The Role of Natural Chlorinated Hydroquinone Metabolites in Ligninolytic Fungi

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The Role of Natural Chlorinated Hydroquinone Metabolites in Ligninolytic Fungi

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Thesis Wageningen. - With summary in Dutch

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Stellingen

1. Ondanks het feit dat Cai en Tien hebben aangetoond dat LiP compound III* een artefact is, en geen complex tussen LiP compound III en waterstofperoxide, wordt LiP compound III* toch nog regelmatig als zodanig in publicaties vermeld.
   Cai, D., and M. Tien (1992) J. Biol. Chem. 267:11149-11155.

2. Het succes van de "directed enzyme evolution"- methode is direct afhankelijk van de gebruikte screenings- of selectiemethode.

3. De door Verger en de Haas gegeven definitie van lipases is achterhaald, aangezien een *Bacillus subtilis* lipase beschreven is zonder aantoonbaar verhoogde activiteit in interfaces.
   Verger, R., and G. H. de Haas (1976) Annu. Rev. Biophys. Bioeng. 5:77-117.
   Lesuisse, E., K. Schanck, and C. Colson (1993) Eur. J. Biochem. 216:155-160.

4. De suggestie van Candeias en Harvey dat de binding van het veratrylalcohol kationradicaal aan lignine peroxidase niet alleen de levensduur van het kationradicaal verlengt, maar tevens de reactiviteit, is in strijd met algemeen gangbare chemische principes.
   Candeias, L. P., and P. J. Harvey (1995) J. Biol. Chem. 270:16745-16748.

5. De verwijdering van het *DNA adenine methylase*-gen uit *Salmonella typhimurium* heeft volgens Heithoff *et al.* geleid tot de ontwikkeling van een levend vaccin. Echter, zolang niet duidelijk is hoe dit micro-organisme zijn ziekmakend vermogen is kwijtgeraakt, is het vaccin niet toepasbaar.
   Heithoff, D. M., R. L. Sinsheimer, D. A. Low, and M. J. Mahan (1999). Science 284:967-970.

6. De ontdekking van *Thiomargarita namibiensis*, de "Namibische zwavelparel" door Schulz *et al.* vergroot niet alleen de kennis omtrent de aanpassing van micro-organismen aan hun omgeving, maar ook letterlijk de gemiddelde grootte van bacteriën.
   Schulz, H. N., T. Brinkhoff, T.G. Ferdelman, M. Hernández Mariné, A. Teske, and B. B. Jørgensen (1999). Science 284:493-495.

7. De milieunormen in Nederland houden onvoldoende rekening met de natuurlijke productie van gechloreerde verbindingen.
   Dit proefschrift

8. Het falen van het bursaalsysteem voor promovendi toont aan dat universiteiten niet met hun tijd meegaan.

9. Indien men echt belangrijk is, kan men het zich permitteren geen mobiele telefoon te hebben.

10. Het klonen van Dolly's en menselijke embryo's zet de biotechnologie als geheel in een kwaad daglicht.

11. In Nederland wordt een voltijdwerkende man met kinderen allereerst beschouwd als kostwinner en niet als slechte ouder. Dit geldt niet voor een voltijdwerkende vrouw met kinderen.

12. Ook vrouwen kunnen op zondag het vlees snijden.

13. Een goede wetenschapper toetst een theorie op basis van zijn/haar resultaten en niet andersom.

14. "Pro-life" activisten tonen geen respect voor het (on)geboren leven, indien ze aanslagen plegen op abortusklinieken.

15. Het feit dat extreem rechts weinig politieke invloed heeft in Nederland betekent niet dat Nederlanders tolerant zijn ten opzichte van buitenlanders.

16. Het publiceren in een wetenschappelijk tijdschrift wordt aanzienlijk vergemakkelijkt als één van de auteurs een zekere mate van naamsbekendheid heeft.

Stellingen behorende bij het proefschrift, getiteld "The Role of Natural Chlorinated Hydroquinone Metabolites in Ligninolytic Fungi". Pauline Teunissen, Wageningen, woensdag 16 juni 1999.

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1

General Introduction

Organohalogen sources

Organohalogens, which are organic compounds containing one or more chlorine, bromine, iodine or fluorine atoms, have generally been regarded as toxic xenobiotic compounds. Chlorinated aromatic compounds like pentachlorophenol (PCP), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), and polychlorinated dioxins/furans are well-known environmental pollutants. These chloroaromatic compounds have caused a great deal of public concern, because of their persistence in the environment, threat to public health and animal populations, as well as their ability to bioaccumulate in animal fat which can build up to toxic levels (73, 141). However, the public's attitude towards organohalogens is also based on the belief that organohalogens are mainly derived from anthropogenic sources, such as industrial wastes, agriculture, wood preservation, and cleaning agents (93). To date, compounds such as PCBs, DDT, and PCP have been banned from industry in large parts of the Western world (73, 141).

The presence of xenobiotic organohalogen pollution in the environment is monitored by bulk parameters, such as adsorbable organic halogens (AOX). Surprisingly, the pool of AOX detected in unpolluted environments was recently shown to be at least 300 times greater than which could be accounted for by anthropogenic sources (7, 50). These observations raised questions regarding the origin of these organohalogens and

were the starting point for an extensive research on the occurrence of naturally produced organohalogens. To date, over 2900 different naturally produced organohalogens have been identified (48).

Natural organohalogen production by Basidiomycetes

The largest source of naturally occurring organohalogen compounds is marine life (46). However, an extensive pool of organohalogens was also found in terrestrial environments, especially in forest soils (7, 49). Samples of forest soil collected worldwide contain AOX concentrations of between 20 mg and 360 mg/kg dry weight soil (7). AOX production was shown to take place during the decay of forest litter (8). Therefore, microorganisms that degrade lignocellulosic material are implicated in the production of organohalogens.

Basidiomycete fungi are the ecologically most significant group of organisms responsible for the conversion of lignocellulosic material such as wood, straw, and litter (160). The most rapid and extensive degradation of lignin described to date is caused by white rot fungi (15, 44).

Recently, Verhagen et al. (180) demonstrated that organohalogen production is a ubiquitous capacity among commonly occurring basidiomycete fungi based on an extensive screening program using AOX measurements. Many of the high- and moderate-AOX-producing species are ecologically significant fungi, such as the white rot fungi *Hypholoma fasciculare, Bjerkandera adusta, Armillaria mellea,* and *Mycena* spp..

Types of organohalogens produced by Basidiomycetes

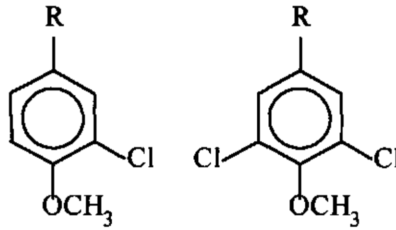
A large number of organohalogen metabolites have been described in Basidiomycetes. Most of the reported metabolites are chlorinated, although some brominated and iodated metabolites have been described. The organohalogen

metabolites can generally be classified as either chlorinated aliphatic or aromatic compounds.

The most important chlorinated aliphatic compounds are chloromethane and the chlorinated nonprotein amino acids (89). To date, seven different halomethane metabolites are known from several genera (62, 77, 152), ranging from chloromethane to dichloroiodomethane and even chloroform was detected. Chlorinated nonprotein amino acids, all of the unsaturated norleucine-type, were isolated from different species of the genus *Amanita* (21, 65, 66, 126, 127).

However, most organohalogens produced by Basidiomycetes have an aromatic structure. The majority of metabolites are simple methyl ethers of chlorophenolic structures such as the chlorinated anisyl metabolites (CAM), and the chlorinated hydroquinone methyl ethers (CHM) (34, 89).

CAM (Figure 1) represent 7 metabolites that are known from 16 genera (35, 89). In laboratory cultures, CAM are produced at relatively high concentrations ranging from 0.2 up to 108 mg/liter (34, 157). Interestingly, CAM-producing fungal strains include ecologically significant species such as *Bjerkandera, Mycena,* and *Hypholoma* (88, 157). The most frequently produced CAM are 3,5-dichloro-*p*-anisyl alcohol (IV) and 3-chloro-*p*-anisaldehyde (II). Recently, 3,5-dichloro-*p*-anisic acid methyl ester (VII) and other CAM related metabolites, chlorinated 4-hydroxybenzoic acid derivatives, were isolated from the extracellular fluid of mycelial cultures of *Bjerkandera* spp. (156).



R		
CH_2OH	I	IV
CHO	II	V
COOH	III	VI
$COOCH_3$		VII

Figure 1. *Chlorinated anisyl metabolites produced de novo by Basidiomycetes. (I) 3-chloro-p-anisyl alcohol (II) 3-chloro-p-anisaldehyde (III) 3-chloro-p-anisic acid (IV)3,5-dichloro-p-anisyl alcohol (V)3,5-dichloro-p-anisaldehyde (VI)3,5-dichloro-p-anisic acid (VII) 3,5-dichloro-p-anisic acid methyl ester*

Several chlorinated hydroquinone metabolites (CHM) have been described in 11 genera (35). The most common structures are shown in Figure 2. The first chloroaromatic metabolite of this category that was identified was tetrachloro-4-methoxyphenol (Figure 2; IX) with a structure very similar to the wood preservative PCP (2, 94). Tetrachloro-4-methoxyphenol, denominated as drosophilin A (DA) was originally isolated from the culture fluid of *Drosophila subatrata,* which is now classified as *Psathyrella subatrata* (94). Further research also showed the biosynthesis of the methylated drosophilin A analog, tetrachloro-1,4-dimethoxybenzene (VIII) (denominated as drosophilin A methyl ether; DAME) by several fungi. DA and DAME have now been detected in 13 species from 9 genera, including *Hypholoma, Bjerkandera* and *Phellinus* spp. (89). Recently DA production was demonstrated in *Hypholoma* spp. (158, 181).

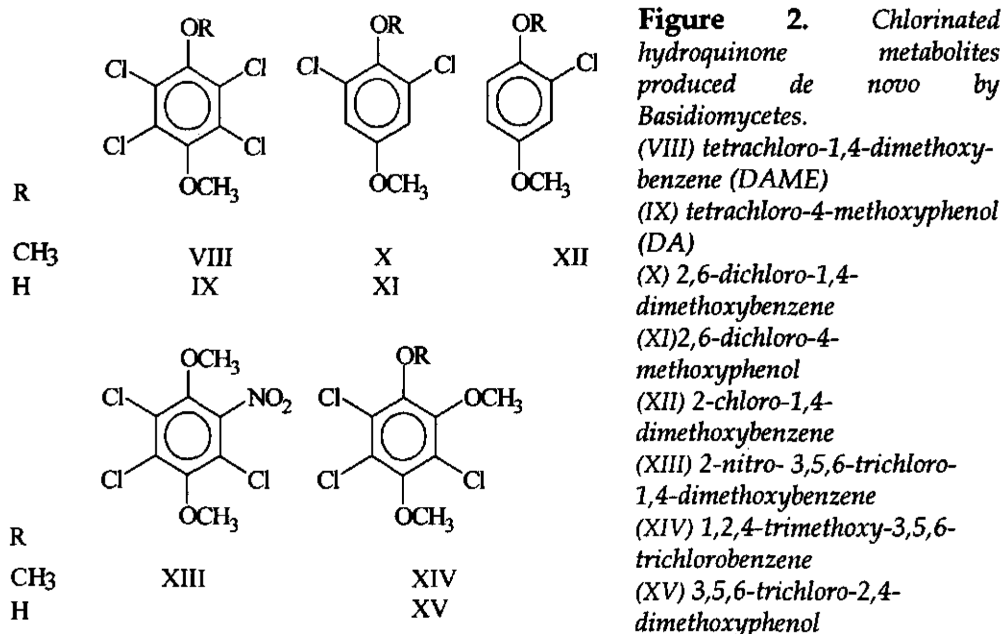


Figure 2. *Chlorinated hydroquinone metabolites produced de novo by Basidiomycetes.*
(VIII) tetrachloro-1,4-dimethoxybenzene (DAME)
(IX) tetrachloro-4-methoxyphenol (DA)
(X) 2,6-dichloro-1,4-dimethoxybenzene
(XI)2,6-dichloro-4-methoxyphenol
(XII) 2-chloro-1,4-dimethoxybenzene
(XIII) 2-nitro- 3,5,6-trichloro-1,4-dimethoxybenzene
(XIV) 1,2,4-trimethoxy-3,5,6-trichlorobenzene
(XV) 3,5,6-trichloro-2,4-dimethoxyphenol

Structurally related to DA and DAME, are 2,6-dichloro-1,4-dimethoxybenzene (X) and the monochlorinated 2-chloro-1,4-dimethoxybenzene (XII). These compounds were isolated from *Bjerkandera* and *Lepista* spp. in trace amounts (76, 152, 156). Also 2,6-dichloro-4-methoxyphenol (XI) was isolated from *Russula subnigricans* (162). *Russula subnigricans* is also known for the production of russuphelins, which are chlorinated biphenyl, triphenyl and tetraphenyl ethers resulting from the polymerization of XI (128, 161, 162). In addition, two other CHM are produced by *Phellinus* spp. *Phellinus robinae* produces a nitrated CHM 1,4-dimethoxy-2-nitro-3,5,6-trichlorobenzene (XIII); whereas, *P. fastuosus* produces 1,2,4-trimethoxy-3,5,6-trichlorobenzene (XIV) (16, 80). Recently, the related structures 3,5,6-trichloro-2,4-dimethoxyphenol (XV) and 3,4,6-trichloro-2,5-dimethoxyphenol were isolated from *Hypholoma elongatum* (158).

In addition to CAM and CHM chlorinated orcinol methyl ethers, chlorinated sesquiterpenes, chlorinated anthraquinones

and strobilurins have been identified (34, 89). Furthermore, new metabolites have been identified since the last published literature review (89). Daferner et al. (26) discovered the presence of tetrachloropyrocatechol and its methyl ether as new natural products from a *Mycena* sp.. From a *Pterula* sp. three new chlorinated metabolites were isolated, pterulone, pterulinic acid and pterulone B (31, 32). Other recently identified chlorinated metabolites from strains in the genus *Bjerkandera* include two novel chlorinated *p*-anisylpropanoid metabolites *erythro*-1-(3′,5′-dichloro-4′-methoxyphenyl)-1,2-propanediol and 1-(3′-chloro-4′-methoxyphenyl)-3-hydroxy-1-propanone, veratrylchloride, and 2,4-dichlorobenzoate (125, 157, 159). Swarts et al. (158) also demonstrated the formation of 2,4,6-trichloro-3-methoxyphenol by *Hypholoma elongatum*.

Environmental impact of natural fungal chloroaromatic production

In the previous section, a variety of organohalogens were reviewed that were produced by many Basidiomycetes in the laboratory. However, a substantial production of organohalogens was also demonstrated in natural environments. Verhagen et al. (182) showed that when *Hypholoma fasciculare*, the most common occurring species of Basidiomycetes in The Netherlands (6), was cultured on forest litter, concentrations of 3,5-dichloro-*p*-anisyl alcohol (IV) reached 204.9 mg per kg (dry weight) of substrate after 84 days of incubation. In natural forest environments, CAM of the white rot fungi *Bjerkandera, Hypholoma* and several other ecologically significant genera have consistently been found in samples of colonized lignocellulosic debris (74, 88). The concentrations of CAM encountered in nature ranged from 15 mg/kg wood produced in the vicinity of *Bjerkandera adusta*, 39 mg/kg litter by *Lepista nuda*, 71 mg/kg wood by *Pholiota squarrosa* to 75 mg/kg wood by *Hypholoma* spp. (88).

The amount of fungal chloroaromatics present in the environment exceed the Dutch and Canadian (British Columbia) hazardous-waste norms for chlorophenols in soil, which are set at 1-10 mg kg^{-1} for mandatory remediation (3, 155). It must be concluded that these defined norms do not take into account the extent of chlorophenol methyl ether production by fungi in the ecosystem.

Physiological impact of natural fungal chloroaromatics

Clearly, Basidiomycetes, and especially white rot fungi, produce significant amounts of chlorinated aromatic metabolites. An important question that remains to be answered is why Basidiomycetes produce these chloroaromatics. The biosynthesis of chloroaromatics is not just a biological accident, some Basidiomycetes even produce organically bound halogens up to 3% of their biomass dry weight (180). Several physiological functions have been proposed for these chloroaromatics.

