Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*

P.J. Harvey, H.E. Schoemaker* and J.M. Palmer

Department of Pure & Applied Biology, Imperial College, Prince Consort Road, London SW7 2BB, England and **Central Laboratory DSM, PO Box 18, NL-6160 MD Geleen, The Netherlands*

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The oxidative capacity of the ligninase from *Phanerochaete chrysosporium* toward monomethoxylated and dimethoxylated aromatic compounds was investigated. Phenylacetic acid derivatives were shown to be decarboxylated by the ligninase, via C–C bond cleavage. Dimethoxylated substrates were much more readily oxidised by the ligninase than were monomethoxylated derivatives, but oxidation of the latter could be stimulated by catalytic amounts of 1,4-dimethoxybenzene or veratryl alcohol. A mechanism based upon the ability of radical cations to function as one-electron oxidants is described. The role of redox mediators in lignin degradation, and the biological significance of veratryl alcohol as a secondary metabolite of *P. chrysosporium* are discussed.

Ligninase *Radical cation* *Redox mediator* *Veratryl alcohol* *Lignin degradation* *Decarboxylation*

1. INTRODUCTION

Ligninase, an extracellular haem protein from *Phanerochaete chrysosporium*, exhibits a broad specificity for aromatic substrates and catalyses a variety of reactions in lignin model substrates [1, 2]. Recent research with the one-electron oxidants tris(phenanthroline)iron(III) and ceric nitrate has led to elucidation of the molecular basis of these reactions: initial one-electron oxidation of the aromatic substrate yields a radical cation which thereafter degrades according to the nature of the substituents on the aromatic ring [3,4]. Ligninase is considered to be a peroxidase catalysing one-electron oxidation of the aromatic substrate in the presence of hydrogen peroxide [3,4] and identification of radical cations generated in the course of the enzymic oxidation of methoxybenzenes [5] as well as recent spectral characterisation of this protein [6,7] support this notion.

Radical cations may act as electron transfer oxidants [8] and are assuming increasing importance as synthetic intermediates in organic electrochemistry [9]. In [4] we suggested that low-M_r aromatic compounds may be oxidised enzymically to produce radical cations that can then act as electron transfer oxidants to induce radical cations in remote and insoluble lignin structures. Here, we describe a novel organic redox system in which enzymically generated and regenerated radical cations, acting catalytically as electron transfer oxidants, effect oxidation of 4-methoxymandelic acid and of anisyl alcohol. We report that the fungal secondary metabolite veratryl alcohol will act as a mediator in this system and discuss the biological significance of this observation.

2. METHODS

Methods for ligninase production, spectrophotometric and HPLC analyses and oxygen consumption were as in [3]. Concentration of H_2O_2 was measured using $E = 0.036\ \mu mol^{-1} \cdot cm^2$ at 240 nm. Oxidation products were identified by

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HPLC analysis using authentic compounds. Rates for 4-methoxymandelic acid and anisyl alcohol oxidation were calculated from the initial increase in absorption due to anisaldehyde formation, using $E = 16.1\ \mu mol^{-1} \cdot cm^2$ at 280 nm; those for homoveratric acid and veratryl alcohol were from the initial increase due to veratraldehyde formation using $E = 9.3\ \mu mol^{-1} \cdot cm^2$ at 310 nm; for 1,4-dimethoxybenzene oxidation, rates were calculated from the increase in absorption at 245 nm using $E = 17.75\ \mu mol^{-1} \cdot cm^2$ corresponding to 1,4-benzoquinone formation. In co-oxidation analyses, anisaldehyde formation was monitored at 275 nm at which point dimethoxybenzene oxidation was isosbestic and was calculated using $E = 15.385\ \mu mol^{-1} \cdot cm^2$.

3. RESULTS AND DISCUSSION

Both 3,4-dimethoxyphenylacetic acid (homoveratric acid) and 4-methoxyphenyl-α-hydroxyacetic acid (4-methoxymandelic acid) were decarboxylated by the ligninase to yield the corresponding methoxylated benzaldehydes, as indicated in table 1a. Decarboxylation reactions such as these may be mediated via C–C bond cleavage in radical cations [10,11], therefore this result is in full accord with the concept that ligninase reactions proceed via enzymically generated radical cations. However, the rate of anisaldehyde formation from 4-methoxymandelic acid was much less rapid than de corresponding rate for veratraldehyde formation from homoveratric acid (table 1a). The mechanism proposed for ligninase catalysis involves initial enzyme-mediated, one-electron oxidation of the substrate, followed by non-enzymic side chain fragmentation in the ensuing radical cation. 4-Methoxymandelic acid has a Cα-hydroxyl substituent which is predicted to increase the rate of side chain cleavage [13] and to simplify the pathway leading to aldehyde production. The fact that the rate of aldehyde formation from 4-methoxymandelic acid was less rapid than anticipated points, therefore, to differences in the relative ease of the initial enzyme-mediated electron transfer step. This step, in turn, seems to be related to the level of methoxylation, dimethoxylated compounds being better electron donors than monomethoxylated. To confirm this deduction, we examined the influence of the enzyme upon anisyl alcohol, a monomethoxylated compound and compared this with the enzymic oxidation of veratryl alcohol, a dimethoxylated homologue. Both compounds were oxidised to the corresponding aldehydes, but whilst veratryl alcohol was rapidly oxidised to yield veratraldehyde, the rate of anisaldehyde formation from anisyl alcohol was even less rapid than that obtained for 4-methoxymandelic acid (table 1a). From these results we conclude that the catalytic capacity of the ligninase is inefficient with regard to the one-electron oxidation of monomethoxylated substrates.

