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ENZYME-CATALYSED OXIDATION OF NON-PHENOLIC AROMATIC COMPOUNDS

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Key Word Index—Ligninase; enzymic oxidation; alkoxybenzenes; benzyl alcohols.

Abstract—It has been shown that the enzyme ligninase is able to catalyse the oxidation of a number of non-phenolic aromatic compounds. The characterisation of two new products from 1,4-dimethoxybenzene and 1,2,3,5-tetramethoxybenzene is also reported. The ability of di-, tri- and tetramethoxybenzene derivatives to facilitate the oxidation of less readily oxidised substrates such as anisyl alcohol and 4-methoxymandelic acid has also been studied.

INTRODUCTION

It has been shown that the enzyme ligninase isolated from the white-rot fungus *Phanerochaete chrysosporum* is able to degrade lignin and catalyses the oxidation of a number of lignin-related model compounds [1, 2]. Ligninase is one of two enzymes which have been isolated from the fungus and is unusual in that it operates at an optimum pH of 3.0. Although H_2O_2 is essential for its activity, a high concentration of H_2O_2 is inhibitory. The enzyme catalyses a wide range of oxido-reduction reactions which use H_2O_2 as oxidant. However, it is unique in being able to oxidise both phenolic and non-phenolic aromatic substrates.

We have studied the ligninase catalysed oxidation of a series of simple mono-, di-, tri- and tetramethoxybenzene derivatives. We have also studied the ability of these compounds to increase the rate of oxidation of less readily oxidised substrates. Compounds which are able to perform this role are defined as mediators. Veratryl alcohol has been previously shown to act as a mediator in the oxidation of substrates such as anisyl alcohol and 4-methoxymandelic acid [3–6].

The purified enzyme was isolated and stored at −18°. The enzyme concentration was determined by UV spectroscopy and its activity estimated by monitoring the rate of formation of veratraldehyde from veratryl alcohol by UV spectroscopy. Reactions were carried out at pH 3.0 and were initiated by addition of H_2O_2. The oxidations were followed by HPLC using a 25 cm Spherisorb ODS2 (5 μ) column eluted with acetonitrile–water (2:3), and using benzonitrile as internal standard. The products were identified by HPLC comparison with authentic samples or, where necessary, by fraction collection followed by solvent extraction and correlation of UV and mass spectral data.

RESULTS AND DISCUSSION

Oxidation of individual alkoxybenzenes

Of the alkoxybenzenes **1**–**7**, compounds **3**, **5** and **6** underwent oxidation to give products which could be detected by HPLC. In the case of compounds **1**, **4** and **7**, although slow disappearance of the substrate was observed, no products were detected using the above HPLC system.

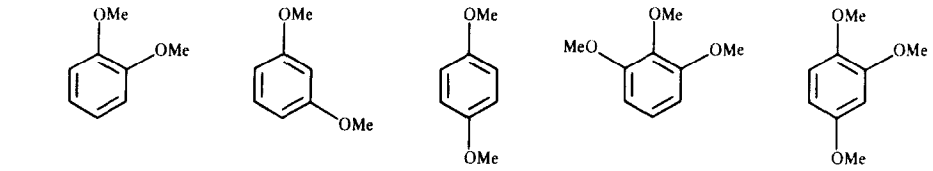
The oxidation of 1,4-dimethoxybenzene **3** gave, in addition to benzoquinone (**14**) [7] (Scheme 1) which could be readily identified, a second product which was less polar than the starting material. The UV spectrum of this compound was determined using a diode-array detector and had maxima at 204 and 280 nm which were unchanged on addition of sodium methoxide solution. Its mass spectrum, determined on a sample obtained by collecting the HPLC fractions, followed by extraction with methylene chloride, gave a molecular ion at *m/z* 244.0738 corresponding to $C_{14}H_{12}O_4$. On the basis of this data and by comparison with data in the literature, this product was identified as 2,6-dimethoxydibenzodioxin (**13**) [10, 11].

The oxidation of 1,2,3,5-tetramethoxybenzene (**6**) gave none of the corresponding quinone, but instead yielded a product having a molecular ion at *m/z* 380.1486 ($C_{19}H_{24}O_8$), corresponding to coupling of two molecules of **6** with formal loss of CH_4. This compound was more polar than the starting material but nevertheless showed no change in its UV spectrum (λ_{max} 242 nm) on addition of sodium methoxide solution. It was therefore identified as a diphenyl ether having one of the isomeric structures **15**–**17**.