The production of several chloroaromatics by Basidiomycetes has been shown to be induced in response to an antagonizing fungus, indicating that chloroaromatics are possible antibiotic agents (151). In fact, many of the chloroaromatics produced by Basidiomycetes have strong antibiotic effects. Antimicrobial activity of DA and DAME has been reported (94). As well CAM as CHM inhibit chitin synthase (138), whereas CAM have also been shown to inhibit the seed germination in plants (68).

Apart from their antibiotic properties chloroaromatics have properties in fungal metabolism as well. Most Basidiomycetes that produce CAM are white rot fungi, and CAM alcohols are important metabolites in the ligninolytic system of white rot fungi (89). Almost all fungi that synthesize CAM produce extracellular aryl alcohol oxidases (AAO) (87, 135). The CAM alcohols are much better substrates for AAO compared to the structurally similar nonhalogenated secondary metabolites veratryl alcohol (3,4-dimethoxybenzylalcohol) and anisyl

alcohol (87). As shown in Figure 3, the oxidases oxidize the alcohols in order to reduce O_2 to hydrogen peroxide (H_2O_2), which is a necessary electron acceptor for the peroxidases in the ligninolytic system. The aldehydes that are formed are intracellularly reduced back by NADPH-dependent aryl-alcohol dehydrogenases to the alcohols which generates a physiological cycle (52, 87, 121).

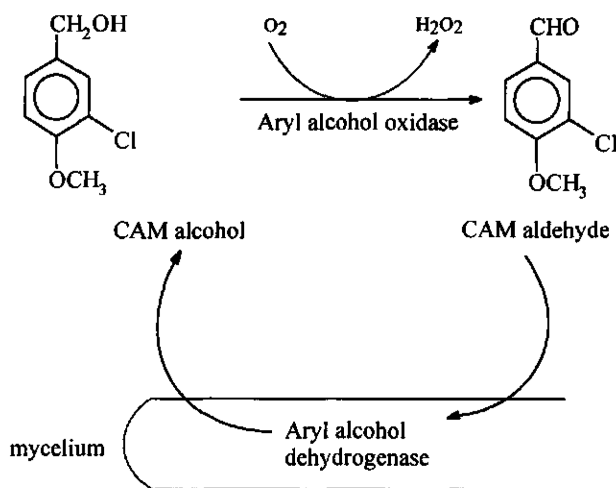


Figure 3. *The proposed physiological redox cycle for the chlorinated anisyl metabolites (CAM) as substrates of extracellular aryl alcohol oxidase generating H_2O_2 for ligninolytic peroxidases. The aldehyde is reduced back by an intracellular NADPH dependent aryl-alcohol dehydrogenase.*

The chlorinated anisyl alcohols are much better protected against decay by aggressive ligninolytic peroxidases than their non-chlorinated counterparts, because the electron-withdrawing character of the chloro group increases the oxidation potential of the methoxy benzyl ring (87).

Although aryl alcohol oxidases are not the only enzymes available for H_2O_2 generation, CAM may be important under the natural physiological conditions of wood degradation. These chlorinated compounds have extremely low K_m values for AAO compared to other nonchlorinated substrates for the various extracellular fungal oxidases. Thus, chloroaromatics are purposeful for ecologically significant processes such as lignin degradation. The next sections will give more details about the

ligninolytic system of white rot fungi and the possible role of chloroaromatics in this enzyme system.

Lignin degradation

LIGNIN DEGRADATION BY WHITE ROT FUNGI

Lignin, one of the most abundant natural aromatic polymers on earth, is found in all higher plants. It comprises 20-30% of woody plant cell walls and by forming a matrix surrounding the cellulose and hemicelluloses, it provides strength and protection to the plant cell walls (145). Lignin is a heterogeneous, three-dimensional, hydrophobic structure that is highly resistant to biodegradation (15, 100, 145). White rot fungi are the most efficient degraders of the polymer due to a nonspecific extracellular, oxidative process. Lignin is not used as a sole carbon source by white rot fungi, although they are able to completely mineralize it to carbon dioxide (100). The real purpose of ligninolysis is presumably to get better access to the polysaccharides protected by the lignin.

Several hundreds of white rot species have been identified so far. The lignin degrading enzyme system of one species, *Phanerochaete chrysosporium*, has been studied extensively (44, 143). Recently, the ligninolytic system of other species such as *Bjerkandera adusta, Trametes versicolor, Pleurotus spp., Phlebia radiata, Ceriporiopsis subvermispora, Lentinus tigrinus* and *Dichomitus squalens* were characterized as well (109, 112, 114, 120, 137, 142, 179). In our laboratory, the ligninolytic system of *Bjerkandera* sp. BOS55 and its secondary metabolites is extensively studied (116, 117).

LIGNINOLYTIC SYSTEM OF WHITE ROT FUNGI

The ligninolytic system of white rot fungi is composed of extracellular enzymes, such as ligninolytic peroxidases and H_2O_2 generating oxidases, and low molecular weight cofactors. The components of the ligninolytic system are summarized in Table 1.

Extracellular enzymes

The main ligninolytic enzymes are lignin peroxidase (LiP) and manganese peroxidase (MnP). Both enzymes are classical hemeprotein peroxidases, which require H_2O_2 for their activity. The required H_2O_2 is provided by H_2O_2 generating oxidases, such as glucose oxidase, glyoxal oxidase and aryl alcohol oxidase (Table 1). White rot fungi which lack H_2O_2 -generating oxidases rely on the oxidation of organic acids such as oxalate and glyoxylate which results in superoxide anion radicals that dismutate to H_2O_2 (174).

Table 1. *Extracellular components of the ligninolytic system of white rot fungi*

LIGNINOLYTIC PEROXIDASES	lignin peroxidase (LiP) manganese dependent peroxidase (MnP) manganese/lignin peroxidase hybrid enzyme (MnP/LiP hybrid enzyme) manganese independent peroxidase (MiP)
HYDROGEN PEROXIDE GENERATING OXIDASES	aryl-alcohol oxidase glucose oxidase glyoxal oxidase
PHENOL OXIDASE	laccase
LOW MOLECULAR WEIGHT COFACTORS	veratryl alcohol (chlorinated) anisyl metabolites organic acids manganese

Recently, other peroxidases that might be involved in lignin degradation have been described as well, such as manganese independent/inhibited peroxidase (MiP) and a MnP/LiP hybrid enzyme (83, 117, 178).

LiP and MnP were first discovered in *P. chrysosporium* (39, 107, 166). The catalytic cycle of LiP and MnP is like those of other peroxidases (Figure 4). The native enzyme is activated in the presence of H_2O_2 to form compound I, a two-electron oxidized intermediate. Compound I oxidizes a substrate by one electron

to produce a free radical product and a one-electron oxidized intermediate, compound II. Transfer of a second electron from an appropriate substrate to compound II is the final step of the catalytic cycle, reducing the enzyme back to its native state (143, 167). If no appropriate substrate is present or in the presence of excess H_2O_2, compound II can undergo a series of reactions with H_2O_2 to form compound III, an inactive form of the enzyme (17, 185).

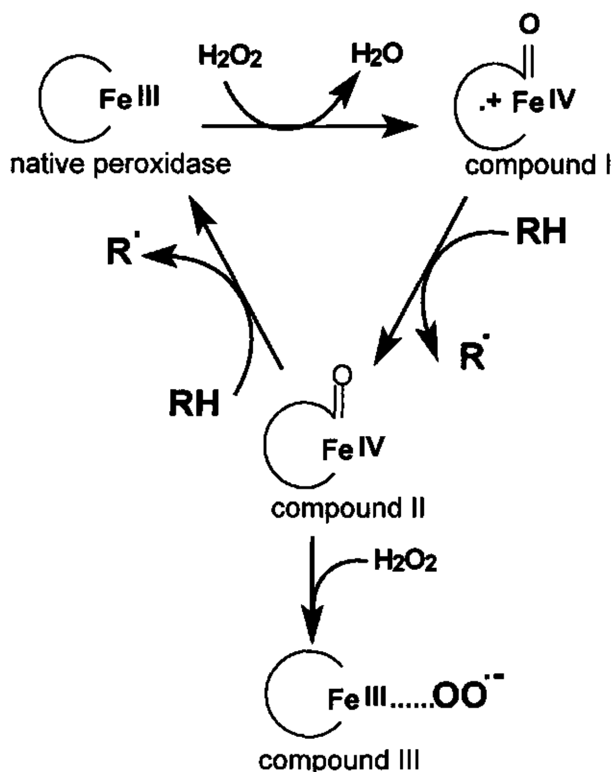


Figure 4. *The catalytic cycle of peroxidases. One molecule of H_2O_2 oxidizes the native peroxidase enzyme withdrawing two electrons to enzyme compound I. Compound I is reduced back by an appropriate substrate (RH) in two electron oxidation steps to the native enzyme via compound II. In absence of appropriate substrate compound II reacts with H_2O_2 to the inactive enzyme compound III.*

A major difference between MnP and LiP is that MnP exhibits an absolute dependence on Mn(II) as a substrate for compound

II (184). The expression of MnP is strongly regulated by the presence of manganese in the culture fluid (14, 115, 134). The principle function of MnP is to oxidize Mn(II) to Mn(III) in the presence of H_2O_2 and organic chelators, such as oxalate or malonate (106, 136). Mn(III) generated by MnP acts as a mediator in the oxidation of various phenolic compounds (131, 189).

A unique feature of LiP is that the enzyme can oxidize compounds with a higher ionization potential than other peroxidases (96). This capability enables the enzyme to oxidize nonphenolic methoxylated aromatic compounds, such as veratryl alcohol and 1,4-dimethoxybenzene (55, 95). LiP can also directly catalyze the oxidation of recalcitrant xenobiotics, such as polycyclic aromatic hydrocarbons (54), dioxins (177), chlorophenols (57), and azo-dyes (71, 133).

Role of low molecular weight cofactors

Various low molecular weight compounds (86, 111, 116, 190) play vital roles in the ligninolytic enzyme system of white rot fungi (Table 1).

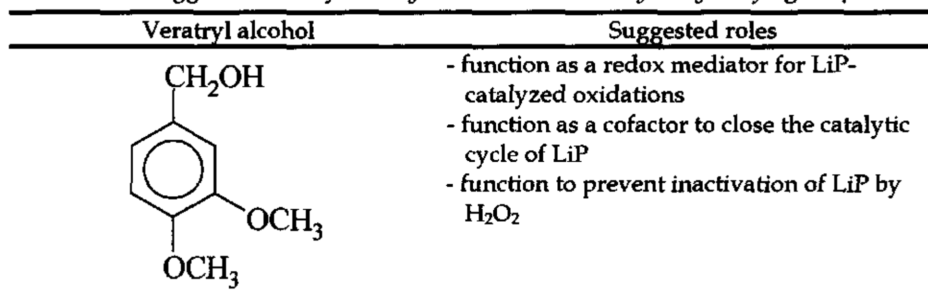
Manganese is the essential cofactor of MnP, since only Mn(II) can reduce compound II back to the native enzyme, which closes the catalytic cycle of the enzyme (184, 186). The oxidation of Mn(II) is dependent on the presence of organic acids, such as lactate, malonate and oxalate (40, 41). Organic acids chelate enzymatically generated Mn(III), stabilizing this species in aqueous solution (41, 131, 186) and ensuring the efficiency of Mn(II) oxidation by enhancing the removal of Mn(III) from the enzyme (101). The Mn(III)-organic acid complex is able to oxidize phenols, dyes, and phenolic lignin compounds (40, 189).

Another important low molecular weight compound is 3,4-dimethoxybenzyl alcohol (veratryl alcohol; VA). The function and biosynthesis of VA is extensively studied throughout the years (63, 103, 111, 149, 175). VA is biosynthesized by many

fungi that also produce LiP (86) and VA appears to be the preferred natural substrate for LiP.

The discovery that VA stimulated the LiP-catalyzed oxidation of monomeric monomethoxylated aromatic compounds like *p*-anisyl alcohol and 4-methoxymandelic acid provided the first experimental evidence for a special role of VA in the catalytic cycle of LiP (63). Further research showed that polymeric lignin is degraded only in the presence of VA (59). To date, three suggested roles of VA have been described (Table 2).

Table 2. *Suggested roles of veratryl alcohol in the catalytic cycle of lignin peroxidase*

Veratryl alcohol	Suggested roles
	- function as a redox mediator for LiP-catalyzed oxidations - function as a cofactor to close the catalytic cycle of LiP - function to prevent inactivation of LiP by H_2O_2

VA acts as a cation radical redox mediator in the LiP-catalyzed oxidation of substrates with lower redox potential than the VA cation radical, such as guaiacol, chlorpromazine, 4-methoxymandelic acid, and oxalate (1, 45, 104, 169).

In the proposed mechanism (Figure 5), LiP catalyzes the one-electron oxidation of VA to form a VA cation radical. The VA cation radical would then be capable of diffusing from the active site (63) and oxidizing the substrates to their corresponding cation radicals, reducing the VA cation radical back to VA. Recent debate is focused on the fact that the free VA cation radical with a half-life of 0.5 ms (97) is too short-lived to diffuse away from the active site (92, 97). Instead, it was suggested that a more stable enzyme-bound radical is formed

with a longer lifetime and a higher redox potential than the free VA cation radical (20). Indeed, Khindaria et al. (97) demonstrated that the LiPII-VA cation radical complex decays with a half-life of 0.54 s.

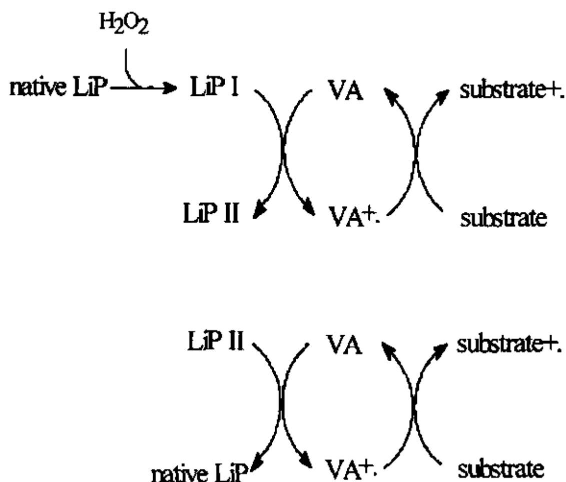


Figure 5. *Proposed redox mediator function of veratryl alcohol (VA) in lignin peroxidase catalyzed oxidations. Native lignin peroxidase (LiP) is oxidized to the two electron oxidized form LiP I. LiP I oxidizes VA to the corresponding cation radical which can oxidize a substrate molecule, such as guaiacol, chlorpromazine, 4-methoxy-mandelic acid, or oxalate. As a result the VA cation radical is reduced back to VA. VA can also be oxidized by LiP II which can result in the indirect oxidation of the substrate.*

An alternative role that was assigned to VA is that VA acts as a cofactor, necessary to close the catalytic cycle of LiP (Figure 6). Koduri and Tien (103) showed that the methoxylated nonphenolic compound anisyl alcohol could only be oxidized by LiP compound I, thus being stuck at LiP compound II. Inclusion of VA results in its oxidation by compound II allowing the enzyme to complete the catalytic cycle.

A third role was suggested by Valli et al. (175). According to them, VA prevents the H_2O_2 inactivation of LiP. They showed that in the presence of excess H_2O_2 and VA, the enzyme was present in the active compound II form, but when the VA was

replaced by anisyl alcohol, the enzyme was present in the inactive compound III form (175, 188). This role can partly be explained by the fact that VA is a good substrate for compound II, which prevents the formation of the inactive compound III (103, 143). In addition, it was shown that VA or the VA cation radicals could convert the inactive LiP compound III back to native enzyme (12, 17).

