2-Chloro-1,4-dimethoxybenzene as a mediator of lignin peroxidase catalyzed oxidations

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Abstract Poly R-478, 4-methoxymandelic acid and oxalic acid were oxidized by lignin peroxidase (LiP) in the presence of the fungal metabolite 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB), whereas no oxidation occurred in the absence of 2Cl-14DMB. These substrates clearly inhibited the consumption of 2Cl-14DMB and the formation of 2-chloro-1,4-benzoquinone from 2Cl-14DMB by LiP. The results suggest that 2Cl-14DMB can replace the function of veratryl alcohol as a redox mediator in lignin peroxidase catalyzed oxidations.

Key words: Lignin peroxidase; 2-Chloro-1,4-dimethoxybenzene; Mediator; Poly R-478; 4-Methoxy mandelic acid; *Bjerkandera* sp. BOS55

1. Introduction

Lignin peroxidase (LiP), an extracellular heme peroxidase produced by several white rot fungi, can catalyze the depolymerization of the aromatic polymer lignin [1] as well as the oxidation of recalcitrant aromatic substrates [2–4]. The H_2O_2 oxidized states of LiP [5] are similar to those of horseradish peroxidase [6].

The fungal secondary metabolite veratryl alcohol (VA) stimulates the LiP oxidation of synthetic lignin [7], dyes [8,9] and a variety of aromatic compounds [10,11].

VA plays important roles in LiP catalysis acting as a redox mediator, aiding in the turnover of the enzyme, and protecting the enzyme from H_2O_2 inactivation [10–15].

In this report, we demonstrate that the fungal secondary metabolite [16,17] 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB) can act as a redox mediator in lignin degradation. Our results provide new evidence for the possible role of other fungal metabolites as redox mediators besides VA in lignin degradation.

2. Materials and methods

2.1. Lignin peroxidase preparation

Bjerkandera sp. strain BOS55 (ATCC 90940) was isolated and maintained as previously described [18]. LiP was produced by growing *Bjerkandera* sp. strain BOS55 in a high-nitrogen medium containing glucose, peptone, yeast extract, VA and mineral nutrients as previously described [19]. LiP was purified from the extracellular fluid of *Bjerkandera* sp. strain BOS55 cultures as previously described [20]. A LiP isozyme mixture was used in these experiments.

2.2. Enzyme assay

LiP activity was measured by monitoring the oxidation of VA to veratraldehyde at 310 nm ($\varepsilon = 9300\ M^{-1}\ cm^{-1}$) as described by Tien and Kirk [21]. One unit of LiP activity was defined as the amount of enzyme required to oxidize 1 μmol of VA per minute.

2.3. Dye decolorization

Decolorization of dyes was monitored at the visible absorbance maximum of each dye, these were tartrazine (426 nm; $\varepsilon = 24800\ M^{-1}\ cm^{-1}$), methyl orange (500 nm; $\varepsilon = 30000\ M^{-1}\ cm^{-1}$), Biebrich scarlet (505 nm; $\varepsilon = 21000\ M^{-1}\ cm^{-1}$) and Poly R-478 (520 nm; $10.5\ (g/l)^{-1}\ cm^{-1}$). Unless otherwise stated in the text, the reaction mixture (1 ml) contained 20–40 μM dye, 0.05 U LiP from *Bjerkandera* sp. BOS55, 2 mM of the mediator compound (VA, 1,4-dimethoxybenzene, or 2Cl-14DMB) and 0.2 mM H_2O_2 in 50 mM sodium tartrate pH 3.0. The reaction was initiated by the addition of H_2O_2 and decrease in absorbance was followed during 5 min. Absorbance was also measured 30 min after initiation. The molar extinction coefficient of each dye was used for calculation of the concentration of the dye in the reaction mixture.