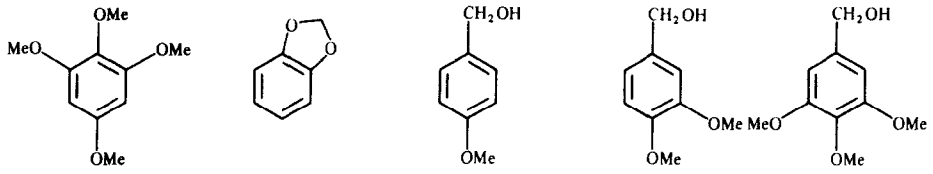
Possible mechanisms leading to the formation of the products formed in the above reactions are shown in Scheme 1. It is quite clear that whilst redox potential plays a part in determining whether alkoxybenzenes are substrates for ligninase, the situation is too complex to allow a simple correlation. Thus **3** ($E_{1/2}$1.34) is oxidised very rapidly but **1** ($E_{1/2}$1.45), **4** ($E_{1/2}$1.42) and **5** ($E_{1/2}$1.12) undergo oxidation only at a moderate rate and **2** ($E_{1/2}$1.49) showed no sign of reaction.

Oxidation of individual benzylic alcohols

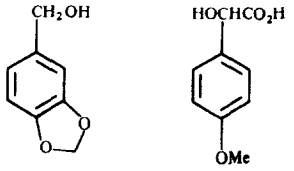
The benzylic alcohols **8**–**12** all underwent oxidation giving rise to products which could be detected by



1 (1 45)* 2 (1 49) 3 (1 34) 4 (1 42) 5 (1 12)

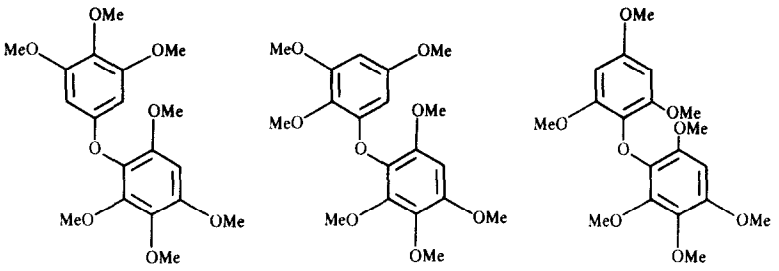


6 (1 09) 7 8 9 10



11 12

* Half - wave potentials obtained by polarography with respect to a saturated calomel electrode [8, 9]



15 16 17

Table 1. Oxidation of anisyl alcohol (**8**) in the presence of alkoxybenzenes

Compound added*	Fate of compound	Anisaldehyde formed	Other products formed
1	Decreased slowly	23%	None
2	Unchanged	—	None
3	Completely destroyed	14%	Benzoquinone + **13**‡
4	Slight decrease	—	None
5	Slight decrease	—	†
6	Decrease	—	**15** (or isomer)
7	Slight decrease	—	None
9	Decrease	5%	Veratraldehyde
10	Slight decrease	7%	Small amount of aldehyde
11	Decrease	—	Piperonal