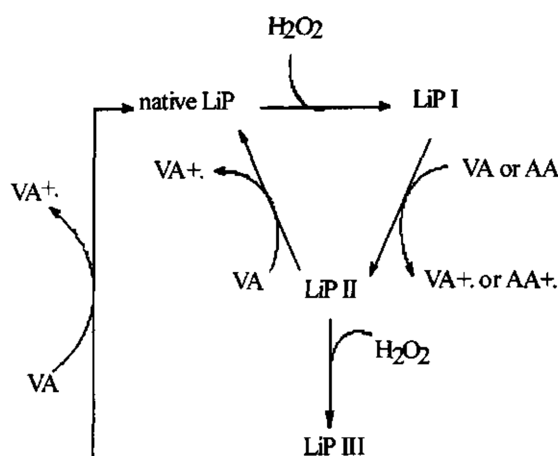


Figure 6. *Proposed cofactor function of veratryl alcohol (VA) in the lignin peroxidase (LiP) catalyzed oxidation of anisyl alcohol (AA). LiP I can oxidize a variety of substrates such as VA and AA. However, LiP II is not reactive with AA. When AA is the only substrate present, the enzyme is stuck at LiP II. LiP II can only react with H_2O_2 which results in the formation of inactive LiP III. If VA is present in the reaction mixture, LiP II can be reduced back to native LiP. Thus, VA closes the catalytic cycle of LiP while as a secondary consequence LiP is protected from H_2O_2 inactivation.*

POSSIBLE ROLES OF CHLOROAROMATICS IN THE LIGNINOLYTIC SYSTEM

In the previous sections it was shown that both peroxidases, LiP and MnP, require their own cofactor/mediator, respectively VA and manganese, for efficient catalysis of oxidative reactions. Since VA is the most frequently occurring secondary metabolite produced by white rot fungi, its physiological relevance in the ligninolytic system has never been questioned. However, recent research showed that other compounds could replace VA as a cofactor of LiP, such as 3,4-dimethoxytoluene, 3,4,5-trimethoxybenzylalcohol and 1,4-dimethoxybenzene (92, 103). These compounds, however, are not de novo metabolites of white rot fungi. Interestingly, 1,4-dimethoxybenzene is

structurally related to CHM, such as 2-chloro-1,4-dimethoxybenzene, 2,6-dichloro-1,4-dimethoxybenzene and tetrachloro-1,4-dimethoxybenzene, which are de novo synthesized by basidiomycetes including white rot fungi (34, 89). 1,4-dimethoxybenzene is oxidized to a relatively stable cation radical (95) implying a possible function as a diffusible redox mediator in LiP oxidations (92). As mentioned earlier, the VA cation radical is too short-lived to act as a diffusible mediator (92, 97). The structural relationship between 1,4-dimethoxybenzene and CHM implies that CHM could serve the same function as VA in LiP catalysis.

Outline of this thesis

The objective of this PhD study was to determine the extent of biosynthesis of chlorinated hydroquinone metabolites among white rot fungi and to elucidate their possible physiological role in lignin biodegradation.

In Chapter 2 ligninolytic basidiomycetes were screened for their ability to synthesize tetrachlorinated hydroquinone metabolites. In Chapter 3, 2-chloro-1,4-dimethoxybenzene, a CHM, was identified as an alternative cofactor for VA in LiP catalysis. In Chapter 4, the reaction mechanism of 2-chloro-1,4-dimethoxybenzene with anisyl alcohol was elucidated and evidence for the formation of a 2-chloro-1,4-dimethoxybenzene cation radical is presented. In Chapter 5, the role of 2-chloro-1,4-dimethoxybenzene as a redox mediator for various lignin peroxidase catalyzed oxidations is described. In Chapter 6 the production of a manganese independent peroxidase from a CHM-producing fungus *Phellinus fastuosus* is described. This manganese independent peroxidase is capable of oxidizing drosophilin A.

2

The de novo Production of Drosophilin A (Tetrachloro-4-methoxyphenol) and Drosophilin A Methyl Ether (Tetrachloro-1, 4-dimethoxybenzene) by Ligninolytic Basidiomycetes

Pauline J. M. Teunissen, Henk J. Swarts, Jim A. Field

Ligninolytic basidiomycetes were screened for their ability to produce the tetrachlorinated hydroquinone metabolites drosophilin A (DA, tetrachloro-4-methoxyphenol) and drosophilin A methyl ether (DAME, tetrachloro-1,4-dimethoxybenzene). Five fungal strains produced these metabolites in detectable amounts, including strains from *Bjerkandera* and *Peniophora,* which are genera not previously known for DA or DAME production. *Phellinus fastuosus* ATCC26.125 had the highest and most reliable production of DA and DAME in peptone medium, respectively 15-60 μM and 4-40 μM. This fungus was used to study culture conditions that could increase DAME production. A fourfold increase in DAME production was found after the addition of hydroquinone to growing cultures of *P. fastuosus*. Therefore, hydroquinone is postulated to be a possible biosynthetic precursor of DAME in the fungus. Antagonizing *P. fastuosus* by adding filter-sterilized culture fluid of a competing fungus, *Phlebia radiata,* increased DAME production significantly by tenfold. This result suggest that DAME production is elicited by compounds present in the culture fluid of *P. radiata,* indicating that DAME has an antibiotic function in *P. fastuosus*.

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Introduction

Basidiomycetes have been reported to produce a wide variety of chloroaromatic compounds as secondary metabolites (34). Among these, chlorinated anisyl metabolites and chlorinated hydroquinone metabolites are the most common, since they are known from several genera of basidiomycetes (34). Methyl ethers of tetrachlorinated hydroquinone metabolites are known as drosophilins (Figure 1). Tetrachloro-4-methoxyphenol, called drosophilin A (DA), was the first halogenated metabolite identified in a basidiomycete (2) and has a structure very similar to the anthropogenic pentachlorophenol. DA has so far been found in *Coprinus plicatilis* and *Psathyrella subastrata* (2, 13). Furthermore, tetrachloro-1,4-dimethoxybenzene, called drosophilin A methyl ether (DAME), was also detected as a de novo metabolite in *Agaricus bisporus, Phellinus fastuosus, Phellinus yucatensis, Phellinus robinae* and *Mycena megaspora* (28, 30, 51, 79, 150).

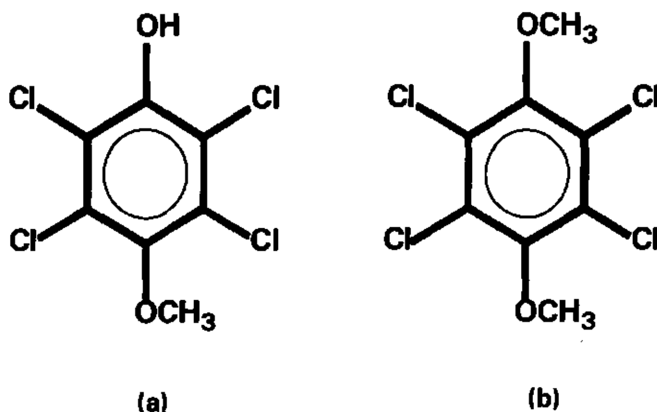

Figure 1.
(a) Drosophilin A and (b) Drosophilin A methyl ether

The purpose behind the biosynthesis of tetrachlorinated hydroquinone metabolites by basidiomycetes is not fully understood. The physiological role of drosophilins may be their antimicrobial activity towards bacteria (2) and fungi (138). It is not known whether DA and DAME are only produced as antibiotics. Previously it was shown that chlorinated anisyl

metabolites had a physiological role in lignin degradation, serving as substrates for the aryl alcohol oxidase involved in extracellular hydrogen peroxide production (87). Another chlorohydroquinone metabolite, 2-chloro-1,4-dimethoxybenzene, is a known substrate of lignin peroxidase (176). Furthermore, 1,4-dimethoxybenzene stimulates the oxidation of monomethoxylated aromatic compounds by lignin peroxidase (63). Consequently, structurally related DA and DAME may also have a physiological role in lignin degradation.

The objective of this study was to identify ligninolytic basidiomycete strains capable of DA and DAME biosynthesis in different culture conditions. The production of DA and DAME was quantified. Furthermore, culture conditions enhancing the production of drosophilins were investigated. The effects of adding both biosynthetic precursors and culture fluids of antagonizing fungi were evaluated.

Materials and methods

ORGANISMS AND CULTURE CONDITIONS

The selection of the 92 fungal strains screened in this study was based, for the most part, on their ability to produce absorbable organic halogens reported by Verhagen et al. (180). Fungi with absorbable organic halogen production exceeding 0.5 mg/l were included in the screening. Twenty-seven fungal strains were obtained from different culture collections: American Type Culture Collection, Rockville, Maryland, USA (ATCC); Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (CBS); Culture Collection of Industrial Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands (CIMW); Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain (IJFM). Twelve strains were kindly provided by H. Anke, Department of Biotechnology, University of Kaiserslautern, Kaiserslautern, Germany (ANKE). 53 fungal strains were isolated by our laboratory in the period 1992-1994 from the tissue or spores of fruiting bodies (BEUK; EPE; RHEN; WAG).

Fungal strains were maintained at 4°C on one of two different agar slants, depending on the strain. One agar medium contained (l^{-1}) 20 g glucose, 5 g mycological peptone (Oxoid Ltd., Basingstoke,

Hampshire, UK), 2 g yeast extract (Gibco BRL, Life Technol. Ltd., Paisley, Scotland, UK), 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 15 g agar. The other agar medium contained (l^{-1}) 5 g glucose, 3.5 g malt extract (Oxoid), and 15 g agar. Fungal strains were grown in a high nitrogen peptone medium according to Kimura et al. (99), containing (l^{-1}) 20 g glucose, 5 g mycological peptone, 2 g yeast extract, 1 g KH_2PO_4 , 0.5 g $MgSO_4.7H_2O$, with the addition of 0.058 g NaCl. Fungi were also grown in a low nitrogen mineral medium containing glucose (1% w/v), diammoniumtartrate (0.20 g l^{-1}), 2,2-dimethylsuccinic acid (20 mM), thiamin.HCl (200 mg l^{-1}) and BIII mineral medium (168).

Either 30-ml or 300-ml serum bottles, containing 5 ml medium, were inoculated with a cylindrical agar plug (diameter 5 mm), which was taken from the outer periphery of an agar medium covered with mycelium of the fungal strain. Duplicate fungal cultures were incubated statically in the dark at 25°C. Controls were set up in parallel in sterile medium inoculated with a sterile agar plug. Samples were taken at different times to determine the time course of the aromatic metabolite production. Separate fungal cultures were set in for each assay time. Assay times were determined by the visual growth of the fungus.

PLATE SCREENING

A plate screening was conducted to indicate ligninolytic enzyme activity in the basidiomycete strains that were found to produce DA or DAME. This plate screening is based on the ability of ligninolytic fungi to decolorize the polyanthraquinone dye Poly R-478 or Azure B dye and to discolor guaiacol plates. Two different agar media, supplemented with either Poly R-478 (0.2 g l^{-1}), Azure B (0.1 g l^{-1}) or guaiacol (0.1 g l^{-1}), were used for the plate screening. One of these was hemp stem wood (HSW) agar medium, containing 2 g l^{-1} 100 - 1000 μm classified particles of hemp stem wood (*Cannabis sativa* Fibrimon) and 15 g l^{-1} agar in 10 mM 2,2-dimethylsuccinic acid at pH 4.5. The other one was peptone agar medium, containing (l^{-1}) 20 g glucose, 5 g mycological peptone, 2 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and 15 g agar. Plates were inoculated with one cylindrical agar plug (diameter 5 mm). Decolorization of the Poly R-478 plates indicates the presence of peroxidases (33, 43); whereas, decolorization of Azure B plates indicates the presence of lignin peroxidase (4). The formation of colored zones on guaiacol plates indicates the presence of laccases and/or peroxidases (124).

STIMULATION OF DA/DAME PRODUCTION BY ADDITION OF POSSIBLE PRECURSORS

Culture bottles (300 ml) containing 5 ml high nitrogen peptone medium were inoculated with a cylindrical agar plug (diameter 5 mm) of *Phellinus fastuosus* ATCC26.125. Cultures were incubated in the dark at 25°C. After 6 days of growth, one of the following compounds was

added, in a concentration of 1 mM, to the culture: phenylalanine, tyrosine, tryptophan, 3-chloro-4-methoxybenzoic acid, 3-chloro-4-methoxybenzaldehyde, 1,4-dimethoxybenzene, 4-methoxy benzoic acid, 4-methoxybenzaldehyde, 4-methoxybenzyl alcohol, 1,4-benzoquinone, hydroquinone, acetate and 2,6-dichlorobenzoquinone. Chemicals were purchased from Acros Chimica (Geel, Belgium), Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany). 3-chloro-4-methoxybenzaldehyde was prepared as described previously (84).

STIMULATION OF DA/DAME PRODUCTION BY ANTAGONISM

Culture bottles (300 ml) containing 5 ml of high nitrogen peptone medium were inoculated with a cylindrical agar plug (diameter 5 mm) of *Heterobasidium annosum* EPE93.1, *Phlebia radiata* CBS184.83 and *Penicillium simplicissimum* CBS170.90. The fungi were cultivated for 8 days as described previously for *P. fastuosus* ATCC26.125. Filter-sterilized culture fluids (500 µl; 0.2 µm pore size; Schleicher & Schuell, Dassel, Germany) of these fungi were added to the 8-day-old cultures of *P. fastuosus* ATCC26.125. Metabolites were subsequently analyzed by HPLC at selected times.

GC ANALYSIS

Filtration and extraction of the extracellular fluid of the fungi were performed according to Swarts et al. (156). The remaining residue was dissolved in 0.25 ml of ethyl acetate containing 112 mg l^{-1} 4-bromoanisole as the internal standard. The identification of DA and DAME was based on the mass spectrum and matching retention times with the respective reference compounds {*DAME:* 274 $[M]^+$(75), 259 $[M - CH_3]^+$(100), 209 (42), 181 (13), 153 (5.5), 87 (27); *DA:* 260 $[M]^+$(45.2), 245$[M - CH_3]^+$(91.1), 217 (12.2), 209 (6.3), 181 (9.5), 87 (36.5)}. All measurements were done in duplicate from a duplicate set of cultures.

GC analyses were performed as described by Swarts et al. (156), using a Varian 3600 GC with a J & W Scientific DB17 column (film thickness 0.25 µm, 30 m x 0.25 mm inner diameter). Parallel detection was carried out by flame ionization and electron capture. The injector temperature was 200°C. The initial oven temperature was 70° C (0 min hold) with a gradient of 7°C min^{-1} to 250°C (20 min hold). The carrier gas was N_2, and the flow rate 1.2 ml min^{-1}. GC/mass spectroscopy was performed on an Hewlett Packard 5890 GC with a J & W Scientific DB17 column and an Hewlett Packard mass spectrometer (Waldbronn, Germany). The injection port was maintained at 220° C. The temperature program was identical to that used by GC. The carrier gas was He, and the flow rate 1.1 ml min^{-1}. Mass spectra were obtained with electron impact of 70 eV.

HPLC ANALYSIS

Acetonitrile (10 ml) was added to the culture bottles (5 ml medium, corrected for water evaporation losses), sealed with Teflon-lined silicone septa and extracted for 1 h on a shake table (250 strokes min^{-1}; 1 stroke = 2 cm). After centrifugation of the extract, 50 µl was injected into the HPLC.

Supernatants (50 µl) were routinely analyzed on a Hewlett Packard HPLC Chemstation (Pascal series) (Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector and a HP9000-300 data processor. The column (200 mm x 3 mm) filled with ChromSpher C18-PAH (5 µm particles), was from Chrompack (Middelburg, The Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30°C): 90:10, 0:100 and 0:100 H_2O: CH_3CN at 0, 15 and 25 minutes respectively. The UV absorbance was monitored at wavelength intervals of 2 nm from 200 to 400 nm. Compound identification was carried out by matching UV spectra and the retention times of found metabolites with their standards. (DAME UV_{max}: 209 nm, 294 nm; DA UV_{max}: 217 nm, 322 nm).

REFERENCE COMPOUNDS

Standards for DA and DAME were kindly provided by Dr. J. Knuutinen, Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland. Standards for 4-methoxy-3,5-dichlorobenzyl alcohol and 4-methoxy-3,5-dichlorobenzaldehyde were kindly provided by Prof. Kawagishi, Department of Applied Biological Chemistry, Faculty of Agriculture, Schizuoka University, Ohya, Shizuoka, Japan.

Results

Of the 92 screened basidiomycete strains, 5 were found to produce DA and DAME. These compounds were detected in the extracellular culture fluid of the fungal strains belonging to four different genera of basidiomycetes (Table 1). *Agaricus arvensis, Peniophora pseudopini and P. fastuosus* only produced DA and DAME in peptone medium; whereas, *Bjerkandera adusta* BEUK47 and *B. adusta* RHEN93.4 also produced the metabolites in mineral BIII medium. Another chlorinated metabolite was detected for the first time in *B. adusta* RHEN93.4, namely 4-chloro-3,5-dimethoxybenzaldehyde at a concentration of 2 µM in peptone medium. Concentrations of DA and/or DAME found in the fungi are recorded as ranges, since they varied

over the several assay times. DA and DAME were detected in low amounts in *Bjerkandera, Agaricus* and *Peniophora* strains. *P. fastuosus* had the highest production of DA and DAME in the screening.

