Do the extracellular enzymes cellobiose dehydrogenase and manganese peroxidase form a pathway in lignin biodegradation?

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Received 29 March 2000; received in revised form 2 June 2000

Edited by Shozo Yamamoto

Abstract The extracellular enzyme manganese peroxidase is believed to degrade lignin by a hydrogen peroxide-dependent oxidation of Mn(II) to the reactive species Mn(III) that attacks the lignin. However, Mn(III) is not able to directly oxidise the non-phenolic lignin structures that predominate in native lignin. We show here that pretreatment of a non-phenolic lignin model compound with another extracellular fungal enzyme, cellobiose dehydrogenase, allows the manganese peroxidase system to oxidise this molecule. The mechanism behind this effect is demethoxylation and/or hydroxylation, i.e. conversion of a non-phenolic structure to a phenolic one, mediated by hydroxyl radicals generated by cellobiose dehydrogenase. This suggests that cellobiose dehydrogenase and manganese peroxidase may act in an extracellular pathway in fungal lignin biodegradation. Analytical techniques used in this paper are reverse-phase high-pressure liquid chromatography, gas chromatography connected to mass spectroscopy and UV-visible spectroscopy.

Key words: Lignin biodegradation; Cellobiose dehydrogenase; Hydroxyl radical; Manganese peroxidase; *Phanerochaete chrysosporium*

1. Introduction

Lignin is a complex branched aromatic polymer of coniferyl, sinapyl and *p*-coumaryl alcohols occurring in cell walls of higher plants [1]. As a biopolymer it is interesting because of its partly random and racemic structure, believed to be caused by radical polymerisation [1]. Native lignin can be divided into phenolic and non-phenolic structures, the non-phenolic ones being dominant in most woods [2,3]. Lignin is an obstacle to biological degradation of wood, but many different types of wood-degrading microorganisms can degrade, or at least modify, lignin. The best known class of such organisms is the basidiomycetic white rot fungi. The white rot fungus *Phanerochaete chrysosporium* has been the preferred model organism for lignin biodegradation and secretes lignolytic enzymes, among others, the heme-containing lignin peroxidases (LP, EC 1.11.1.14) and manganese peroxidases (MnP, EC 1.11.1.13) under lignolytic conditions, i.e. nitrogen starvation [4–6]. The latter reduces hydrogen peroxide and oxidises Mn(II) to Mn(III). Mn(III) is able to perform a one-electron oxidation of lignin [7]. The MnP system can thus oxidise lignin in an indirect mode without direct contact between the lignin and the enzyme. This is important, since the lignin makes the structure of a wood cell wall so compact that a large molecule, such as a protein, cannot penetrate it and thereby get access to the substrate. Manganese is present in most woody tissues [8] and can be further enriched up to 100-fold by transportation into the fungal mycelium [9]. In crystallised MnP the manganese ion is hexa-coordinated and loosely bound to the enzyme [10]. When diffusing away from the enzyme the normally unstable Mn(III) can be chelated and thus stabilised by an organic acid such as oxalic acid [11–13] which is naturally present in lignin degradation [2]. The manganese(III)oxalate complex is known to oxidise many phenolic substances [3]. Neither MnP nor Mn(III)oxalate can, however, oxidise non-phenolic lignin structures directly [3]. Different ways for the fungus to overcome this problem have been suggested, e.g. the involvement of a reaction with an unsaturated fatty acid [14,15]. Another possibility is that MnP cooperates with another enzyme, cellobiose dehydrogenase (CDH, EC 1.1.99.18). This protein is secreted by *P. chrysosporium* under cellulolytic conditions [16], that is, different growth conditions from those resulting in MnP and LP production. In vivo, however, the enzymes are probably present simultaneously [17,18]. CDH oxidises cellodextrins and mannodextrins (preferably cellobiose) to their corresponding lactones. The exact biological role of CDH is not yet fully understood. It is suggested that it (i) prevents product inhibition during cellulose degradation by oxidising cellobiose [16,19]; (ii) produces hydroxyl radicals through a Fenton-type reaction that can take part in lignin and polysaccharide degradation [20]; (iii) inhibits polymerisation of aromatic radicals produced by LP [21]; and (iv) provides MnP with Mn(II) by reducing MnO_2 precipitates [13]. Nothing excludes, of course, that several of the above suggestions might be relevant. In this article we will focus on the hydroxyl radical-producing ability of CDH and its possible interaction with the MnP system.

