high-spin hexacoordinate of LiP [13,14,24]. It is generally accepted that two different haem proteins having identical co-ordination environments will exhibit wavelength variations no greater than ± 5 nm for any given transition in their electronic absorption spectra. The g values of 6.10, 5.64 and 1.99 calculated from the EPR spectrum of PoP [19], the absorption band at 511 nm and the high-spin marker band at 636 nm support the high-spin ferric assignment for the native PoP. The absorption spectral pattern of the cyano adduct of PoP is clearly distinct from that of the high-spin form. The absorption maxima (360, 420 and 536 nm) of the ferric haem cyano adduct of PoP are virtually identical to that of the corresponding low-spin LiP, MnP and HRP forms [13,14,24]. The ferrous enzyme reveals its Soret and visible bands at 432 and 558 nm respectively, which are in good agreement with the analogous bands of the ferrous forms of LiP, MnP and HRP. From the above discussions it is strongly suggested that the fifth ligand of the haem iron of PoP could be a histidine or a histidinate.

Figure 3 Chromatograms of the oxidation products of the model compound (i) analysed by means of GC

Ammonium cerium (IV) nitrate (1 mM) (A) and 0.1 μM PoP (B) were added to the reaction mixture containing 1 mM model compound (i) in 20 mM sodium acetate buffer (pH 4.0).

PoP was completely converted into compound III within 3 min of the addition of a 30-fold excess of H_2O_2 , and compound III revealed a Soret band at 414 nm and visible absorption bands at 528 and 557 nm. The amount of H_2O_2 required to produce compound III of PoP was similar to that required to produce compound III of LiP and MnP, but considerably less than that required to produce compound III of HRP [10,15,25]. In the case of LiP, in the presence of more than 30 equivalents of H_2O_2 , the formation of compound III is followed by irreversible inactivation and the disappearance of Soret and visible absorption maxima; the addition of veratryl alcohol is then required for the reversion of compound III to the native form [26,27]. In contrast, compound III of PoP spontaneously reverted to the native form (Figure 2A). The reversion of compound III to the native form was monitored by the increase of the Soret absorption at 402 nm of the native PoP. The concentration effect of H_2O_2 , which was used to make compound III, on the reversion rate of compound III to native PoP is shown by the natural log of the difference between the absorbance (at 402 nm) at each time point and that of native PoP plotted against time (Figure 2B). The reversion rate constant, k , was 0.036, 0.033 and 0.022 min^{-1} when concentrations of 51, 170 and 850 μM H_2O_2 respectively were used to convert 1.7 μM PoP into compound III. These results indicate that the reversion of compound III to the native form is inhibited by the high concentration of H_2O_2 .

Single-electron transfer process

It is known that lignin degradation is brought about by an initial single-electron transfer process [28]. To confirm this process, the reaction products of model compound (i), oxidized with a metal-centred one-electron oxidant, were analysed by means of GC. As shown in Figure 3, the chromatogram of the oxidation products

Figure 4 EPR spectra of the free radicals produced upon oxidation of (A) the model compound (i) and (C) acetosyringone by PoP

(B) and (D) are the simulated spectra of (A) and (C) respectively. The conditions of reaction and EPR spectroscopy are as described in the text.

of the model compound (i) after the treatment of PoP was nearly identical to that in the case of treatment with ammonium cerium (IV) nitrate, indicating that PoP mediates single-electron transfer

between the substrate molecule and a high-potential oxyferryl haem complex of the enzyme. The reaction proceeds further by a non-enzymic mechanism. The chemical nature of subsequent products may depend on that of the substituents in the aromatic ring.