Table 1. *Drosophilin A and Drosophilin A methyl ether production in ligninolytic basidiomycetes, analyzed by HPLC and GC.* Peptone *high nitrogen medium,* Mineral *low nitrogen medium,* ND *not detected. Concentrations ranges are based on duplicate samples of the fungi, taken at different assay times in growth.*

Fungal strain	medium	Drosophilin A (μM)	Drosophilin A methyl ether (μM)	Detection method
Agaricus arvensis WAG93.1	peptone	ND	0.5 -2	GC-MS
Bjerkandera adusta Beuk47	mineral	0.5 - 2	0.5 - 2	GC-MS
	peptone	0.5 - 2	0.5 - 2	GC-MS
Bjerkandera adusta RHEN93.4	mineral	0.5 - 2	2 - 4	GC-MS
	peptone	ND	0.5 - 5	HPLC
Peniophora pseudopini CBS162.65	peptone	2 - 4	2 - 4	GC-MS
Phellinus fastuosus ATCC26.125	peptone	15-60[a]	4 - 40	HPLC/ GC-MS

[a] *Drosophilin A was only detected in two experiments, in very high concentrations (15 - 60 μM) and in other experiments it was not detected at all, in contrast to Drosophilin A methyl ether, which was detected in all experiments*

The DA- and DAME-producing fungi were also screened for the presence of ligninolytic enzymes by plate screening (Table 2). Two different culture conditions were used in this screening with their ligninolytic indicators. All five fungal strains capable of producing DA and DAME produced colored zones on the guaiacol-containing plates, indicating the presence of laccase and/or peroxidase activity. Four strains decolorized Poly R-478, indicating peroxidase activity. *A. arvensis* only decolorized Poly R-478 in peptone medium, whereas, *P. fastuosus* decolorized Poly R-478 in hemp stem wood medium. Both *B. adusta* strains decolorized Poly R-478 in both media. Only three strains decolorized Azure B, indicating lignin peroxidase activity. *A. arvensis* and both *B. adusta* strains decolorized Azure B in

peptone medium; whereas, *B. adusta* Beuk47 also decolorized Azure B in hemp stem wood medium.

Table 2. *Plate screening as an indication for the presence of ligninolytic enzymes in drosophilin A and drosophilin A methyl ether-producing fungi. Poly R-478 decolorization indicates the presence of peroxidase activity. Azure B decolorization indicates the presence of lignin peroxidase activity. Discolorization (browning) of guaiacol indicates the presence of laccase and/or peroxidase activity.* HSW *low nitrogen medium;* peptone *high nitrogen medium. - No decolorization; + decolorization; ++ fast decolorization. In the case of guaiacol symbols refer to discolorization.*

Fungal strain	Poly R-478		Azure B		guaiacol	
	HSW	peptone	HSW	peptone	HSW	peptone
Agaricus arvensis WAG93.1	-	+	-	+	+	+
Bjerkandera adusta Beuk47	++	++	++	++	+	+
Bjerkandera adusta RHEN93.4	++	++	-	+	+	-
Peniophora pseudopini CBS162.65	-	-	-	-	+	+
Phellinus fastuosus ATCC26.125	++	-	-	-	+	-

P. fastuosus ATCC26.125 was used in further experiments investigating DA and DAME production, since it was the most reliable producer of these metabolites. To stimulate the production of DA and DAME in *P. fastuosus* ATCC26.125, one of several compounds that were suspected to be biosynthetic precursors, was added to the culture fluid of the fungus on day 6 at a concentration of 1 mM. Analysis of DA and DAME production was done by HPLC at different times. The addition of 1 mM hydroquinone increased DAME production at least four-fold over the non-supplemented control, this result could be reproduced in three separate experiments (results not shown).

Since 1 mM of hydroquinone was capable of increasing DAME production, a concentration range was used to demonstrate the stimulating effect of hydroquinone on DAME production (Figure 2). These results show an increase in DAME production from 6 μM (no addition of hydroquinone) to a maximum of 43

μM (0.25 mM hydroquinone), when DAME production was measured on day 20. Not only did 0.25 mM hydroquinone have a stimulating effect but 0.5 mM and 1.0 mM hydroquinone also increased DAME production when measured on day 20. Addition of 2 - 10 mM hydroquinone did not increase the production of DAME on day 20, because these concentrations were partially inhibitory and delayed fungal growth. The high hydroquinone supplements did stimulate DAME production after 30 days, increasing the metabolite concentration to 32 μM and 34 μM when 2 and 5 mM hydroquinone were added, respectively.

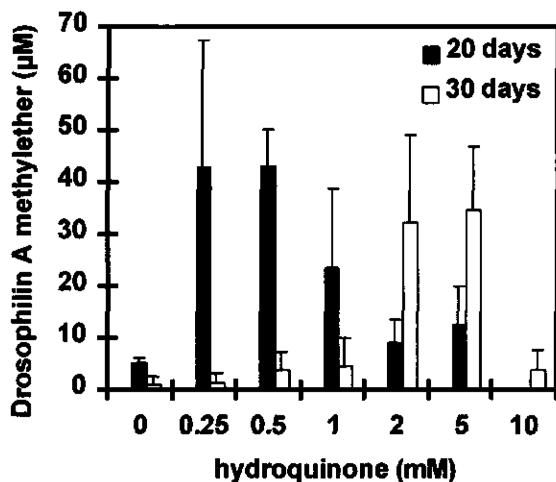

Figure 2. *Drosophilin A methyl ether production in P. fastuosus measured after 20 and 30 days. A concentration range (0 - 10 mM) of hydroquinone was added to P. fastuosus on day 6 of fungal growth. Average values of three separate cultures are shown with their standard deviations*

Another approach to increase DAME production, based on antagonizing *P. fastuosus* was used. Filter-sterilized culture fluids of *Heterobasidium annosum, Phlebia radiata* and *Penicillium simplicissimum* were added to the 8-day-old cultures of *P. fastuosus.* Measurements were taken on three separate days: days 14, 18 and 20 (Figure 3). The production of DAME in *P. fastuosus* increased ten times over that in the non-supplemented control when filter-sterile culture fluid of *P. radiata* was added to the fungus. The effect was not only observed on one day, but

lasted during all the days of analysis, indicating that DAME production was elicited by metabolites secreted by *P. radiata*. The filter-sterile culture fluid of *P. radiata* did not contain DAME or possible precursors such as hydroquinone or benzoquinone. *H. annosum* and *P. simplicissimum* did not have a significant effect on DAME production by *P. fastuosus*.

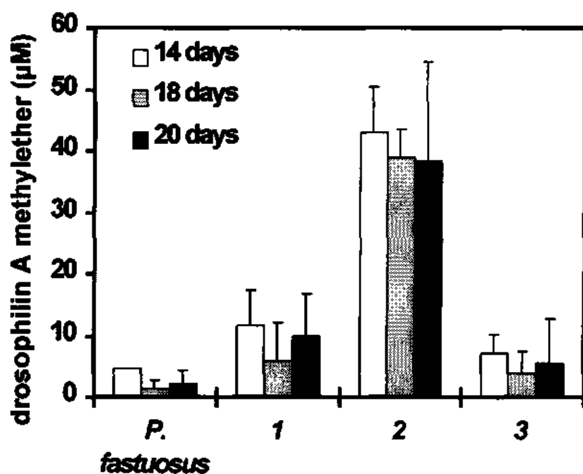

Figure 3. *Drosophilin A methyl ether production in P. fastuosus ATCC26.125 measured after 14, 18 and 20 days. Sterilized extracellular culture fluid of 8-day-old Heterobasidium annosum (1), 8-day-old Phlebia radiata (2) and 8-day-old Penicillium simplicissimum (3) was added to P. fastuosus on day 8. Average values of three separate cultures are shown with their standard deviations.*

Discussion

This study indicates that several ligninolytic basidiomycetes are capable of de novo DA and DAME production. These metabolites were observed for the first time in several fungal species. Although *Bjerkandera* species and *Peniophora pseudopini* are known to produce chlorinated anisyl metabolites (88, 157), this is the first time tetrachlorohydroquinone metabolites (DA and DAME) were detected in the genera *Bjerkandera* and *Peniophora*. However, 2-chloro-1,4-dimethoxybenzene has previously been identified in *Bjerkandera adusta* (152, 156). DA and DAME were only detected in low amounts in these strains, up to 4 µM. Also, another chlorinated metabolite, 4-chloro-3,5-dimethoxybenzaldehyde, was found in the genus *Bjerkandera* for the first time. This metabolite was previously detected in

cultures of *Hericium erinaceum* (129). *A. bisporus* is known to produce DAME as a secondary metabolite (51). Here we show that *A. arvensis* is a new species of this genus capable of DAME production. Although *P. fastuosus* has already been reported to produce DAME in the fruiting body (150), here we report that this fungus is able to produce both DA and DAME in liquid mycelium cultures. *P. fastuosus* was the highest producer of DA and DAME.

Since ligninolytic activity was expressed together with DA and/or DAME production in all positive strains, a possible physiological role of DA and/or DAME in lignin degradation should be investigated in further research. Although *P. fastuosus* did not decolorize Poly R plates on peptone agar while DA and DAME production was found only in peptone liquid medium, we did detect high extracellular peroxidase activity towards the assay substrate 2,6-dimethoxyphenol in peptone liquid medium (results not shown).

To identify physiological factors responsible for DA and/or DAME production in *P. fastuosus,* several compounds were added to the culture fluid of the fungus. Only hydroquinone stimulated DA production. It is possible that hydroquinone is an intermediate in the biosynthesis of DAME and DA. Although no chlorinating enzymes have yet been found in white-rot fungi, it is not unlikely that hydroquinone is chlorinated by a chloroperoxidase. Compounds like phenols and their derivatives are good substrates for chloroperoxidases (37). Subsequently, methylation of the chlorinated hydroquinone could occur by one of the two known methylating systems in ligninolytic fungi, utilizing either S-adenosylmethionine or chloromethane as the methyl donor (25, 61). Chloromethane is known to be produced by *Agaricus* and *Phellinus* species (60, 173). Tetrachlorohydroquinone was shown to be methylated by several *Mycena* species producing DA (105).

Antagonizing *P. fastuosus* by adding filter-sterilized culture fluid of the basidiomycete *P. radiata* also resulted in increased DAME production. The culture fluid of *Phlebia radiata* probably contains metabolites responsible for the induction of DAME production in *P. fastuosus*. Sonnenbichler et al. (151) showed that the addition of sterile culture fluid of *Gloeophyllum abietinum* increased the production of the chlorinated metabolite, melledonal C, a chloro-orsenillate sesquiterpene ester, in *Armillaria ostoyae*. These fungi display antagonistic behavior when they are grown in the same medium (78). The toxins that were produced by the antagonist are suggested to be responsible for the induction of melledonal C in *A. ostoyae* (151). In a similar fashion, it was shown that 3,4-dichloroaniline induced the de novo production of DA in *Schizophyllum commune*, although DA is not a biotransformation product of 3,4-dichloroaniline (146). Therefore, stimulation of DAME production could be due to the reaction of *P. fastuosus* to an unknown compound in the culture fluid of *P. radiata*. This observation indicates clearly that DAME has an antibiotic function in *P. fastuosus*.

Acknowledgments

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3

2-Chloro-1,4-Dimethoxybenzene as a Novel Catalytic Cofactor for Oxidation of Anisyl Alcohol by Lignin Peroxidase

Pauline J. M. Teunissen and Jim A. Field

2-chloro-1,4-dimethoxybenzene (2Cl-14DMB) is a natural compound produced de novo by several white rot fungi. This chloroaromatic metabolite was identified as a cofactor superior to veratryl alcohol (VA) in the oxidation of anisyl alcohol (AA) by lignin peroxidase (LiP). Our results reveal that good LiP substrates, such as VA and tryptophan, are comparatively poor cofactors in the oxidation of AA. Furthermore, we show that a good cofactor does not necessarily serve a role in protecting LiP against H_2O_2 inactivation. 2Cl-14DMB was not a direct mediator of AA oxidation, since increasing AA concentrations did not inhibit the oxidation of 2Cl-14DMB at all. However, the high molar ratio of anisaldehyde formed to 2Cl-14DMB consumed, up to 13:1, indicates that a mechanism which recycles the cofactor is present.

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Introduction

White-rot fungi are involved in the extensive degradation of lignin by means of their extracellular ligninolytic system (100). Key enzymes involved in the lignin-degrading system are extracellular peroxidases which are directly responsible for the oxidative depolymerization of lignin (59). Lignin peroxidase (LiP) plays an important role in the degradative ability. LiP can oxidize substrates with a higher ionization potential than other peroxidases (96). This capability enables the enzyme to oxidize nonphenolic methoxylated aromatic compounds (95). LiP also catalyzes the oxidation of recalcitrant xenobiotic compounds such as polycyclic aromatic hydrocarbons (56), dioxins (177), chlorophenols (57) and azo-dyes (130, 133).

The catalytic cycle of LiP is like those of other peroxidases. LiP is activated in the presence of H_2O_2 to form compound I. This intermediate is able to catalyze a one-electron oxidation of numerous substrates, forming compound II. The cycle is completed by an additional one-electron oxidation of a limited number of substrates, causing the reduction of compound II back to the enzyme's ferric state (167). However, in the absence of reducing substrate, compound II can undergo a series of reactions with H_2O_2 to form compound III, an inactive form of the enzyme (185).

In the presence of veratryl alcohol (VA), a secondary metabolite of several ligninolytic white-rot fungi (86, 111), the formation of compound III is prevented (188). VA is a favorable substrate for compound II and converts it to the resting state, completing the catalytic cycle (103, 143). Nonphenolic monomethoxylated lignin model compounds, such as anisyl alcohol (AA), are poorly oxidized by LiP. Inclusion of VA in the reaction mixture accelerated the oxidation of AA (63). Koduri and Tien (103) showed that AA can be oxidized only by compound I. VA is required as an essential cofactor for oxidation by compound II,

allowing the enzyme to return to its ferric state (103). Furthermore, a secondary consequence of the cofactor role is that VA prevents the inactivation of the enzyme by excess H_2O_2 (185).

VA has also been implicated as a redox mediator of LiP for substrates with a lower ionization potential than VA itself. VA is oxidized by one electron to form the veratryl alcohol cation radical ($VA^{+\cdot}$) (20, 63). $VA^{+\cdot}$ was suggested to oxidize other substrates at a distance from the active site of the enzyme (63). However, the free VA cation radical is too short-lived (half-life 0.5 ms) to diffuse away from the enzyme (92, 97). Therefore, it was suggested that an enzyme-bound radical was formed (20). Khindaria et al. (97) demonstrated the presence of a LiPII-$VA^{+\cdot}$ complex, which has a half-life of 0.54 s, implying a more stable VA cation radical. The enzyme-bound radical could be more reactive because it is longer-lived or because it has a higher oxidation potential than the free $VA^{+\cdot}$ (20).

It is possible that white-rot fungi produce alternative metabolites which could serve as cofactors or mediators of LiP catalysis. Several other compounds were found to substitute the function of VA as reducing agents for compound II in AA oxidation: 3,4-dimethoxytoluene, 1,4-dimethoxybenzene (14DMB) and 3,4,5-trimethoxybenzyl alcohol (92, 103). Collins et al. (22) also introduced tryptophan (Trp) as an alternative cofactor for VA in LiP catalysis; Trp stabilizes the enzyme against H_2O_2 inactivation even better than VA. Moreover, 14DMB is oxidized to a cation radical, as was demonstrated by electron spin resonance spectroscopy, indicating the relative stability of this cation radical (95) and implying a possible function as diffusable mediator in LiP oxidations (92). However, 14DMB is not naturally produced by white rot fungi.

Chlorinated 1,4-dimethoxybenzenes, structurally related to 14DMB, are naturally produced by several white-rot fungi with

ligninolytic activity. Examples of such metabolites include: 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB), 2,6-dichloro-1,4-dimethoxybenzene (26DCl-14DMB), tetrachloro-1,4-dimethoxybenzene (designated drosophilin A methyl ether [DAME]) and tetrachloro-4-methoxyphenol (designated drosophilin A [DA]) (34, 152, 156, 163). Consequently, chlorinated 1,4-dimethoxybenzenes can possibly serve the same function as VA in LiP catalysis. In this report we have identified 2Cl-14DMB as an alternative for VA in LiP catalysis. We demonstrate that 2Cl-14DMB is a cofactor superior to VA and 14DMB in the LiP-catalyzed oxidation of AA.

