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Horseradish peroxidase oxidizes 1,2,4,5-tetramethoxybenzene by a cation radical mechanism

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ABSTRACT

Recent studies show that ligninase oxidizes various non-phenolic substrates by one electron to give cation radical intermediates. To investigate the possibility that other peroxidases my have a similar mechanism, we tested various methoxybenzenes as substrates with horseradish peroxidase and H_2O_2. We found that 1,2,4,5-tetramethoxy-benzene is a good substrate. The corresponding cation radical intermediate was detected directly by ESR spectroscopy, and product analyses indicated that 2.5-dimethoxy-*p*-benzoquinone is a final product of the oxidation. Experiments with $H_2{}^{18}O$ showed that the quinone oxygens come from water. These results are discussed in relation to the oxidation potentials of peroxidases.

INTRODUCTION

From a physical standpoint (Kuila *et al* 1986; Renganathan, Gold, 1986) and kinetic standpoint (Tien *et al.* 1986), ligninase of *Phanerochaete chrysosporium* can be classified as a peroxidase. Ligninase, however, catalyzes reactions not normally associated with other peroxidases: C_α oxidation, C_α- C_β cleavages, ether bond cleavages, and even aromatic ring cleavage of non-phenolic lignin model compounds (Higuchi, 1986; Renganathan *et al.,* 1985; Tien, Kirk, 1984). These various reactions can be explained on the basis of substrate cation radical formation as a consequence of one electron oxidation by the enzyme (Hammel *et al.,* 1985; Kersten *et al.,* 1985; Kirk *et al.,* 1986; Renganathan *et* al., 1985; Shoemaker *et al.,* 1985). If a phenolic hydroxyl group is present, ligninase oxidizes the substrate to a phenoxyl radical as do other peroxidases (Odier *et al.,* this Seminar). Thus substrate structure determines whether a cation radical is observed.

Our purpose in this study was to determine whether cation radical intermediates can be detected during oxidation of a non-phenolic substrate by a peroxidase other than ligninase, and if so, whether final products are consistent with the mechanism proposed for

ligninase. We also sought to determine whether peroxidases might be characterized on the basis of the redox potentials of the substrates they oxidize, thereby reflecting differences in redox potentials of the enzymes.

The complete family of methoxybenzenes, of which there are twelve congeners, were particularly useful as substrates in elucidating the cation radical mechanism of ligninase (Kersten *et al.,* 1985). In the presence of H_2O_2, ligninase can oxidize at least 9 of the 12 congeners, forming from several of them relatively stable cation radicals (Kersten *et al.,* 1985). From the kinetics of radical formation, and results of product analyses, we concluded that oxidation of 1,4-dimethoxybenzene by ligninase results in demethoxylation to give *p*-benzoquinone and methanol as products. The proposed mechanism indicated that the quinone oxygens were derived from solvent H_2O. We have substantiated this with $H_2{}^{18}O$-labelling studies (unpublished results). Interestingly, Meunier and Meunier (1985) recently reported that *O*-demethylation of 9-methoxyellipticine and N^2-methyl-9-methoxyellipticinium acetate is catalyzed by horseradish (HRP) peroxidase and that the quinone oxygens in the quinone-imine products are also derived from water. In this case, however, a cation radical intermediate was not proposed.

Another advantage of using methoxybenzenes as substrates is that the half-wave potentials of these compounds have been determined; they range from 0.81 V to 1.76 V (Zweig *et al.,* 1964). This allows possible correlations to be made between the redox potential of the substrates and the ability of peroxidases to oxidize them. Hammel *et al.* (1986) reported that ligninase could oxidize polycyclic aromatics with ionizing potentials of $\leq$ ~7.55 eV. Similarly Cavalieri and Rogan (1955) reported that HRP will oxidize poly-cyclics with ionization potentials below 7.35 eV.

RESULTS AND DISCUSSION

To investigate the possibility that methoxybenzenes may be substrates for HRP (Type VI, Sigma Chemical Co., St Louis, MO, USA), we looked for UV-visible spectral changes with the compounds in the presence of the enzyme and H_2O_2. Of the twelve methoxybenzenes, only the 1,2,4,5-congener gave strong spectral changes; three others gave weak spectral changes and the remaining gave no detectable change (Table 1). The spectral changes with 1,2,4,5-tetramethoxybenzene are shown in figure 1; this reaction was further characterized

Consistent with our earlier observations with ligninase, we detected the cation radical of 1,2,4,5-tetramethoxybenzene during its oxidation of the substrate by HRP (+H_2O_2). ESR measurements of the magnetic parameters were as previously described (Kersten *et al.* 1985). Based on our previous results with ligninase, an expected product from the oxidation of 1,2,4,5-tetramethoxybenzene by HRP was 2,5-dimethoxy-*p*-

benzoquinone. In accord with this, the quinone (as its hydroquinone diacetate) was identified by gas chromatography / mass spectrometry as a product of the reaction. Furthermore, $H_2{}^{18}O$-labelling experiments indicated that the quinone oxygens are derived from water, consistent with the hypothetical scheme of figure 2.

Table 1. Oxidation of methoxybenzenes by ligninase and horseradish peroxidase.