The Fenton reaction (1) is dependent on the presence of

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Abbreviations: CDH, cellobiose dehydrogenase; GC, gas chromatography; GC–MS, gas chromatography connected to mass spectroscopy; LP, lignin peroxidase; MnP, manganese peroxidase; MS, mass spectroscopy; MSA, α-methylsyringylalcohol; NIST, National Institute of Standards and Technology; RP-HPLC, reverse-phase high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; VG, veratryl glycol

0014-5793/00/$20.00
PII: S0014-5793(00)01757-9

ferrous iron in the solution. Catalytic amounts are, however, enough.

$$H_2O_2 + Fe(II) \rightarrow OH^{\bullet} + OH^{-} + Fe(III) \quad (1)$$

In an aerobic environment Fe(II) readily oxidises to Fe(III) [22], so for the Fenton reaction to have any significance, Fe(II) has to be produced continuously. One possible means to produce the unfavoured Fe(II) is by the continuous flow of electrons from cellobiose modified by CDH [22]:

$$\text{Cellobiose} + 2Fe(III) \xrightarrow{CDH} \text{Cellobionolactone} + 2Fe(II) \quad (2)$$

Hydrogen peroxide is produced by CDH itself but can also be produced by other oxidases from the fungus [22]. Hydroxyl radicals are very reactive and can hydroxylate both phenolic and non-phenolic lignin model compounds to a multitude of products [23]. In another work [24] we have shown that the CDH system can perform reactions that suggest that β-ether linkages in lignin can be broken and aromatic rings can be demethoxylated. Both of these reactions introduce new hydroxyl groups into the lignin. In order to examine whether these reactions can open up for oxidation by the MnP system we have here treated a non-phenolic model compound, veratryl glycol (VG) (Fig. 1), with the CDH system and the MnP system in two separate steps. For comparison we also treated a phenolic model compound, α-methylsyringylalcohol (MSA) (Fig. 1), with the MnP system.

2. Materials and methods

2.1. Chemicals, enzymes and solutions

VG was prepared from bromo-acetoveratrone by acetylation and reduction of the acetate with lithium aluminium hydride [25]. MSA was obtained from the collection of chemicals at the Institution of Pulp and Paper Chemistry and Technology at the Royal Institute of Technology, Stockholm, Sweden. The water was double-distilled (ddH_2O). The silylation kit (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide+trimethylchlorosilane (BSTFA+TMCS), 99:1) was from Supelco Park, Bellefonte, PA, USA. All other chemicals were of analytical quality.

MnP, purchased from Tienzyme, USA, was 75 U/ml. MnP rendered a single band in a 10% SDS–PAGE. CDH was purified from a cellulolytic culture filtrate of *P. chrysosporium* strain K3 as described previously [26,27].

A 2 mM Mn-oxalate solution, pH 5 was made by mixing $Mn(II)SO_4$ to a final concentration of 2 mM in 40 mM oxalic acid adjusted to pH 5 with NaOH. The model compounds were 10 mM in water. Ferriacetate solution was made by dissolving 0.1 mmol $FeCl_3 \cdot 6H_2O$ in 1000 μl 1 M acetic acid and then diluted in a solution to 60 ml. The pH was then adjusted to 4 with NH_3 and the solution was diluted to 100.0 ml.

2.2. Spectroscopic analysis of Mn(III)oxalate accumulation in the presence of model compounds

Spectrophotometric analysis was conducted on MSA and VG but also on differently CDH/MnP-processed VG. Comparison of the spectrophotometric behaviour of the raw model components and the products of the CDH/MnP processing gave information about the type of processing that had occurred. The solutions were rapidly mixed in the cuvette and the absorbance at 500 nm was measured immediately at 25°C with a Shimadzu UV-160 spectrophotometer. The concentration of the Mn(III)oxalate formed was calculated using the extinction coefficient $\varepsilon_{500} = 0.29\ mM^{-1}\ cm^{-1}$ [28].