Materials and methods

ORGANISMS AND MEDIA

Bjerkandera sp. strain BOS55 (ATCC 90940) was isolated and maintained as previously described (85). Inoculum was prepared on malt extract plates containing, per liter, 5 g of glucose, 3.5 g of malt extract (Oxoid Ltd., Basingstoke, Hampshire, UK) and 15 g of agar. To obtain high LiP activity, *Bjerkandera* sp. strain BOS55 was grown in a high nitrogen medium containing, per liter, 10 g of glucose, 5 g of mycological peptone (Oxoid), 1 g of yeast extract (Gibco BRL, Paisley, UK), 0.2 g of diammonium tartrate, 20 mmol of 2,2-dimethylsuccinate (DMS), 2 mmol of VA, 200 mg of thiamine, and 100 ml modified BIII mineral medium (168). The modified BIII medium contained 54.4 g of KH_2PO_4 per liter and no manganese. After autoclaving, the pH was adjusted to 6.0 with 2 M autoclaved NaOH.

CULTIVATION CONDITIONS ON LIQUID MEDIUM

For the purification of LiP, Erlenmeyer flasks (5 liter) each containing 1 liter of standard medium were inoculated with five cylindrical agar plugs (diameter, 5 mm), which were taken from the outer periphery of a malt extract agar plate covered with mycelium of *Bjerkandera* sp. strain BOS55 incubated at 30°C for 6 days. The Erlenmeyer flasks were left to grow statically in the dark at 30 °C for 20 days.

LIP PREPARATIONS

LiP was purified from the extracellular fluid of *Bjerkandera* sp. strain BOS55 cultures. The proteins were concentrated by ammonium sulfate precipitation (85% saturation). The concentrate was dialyzed against 10 mM sodium acetate buffer (pH 6.0). The dialyzed fraction was further purified on a Resource Q anion-exchange column (Pharmacia, Woerden, The Netherlands) with a gradient of 10 mM to 1 M sodium

acetate (pH 6.0). The first LiP containing fraction was further purified on a Source S cation-exchange column (Pharmacia) in order to separate LiP from aryl alcohol oxidase. The purity of the LiP isozyme was confirmed by SDS-PAGE. Also a partially purified LiP preparation from *Phanerochaete chrysosporium* obtained from Tienzyme, Inc. (State College, Pa, USA) was used in several experiments. LiP activity is expressed in units. One U of LiP activity was defined as the amount of enzyme required to oxidize 1 µmol of VA per min.

CHLORINATED COMPOUNDS AS SUBSTRATES OF LiP

VA, 14DMB, 2Cl-14DMB, 26DCl-14DMB, DAME and DA were tested as substrates for LiP. 14DMB and the chlorinated 14DMB derivatives were dissolved in acetone. The maximum acetone concentration in the final reaction mixture of the experiments was 5%. The reaction mixture was composed of the following: 500 µM substrate, 0.1 units purified LiP of *Bjerkandera* sp. BOS55, 0.25 mM H_2O_2 in 20 mM sodium succinate pH 3.0. Assay volumes were adjusted to 1 ml with distilled H_2O. After 90 minutes incubation at 30°C, the reaction was stopped with the addition of 1 ml of acetonitrile and the products were analyzed by HPLC.

H_2O_2 INACTIVATION

The protective effects of VA and 2Cl-14DMB against LiP inactivation by high concentrations of H_2O_2 were examined. Assay mixtures were composed of the following: 2Cl-14DMB (25, 50, 100, 500, or 2,000 µM), LiP (initial activity 0.1 U/ml), H_2O_2 (final concentration in assay mixture, 0.1 mM) and 100 mM sodium acetate buffer (pH 5.0). Assay volumes were adjusted to 1 ml with distilled H_2O. After 0, 2, 5, 8, 11, 14, 17, and 20 min. of incubation at room temperature, 100 µl aliquots were removed from assay mixtures and their VA-oxidizing activities were measured (as described in "Enzyme assays"). Data are expressed as percentages of the initial LiP activities remaining.

AA OXIDATION

The stimulating effect of 2Cl-14DMB on the oxidation of AA was examined. The assay mixture was composed of 500 µM AA, 20 mM sodium succinate (pH 3.0) (99% purity), 2 to 500 µM 2Cl-14DMB, 0.1 U of purified LiP from *Bjerkandera* sp. strain BOS55 and 250 µM H_2O_2. The assay volume was adjusted to 1 ml. The incubation time of the assay mixture was 90 min. The reaction was stopped by the addition of 1 ml acetonitrile to the assay mixture, and the samples were analyzed by HPLC.

COFACTOR RECYCLING

The effects of succinic acid, acetone and bovine serum albumin (BSA) on the recycling of 2Cl-14DMB were examined. The assay mixture was

composed of 500 μM AA, 0 to 40 mM sodium succinate (pH 3.0) (99% purity), 50 μM 2Cl-14DMB, 0.1 U of purified LiP from *Bjerkandera* sp. strain BOS55, and 250 μM H_2O_2. The reaction was stopped by the addition of 1 ml acetonitrile to the assay mixture, and the samples were analyzed by HPLC for succinic acid elimination or by GC for CO_2 production. When acetone (0.05% to 5% of assay volume) or BSA (0 to 50 mg/liter) was added, 20 mM sodium succinate was used. The assay volume was adjusted to 1 ml. The incubation time of the assay mixture was 90 min. The reaction was stopped by the addition of 1 ml acetonitrile to the assay mixture, and the samples were analyzed by HPLC.

ENZYME ASSAYS

LiP activity was measured by monitoring the oxidation of VA to veratraldehyde (VAld) at 310 nm (ε = 9,300 M^{-1} cm^{-1}) as described by Tien and Kirk (168) and corrected for background VA oxidase activity. VA oxidase activity was measured by monitoring the oxidation of VA to VAld at 310 nm without the addition of 0.4 mM H_2O_2.

GAS CHROMATOGRAPHIC ANALYSIS

The methanol concentration was measured on a gas chromatograph. Samples were adjusted to pH 2.0 with 3% formic acid and centrifuged for 5 min (at 17000 x *g*). Methanol was determined using a Packard Becker model 417 (Delft, The Netherlands) gas chromatograph equipped with a 6-m by 2-mm glass column packed with a Supelco port (Bellefonte, Pa.), 100 -/120 mesh, coated with 10% Fluorad FC431 (3M, St. Paul, Minn.). The flow rate of the carrier gas (nitrogen saturated with formic acid) was 30 ml min^{-1}, and column pressure was ± 3 x 10^5 Pa. The column temperature was 70°C, the injection port and the detector were at 220 and 280°C, respectively.

CO_2 concentrations were determined by using a Packard model 427 gas chromatograph with a Hayesep Q column (Chrompack, Middelburg, The Netherlands). Headspace samples (50 μl) were analyzed.

HPLC ANALYSIS

Fifty microliters of the incubation mixtures was analyzed for products by HPLC as described previously (163) with the column (200 by3 mm) filled with ChromSpher C18-PAH (5-μm particles) (Chrompack). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30°C): 90:10, 0:100 and 0:100 H_2O: CH_3CN at 0, 15, and 20 min, respectively. The UV absorbance was monitored at 2-nm wavelength intervals from 200 to 400 nm. Compound identification was carried out by matching UV spectra and the retention times of the observed products with their standards.

ORGANIC ACID DETERMINATION

Organic acid concentration was measured by HPLC. HPLC analysis was performed on an Aminex HPX-87H column (Biorad, Veenendaal, The Netherlands). Samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min at 40°C, and detection was at 210 nm. Compound identification was carried out by matching retention times of samples with their standards.

REFERENCE COMPOUNDS

Standards for 26DCl-14DMB, DA and DAME were kindly provided by Dr. J. Knuutinen, Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland. 2Cl-14DMB and 14DMB were obtained from Janssen Chimica. VA was obtained from Aldrich.

STATISTICAL PROCEDURES

In all experiments, measurements were carried out in triplicate. Values reported are means with standard deviation values.

Results

METHOXYBENZENES AS SUBSTRATES FOR LIP

VA, 14DMB, and several chlorinated derivatives of 14DMB were compared as substrates of semipurified LiP from *Phanerochaete chrysosporium* and purified LiP from *Bjerkandera* sp. strain BOS55 (Table 1). Of the nonphenolic compounds tested, VA, 14DMB, and 2Cl-14DMB were found to be substrates of LiP. VA and, to a lesser extent, 14DMB were observed to be relatively good substrates, whereas, 2Cl-14DMB was a poor substrate. The more chlorinated nonphenolic compounds, 26DCl-14DMB and DAME, were not oxidized by the LiP preparations. Inclusion of VA (50 μM or 500 μM) in the reaction mixture did not enable these compounds to become oxidized by LiP, nor did VA alter the extent of 14DMB and 2Cl-14DMB oxidation (results not shown). The theoretical maximum conversion of the nonphenolic compounds (donating two electrons per molecule) was 50%, since they were supplied in a twofold excess of the H_2O_2 (accepting two electrons per molecule). The fact that VA was oxidized up to 63% indicates that there was probably either some endogenous production of peroxide or another electron acceptor during the reaction. The only phenolic compound tested, DA, was a good substrate for the LiP preparations.

Table 1. *Oxidation of VA, 14DMB, and several chlorinated derivatives of 14DMB by semipurified LiP from P. chrysosporium and a purified LiP isozyme of Bjerkandera sp. strain BOS55.*

compound	% oxidation[a]		identified product(s)	molar product yield (%)[b]	
	semipurified LiP	purified LiP		semipurified LiP	purified LiP
VA	54	63	veratraldehyde	100	83
14DMB	41	24	1,4-benzoquinone	100	69
			methanol	NM	160
2Cl-14DMB	13	11	2-chloro-1,4-benzoquinone	100	35
			methanol	NM	76
26DCl-14DMB	0	0[c]	not detected		
DAME	0	0		0	0
DA	73	53	unidentified		

[a] *Calculated as (initial concentration of substrate - final concentration of substrate/initial concentration of substrate)x 100.*
[b] *Calculated as (product concentration/initial concentration of substrate - final concentration of substrate) x 100 , NM, not measured.*
[c] *in some incubations, 2 to 5% 26DCl-14DMB was consumed*

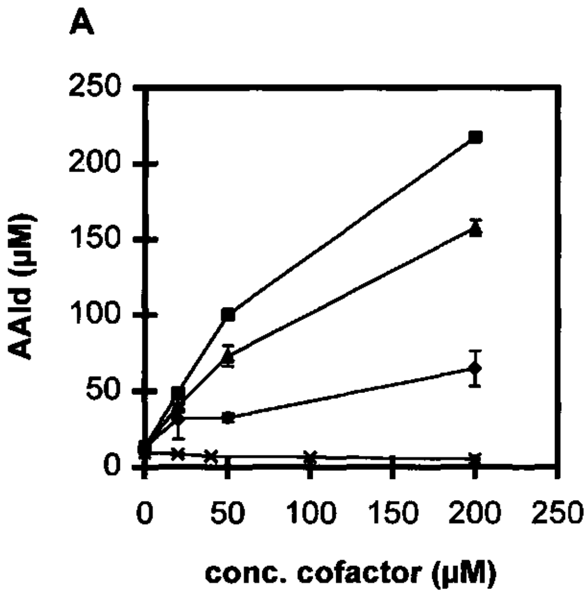
The major product of VA was VAld, whereas the major products of the 14DMB derivatives were the corresponding quinones (1,4-benzoquinone and 2-chloro-1,4-benzoquinone) and methanol. The molar ratio of methanol to quinone formed was approximately 2:1.

The molar product yields obtained from semipurified LiP and purified LiP were different. The results with semipurified LiP showed a complete conversion of the consumed substrates to the identified products (molar product yield, 100%). The comparative product yield with purified LiP was lower, indicating the formation of other products besides those that were identified in these experiments. No products were detected in reactions lacking either enzyme or H_2O_2 or with boiled enzyme.

2CL-14DMB IN AA OXIDATION

The abilities of LiP substrates to act as cofactors in the catalysis of purified LiP were examined. Relatively good substrates such as VA and 14DMB (Table 1), as well as tryptophan (22), were compared with the poor substrate 2Cl-14DMB. These compounds were tested at several concentrations in the range from 20 to 200 μM as shown in Figure 1. Trp, which was clearly the best LiP substrate, had no role in improving the background level of AA oxidation. The next best substrate, VA, supported limited enhancement of AA oxidation up to 50 μM; however, there was no significant increase in the amount of anisaldehyde (AAld) formed as the VA concentration was increased further up to 200 μM. Both 14DMB and 2Cl-14DMB were better cofactors. The extent to which AA was oxidized increased with the cofactor concentration. 2Cl-14DMB was clearly the worst LiP substrate and the best cofactor, supporting the highest conversion of AA at any given cofactor concentration. 26DCl-14DMB, which is not a substrate of LiP, was also tested and was found not to have any cofactor effect (results not shown).

The stoichiometric relationship between the AAld formed (corrected for the background formation) and the cofactor consumed is given in Table 2. This ratio reached a maximum of 2 for VA and 14DMB at the lowest cofactor concentration tested. But the ratio approached 1 or less at higher cofactor concentrations, indicating that VA and 14DMB are for the most part noncatalytic cofactors (just one turnover). On the other hand, this ratio was distinctly higher for 2Cl-14DMB, ranging between 3 and 13 in various experiments. Thus, 2Cl-14DMB is a cofactor which is catalytic; each molecule consumed supports multiple turnovers of the enzyme for AA oxidation.



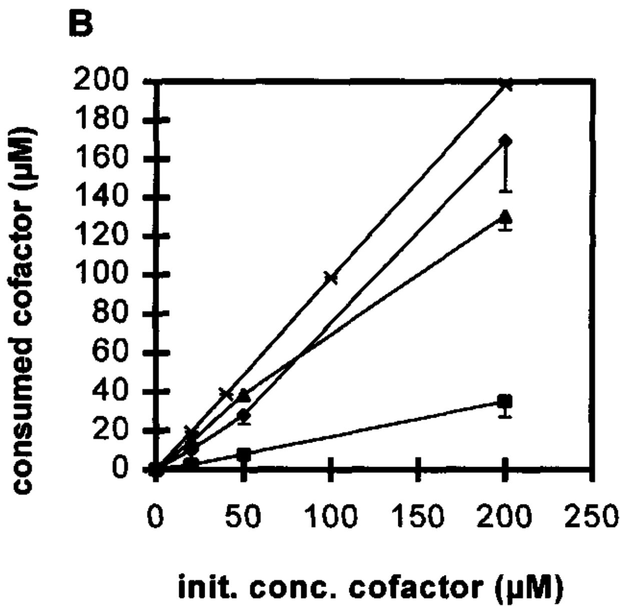


Figure 1. *Effects of the concentrations of VA (◆), 14DMB (▲), 2Cl-14DMB (■), and Trp (×) AAld formation (A) and the concomitant consumption of the cofactors (B) during AA oxidation.*

Table 2. *Effect of increasing concentrations of veratryl alcohol (VA), 1,4-dimethoxybenzene (14DMB) and 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB) on the oxidation of anisyl alcohol (AA) and the stoichiometric ratio of anisaldehyde (Aald) versus consumed cofactor.*

Cofactor and conc. (μM)	% AA oxidation	% Oxidation of cofactor	stoichiometric ratio, AAld formation: consumed cofactor[a]
none	2		
VA			
20	3	50	2 : 1
50	4	60	1 : 1.5
200	10	80	1 : 3
14DMB			
20	5	74	2 : 1
50	11	81	1.5 : 1
200	26	71	1 : 1
2Cl-14DMB			
20	10	22	8 : 1 (11 : 1)
50	21	25	5 : 1 (13 : 1)
200	40	40	5 : 1 (3 : 1)

[a] *Values in parentheses are results observed in additional experiments*

The cofactor role of 2Cl-14DMB was examined further by testing a larger range of concentrations and comparing the oxidation of substrates with the consumption of H_2O_2 (Figure 2). AA oxidation increased with elevated 2Cl-14DMB concentrations up to 200 μM. Thereafter, the H_2O_2 supply became limiting and further increases in the cofactor concentration only had the effect of stealing H_2O_2 away from AA oxidation, thereby causing some decreases in the AAld formed. Throughout the entire cofactor concentration range considered, the sum of cofactor and AA oxidized was approximately 20% higher than the H_2O_2 consumption, suggesting that some endogenous production of H_2O_2 was occurring or that an alternative electron acceptor was present. The molar yield of 2-chloro-1,4-benzoquinone per mol of 2Cl-14DMB consumed was 100% at low cofactor concentrations but decreased to 70% at higher concentrations.