2.4. Mediation of Poly R-478 decolorization

The reaction mixture (0.5 ml) contained 0–0.08 g/l Poly R-478, 50 mM sodium tartrate pH 3.0, 0 to–mM 2Cl-14DMB, 0.05 U LiP and 0.2 mM H_2O_2. Initial decolorization rate and percentage of decolorization after 30 min were measured. The reaction was stopped by the addition of 0.5 ml acetonitrile to the reaction mixture and the samples were analyzed for 2Cl-14DMB consumption and 2-chloro-1,4-benzoquinone (2Cl-14BQ) formation, an oxidation product of 2Cl-14DMB, by HPLC.

2.5. Oxalate oxidation by 2Cl-14DMB

The reaction mixture (0.5 ml) contained 0–50 mM oxalate, 20 mM sodium tartrate pH 3.0, 0.05 U LiP, 1 mM 2Cl-14DMB and 0.2 mM H_2O_2. After 60 min, CO_2 production was measured in the headspace (headspace volume 1.3 ml) by GC. Furthermore, the samples were analyzed for 2Cl-14BQ formation by HPLC.

2.6. 4-Methoxymandelic acid oxidation

The reaction mixture (0.5 ml) contained 1 mM 4-methoxymandelic acid (4-MMA), 20 mM sodium succinate pH 3.0 (99% purity), 0–2 mM 2Cl-14DMB, 0.1 U LiP and 0.2 mM H_2O_2. The reaction was stopped after 30 min by the addition of 0.5 ml acetonitrile to the assay mixture and the samples were analyzed for *p*-anisaldehyde production, the oxidation product of 4-MMA, by HPLC.

2.7. HPLC analysis

50 μl of the incubation mixtures was analyzed for products by high pressure liquid chromatography as described before [22] with the column (200 mm × 3 mm) filled with ChromSpher C18-PAH (5 μm particles) (Chrompack, Middelburg, The Netherlands). Product identification was based on matching retention times with UV spectra of commercially available standards.

2.8. GC analysis

CO_2 concentration in the head space was determined using a Pack-

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Abbreviations: LiP, lignin peroxidase; 2Cl-14DMB, 2-chloro-1,4-dimethoxybenzene; VA, veratryl alcohol; 2Cl-14BQ, 2-chloro-1,4-benzoquinone; 4-MMA, 4-methoxymandelic acid; 14DMB, 1,4-dimethoxybenzene; AAld, anisaldehyde

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Table 1
Percentage decolorization of tartrazine, Biebrich scarlet, methyl orange and Poly R-478 by LiP after 30 min in the absence and presence of 2 mM VA, 14DMB or 2Cl-14DMB

Dye	Decolorization (%)			
	No mediator	VA	14DMB	2Cl-14DMB
Tartrazine	5.1 ± 0.4	89.0 ± 0.3	99.6 ± 0.2	100
Methyl orange	21.5 ± 0.9	84.4 ± 6.7	86.6 ± 4.2	64.0 ± 3.3
Biebrich scarlet	7.0 ± 0.6	97.9 ± 0.2	100	96.8 ± 1.1
Poly R-478[a]	0	55.4 ± 0.17	52.2 ± 1.8	73.1 ± 0.5

Reactions were performed at pH 3.0 and monitored at the absorption maximum of each dye as mentioned in the text.
[a]Poly R-478 concentration in reaction mixture was 24 mg/l.

ard model 427 gas chromatograph (Packard, Delft, The Netherlands) with a Hayesep Q column (Chrompack, Middelburg, The Netherlands). 50 μl headspace samples were analyzed.

2.9. Chemicals

Poly R-478 was obtained from Sigma (St. Louis, MO, USA). Methyl orange was obtained from Acros (Geel, Belgium). Biebrich scarlet and tartrazine were obtained from Aldrich (Steinheim, Germany).

3. Results

3.1. LiP mediated dye decolorization by 2Cl-14DMB

Two classes of dyes were used in the experiments, either azo dyes (tartrazine, Biebrich scarlet and methyl orange) or the polymeric anthraquinone dye, Poly R-478. LiP was used directly or together with various mediators, such as VA, 1,4-dimethoxybenzene (14DMB) or 2Cl-14DMB, to decolorize the dyes.