If PoP mediates the first step of the oxidation of the model compound (i), free radicals must be produced by PoP as reaction intermediates. As realized in the EPR spectra of Figure 4, PoP catalysed the formation of the free radical intermediates during the oxidation of the model compound (i) and acetosyringone as a reference compound. From the hyperfine splitting constants [$a^H = 0.145$ mT (6 equivalent protons), $a^H = 0.145$ mT (2 equivalent protons), $a^H = 0.031$ mT (3 equivalent protons)], which are consistent with the previously reported values [29] and computer simulation, the free radical from the oxidation of acetosyringone was identified as the phenoxy radical as shown in Figures 4(C) and 4(D). The EPR spectrum obtained upon oxidation of the model compound (i) by PoP showed three resolvable hyperfine splitting constants [$a_\alpha^H = 0.91$ mT (1 proton), $a_\beta^H = 0.282$ mT (2 equivalent protons), $a^H = 0.144$ mT (4 equivalent protons of the aromatic ring); (Figure 4A)] and unresolvable hyperfine splitting constants. This radical was not the phenoxy radical derived from one-electron oxidation of the model compound (i), but the fragment carbon-centred radical formulated in Figure 4(A). The phenoxy radical from the model compound (i) might be so unstable that it could not be detected. On the other hand, the fragment radical was stable enough to be detected under these conditions. As PoP can attack only various phenolic compounds [19] and catalyse the formation of phenoxy radical (as shown in the case of acetosyringone), this fragment radical is considered to be derived from C_α - C_β cleavage of phenoxy radical primarily produced by PoP. From the above results, we conclude that PoP mediates the oxidation of phenolic compounds by producing phenoxy radicals, and these radicals undergo further reaction giving rise to various final reaction products.

Oxidation of the model compound (i)

The degradation products were analysed by GC/MS, either with their free forms or with their TMS-derivatives. Model compound (i) was oxidized by PoP to yield six identifiable products. Dimeric ketone (ii), 2,6-dimethoxyhydroquinone (iii), phenoxypropanal (iv), phenoxypropanoic acid (v), 2,6-dimethoxy-1,4-benzoquinone (vi) and guaiacol (vii) were identified on the basis of mass spectral data of Table 2. When dimeric ketone (ii) was oxidized by PoP, four identifiable products [2,6-dimethoxyhydroquinone (iii), phenoxypropanoic acid (v), 2,6-dimethoxy-

Table 2 GC/MS data of products

Products (iii) and (v) were analysed after TMS derivatization and others were analysed as native substances. The numbers in parentheses mean the relative intensity of the peak as a percentage of the highest peak.

Product	Retention time (min)	Mass spectrum m/z (relative intensity)
(ii)	28.20	348 (M^+ , 1.9), 318 (0.6), 198 (8.3), 181 (100), 167 (3.1), 150 (16.8)
(iii) (di-TMS ether)	10.99	314 (M^+ , 64.3), 285 (24.7), 284 (100), 135 (12.8), 127 (13.4), 89 (16.2), 75 (10.9), 73 (79.8), 59 (14.9)
(iv)	7.65	196 (M^+ , 3.9), 149 (26.6), 124 (27.2), 123 (20.7), 109 (56.2), 108 (14.7), 95 (55.3), 92 (27.5), 81 (51.6), 77 (100), 67 (17.5), 65 (56.4), 63 (38.5), 55 (15.7), 53 (39.6), 52 (94.5)
(v) (di-TMS ether)	14.06	356 (M^+ , 19.95), 196 (11.3), 181 (18.5), 166 (20.3), 149 (35.2), 147 (19.8), 124 (11.6), 103 (12.3), 75 (14.0), 74 (10.1), 73 (100)
(vi)	7.13	168 (M^+ , 74.3), 138 (25.7), 125 (16.2), 112 (10.9), 104 (14.3), 97 (14.3), 80 (46.2), 76 (20.9), 69 (100), 59 (21.4), 53 (26.2), 45 (13.3)
(vii)	1.82	124 (M^+ , 15.5), 109 (37.7), 81 (100), 65 (11.0), 63 (12.9), 53 (43.8)

Figure 5 Proposed pathway for C_α - C_β oxidation, alkyl-phenyl cleavage and C_α -oxidation of the model compound (i) by PoP