Table 1

Rates of aromatic aldehyde production from different substrates after catalysis by ligninase

Substrate	Rate of aromatic aldehyde production (μmol per unit[a])
(a) Single substrates	
Homoveratric acid	0.77
4-Methoxymandelic acid	0.04
Veratryl alcohol	1.00
Anisyl alcohol	0.01
(b) Co-oxidation with 1,4-dimethoxybenzene	
4-Methoxymandelic acid	0.60
Anisyl alcohol	0.39

[a] One unit oxidizes 1 μmol veratryl alcohol per min

Substrates were present at 800 nmol/ml, apart from 1,4-dimethoxybenzene which was present at 40 nmol/ml

To circumvent this limitation on the oxidative capacity of the ligninase, we considered the possibility that stable radical cations generated by the enzyme might act as mediators to enhance the oxidation of unfavourable substrates [8,9,11,13]. Since the ligninase generates relatively stable radical cations from 1,4-dimethoxybenzene [5], we supplied a catalytic amount of this substrate to an enzyme reaction mixture containing 4-methoxymandelic acid. As shown in table 1b, the rate of aldehyde formation from 4-methoxymandelic acid was now accelerated 15-fold over that obtained with the enzyme alone. Furthermore, anisaldehyde was the only product of oxidation detected and was obtained in excess of the amount of 1,4-dimethoxybenzene supplied (table 2). Addition of 1,4-dimethoxybenzene with anisyl alcohol also ac-

Table 2

The effect of mediation on the oxidation of monomethoxylated substrates

	Substrate	Yield of anisaldehyde (nmol)
No mediator	4-methoxymandelic acid	36
	anisyl alcohol	20
1,4-Dimethoxybenzene as mediator	4-methoxymandelic acid	244
	anisyl alcohol	142
Veratryl alcohol as mediator	4-methoxymandelic acid	548
	anisyl alcohol	82

Substrates (800 nmol) were incubated with 0.06 U enzyme and 230 nmol H_2O_2 in the presence or absence of 20 nmol mediator. After 3 h, when the reactions with mediators were complete, samples were analysed by HPLC

celerated the initial rate of anisaldehyde formation from this substrate (tables 1 and 2).

We propose that in these experiments, the concentration of one-electron oxidant is effectively increased by virtue of the relatively stable radical cations of 1,4-dimethoxybenzene which are generated readily in the course of enzymic oxidation of 1,4-dimethoxybenzene. These radical cations undergo rapid one-electron reduction as a result of electron transfer from the less readily oxidisable substrates, 4-methoxymandelic acid and anisyl alcohol. Ensuing oxidation of 4-methoxymandelic acid or anisyl alcohol yields radical cations which, by contrast to the 1,4-dimethoxybenzene radical cations, react rapidly and irreversibly via C–C or C–H bond cleavage respectively. 1,4-Dimethoxybenzene is at the same time regenerated and can therefore act catalytically as a mediator.

Veratryl alcohol, a natural secondary metabolite of *P. chrysosporium*, has been reported to increase the initial rate of lignin degradation to CO_2 when supplied to cultures of this fungus [14,15]. It also increases the rate of ethylene production from α-keto-γ-methiol butyric acid (KTBA) [18]. Veratryl alcohol can also be readily oxidised by the ligninase to yield veratraldehyde [1] yet at the same time its accumulation in vivo coincides with the appearance of ligninolytic activity [14,16,17]. Since 1,4-dimethoxybenzene could act as a mediator with the ligninase, it seemed likely that these observations could be reconciled if veratryl alcohol also functioned as a mediator with the ligninase. To test this possibility, we investigated the effect of the enzyme on the oxidation of 4-methoxymandelic acid in the presence of a catalytic amount (20 nmol) of veratryl alcohol. As shown in table 2, the yield of anisaldehyde (548 nmol) now exceeded by 15-fold that amount obtained by the enzyme alone (36 nmol). This effect is also illustrated in fig.1. With anisyl alcohol, the yield of anisaldehyde obtained under similar conditions was lower (80 nmol) but nevertheless in 4-fold excess of the amount obtained by the enzyme alone (table 2).