The Mn(III)oxalate accumulation in the presence of the model compounds was examined as follows: Mn-oxalate (to 1.6 mM), H_2O (q.s.), MSA or VG (to 1 mM), MnP (0.15 U) and H_2O_2 (to 0.1 mM) were added to the cuvette, in that order, to a final volume of 500 μl. Controls were: (1) MSA, Mn-oxalate, H_2O, MnP; (2) VG, Mn-oxalate, H_2O, MnP; (3) Mn-oxalate, H_2O, MnP; and (4) Mn-oxalate, H_2O, MnP, H_2O_2. The solutions were mixed rapidly in the cuvette and the absorbance at 500 nm was measured immediately in the time scan mode.

2.3. Chromatographic analysis of the reaction products

Reverse-phase high-pressure liquid chromatography (RP-HPLC) analysis of the reaction products was performed after isolating them from the proteins using Nanosepp 10 kDa cut-off filters. RP-HPLC separations were carried out on a Hichrom 5C-18 column (4.6×150 mm) at a flow rate of 1 ml/min. The injected volume of each sample was 10 μl. The system was washed with water containing 0.1% trifluoroacetic acid for 0.5 min and thereafter with an increasing linear gradient of methanol with 0.1% trifluoroacetic acid (0–100%) for 5 min and eventually with 0.1% trifluoroacetic acid in methanol. Absorbance at 280 nm was monitored.

The gas chromatography–mass spectrometry (GC–MS) analysis was carried out employing a Hewlett 5890A gas chromatograph (GC). The GC was linked to a VG 70-250 SE high-resolution mass spectrometer. High-purity helium was used as carrier gas at a flow of 2 ml/min. Before the analysis the samples were silylated with trimethylsilane as follows: dried material was dissolved in dried pyridine at a concentration of 8 mg/ml. 8 mg BSTFA+TMCS, 99:1 was added and the sample was incubated at 70°C for 1 h. The sample was thereafter dried under nitrogen and dissolved in dried dichloromethane.

For the GC separations a fused silica capillary column, CP SIL 8CB/MS (60 m), with an inner diameter of 0.32 mm and a film thickness of 0.25 μm was employed. The temperature programme applied in all GC analyses was 50°C for 2 min and then 5°C/min to 310°C for 6 min. The MS interface temperature was 290°C. 1 μl of sample was injected without splitting.

The MS instrument was operated in the electron ionisation mode using an electron energy of 70 eV. The resolution was set at 1000 and the ion source temperature was held at 180°C. A scan range of 40–700 *m/z* and a scan rate of 1 scan/s were used throughout the study.

2.4. CDH treatment

Pretreatment of VG with CDH was conducted in a water bath at 35°C in the following manner and with the following concentrations: ammonium acetate buffer (46 mM), CDH (0.4 μM), VG (0.8 mM) and ferroacetate (5 μM) were added in that order to a final volume of 1000 μl. The same mixture, but without CDH, was used as a blank. To these solutions was thereafter added 20 μl of 100 mM cellobiose and 10 μl of 10 mM H_2O_2 at 8 h intervals over 24 h. The solutions were finally filtered with a Nanosepp 10 kDa cut-off filter to remove the CDH. The filtrate was either directly analysed spectrophotometrically as described above (with the same concentration as the model compounds used there) or was freeze-dried before RP-HPLC and GC–MS analysis. Some of the sample was treated further with MnP as described below.

2.5. MnP treatment

The CDH-treated VG was incubated with MnP at 35°C using the following final concentrations (volume 1000 μl): Mn-oxalate 0.1 mM, ddH_2O q.s., ammonium acetate buffer pH 5 10 mM, CDH-treated VG (8 mM), MnP (0.2 U/ml) and H_2O_2 in 0.1 μmol portions totally 30 times (totally 3 mM) separated by 15 min intervals to protect the system from H_2O_2 accumulation. Controls were: (1) VG treated with MnP, (2) MSA treated with MnP blank, (3) MSA treated with MnP, (4) MSA in only the ammonium acetate buffer and without addition of H_2O_2 and (5) VG treated with CDH blank and then treated with MnP. The filtrates were freeze-dried before RP-HPLC and GC–MS analysis.