The azo dyes were not dependent on the presence of a mediator to be oxidized by LiP; however, the mediators greatly stimulated their initial decolorization rate and extent (Table 1). The initial decolorization rate for the azo dyes was improved from 1–2 μM/min in the absence of a mediator to 7–30 μM/min in the presence of a mediator. 2Cl-14DMB stimulated the LiP catalyzed decolorization of the azo dyes in a similar fashion as VA and 14DMB (Table 1). The oxidation of Poly R-478 was completely dependent on the presence of mediators. Initial decolorization rates of Poly R-478 were comparable in the presence of VA, 14DMB or 2Cl-14DMB, ranging from 20–23 mg/l/min. However, after 30 min of incubation, the extent of Poly R-478 decolorization was distinctly higher in the presence of 2Cl-14DMB compared to VA and 14DMB (Table 1).

Experiments were conducted to determine the effect of various 2Cl-14DMB concentrations on Poly R-478 decolorization. Maximum decolorization after 30 min was obtained with only 0.1 mM 2Cl-14DMB (results not shown). An interesting observation was that some decolorization was already evident when as little as 10 μM 2Cl-14DMB was used. No decolorization occurred in the absence of either 2Cl-14DMB, LiP, H_2O_2 or if the enzyme was boiled.

The net consumption of 2Cl-14DMB was inhibited in the presence of 24 mg/l Poly R-478 (Fig. 1A). A significantly

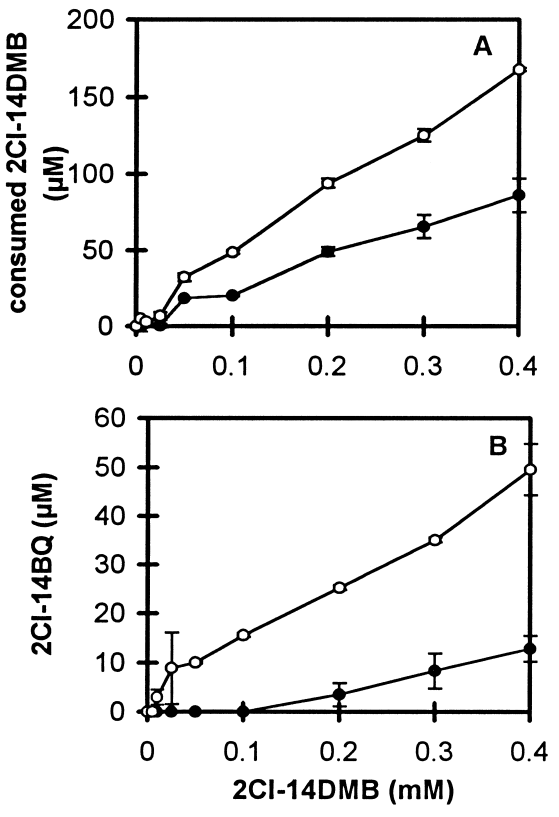

Fig. 1. Effect of the presence (●) and absence (○) of Poly R-478 (24 mg/l) on the oxidation of 2Cl-14DMB by LiP (0.05 U/ml). A: Consumption of 2Cl-14DMB. B: Formation of 2Cl-14BQ, an oxidation product of 2Cl-14DMB.

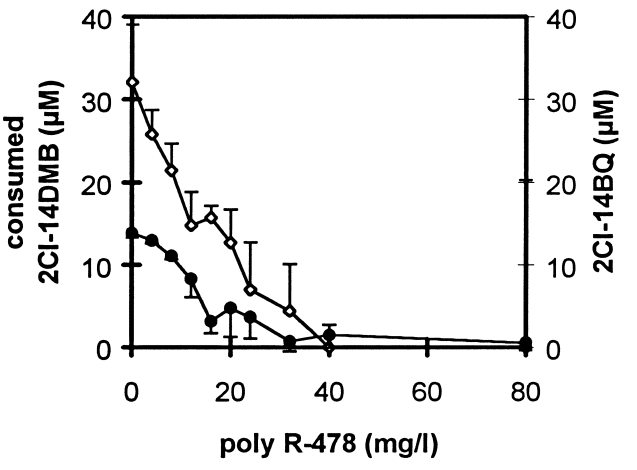

Fig. 2. Effect of varying Poly R-478 concentrations on the oxidation of 2Cl-14DMB (0.1 mM) by LiP (0.05 U/ml). The amount of consumed 2Cl-14DMB is shown (◇) together with the formation (●) of the oxidation product, 2Cl-14BQ.