These results support our contention that the role of veratryl alcohol in lignin degradation is as a mediator with the enzyme. Veratryl alcohol is oxidised to a radical cation which, in acidic media, is not rapidly degraded [13]. Therefore in the presence of a second oxidisable substrate it can act as a one-electron oxidant and, in the process of electron transfer, is regenerated. The production of ethylene from KTBA in the presence of veratryl alcohol could therefore be the result of oxidation by the radical cation of veratryl alcohol, with veratryl alcohol acting catalytically as a mediator; in our opinion, the radical of veratryl alcohol is unlikely to function as an oxidant [7]. The stimulatory effect of veratryl alcohol on the oxidation of 4-methoxymandelic acid or anisyl alcohol recalls observations of the stimulatory effects of phenols on NADH oxidation by peroxidases [19–22]. One theory put forward to explain the latter stimulating effect concerns a redox mediation by phenols [19,20]; more recently, it has been proposed that they serve to prevent formation of compound III which is enzymically inactive in NADH oxidation [21,22].

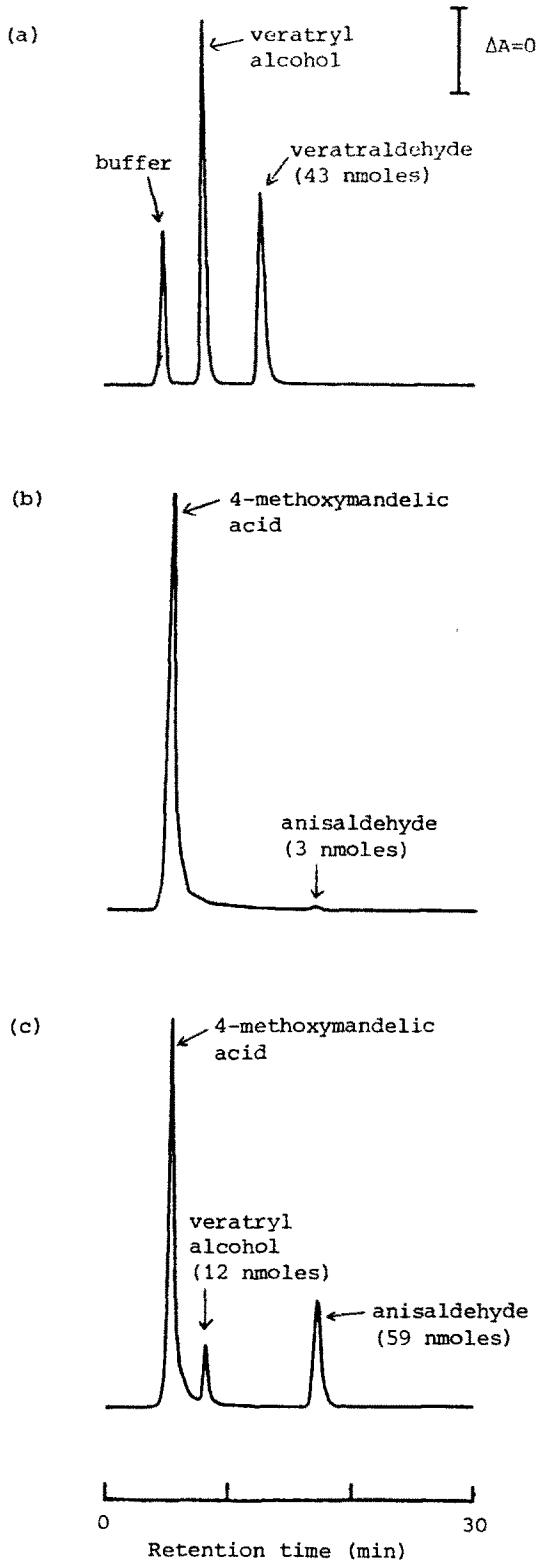
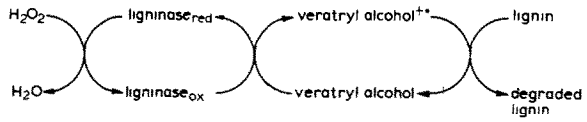


Fig.1. The effect of veratryl alcohol as mediator on the oxidation of 4-methoxymandelic acid. Samples (200 μl) containing 0.01 U ligninase and 47 nmol H_2O_2 were incubated with either 160 nmol veratryl alcohol (a), 160 nmol 4-methoxymandelic acid (b), or 160 nmol 4-methoxymandelic acid with 12 nmol veratryl alcohol as mediator (c). After 3 h, samples were analysed by HPLC.

Veratryl alcohol is thought to play a role in inducing the ligninase [15]. We propose that veratryl alcohol also plays an important role in lignin degradation by the ligninase, as outlined in scheme 1. Ligninase can oxidise a wide variety of substrates directly. Our data show that it can also oxidise substrates indirectly using veratryl alcohol as a mediator. As a consequence, the ligninase enzymes could be located on the surface of the fungal hyphae, in the vicinity of sources of H_2O_2 [23] required for their function, and veratryl alcohol could mediate the oxidation of lignin at a distance from the active site of the enzyme.

Furthermore, charge transfer could take place between aromatic rings in the lignin polymer, which is reported to have an ordered structure [24], up to the point at which side chain cleavage is favoured over charge transfer. Consequently the lignin polymer could be depolymerised at a site removed from the initial site of oxidation.



Scheme 1. Degradation of lignin by ligninase with veratryl alcohol as mediator.

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