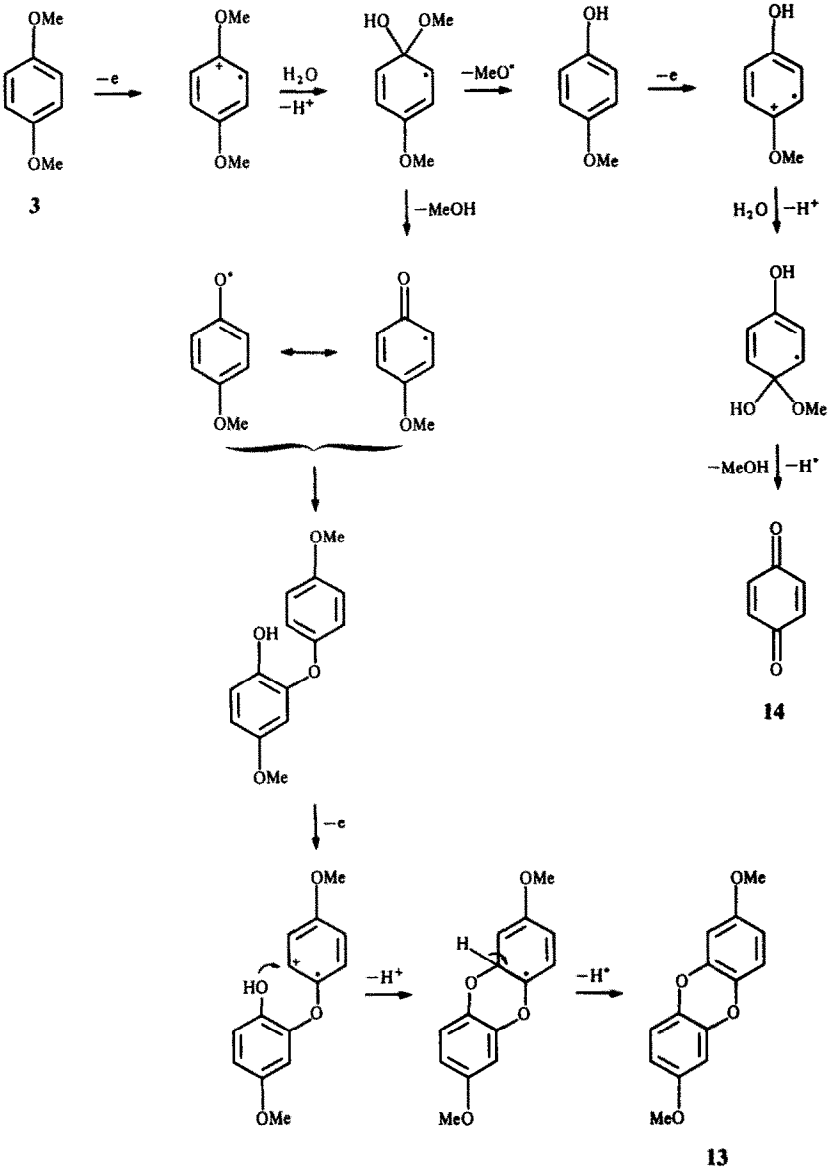
*Ratio of anisyl alcohol to aromatic compound = 2:1 in each case.
†Small amount of unidentified product formed.
‡Benzoquinone alone obtained when 5:1 ratio used

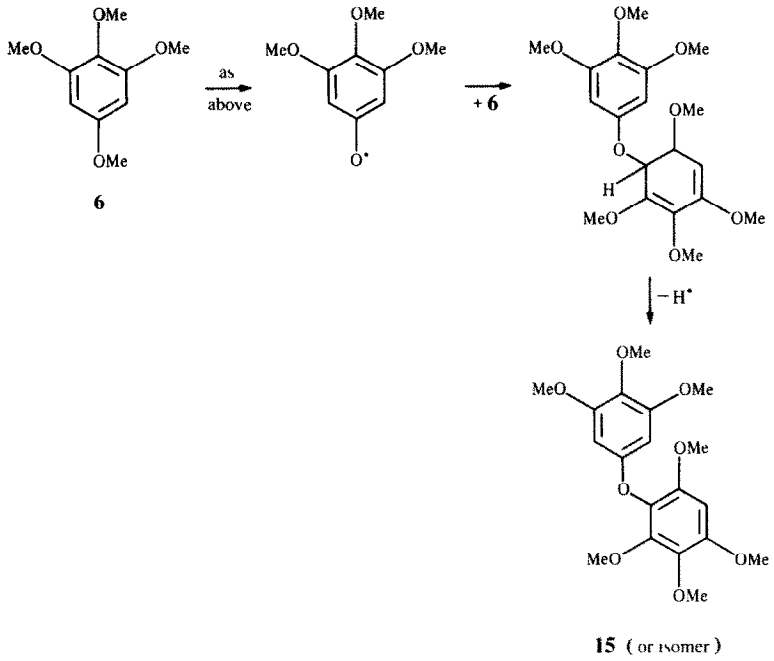
HPLC. However, while veratryl alcohol (**9**) and piperonyl alcohol (**11**) underwent relatively rapid oxidation, anisyl alcohol (**8**), 3,4,5-trimethoxybenzyl alcohol (**10**) and 4-methoxymandelic acid (**12**) were oxidised only very slowly, even when large amounts of the enzyme were used.