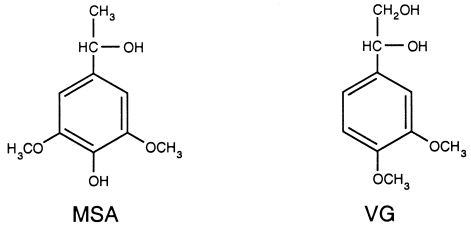

Fig. 1. The two model components used in this work.

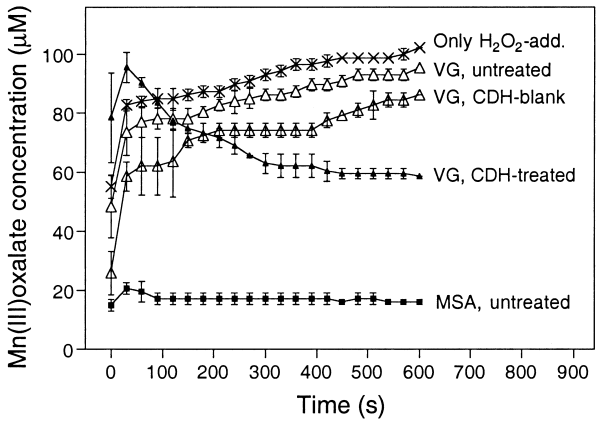

Fig. 2. Mn(III)oxalate accumulation at H_2O_2 addition with no model compound present, with differently treated VG present and with MSA present. 'No model compound' is control 4 mentioned in Section 2.2. MSA is control 1 mentioned in Section 2.2.

3. Results

3.1. Spectroscopic analysis of Mn(III)oxalate accumulation in the presence of model compounds

Without any model compound added (control no. 4, controls described in Section 2.2) the Mn(III)oxalate concentration first rises fast and then enters a slower but still rising mode, resulting in a concentration of 102 μM at 600 s (Fig. 2). The pattern is almost the same when VG is present, but the concentration is generally lower. When MSA is present the pattern is radically altered. The fast rise in the beginning is still present, but the Mn(III)oxalate concentration peaks at 21 μM at 30 s, followed by a slow decrease to a final value of 16 μM at 600 s. Control no. 1 showed a curve similar to, or slightly lower than, the curve for MSA (Fig. 2). The same was true for controls 2 and 3 (data not shown).

3.2. Spectroscopic analysis of CDH-treated VG

Without any CDH present in the CDH system (see Section 2) the VG shows approximately the same pattern as totally unmodified VG (Fig. 2). When CDH was present in the mixture the pattern of the VG changed drastically. The rise of Mn(III)oxalate in the beginning is higher (with a peak at 96 μM at 30 s) and the Mn(III)oxalate concentration at 600 s is lower (59 μM) than in the blank (86 μM). Notable is that the shape of the curve resembles the one for MSA rather than the one for VG (see Fig. 2) indicating that Mn(III)oxalate is being consumed.

3.3. Chromatographic analysis of CDH-treated VG

The RP-HPLC chromatograms of CDH-treated VG (Fig. 3) show, beside the big peak corresponding to unmodified VG, three new peaks at retention times of 7.1, 7.7 and 8.9 min. The peaks do not appear in the CDH blank-treated (only the Fenton system) or in the MnP-treated sample (data not shown). Most important, the peaks are also absent in the sample, which was first CDH-treated and then MnP-treated. The peak at 8.9 min in this sample is, however, not absent but slightly degraded. The remaining controls showed, as expected, that MnP converts MSA and that treatment of VG with only the Fenton system and then with MnP gives the