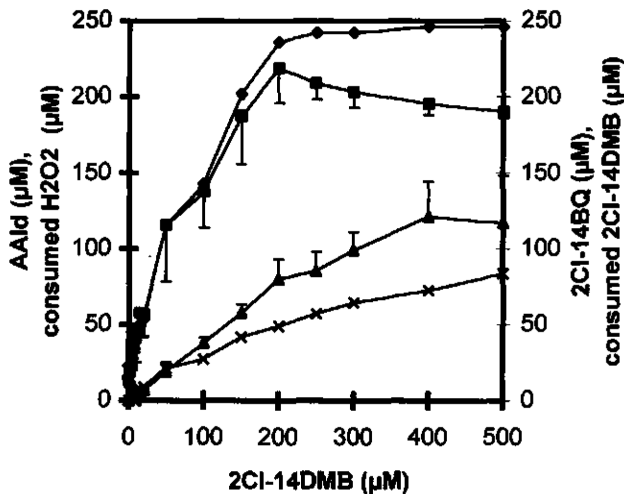


Figure 2. *Effect of varying concentrations of 2Cl-14DMB on AAld formation (■). Also shown are the concomitant consumption of 2Cl-14DMB (▲), the formation of the 2Cl-14DMB oxidation product 2-chloro-1,4-benzoquinone (2-Cl-BQ) (×), and the H_2O_2 used by the system (◆).*

The effect of increasing AA concentrations on the consumption of 50 µM of 2Cl-14DMB by purified LiP was evaluated (Figure 3). Irrespective of the AA concentration from 0 to 2,000 µM, AA had no effect on the extent to which the cofactor was consumed. Thus, AA did not inhibit 2Cl-14DMB oxidation by LiP. However, the production of AAld increased with increasing concentrations of AA.

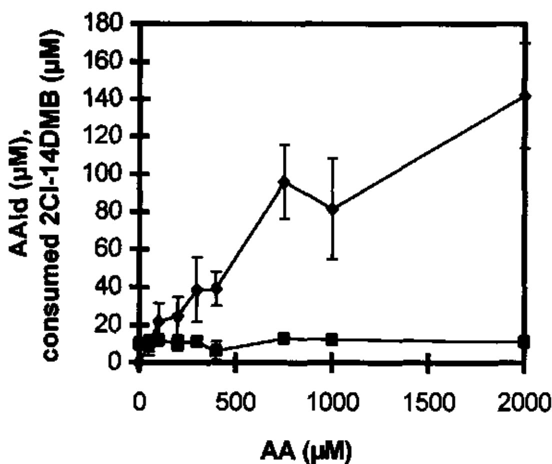


Figure 3. *Effect of varying concentrations of anisylalcohol (AA) on the consumption of 2Cl-14DMB (■) and the formation of AAld (◆).*

EFFECT OF SUCCINIC ACID, ACETONE, AND PROTEIN ON 2CL-14DMB OXIDATION

Assay components were examined for the ability to function as an electron donor for 2Cl-14DMB$^{+\cdot}$, reducing it back to 2Cl-14DMB. The effect of increasing succinic acid concentrations from 0 to 40 mM on the consumption of 50 µM of 2Cl-14DMB was tested. Succinic acid had no effect on the extent to which 2Cl-14DMB was consumed. Furthermore, no CO_2 production or elimination of succinic acid was detected (results not shown). Acetone, which is present as a solvent for 2Cl-14DMB in the assay mixture, could possible act as an electron donor. Therefore, increasing percentages of acetone from 0.05% to 5% of the assay volume were examined. However, none of the tested concentrations had an effect on the consumption of 2Cl-14DMB.

Noncatalytic parts of the protein could chemically interfere with the oxidation of 2Cl-14DMB. However, increasing concentrations of BSA, ranging from 0 to 50 mg/liter, added to the assay mixture did not affect the consumption of 2Cl-14DMB.

2CL-14DMB AS A PROTECTOR AGAINST LiP INACTIVATION BY H_2O_2

Only 2 mM 2Cl-14DMB partially protected LiP against inactivation by high concentrations of H_2O_2 (Figure 4). However, the protective effect was not as good as that of 2 mM VA, which almost completely protected LiP from inactivation in the time period considered. Concentrations below 2 mM 2Cl-14DMB did not have a protective effect on LiP activity at all. On the contrary, 100 µM 2Cl-14DMB decreased LiP activity to a point below that observed when no 2Cl-14DMB was added to the reaction mixture. This was also observed for 25 and 50 µM 2Cl-14DMB . As shown in Figure 5, when no 2Cl-14DMB or VA was added, 80% of the activity of purified LiP was gone after 8 min. but addition of 25, 50, or 100 µM 2Cl-14DMB caused a 90 to 100% inactivation of LiP activity. Similar results were

obtained with semipurified *P. chrysosporium* LiP. Although the inactivation of LiP after 8 min. did not proceed as far as that for purified *Bjerkandera sp.* strain BOS55 LiP, the same pattern of inactivation was observed. These results suggest that small amounts of 2Cl-14DMB stimulate the inactivation of LiP.

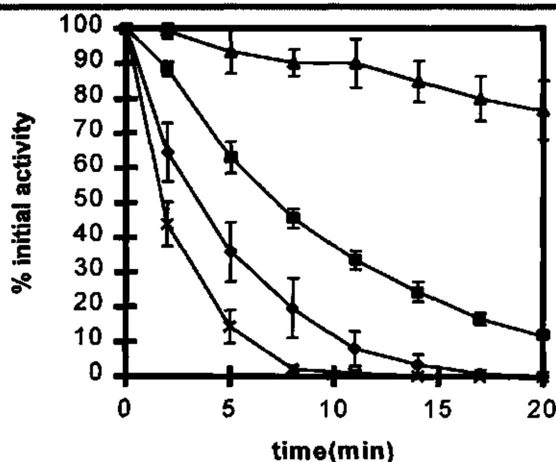


Figure 4. *Protective effects of 100 μM (×) and 2 mM (■) 2Cl-14DMB and of 2 mM VA (▲) against inactivation of purified LiP by 0.1 mM H_2O_2. ◆, no 2Cl-14DMB added to reaction mixture.*

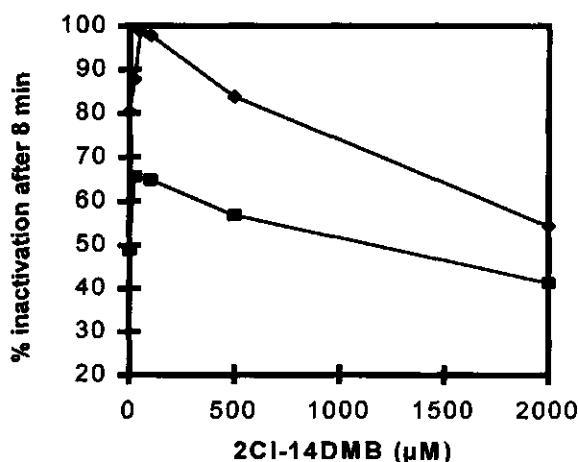


Figure 5. *Inactivation of purified (◆) and semipurified (■) LiP after 8 min of treatment with 0.1 mM H_2O_2 in the presence of varying concentrations of 2Cl-14DMB.*

Discussion

2Cl-14DMB is a 14DMB derivative that is produced de novo by *Bjerkandera adusta* and *Lepista nuda* (76, 152, 156). So far only trace amounts of the compound have been found in the ligninolytic cultures. In *L. nuda* growing on forest litter, up to 0.2 mg of 2Cl-14DMB/kg has been detected (76). In this report we show that this naturally produced chloroaromatic is a cofactor superior to VA and 14DMB in the oxidation of AA.

Naturally produced chlorinated 14DMB derivatives were compared as substrates for LiP with VA and 14DMB. LiP catalyzed the oxidation of VA, 14DMB, 2Cl-14DMB, DA (Table 1). Oxidation of VA by LiP yielded VAld as the major product (83% of oxidized VA), as was also found by Joshi and Gold (92) and others (86). 14DMB and 2Cl-14DMB oxidation yielded the corresponding benzoquinones as major products (respectively 69% and 35% of consumed substrates), although other products, which were not identified in this study, must have been formed as well. Joshi and Gold (92) reported the formation of 2-(2,5-dimethoxyphenyl)-1,4-benzoquinone as a major product of 14DMB, whereas Kersten et al. (95) only reported the formation of 1,4-benzoquinone. Previously, it was observed that 2Cl-14DMB was oxidized to 2-chloro-1,4-benzoquinone as a major product and that, to a lesser extent, 2,5-dimethoxy-1,4-benzoquinone and 3-chloro-4-methoxy-1,2-benzoquinone were formed (176). 26DCl-14DMB and DAME were not oxidized by LiP. Previous research showed that 2,5-dichloro-1,4-dimethoxybenzene also was not oxidized by LiP (90). Obviously, more chloro groups decrease the reactivity of the compound. The ionization potential of dimethoxybenzenes increases when more electron-withdrawing chloro groups are present: values of 8.55, 8.69, 8.81 and 8.95 were calculated for 14DMB, 2Cl-14DMB, 26DCl-14DMB, and DAME, respectively. These values indicate that the one-electron removal from highly chlorinated compounds becomes progressively more difficult.

DA, however, is fairly well oxidized by LiP to a yet unidentified product. It has been shown that pentachlorophenol is also oxidized by LiP, with tetrachlorobenzoquinone as the major product (57, 119). Phenol oxidation proceeds via formation of a phenoxy radical, which occurs more easily than the formation of cation radicals from methoxybenzenes.

Although 2Cl-14DMB was the worst substrate for LiP, it was, surprisingly, found to be the best cofactor in AA oxidation. Our results reveal an inverse relation between the ability to be oxidized by LiP and the ability to act as a cofactor for the oxidation of the monomethoxylated lignin model substrate AA. The good substrates VA and 14DMB were worse cofactors than 2Cl-14DMB, whereas the very excellent LiP substrate Trp (22) was not a cofactor at all (Figure 1). VA was a good cofactor only at low concentrations. Although good substrates can close the catalytic cycle, because they can react with both compound I and compound II, they can also compete with AA oxidation by compound I. Previous research with VA showed that increasing VA concentrations compete with AA for oxidation with compound I, eventually leading in a decrease in AA oxidation (103). Trp is an excellent substrate for LiP: Collins et al. (22) suggested that Trp is even a better substrate for compound II than VA. Trp did not enhance AA oxidation at all, suggesting that AA oxidation was completely competitively inhibited by Trp oxidation. Probably, 2Cl-14DMB cannot compete very well with AA for oxidation at compound I, but is primarily oxidized by compound II.

Good LiP substrates are more effective in protecting LiP against H_2O_2 inactivation. VA has previously been shown to extend the half-life of LiP in fungal cultures (170), whereas Collins et al. (22) showed the superior protective effect of Trp compared to VA. Low concentrations of 2Cl-14DMB, by comparison, did not protect LiP against high H_2O_2 concentrations; only 2 mM 2Cl-14DMB partially protected LiP (Figure 4). Our results show that

a good cofactor does not necessarily serve a role in protecting against H_2O_2 inactivation as proposed by Valli et al. (175).

In fact, we found that low 2Cl-14DMB concentrations even increased LiP inactivation by H_2O_2. One possible explanation for this phenomenon is that 2Cl-14DMB is a much better substrate for compound I than for compound II. At low concentrations, 2Cl-14DMB stimulates the formation of compound II, which in turn reacts with H_2O_2 to form compound III, whereas, at high concentrations, 2Cl-14DMB progressively becomes a better reductant for compound II and likewise there is more cation radical available to restore compound III, as was shown for $VA^{+\cdot}$ (11). $VA^{+\cdot}$ can overcome compound III accumulation by converting it back to active ferric LiP (11). This was also shown for the 1,2,4,5-tetramethoxybenzene cation radical (12).

Although 2Cl-14DMB did not have a protective effect on LiP, the compound clearly stimulated AA oxidation. Unlike VA and 14DMB, 2Cl-14DMB is a catalytic cofactor; each molecule consumed supported multiple turnovers of the enzyme for AA oxidation. Our results show that 2Cl-14DMB is not a direct mediator in AA oxidation. If mediation had occurred, the presence of increasing AA concentrations should have completely inhibited the consumption of 2Cl-14DMB oxidation, as was found for VA in the oxidation of guaiacol, 4-methoxymandelic acid, and chlorpromazine (45, 104, 169). However, 2Cl-14DMB consumption was not inhibited by AA at all (Figure 3). This result also suggests that 2Cl-14DMB and AA do not have the same binding site.

The molar ratio of AAld formed to cofactor consumed ranged from 3 to 13 (Table 2). Probably a mechanism is present which recycles the 2Cl-14DMB cation radical ($2Cl\text{-}14DMB^{+\cdot}$) back to 2Cl-14DMB. As indicated above, 2Cl-14DMB did not directly mediate the oxidation of AA. A second possibility is that H_2O_2

reacts with 2Cl-14DMB$^{+\cdot}$, as was described for VA$^{+\cdot}$ (10). The one-electron reduction of the cation radical back to 2Cl-14DMB would result in net O_2 production from H_2O_2 (10). In such a case, the H_2O_2 consumption would exceed the sum of oxidized 2Cl-14DMB and AA; however, the H_2O_2 consumption was 20% lower than the sum of the oxidized compounds (Figure 2). Consequently, other assay components which might be able to reduce 2Cl-14DMB$^{+\cdot}$ back to 2Cl-14DMB were considered. However, none of these components, succinate, acetone, and non-catalytic protein, was found to affect the oxidation of 2Cl-14DMB, suggesting that the reduction of 2Cl-14DMB$^{+\cdot}$ is carried out somewhere in the catalytic cycle of LiP or possibly by reduced oxygen radicals, which should be confirmed by further research.

In conclusion, this work demonstrates that 2Cl-14DMB is a catalytic cofactor superior to VA. Although so far only trace amounts of 2Cl-14DMB have been found in ligninolytic cultures (76, 152, 156), we showed that only small amounts of 2Cl-14DMB are necessary to exert a major increase in AA oxidation. As the molar ratio of AA oxidation compared to cofactor oxidation is so high, the cofactor must be recycled in the reaction.

Acknowledgments

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2-Chloro-1,4-Dimethoxybenzene Cation Radical: Formation and Role in the Lignin Peroxidase Oxidation of Anisyl Alcohol

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2-Chloro-1,4-dimethoxybenzene (2Cl-14DMB) oxidation by lignin peroxidase (LiP) proceeds via the formation of the 2Cl-14DMB cation radical as indicated by ESR and UV/vis spectroscopy. The products of the LiP-catalyzed oxidation of 2Cl-14DMB were identified as 2-chloro-1,4-benzoquinone and the dimers dichloro-tetramethoxybiphenyl and chloro(chloro-dimethoxyphenyl)-benzoquinone. The addition of anisyl alcohol (AA) rapidly quenched the 2Cl-14DMB cation radical optical absorption bands, suggesting that the cation radical directly mediates the oxidation of AA. When LiP reactions are conducted in the presence of 50 μM 2Cl-14DMB, the enzyme is inactivated; however, this inactivation can be prevented by the addition of AA. This also suggests that the 2Cl-14DMB cation radical formed in the reaction, in turn, oxidizes AA.

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Introduction

Lignin peroxidase (LiP), an extracellular heme peroxidase produced by several white-rot fungi, has been shown to catalyze the depolymerization of lignin (58) as well as the oxidation of a wide range of recalcitrant aromatic substrates (56, 96, 177). The electronic structure of the native, liganded, and H_2O_2 -oxidized states of LiP (42, 113, 140) are similar to those of horseradish peroxidase (29), indicating a similar catalytic cycle. X-ray crystallographic studies of LiP (139) indicate that it is similar in overall structure to other plant and fungal peroxidases.