Compound	$E_{1/2}$ [a]	Oxidation[b] by Ligninase	HRP
Methoxybenzene	1.76	-	-
1,3- Dimethoxybenzene	ND	-	-
1,3,5-Trimethoxybenzene	1.49	-	-
1,2-Dimethoxybenzene	1.45	+	-
1,2,3-Trimethoxybenzene	1.42	+	-
1,4-Dimethoxybenzene	1.34	+[c]	-
1,2,3,4-Tetramethoxybenzene	1.25	+[c]	-
Hexamethoxybenzene	1.24	+[c]	-
1,2,4-Trimethoxybenzene	1.12	+	w
1,2,3,5-Tetramethoxybenzene	1.09	+	w
Pentamethoxybenzene	1.07	+	w
1,2,4,5-Tetramethoxybenzene	0.81	+[c]	+[c]

[a] *$E_{1/2}$ values are literature values (Zweig et al., 1964) obtained by polarography with respect to a saturated calomel electrode. "ND" indicates that the $E_{1/2}$ was not determined.*
[b] *A "+" indicates that UV-visible spectral changes were observed with enzyme and H_2O_2 while "-" indicates that they were not. A "w" indicates that the spectral changes were weak.*
[c] *Cation radical detected directly by ESR.*

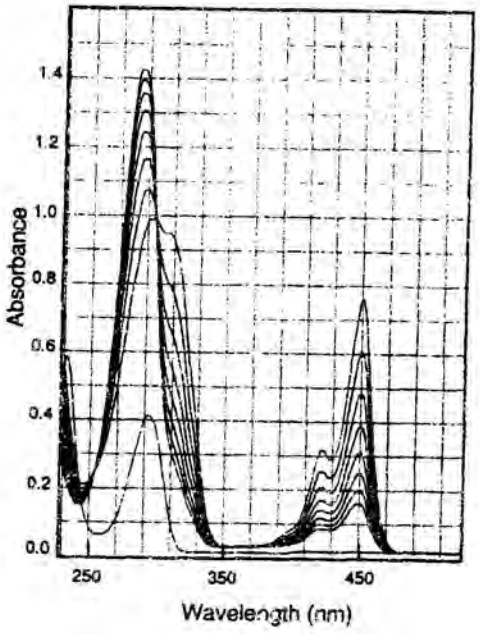

Figure 1. UV-visible spectral changes in 1,2,4,5-tetramethoxybenzene on incubation with HRP and H_2O_2. Upon initiation of the reaction with H_2O_2, the spectrum of the starting material (290 nm λ_{max}, lowest spectrum) was quickly replaced with a new spectrum of λ_{max} 290 nm (shoulder: 305 nm) and λ_{max} 450 nm. The absorbance at 450nm and at 305 nm then slowly decreased to give a new peak at 282 nm. Cycle time was 3.12 min. The reaction was run in 25 mM Na-tartrate, pH 3, with 8 x 10^{-5} 1,2,4,5-tetramethoxybenzene, 10 µg HRP per ml reaction and 3 3 x 10^{-4} H_2O_2.

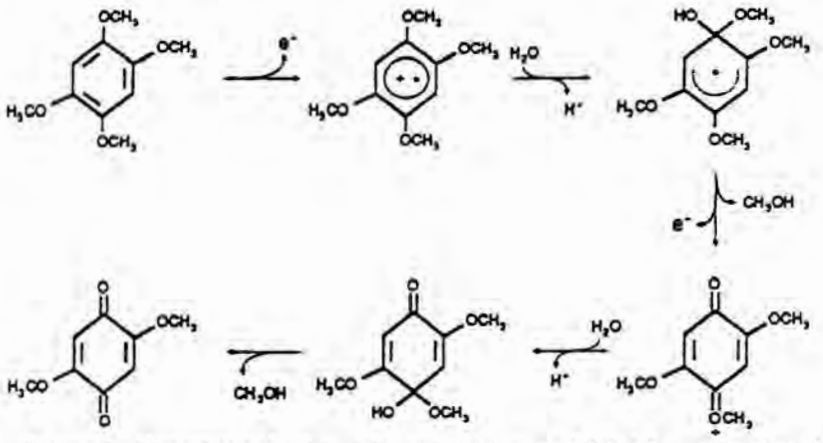

Figure 2. Hypothetical scheme for the oxidation of 1,2,4,5-tetramethoxybenzene by horseradish peroxidase.

CONCLUSIONS

From the results of our product analyses, $H_2{}^{18}O$-labeling studies, and ESR-spectral studies, we conclude that HRP (+ H_2O_2) oxidizes 1,2,4,5-tetramethoxybenzene to its cation radical. Subsequent reactions include nucleophilic attack by solvent H_2O and ultimately result in demethoxylation of the aromatic substrate. Furthermore, the ability of ligninase to oxidize methoxybenzenes of higher redox potential than those oxidized by HRP suggests that a key difference between the two peroxidases is a higher redox potential for ligninase.

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RÉSUMÉ

Des études récentes montrent que la ligninase oxyde divers substrats non phénoliques suivant une réaction à un électron pour donner des intermédiaires de type radical cation. Afin de vérifier que d'autres péroxydases peuvent fonctionner suivant la même réaction, on a recherché l'action de péroxydase de raifort vis-à-vis de différents méthoxybenènes en présence de H_2O_2. Le 1,2,4,5-tétraméthoxybenzène est un bon substrat pour la péroxydase de raifon. Le radical cation corespondant a été détecté comme intemédiaire par spectroscopie RPE et l'analyse des produits frnaux de la réaction a permis d'identifer la 2,5-deméthoxy-*p*-benzoquinone. Les résultats des expériences d'incorporation avec $H_2{}^{18}O$ montrent que l'oxygène de la quinone provient de l'eau. L'ensemble de ces résultats sont discutés en relation avec le potentiel d'oxydation des péroxydases.

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