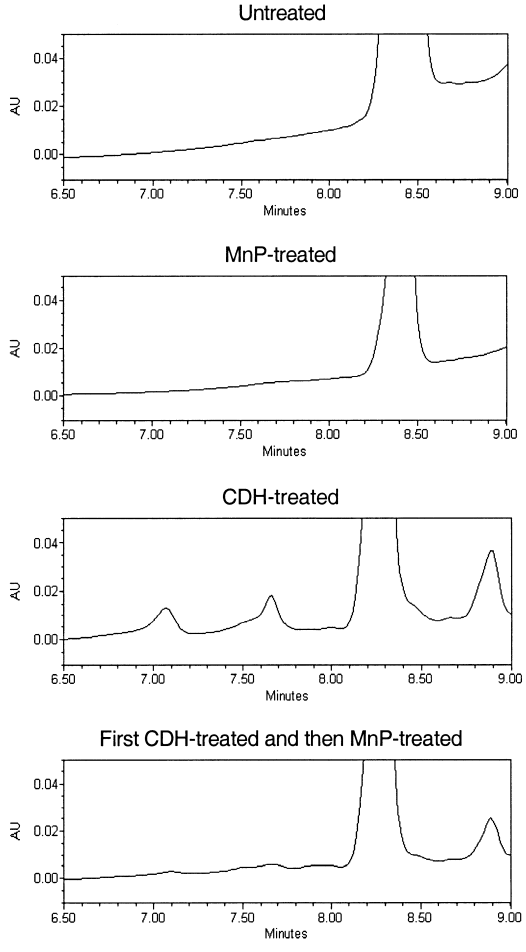

Fig. 3. HPLC chromatograms for differently treated VG.

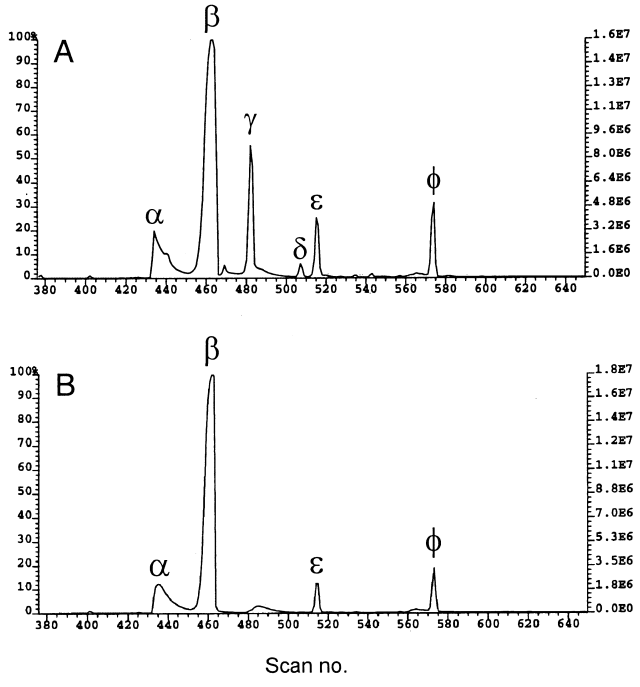

Fig. 4. GC chromatograms. (A) VG, CDH-treated. (B) VG, CDH- and then MnP-treated.

same result as only MnP treatment (data not shown). The chromatograms indicates that the three new peaks are CDH-related but that the 8.9 min peak cannot be degraded efficiently by MnP.

The GC curve for the VG treated with CDH shows six main peaks (marked α–ϕ in Fig. 4A). The γ and δ peaks are not present in the CDH blank (CDH omitted), indicating that these two peaks are CDH-related. The MS data for the γ peak in Fig. 4A reveal (Fig. 5) that the largest substance present in that peak has a mass of 400 Da. The MS data for the δ peak indicate a mass of 458 Da (Fig. 5). After comparison with reference data in the NIST (National Institute of Standards and Technology) library (Fig. 5) it is reasonable to draw the conclusion that the γ peak is one, or both (they are identical in NIST), of the two isomers of VG with one of the methoxy