The fungal secondary metabolite veratryl alcohol (VA) plays an important role in stimulating the LiP oxidation of a wide range of recalcitrant substrates, including small aromatic compounds (63, 91, 175), lignin (58), and proteins (148). A variety of proposals have been put forth to account for the stimulation of LiP oxidations by VA. These include acting as a redox mediator, aiding in the turnover of the enzyme, and protecting the enzyme from H_2O_2 inactivation (45, 63, 103, 148, 175, 188). Previous optical absorption and EPR studies showed that a variety of methoxybenzenes and other aromatics, including VA, are oxidized by LiP to their corresponding aryl cation radicals via one-electron reactions (20, 95, 96, 98).

The fungal secondary metabolite 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB) can replace VA as a cofactor in the LiP-catalyzed oxidation of anisyl alcohol (AA) (164). Indeed, under some conditions, 2Cl-14DMB stimulated AA oxidation better than VA (164). In the present study, we examine the mechanism by which 2Cl-14DMB forms a stable cation radical and stimulates AA oxidation, and we examine the mechanism by which AA protects LiP from inactivation by 2Cl-14DMB.

Materials and methods

ENZYME PURIFICATION

LiP isozyme (H8) was purified from the extracellular medium of agitated, acetate-buffered cultures of *Phanerochaete chrysosporium* wild-type strain OGC101 as previously described (42, 188). The purified enzyme was electrophoretically homogeneous and had an RZ value (A_{408}/A_{280}) of 5.0. The enzyme concentration was determined at 408 nm using an extinction coefficient of 133 mM^{-1} cm^{-1} (42).

CHEMICALS

Hydrogen peroxide (30% solution) was obtained from Sigma. The concentration of H_2O_2 solutions was determined as described (21), using an extinction coefficient at 240 nm of 39.6 M^{-1} cm^{-1}. 2Cl-14DMB, 1,4-dimethoxybenzene (14DMB), AA, and benzyl alcohol (BA) were obtained from Aldrich. All solutions were prepared using deionized water from a Milli-Q 50 system (Millipore).

OPTICAL ABSORPTION SPECTRA

Enzyme reaction mixtures (1 ml) contained 500 μM 2Cl-14DMB and 0.125 μM LiP in 20 mM sodium succinate, pH 3.0. The reaction was initiated by the addition of 100 μM H_2O_2. Where indicated, reaction mixtures also contained 2 mM AA or 2 mM BA.

EPR SPECTROSCOPY

EPR spectra were obtained on a Varian E109 Century Series spectrometer equipped with a Varian E-102 microwave bridge and an Air Products helium cryostat.The reaction mixture (1 ml) contained 100 μM LiP, 150 μM H_2O_2, and 500 μM 2Cl-14DMB in 20 mM sodium succinate, pH 3.0.

ENZYME OXIDATION PRODUCTS

Reaction mixtures (1 ml) contained LiP (0.05 μM), 2Cl-14DMB (100 μM), and H_2O_2 (200 μM) in 20 mM sodium succinate, pH 3.0. Reactions were carried out at 25°C for 5 min. Products were reduced with dithionite, extracted with ethyl acetate at pH 2.0, dried over anhydrous sodium sulfate, evaporated under N_2, acetylated, and analyzed by GC/MS as described (91, 175). Dimeric products were identified based on their mass spectra. GC/MS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 30-m fused Silica column (DB-5, J&W Scientific). The oven temperature was programmed to increase from 70 to 320°C at 10°C/min.

LIP INACTIVATION

Reaction mixtures (1 ml) contained LiP (2.4 μM) and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0. Reactions were carried out at 25°C, with or without 2Cl-14DMB (50-500 μM) and/or AA (1 mM) or BA

(1 mM), as indicated. Aliquots (20 μl) were removed at the indicated intervals, and LiP activity was assayed using VA (2 mM) and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0, as previously described (44, 100). One unit of LiP activity was defined as the amount of enzyme required to oxidize 1 μmol of VA/min at 25°C and pH 3.0.

Results

The EPR spectrum of the LiP-generated 2Cl-14DMB cation radical is shown in Figure 1. Rapid freezing upon addition of H_2O_2 to the reaction mixture containing LiP and 2Cl-14DMB in pH 3.0 sodium succinate yielded a low temperature EPR spectrum with an intense signal at g = 2, characteristic of an organic cation radical. This EPR signal, also observed when 2Cl-14DMB was oxidized with cesium ammonium nitrate (data not shown), is assigned to the 2Cl-14DMB cation radical. The EPR spectrum of the LiP reaction mixture exhibited an additional feature at lower field which was not observed in the chemically generated cation radical. The enzymatically generated cation radical was relatively stable. The radical signal was present for at least 30 min at 15 K.

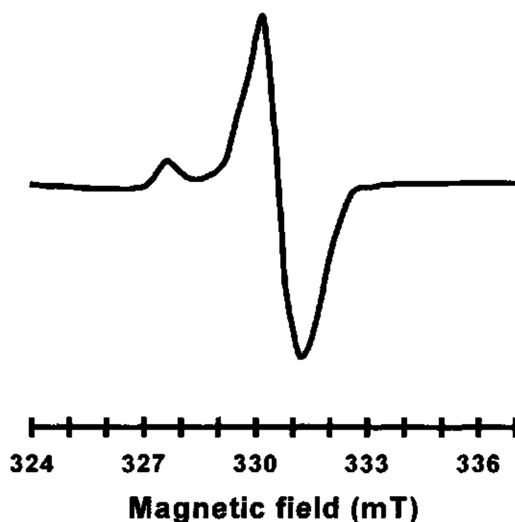

Figure 1. *EPR spectrum of 2Cl-14DMB. Experimental conditions: frequency, 9.34 GHz; modulation amplitude, 0.8 mT; microwave, 20 mW; temperature, 15 K. The reaction conditions were as described in the text. The reactants were mixed and immediately immersed in a methanol liquid N_2 slurry (100 K) and then transferred to the EPR cavity.*

The optical absorption spectrum of the intermediate formed during the oxidation of 2Cl-14DMB by LiP is shown in Figure 2A. Oxidation of 2Cl-14DMB resulted in the rapid formation of a stable intermediate exhibiting absorption maxima at 441 and 467.6 nm. After 2 min, approximately 90% of the intermediate remained (Figure 2A). Earlier, the radical cation intermediates generated by the LiP oxidation of tetramethoxybenzene and VA were reported to have an absorption maxima at 450 nm (96) and 430 nm (20), respectively.

Upon the addition of AA to the reaction mixture, a rapid decrease in the absorption bands at 441 and 476.6 nm was observed (Figure 2B and Figure 3). In contrast, the addition of BA to the reaction mixture containing the 2Cl-14DMB cation radical did not affect the appearance of the cation radical (Figure 2C). The rate of decay of the 2Cl-14DMB cation radical, either alone or in the presence of BA, is 3×10^{-5} s^{-1}, whereas in the presence of 1 mM AA the rate is 9×10^{-4} s^{-1} (Figure 3).

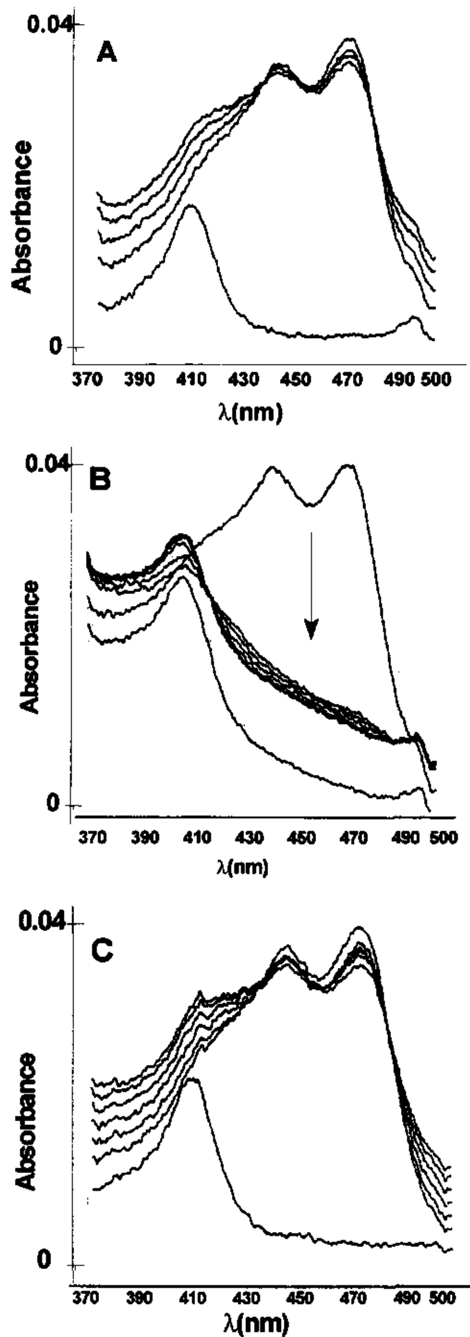

Figure 2. *Effect of AA and BA on the presence of the 2Cl-14DMB cation radical. Reactions were monitored every 30 s by following the changes in the visible spectrum of the 2Cl-14DMB cation radical. All reactions contained 2Cl-14DMB (500 µM) and LiP (0.125 µM) in 20 mM sodium succinate pH 3.0. The 2Cl-14DMB cation radical was formed after the addition of 100 µM H_2O_2. The initial absorption spectrum is that of the enzyme with a Soret maximum at ~ 405 nm. After the addition of H_2O_2, new maxima appear at 441 nm and 467.6 nm, owing to the formation of the 2Cl-14DMB cation radical. Subsequently, 2 mM AA or BA or nothing was added to the reaction mixture.* **(A)** *500 µM 2Cl-14DMB.* **(B)** *500 µM 2Cl-14DMB and 2 mM AA.* **(C)** *500 µM 2Cl-14DMB and 2 mM BA and quenching of the radical by AA (2B) is observed.*

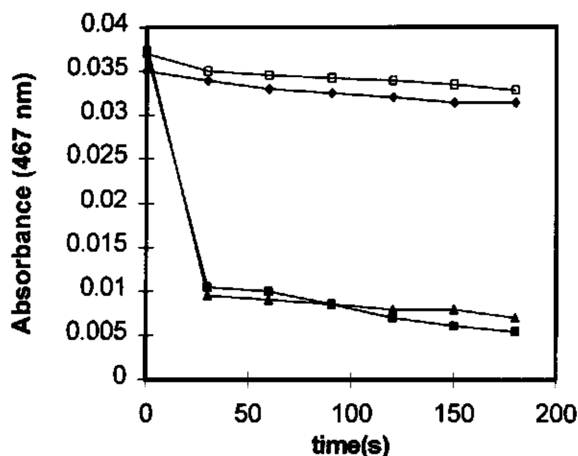

Figure 3. *Decay of the 2Cl-14DMB cation radical. The decrease in absorbance (467 nm) over time was measured. Reaction mixtures contained LiP (0.125 μM) and 2Cl-14DMB (500 μM) in 20 mM sodium succinate pH 3.0. The 2Cl-14DMB cation radical was formed after the addition of 100 μM H_2O_2. Subsequently, no AA or BA (♦), 1 mM AA (■), 2 mM AA (▲), or 2 mM BA (□) was added to the reaction mixture.*

The three products produced during the oxidation of 2Cl-14DMB are shown in Figure 4. The mass spectra and GC retention times of these products are shown in Table 1. Product II is 2-chloro-1,4-benzoquinone. Products III and IV are the dimers dichlorotetramethoxybiphenyl and chloro (chlorodimethoxyphenyl) benzoquinone. Similar products are formed during the oxidation of 14DMB (92). While a number of isomers of the dimeric products are possible, the most likely isomers are 3,3′-dichloro-2,2′,5,5′-tetramethoxybiphenyl and 2-chloro-6-(3-chloro-2,5-dimethoxyphenyl)-1,4-benzoquinone.
The formation of the dimers also suggests that the cation radical is long-lived as described for 1,4DMB (92).

Table 1. *Mass spectra of LiP oxidation products of 2-chloro-1,4-dimethoxybenzene*

Substrate or product	GC retention time (min)	Mass spectrum m/z (relative intensity)
2-chloro-1,4-dimethoxy-benzene	7.14	174 (28.4), 172 (86.5), 159 (41.2), 157 (100), 131 (8.9), 129 (27.4)
2-chloro-1,4-diacetoxy-benzene[a]	9.16	230 (1.0), 228 (3.6), 188 (6.5), 186 (19.5), 146 (44.3), 144 (100)
dichlorotetramethoxybiphenyl	19.44	346 (11.5), 344 (66.3), 342 (100), 331 (0.8), 329 (4.8), 327 (8.3), 294 (20.4), 292 (44.6), 279 (7.4), 277 (22.5), 209 (13.9), 207 (42.7)
chloro(chlorodimethoxyphenyl) benzoquinone	20.15	402 (2.1), 400 (13.5), 398 (19.9), 360 (0.7), 358 (3.2), 356 (7.6), 318 (12.7), 316 (65.8), 314 (100), 266 (4.9), 264 (15.0)

[a]*Reaction mixtures, derivatization, and analysis of products were as described in the text.*

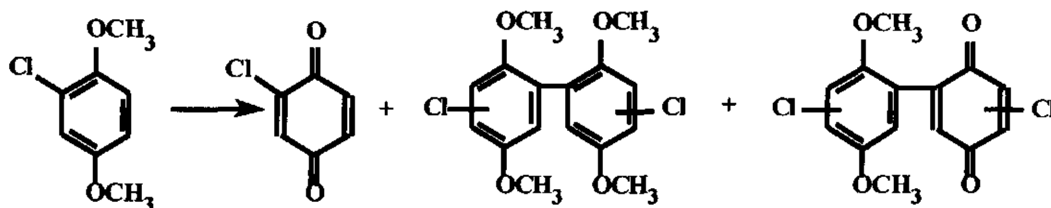

Figure 4. *Products identified from the oxidation of 2Cl-14DMB by LiP. Reaction conditions and product analysis were as described in the text. Mole (%) yields of substrate and product are shown in parentheses.*

The effects of AA and 2Cl-14DMB on the H_2O_2-induced inactivation of LiP were investigated (Figure 5). When LiP (2.4 µM) was incubated with 100 µM H_2O_2 at pH 3.0 and 25°C, almost all LiP activity was lost within 20 min, as previously observed (148). The addition of 50 µM 2Cl-14DMB to the reaction mixture did not prevent this inactivation. On the contrary, LiP activity decreased more rapidly in the presence of

this concentration of 2Cl-14DMB. In contrast, when 50 μM 2Cl-14DMB and 1 mM AA were both present in the incubation mixture, maximal LiP activity was retained for at least 20 min, whereas AA alone did not protect LiP from H_2O_2 inactivation (175, data not shown). In the presence of 50 μM 2Cl-14DMB and 1 mM BA (the latter is not oxidized by LiP), the decrease in LiP activity was the same when only 50 μM 2Cl-14DMB was present. Finally, the addition of 500 μM 2Cl-14DMB protected LiP from inactivation, although not as well as when 1 mM AA and 50 μM 2Cl-14DMB both were present in the reaction mixture.

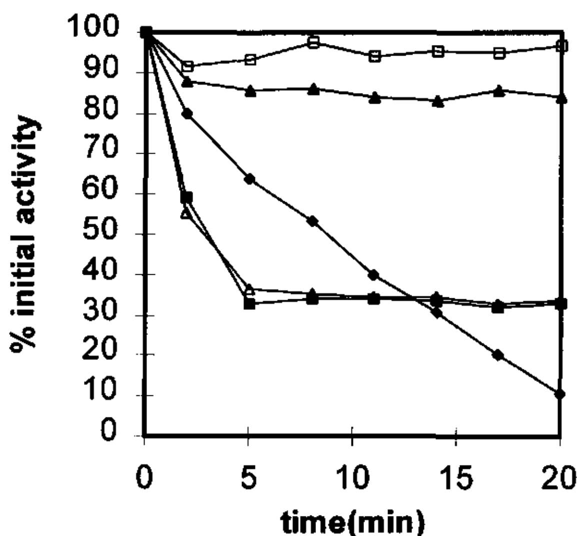

Figure 5. *Effects of AA and BA on LiP inactivation by H_2O_2 and/or 2Cl-14DMB cation radical. LiP (2.5 □M) was incubated with H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0, alone (♦), with 50 μM 2Cl-14DMB (■), with 500 μM 2Cl-14DMB (▲), with 50 μM 2Cl-14DMB and 1 mM AA (□), and with 50 μM 2Cl-14DMB and 1 mM BA (Δ). At the indicated intervals, aliquots (20 μl) were removed and assayed for LiP activity, as described in the text.*

Discussion

As reported for other methoxybenzenes (20, 95, 96, 98), our EPR results demonstrate that LiP oxidizes 2Cl-14DMB to form a relatively long-lived aryl cation radical (Figure 1). 2Cl-14DMB oxidation yields 2-chloro-1,4-benzoquinone and the two dimers shown in Figure 4. The dimeric product III is formed by the coupling of the aryl cation radical and a neutral species, as previously reported for 1,4-DMB (92). The dimeric product IV

is formed by the subsequent oxidation of III (92). The formation of these dimeric products suggests that the cation radical is sufficiently long-lived to diffuse away from the enzyme active site, enabling it to couple with a neutral molecule. In contrast, VA oxidation does not lead to the formation of dimeric products (92).