1,4-benzoquinone (vi) and guaiacol (vii)] were produced in smaller amounts compared with the oxidation of model compound (i). These results suggest that the phenolic β -O-4 lignin substructure was readily oxidized by PoP to produce various products via C_α - C_β cleavage, C_α -oxidation and alkyl-phenyl cleavage. 2,6-Dimethoxyhydroquinone (iii) was oxidized to 2,6-dimethoxy-1,4-benzoquinone (vi) by PoP, but the latter compound was not changed by the reaction with PoP. Further reactions from 2,6-dimethoxy-1,4-benzoquinone (vi) may be carried out by other enzyme systems including an enzyme responsible for the quinone ring cleavage. From the above results, the oxidation pathway of β -O-4 lignin model compound (i) mediated by PoP can be proposed, as shown in Figure 5. PoP abstracts an electron from the phenol ring to produce the phenoxy radical which occurs in chemical resonance with the cyclohexadienyl radical (i'). This radical would produce a variety of products via C_α - C_β cleavage, C_α -oxidation and alkyl-phenyl cleavage. C_α - C_β cleavage produced 2,6-dimethoxy-1,4-benzoquinone (vi) and guaiacol (vii). In this mechanism, phenoxy radical (i') would undergo C_α - C_β bond cleavage, giving rise to a quinone methide and a fragment C_6 - C_2 ether radical. Although syringaldehyde was not identified in this experiment, probably due to its low yield, it is expected that rearrangement of the quinone methide would result in the formation of syringaldehyde, which in turn would be oxidized to 2,6-dimethoxy-1,4-benzoquinone (vi) as described previously [30]. This pathway is supported by the fact that syringaldehyde is oxidized by PoP, generating 2,6-dimethoxy-1,4-benzoquinone (vi). The C_6 - C_2 ether radical would decompose as described previously [18,30] to form guaiacol (vii). Alkyl-phenyl cleavage produced 2,6-dimethoxyhydroquinone (iii), 2,6-dimethoxy-1,4-benzoquinone (vi) and phenoxypopropanal (iv). The alternative resonance form of phenoxy radical, a cyclohexadienyl radical (i''), would be oxidized to the corresponding cation (i''), which is susceptible to attack by H_2O and would undergo alkyl-phenyl bond cleavage to yield 2,6-dimethoxyhydroquinone (iii) and phenoxypopropanal (iv). 2,6-Dimethoxyhydroquinone (iii) is readily oxidized by PoP to form 2,6-dimethoxy-1,4-benzoquinone (vi). Alternatively, the cation intermediate (i'') would undergo C_α -oxidation, through the formation of a quinone methide intermediate following the loss of an α -proton, to yield dimeric ketone (ii). This dimeric ketone (ii), in turn, would be oxidized by PoP to the corresponding phenoxy radical (ii'), which would undergo C_α - C_β cleavage to yield 2,6-dimethoxyhydroquinone (iii), phenoxypropanoic acid (v), and 2,6-dimethoxy-1,4-benzoquinone (vi), and alkyl-phenyl cleavage to yield 2,6-dimethoxy-1,4-benzoquinone (vi) and guaiacol (vii), as shown in the case of the oxidation of model compound (i).

As described above, phenolic β -O-4 lignin substructure is oxidatively degraded by PoP. The reaction mechanisms proposed herein, which are similar to those of MnP [30], are initiated by a one-electron oxidation to generate phenoxy radical followed by radical rearrangement and other non-enzymic reactions. As results of these processes, C_α - C_β cleavage, C_α -oxidation and alkyl-phenyl cleavage of dimeric β -O-4 lignin substructure occur, resulting in the formation of quinone and other monomeric

compounds. Since white-rot fungi effectively degrade quinones and hydroquinones [31,32], this unique feature may be important for the degradation of lignin by white-rot fungi. Our results indicate that PoP can oxidize lignin substructures leading to the depolymerization of native lignin.

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