higher 2Cl-14DMB consumption was obtained in the absence of Poly R-478, for every 2Cl-14DMB concentration up to 0.4 mM. This was also the case for concentrations up to 2 mM 2Cl-14DMB (results not shown). Likewise, the yield of the predominant oxidation product of 2Cl-14DMB, 2Cl-14BQ, was lowered when Poly R-478 was present in the reaction mixture (Fig. 1B).

Increasing Poly R-478 concentrations decreased the consumption of 2Cl-14DMB. At concentrations higher than 40 mg/l, Poly R-478 completely prevented 2Cl-14DMB consumption. These results were confirmed by the inhibition of formation of 2Cl-14BQ (Fig. 2).

3.2. 2Cl-14DMB as a mediator in the oxidation of 4-MMA

The effect of 2Cl-14DMB on the oxidation of 4-MMA (1 mM) was examined. In the absence of the mediator, 4-MMA was not oxidized by LiP. The oxidation of 4-MMA to *p*-anisaldehyde (AAld) increased in a linear fashion with increasing concentrations of 2Cl-14DMB up to 0.3 mM (Fig. 3A). The molar yield of AAld at 0.3 mM 2Cl-14DMB was approximately 100%. Further increases in 2Cl-14DMB concentration resulted in slightly less than optimal yields of AAld. A 4-MMA concentration range up to 0.3 mM rapidly decreased the consumption of 1 mM 2Cl-14DMB, whereas

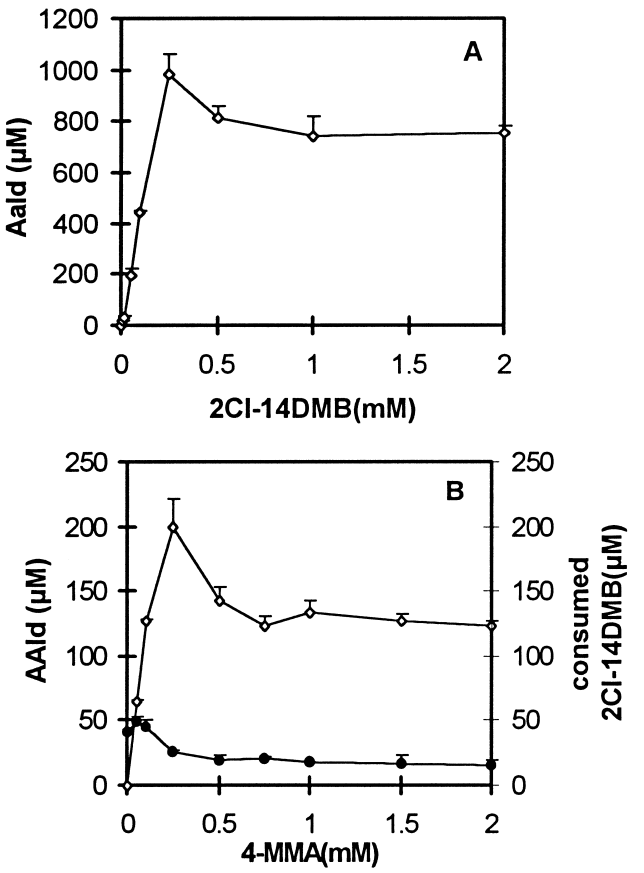

Fig. 3. A: Effect of varying 2Cl-14DMB concentrations on the oxidation of 4-MMA (1 mM) by LiP (0.05 U/ml). The formation of *p*-anisaldehyde (AAld) from 4-MMA is shown. B: Effect of varying 4-MMA concentrations on the oxidation of 2Cl-14DMB (1 mM) by LiP (0.05 U/ml). The consumption of 2Cl-14DMB (●) and the formation of AAld (◇) is shown.