Table 2. Oxidation of aromatic compounds in the presence of veratryl alcohol

Compound added*	Fate of compound	Formation of veratraldehyde†	Other products formed
1	Unchanged	Rapid	None
2	Unchanged	Yes	None
3	Destroyed	Rapid	Benzoquinone‡
4	Unchanged	Rapid	None
5	Slight decrease	No§	‖
6	Decrease	No	**15** (or isomer)
7	Slight decrease	Yes	None
10	Slight decrease	Yes	Small amount of aldehyde
11	Decrease	Yes	Piperonal

*Ratio of veratryl alcohol to compound = 5:1 in each case.
†Monitored by UV spectroscopy at 310 nm.
‡Compound **13** also formed when 1:1 ratio of veratryl alcohol to **3** used.
§If more enzyme added then veratraldehyde is formed.
‖Small amount of unidentified product formed.





Scheme 1.

Table 3. Oxidation of 4-methoxymandelic acid (**12**) in the presence of alkoxybenzenes

Compound added*	Fate of compound	Anisaldehyde formed	Other products formed
1	Slight decrease	11%	None
2	Unchanged	—	None
3	Completely destroyed	21%	Benzoquinone
6	Decreased	—	**15** (or isomer)
7	Unchanged	—	None
9	Decreased	18%	Small amount of aldehyde
11	Slight decrease	13%	Small amount of aldehyde

*Ratio of 4-methoxymandelic acid to compound = 5:1 in each case.

Oxidation of anisyl alcohol (**8**) *in the presence of aromatic compounds*

Compounds **1**, **3**, **9** and **10** all increased the rate of formation of anisaldehyde (Table 1). It is particularly noticeable that in the case of 1,2-dimethoxybenzene (**1**) a relatively large amount of anisaldehyde is formed without the dimethoxybenzene itself undergoing significant degradation. Thus, while the use of either 1,2- or 1,4-dimethoxybenzene led to a 15–25-fold increase in the rate of anisaldehyde formation compared to the use of the enzyme alone [3–6], the 1,4-isomer was rapidly destroyed by oxidation to benzoquinone whereas the 1,2-isomer was largely unchanged. It is noticeable that the enzyme reacted rapidly with 1,2,3,5-tetramethoxybenzene (**6**) to give the diphenyl ether (e.g. **15**) and in this case little or no anisaldehyde was formed. Veratryl alcohol **9** and piperonyl alcohol (**11**) also underwent oxidation by the enzyme in the presence of anisyl alcohol and, in the case of **11** in particular, very little anisaldehyde was obtained.

The trimethoxybenzenes **4** and **5** were not rapidly oxidised alone (see earlier) and also were not able to act as a mediator for the oxidation of anisyl alcohol. The comparison of 1,2-dimethoxybenzene (**1**) and 1,3-benzodioxole (**7**) is also of interest because the benzodioxole is unable to act as a mediator for anisaldehyde formation, despite the similarity of its structure to that of **1**.

1,2-Dimethoxybenzene may therefore find a role as a mediator of oxidation of aromatic compounds by ligninase. Unlike veratryl alcohol, it is not itself destroyed by the enzyme and could give less ambiguous results. Thus 1,2-dimethoxybenzene gives 23% of anisaldehyde compared with 5% with veratryl alcohol under the same conditions.

Use of veratryl alcohol as mediator

In most cases the oxidation of veratryl alcohol itself was unaffected by the presence of a second aromatic compound (Table 2). Thus, for example, in the case of

compounds such as **1** and **3**, a decrease in the rate of formation of veratraldehyde was only detected when large amounts of **1** and **3** were present. This is presumably due to competition between the alkoxybenzene and veratryl alcohol for reaction with the enzyme. However, in the case of **5** and **6**, which are also substrates for the enzyme, the formation of veratraldehyde is apparently blocked and only products resulting from oxidation of **5** and **6** were detected. It is noticeable that even under these circumstances, **5** and **6** are not completely or rapidly degraded. Furthermore, when a small amount of fresh enzyme was added 5–10 hr after the start of the reaction, veratraldehyde formation occurs. It would seem, therefore, that in those cases the veratryl alcohol may be acting as a mediator without undergoing oxidation itself. It is also possible that the products of oxidation of **5** and **6** may be acting as inhibitors of the enzyme or that there is an interaction between the substrates themselves and the enzyme which reduces its ability to oxidise veratryl alcohol.