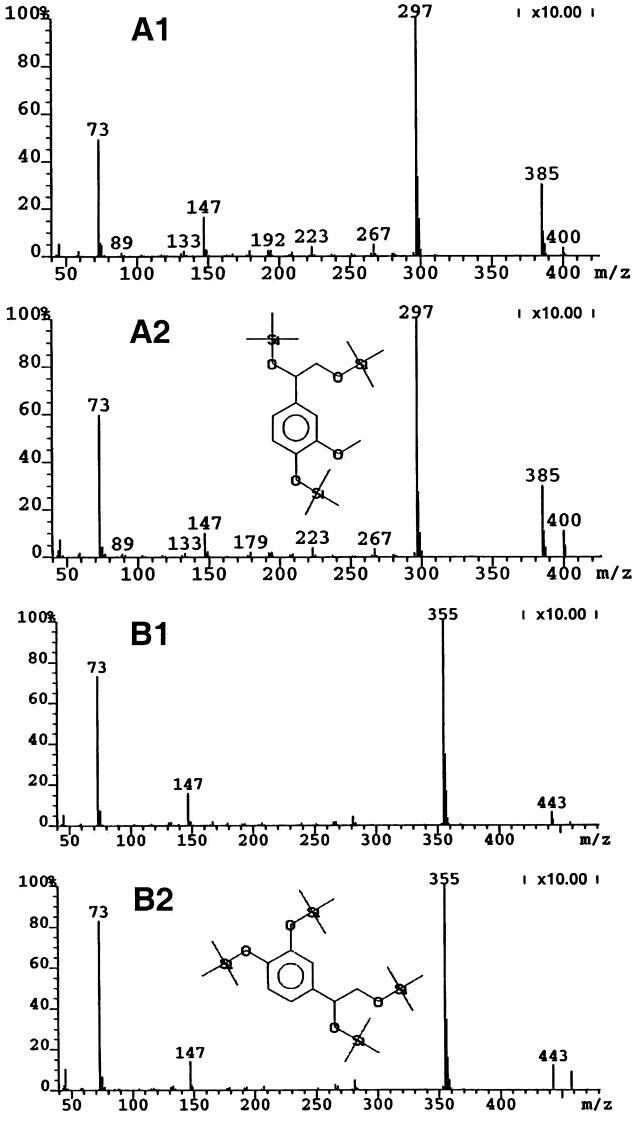

Fig. 5. MS data for the γ and δ peaks in Fig. 4 and control substances in the NIST library. A1: The γ peak in Fig. 4. A2: Silylated VG with one demethoxylation/hydroxylation, CAS: 68595-81-3 in the NIST library, MW 400. B1: The δ peak in Fig. 4. B2: Silylated VG with two demethoxylations/hydroxylations, CAS: 56114-62-6 in the NIST library, MW 458. The purity is 990 of 1000 and 998 of 1000 for A1 compared to A2 and B1 compared to B2 respectively.

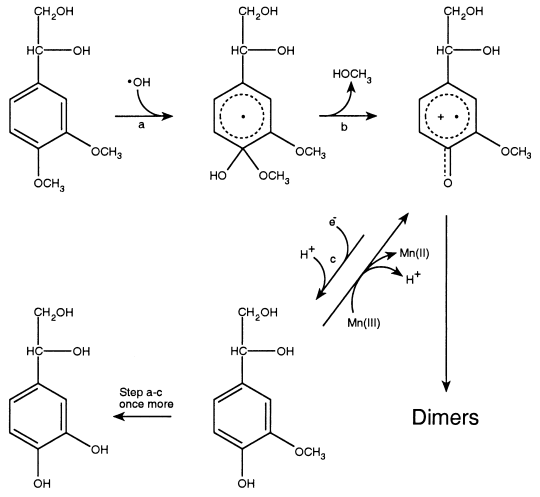

Fig. 6. Suggested reaction, generated by the CDH system, for replacement of methoxy with hydroxyl groups on the VG. Step a: a hydroxyl radical is attached and the free electron is delocalised on the ring; step b: a methanol leaves the VG; step c: a proton and an electron are donated (probably by the $FADH_2$ group in CDH). Steps a–c can then be repeated to generate another demethoxylation. In addition to this further demethoxylation, the MnP system can possibly generate dimers after step c.

groups replaced by a hydroxyl group and that the δ peak is VG with both of the methoxy groups replaced by hydroxyl groups. It should be mentioned that the δ peak is comparatively small and the conclusions drawn above are thus more uncertain than in the case of the γ peak. The ε and ϕ peaks are degradation products from the added cellobiose and the α peak is a broad peak consisting of VG where the trimethylsilyl has lost methyl groups to varying degrees.