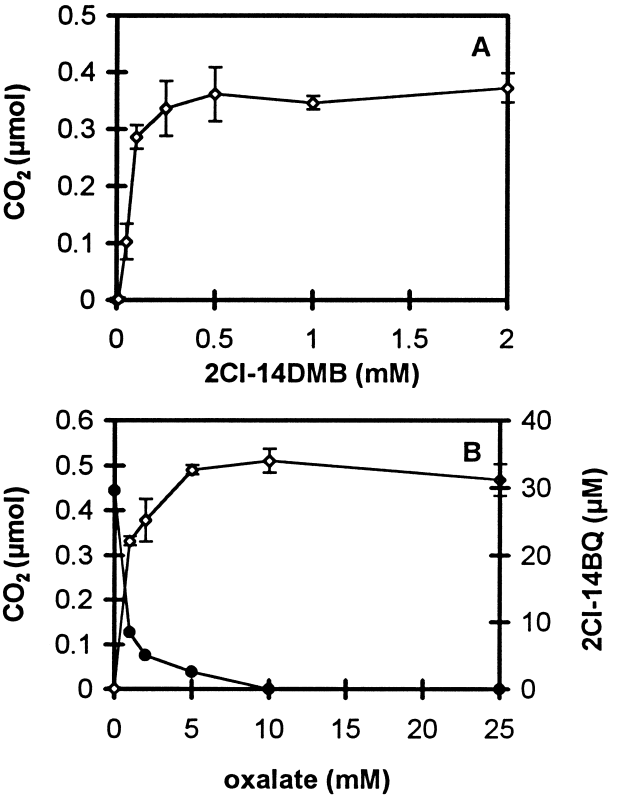

Fig. 4. A: CO_2 production (◇) derived from 2 mM oxalate in 0.5 ml by LiP (0.05 U/ml) in the presence of varying 2Cl-14DMB concentrations. B: Effect of varying oxalate concentrations on the CO_2 production (◇) and the formation of 2Cl-14BQ (●) from 2Cl-14DMB supplied at 1 mM.

AAld production increased up to only 0.3 mM 4-MMA (Fig. 3B). The AAld production decreased when 4-MMA exceeded 0.3 mM.

3.3. Effect of 2Cl-14DMB on oxalate oxidation

The role of 2Cl-14DMB on mediating the LiP catalyzed oxidation of oxalate to CO_2 was examined by applying a range of 2Cl-14DMB concentrations (0–2 mM). Oxalate was not significantly converted to CO_2 in the absence of 2Cl-14DMB. As little as 50 µM 2Cl-14DMB mediated significant oxidation of oxalate which increased up to 250 µM 2Cl-14DMB (Fig. 4A). Higher mediator concentrations did not improve the oxidation of oxalate. Furthermore, increasing oxalate concentrations from 0–50 mM with a fixed 2Cl-14DMB concentration (1 mM) resulted in increasing CO_2 production (Fig. 4B). No CO_2 production was detected in the absence of enzyme or H_2O_2. Formation of 2Cl-14BQ from 2Cl-14DMB decreased to zero with increasing concentrations of oxalate (Fig. 4B).

4. Discussion

2Cl-14DMB is a 1,4-dimethoxybenzene derivative, which is naturally produced by white-rot and litter degrading fungi, such as *Bjerkandera adusta* and *Lepista nuda* [16,17,23]. In this report, we provide evidence that this natural metabolite can act as a redox mediator in the LiP catalyzed oxidation of Poly R-478, 4-MMA and oxalate.

2Cl-14DMB was compared as a mediator with VA and 14DMB in the LiP catalyzed oxidative decolorization of azo and polymeric dyes. The addition of VA, 14DMB or 2Cl-14DMB in the reaction mixture enormously stimulated the decolorization of azo dyes; although, azo dyes were slowly decolorized by LiP in the absence of a mediator (Table 1). Poly R-478 decolorization was completely dependent on the presence of mediators as was found previously with VA [8,9].