The presence of veratryl alcohol did not apparently increase the rate of oxidation of most of the compounds studied. Thus, in most cases although veratryl alcohol may be involved in the electron transfer process, it does not dramatically alter the outcome of the oxidation reaction.

Oxidation of 4-*methoxymandelic acid* (**12**) *in the presence of aromatic compounds*

Compounds **1**, **3**, **9** and **11** increased the rate of anisaldehyde formation from **12** by 15–20-fold over that observed with the enzyme alone (Table 3). Indeed, **9** and **11** appear to act significantly better in this case than in the corresponding reaction with anisyl alcohol (see above). Although veratryl alcohol **9** and piperonyl alcohol **11** are readily oxidised by ligninase alone, the main products observed here are derived from the 4-methoxymandelic acid, suggesting that **9** and **11** are actively involved in mediating anisaldehyde formation rather than themselves undergoing oxidation. Thus, both **9** and **11** were degraded more slowly in the system containing the 4-methoxymandelic acid.

CONCLUSIONS

Ligninase is clearly able to oxidise a wide variety of non-phenolic aromatic substrates directly. Although the ease of oxidation may in general be connected with the oxidation potential of the compound concerned, the correlation is not a simple one. It is also able to oxidise a number of other compounds such as anisyl alcohol and 4-methoxymandelic acid using simple molecules such as 1,2- or 1,4-dimethoxybenzene as mediator. However, 1,4-dimethoxybenzene suffers from the disadvantage that it is rapidly degraded to benzoquinone and the dibenzodioxin **13**. In contrast 1,2-dimethoxybenzene enhances the oxidation of substrates such as anisyl alcohol and remains largely unchanged or is only slowly degraded. 1,2-Dimethoxybenzene may therefore find a role as a mediator of oxidation of aromatic compounds by ligninase. It may be all the more useful in that, unlike veratryl alcohol, it is not itself destroyed by the enzyme.

EXPERIMENTAL

Isolation of the enzyme was carried out as described in the literature [12]. The enzyme preparation was freeze-dried and stored at −18° under which conditions it showed no loss of activity with time. Estimation of the enzyme activity was carried out by UV spectroscopy using veratryl alcohol as substrate [2]. One unit of activity is defined as the amount of enzyme required to oxidise 1 μmol of veratryl alcohol per min.

The assay incubation contained 0.95 ml Pi buffer (pH 2.75), 0.05 ml veratryl alcohol (20 mM), 5 μl enzyme preparation and 30 μl H_2O_2 (10 mM). The absorption was monitored with a UV spectrometer at 310 nm. The enzyme activity was found to be 28.85 μmol aldehyde formed ml^{-1} enzyme min^{-1}. Estimation of the enzyme concentration was determined by measuring the UV absorption at 407 nm. The native protein has a strong absorption at 407 nm and weak maxima at 500 and 632 nm [13, 14]. The enzyme concentration was 21.33 μM.

Typical reaction conditions were as follows. (i) Reaction of substrate alone with enzyme: 800 nmol compound, 10 μl enzyme preparation, 300 nmol H_2O_2 and Pi buffer (pH 3.0) (20 mM); in a final vol. of 1 ml. (ii) Reaction of substrate with enzyme plus veratryl or anisyl alcohol: 100–800 nmol compound, 10 μl enzyme preparation, 1 μmol veratryl alcohol/anisyl alcohol and Pi buffer (pH 3.0) (20 mM); in a final vol. of 1 ml.

Reactions were carried out at room temp. and initiated by the addition of H_2O_2. Reaction products were analysed by HPLC on a reverse phase 25 cm Spherisorb ODS2 (5 μ) column using MeCN–H_2O (2:3) as eluent and monitoring by UV absorption at 240 nm. Analar or HPLC grade solvents were used and degassed before use. In each case the first injection was performed with neither enzyme nor H_2O_2 present, the second after addition of the enzyme and the third after addition of H_2O_2. Subsequent injections were performed at intervals of 1, 3 and 6 hr. In each case aliquots (10 μl) were introduced directly using a 25 μl syringe into a 20 μl loop and injected onto the column using a Rheodyne valve. The HPLC system used comprised a LDC/Milton Roy Constametric III pump, a Spectromonitor D variable wavelength UV detector and CI-10 integrator. The UV spectra of individual components were measured using an LKB 2140 rapid spectral detector connected to personal computer.

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