After MnP treatment the γ and δ peaks in the GC curve disappeared (Fig. 4B) indicating that MnP has degraded or modified the hydroxylated VG. The small remaining bump was also present in a control with the CDH blank treated with MnP (data not shown). The other controls led us to the same conclusions as just described for the RP-HPLC controls (data not shown).

4. Discussion

After CDH treatment, new peaks showed up in both RP-HPLC and GC. RP-HPLC indicated that two of these peaks were more polar and/or smaller than VG and GC–MS suggested that they were mono-demethylated and di-demethylated VG. The CDH-treated VG was able to consume Mn(III)oxalate in a manner similar to MSA. Furthermore the two new peaks disappeared both on HPLC and on GC after CDH treatment followed by MnP treatment. All the above data indicate that the CDH system has modified VG into structures that can be further oxidised by the MnP system. A third peak showed up in HPLC at 8.9 min, after CDH treatment, suggesting the formation of a less polar and/or bigger component than VG. The peak was still present after MnP treatment. No corresponding peak could be identified in GC–MS. The reason for this might be that the phenoxy radicals generated by the oxidation by Mn(III)oxalate polymerise to structures with too high a molecular weight for identifica-

tion with the GC–MS system used in this work. Such polymerisations are expected to be less important in vivo, since the target there is a part of a polymer and not, as here, a water-soluble model compound.

In summary, the MnP system could apparently not oxidise the non-phenolic model compound (VG) contrary to the phenolic model compound (MSA). However, if hydroxyl radicals generated by CDH first react with this component, one or even two hydroxyl groups are introduced at the former positions of the methoxy groups and an oxidation can be performed by Mn(III) generated by the MnP system. In an earlier work we reported similar demethylations of VG as shown above [24]. This was shown by GC–MS studies and RP-HPLC. Gamma radiation of VG gave a similar pattern as CDH treatment suggesting that hydroxyl radicals are the active component. Since the chemistry of the paramethoxy carbon of VG is similar to that of a β carbon in lignin [25], this strongly suggests that CDH-generated hydroxyl radicals can cleave β aryl ethers in lignin. Depolymerisation of synthetic lignin has also been demonstrated [29]. Both the MnP and CDH systems seem to be able to produce radicals that can polymerise into larger structures.

Tanaka et al. have pointed out the possible importance of hydroxyl radicals (partly produced by CDH) in wood degradation by *P. chrysosporium* [30] and demonstrated a correlation between the production of hydroxyl radicals and lignin degradation by MnP in *Trametes versicolor* [31], although the hydroxyl radicals are produced by another mechanism than suggested in this work. In Fig. 6 we present a tentative action pattern for the hydroxyl radicals on a non-phenolic model compound.

In lignin biodegradation, the two enzymes CDH and MnP have the potential to degrade the most common linkages between lignin monomers, both in phenolic and in non-phenolic lignin, by the generation of the reactive species $OH^{\bullet}$ and Mn(III). However, it remains to be demonstrated that a real depolymerisation of lignin takes place under these conditions even though some data already connect CDH to lignin degradation [17,21]. In addition to ether linkages, lignin also contains condensed structures with carbon–carbon bonds between the monomers [1]. It is not known whether the CDH/MnP system can break these linkages, but it is possible that water-soluble lignin containing these structures is oxidised by the extracellular peroxidase LP, which has an unusually high oxidative power [32]. An extracellular pathway for lignin biodegradation may thus be constituted as follows. Firstly, CDH generates hydroxyl radicals which cause demethoxylation and break ether linkages in non-phenolic lignin. Secondly, Mn(III) generated by MnP oxidises the generated and original phenolic lignin structures causing further depolymerisation and thirdly, LP oxidises water-soluble lignin oligomers resulting in a final depolymerisation. The sequence of events in the scheme should, of course, not be taken as absolute; MnP and CDH systems probably alternate on different lignin structures.

Acknowledgements: This work was supported by grants from the School of Chemistry, Royal Institute of Technology to G.H. and by grants from the Swedish Research Council for Engineering Sciences to G.J. We thank David Eaker, Uppsala University for linguistic revision and Josef Giener, Royal Institute of Technology for fruitful discussions.

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