The addition of 2Cl-14DMB significantly increased the extent of Poly R-478 decolorization, compared to VA and 14DMB (Table 1). Only 10 μM 2Cl-14DMB was necessary to obtain decolorization of Poly R-478, indicating that this compound could be important under natural conditions. Over a broad range of 2Cl-14DMB concentrations, 2Cl-14DMB consumption was clearly inhibited in the presence of Poly R-478 (Fig. 1A). The formation of the predominant oxidation product of 2Cl-14DMB, 2Cl-14BQ, was also inhibited by the presence of Poly R-478 (Fig. 1B).

Earlier reports showed the inhibition of VA consumption during the decolorization of Poly R-478 [24,25]. VAld, the oxidation product of VA, did not accumulate until Poly R-478 had been fully decolorized [24,25]. The decay of the VA cation radical, formed in the reaction with LiP [25–27], had the same rate constant as the decolorization of the dye. The VA cation radical, generated by pulse radiolysis, was shown to react with the dye [24,26]. In a similar fashion, we propose that LiP oxidizes 2Cl-14DMB to form a 2Cl-14DMB cation radical, as was shown for other methoxybenzenes [2,28]. The inhibition of 2Cl-14DMB consumption in the presence of the dye suggests that the 2Cl-14DMB cation radical ($2Cl\text{-}14DMB^{+\bullet}$) can oxidize Poly R-478 to regenerate 2Cl-14DMB.

Another substrate that was not directly oxidized by LiP was 4-MMA. However, in the presence of 2Cl-14DMB (Fig. 3A) or VA [29], 4-MMA oxidation is possible. The VA cation radical signal is quenched in the presence of 4-MMA [29], implying a direct mediation role for VA. In addition, net conversion of VA by LiP was inhibited by increasing 4-MMA concentrations in the reaction mixture [29]. Our results with 2Cl-14DMB resemble those obtained with VA in that 4-MMA inhibited the oxidation of 2Cl-14DMB, whereas the presence of excess 2Cl-14DMB does not strongly inhibit 4-MMA oxidation (Fig. 3). Obviously, 4-MMA and 2Cl-14DMB do not compete at the active site of the enzyme, otherwise 4-MMA oxidation would be severely inhibited by high 2Cl-14DMB concentrations. Probably, 4-MMA can effectively reduce the putative $2Cl\text{-}14DMB^{+\bullet}$ back to 2Cl-14DMB, resulting in AAld formation and inhibition of 2Cl-14DMB consumption. Harvey et al. [10] proposed this mediation mechanism for 14DMB.

According to Goodwin et al. [13] redox mediation is important for the oxidation of chemicals, which have lower redox potential than compound I and compound II of LiP, but are unreactive with LiP itself. It was shown that EDTA and oxalate are not oxidized by LiP unless VA is present in the reaction mixture [30,31]. Furthermore, it was shown that EDTA and oxalate reduce the VA cation radical back to VA and the compounds are concomitantly decarboxylated [30–32]. In accordance with these results we found that CO_2 production was dependent on 2Cl-14DMB and the CO_2 production increased with increasing mediator concentrations (Fig. 4A). Furthermore, elevated oxalate concentrations increased CO_2 production (Fig. 4B) and inhibited the formation of 2Cl-14BQ from 2Cl-14DMB. Again, the results could be clarified by the formation of $2Cl\text{-}14DMB^{+\bullet}$, which is reduced back to 2Cl-14DMB by oxalate during LiP catalysis.

In this report we provided evidence that the natural fungal metabolite 2Cl-14DMB can replace VA as a redox mediator in the LiP catalyzed oxidation of dyes, 4-MMA and oxalate. Since only catalytic concentrations (10–50 μM) of 2Cl-14DMB were necessary to mediate the oxidation of the substrates, this fungal metabolite could be important in lignin degradation.

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