The optical spectrum of the putative cation radical exhibits maximum absorption peaks at 441 nm and 467.6 nm (Figure 2A). The absorption maxima for the presumed 2Cl-14DMB cation radical are similar to those exhibited by the tetramethoxybenzene (96) and the VA cation radicals (20). Furthermore, a variety of ring-delocalized cation radicals typically exhibit absorption bands above 400 nm (108). The 2Cl-14DMB cation radical is relatively long-lived; after 2 min, approximately 90% of the cation radical is still present (Figure 2A). The tetramethoxybenzene cation radical had a half-life of 9.9 min (96). The half-life of the free VA cation radical has been reported to be 0.5 ms (97) and 59 ms (20) in two different studies. Regardless, chemical studies suggest that the half-life of the VA cation radical is too short to enable it to diffuse away from its binding site on the enzyme (92). When AA is added to the reaction mixture, the 2Cl-14DMB cation radical signal disappears rapidly (Figures 2B and 3). This suggests that the 2Cl-14DMB cation radical can in turn oxidize AA.

This latter result support earlier work which showed that the molar ratio of AAld formed to 2Cl-14DMB consumed ranged from 3 to 13 (164). This high molar ratio is best explained through the recycling of the 2Cl-14DMB cation radical upon its reduction by AA.

Low 2Cl-14DMB concentrations do not protect LiP against inactivation (Figure 5). In fact, the initial rate of LiP inactivation is increased when 50 μM 2Cl-14DMB is present. This suggests that, if a terminal substrate such as AA is not present and if the concentration of 2Cl-14DMB is not optimal for formation of a

dimer, the cation radical might react with LiP compound II to form LiP compound III which would lead to enzyme inactivation (188). Alternatively, the cation radical could couple with the heme and/or protein leading to inactivation. Addition of sufficient terminal substrate (AA) would lead to the reduction of the 2Cl-14DMB cation radical. Alternatively, addition of excess 2Cl-14DMB would lead to dimer formation through the coupling of a cation radical with a neutral species or via the coupling of two cation radicals (92). In both of these cases, removal of the cation radical would tend to prevent enzyme inactivation. As shown in Figure 5, the presence of 500 μM 2Cl-14DMB or the addition of 1 mM AA to 50 μM 2Cl-14DMB prevents LiP inactivation. In contrast to 2Cl-14DMB, when VA is oxidized by LiP in the absence of a second reducing substrate, the VA cation radical loses a proton to form the benzylic radical (92, 144) and the latter reduces LiP compound II to the native species. This partially explains why LiP is not inactivated in the presence of high concentrations of VA. However, even with low VA concentrations, the presence of a second substrate, which is efficiently oxidized by the VA cation radical, leads to increased protection of the enzyme against H_2O_2 inactivation (148).

Finally, the presence of 50 μM 2Cl-14DMB and H_2O_2 leads to a rapid decrease in LiP activity to 35% of the initial activity, but the remaining activity does not decrease further (Figure 5). In contrast, in the absence of any reducing substrate, LiP activity declines continuously. A possible explanation for this phenomenon is that, in the presence of 2Cl-14DMB, the enzyme turns over, consuming H_2O_2. Thus, less H_2O_2 is available to convert compound II to compound III and compound III* (188).

In conclusion, this work demonstrates that the 2Cl-14DMB cation radical is the major intermediate formed from the oxidation of 2Cl-14DMB and that the major final products are 2-Cl-1,4-benzoquinone and several dimers produced by radical coupling. Furthermore, we show that AA oxidation is directly

mediated by the 2Cl-14DMB cation radical. In addition, we show that when, in turn, AA reduces the 2Cl-14DMB cation radical, the enzyme is protected from inactivation.

Acknowledgments

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2-Chloro-1,4-Dimethoxybenzene as a Mediator of Lignin Peroxidase Catalyzed Oxidations

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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.

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Introduction

Lignin peroxidase (LiP), an extracellular heme peroxidase produced by several white rot fungi, can catalyze the depolymerization of the aromatic polymer lignin (59) as well as the oxidation of recalcitrant aromatic substrates (56, 96, 177). The H_2O_2-oxidized states of LiP (140) are similar to those of horseradish peroxidase (29).

The fungal secondary metabolite veratryl alcohol (VA) stimulates the LiP oxidation of synthetic lignin (58), dyes (130, 133) and a variety of aromatic compounds (63, 175).

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 (45, 63, 103, 148, 175, 188).

In this report, we demonstrate that the fungal secondary metabolite (152, 156), 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.

Materials and methods

LIGNIN PEROXIDASE PREPARATION

Bjerkandera sp. strain BOS55 (ATCC 90940) was isolated and maintained as previously described (85). 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 (164). LiP was purified from the extracellular fluid of *Bjerkandera* sp. strain BOS55 cultures as previously described (69). A LiP isozyme mixture was used in these experiments.

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 (168). One unit of LiP activity was defined as the amount of enzyme required to oxidize 1 μmol of VA per minute.

DYE DECOLORIZATION

Decolorization of dyes was monitored at the visible absorbance maximum of each dye, these were Tartrazine (426 nm; ε = 24800 M^{-1} cm^{-1}), Methyl orange (500 nm; ε = 30000 M^{-1} cm^{-1}), Biebrich Scarlet (505 nm; ε = 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 to 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.

MEDIATION OF POLY R-478 DECOLORIZATION

The reaction mixture (0.5 ml) contained 0 to 0.08 g/l Poly R-478, 50 mM sodium tartrate pH 3.0, 0 to 2 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.

OXALATE OXIDATION BY 2CL-14DMB

The reaction mixture (0.5 ml) contained 0 to 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.

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 to 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.

HPLC ANALYSIS

50 µl of the incubation mixtures was analyzed for products by high pressure liquid chromatography as described before (163) with the column (200 mm x 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.

GC ANALYSIS

CO_2 concentration in the headspace was determined using a Packard model 427 gas chromatograph (Packard, Delft, The Netherlands) with a Hayesep Q column (Chrompack, Middelburg, The Netherlands). 50 µl headspace samples were analyzed.

CHEMICALS

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

Results

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 $\mu M.min^{-1}$ in the absence of a mediator to 7-30 $\mu M.min^{-1}$ 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 $mg.l^{-1}.min^{-1}$. 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.

Table 1. *Percentage decolorization of Tartrazine, Biebrich Scarlet, methyl orange and Poly R-478 by LiP after 30 minutes in the absence and presence of 2 mM VA, 14DMB or 2Cl-14DMB. Reactions were performed at pH 3.0 and monitored at the absorption maximum of each dye as mentioned in the text.*

	% 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

[a] *Poly R-478 concentration in reaction mixture was 24 mg/l.*

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

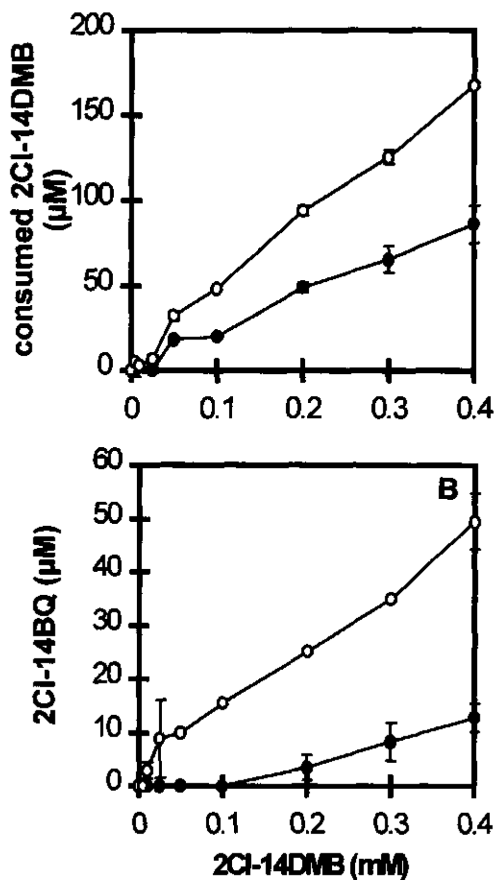


Figure 1. *Effect of the presence (●) and absence (O) of Poly R-478 (24 mg/l) on the oxidation of 2Cl-14DMB by LiP (0.05 U/ml).* **(A)** *consumption of 2Cl-14DMB* **(B)** *formation of 2Cl-14BQ, an oxidation product of 2Cl-14DMB.*

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 (Figure 2).

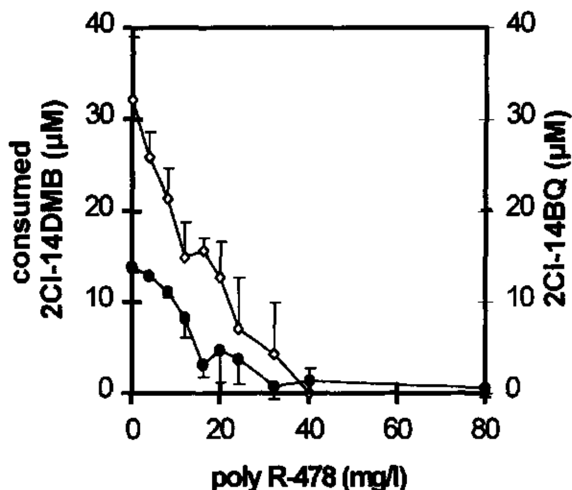


Figure 2. *Effect of varying Poly R-478 concentrations on the oxidation of 2Cl-14DMB (0.1 mM) by LiP (0.05 U/ml). The amount of consumed 2Cl-14DMB is shown (◊) together with the formation (●) of the oxidation product, 2Cl-14BQ.*

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

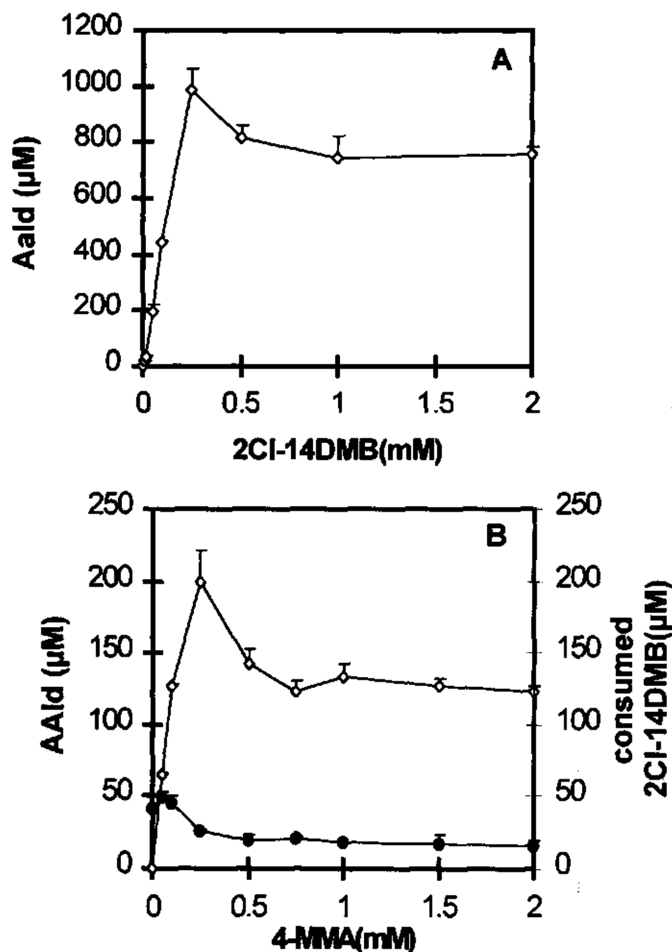
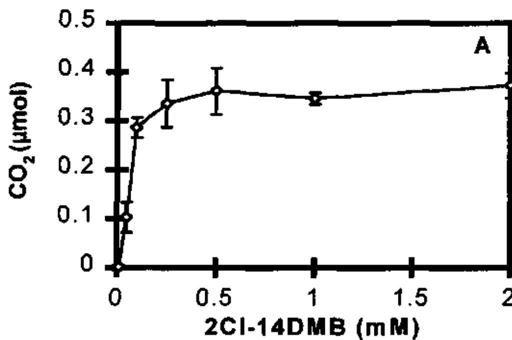


Figure 3. **(A)** *Effect of varying 2Cl-14DMB concentrations on the oxidation of 4-MMA(1 mM) by LiP (0.05 U/ml). The formation of p-anisaldehyde (AAld) from 4-MMA is shown.* **(B)** *Effect of varying 4-MMA concentrations on the oxidation of 2Cl-14DMB (1 mM) by LiP (0.05 U/ml). The consumption of 2Cl-14DMB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

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 (Figure 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 (Figure 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 (Figure 4B).



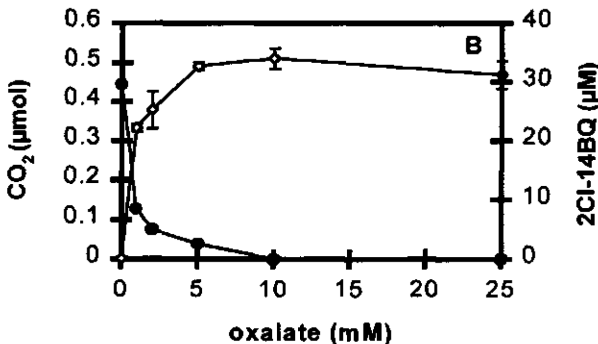


Figure 4. *(A) CO_2 production (◊) derived from 2 mM oxalate in 0.5 ml by LiP (0.05 U/ml) in the presence of varying 2Cl-14DMB concentrations.* ***(B)*** *Effect of varying oxalate concentrations on the CO_2 production (◊) and the formation of 2Cl-14BQ (●) from 2Cl-14DMB supplied at 1 mM.*

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* (76, 152, 156). 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 (130, 133).

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 (Figure 1A). The formation of the predominant oxidation product of 2Cl-14DMB, 2Cl-14BQ, was also inhibited by the presence of Poly R-478 (Figure 1B).

Earlier reports showed the inhibition of VA consumption during the decolorization of Poly R-478 (20, 64). VAld, the oxidation product of VA, did not accumulate until Poly R-478 had been fully decolorized (20, 64). The decay of the VA cation radical, formed in the reaction with LiP (19, 64, 98), 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 (19, 20). In a similar fashion, we propose that LiP oxidizes 2Cl-14DMB to form a 2Cl-14DMB cation radical, as was shown for other methoxybenzenes (95, 96). The inhibition of 2Cl-14DMB consumption in the presence of the dye suggests that the 2Cl-14DMB cation radical ($2Cl\text{-}14DMB^{+\cdot}$) 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 (Figure 3A) or VA (169), 4-MMA oxidation is possible. The VA cation radical signal is quenched in the presence of 4-MMA (169), 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 (169). 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 (Figure 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 $2Cl\text{-}14DMB^{+\cdot}$ back to 2Cl-14DMB, resulting in AAld formation and inhibition of 2Cl-14DMB consumption. Harvey et al. (63) proposed this mediation mechanism for 14DMB.

According to Goodwin et al. (45) 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 (1, 147). Furthermore, it was shown that EDTA and oxalate reduce the VA cation radical back to VA and the compounds are concomitantly decarboxylated (1, 9, 147). 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 (Figure 4A). Furthermore, elevated oxalate concentrations increased CO_2 production (Figure 4B) and inhibited the formation of 2Cl-14BQ from 2Cl-14DMB. Again, the results could be clarified by the formation of $2Cl\text{-}14DMB^{+\cdot}$, 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.

Acknowledgments

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6

Characterization of the Manganese Independent Peroxidase Isozymes from *Phellinus fastuosus* Responsible for the Oxidation of the Natural Metabolite Tetrachloro-4-Methoxyphenol

Pauline J. M. Teunissen, Bart Pieterse, Henk J. Swarts, and Jim A. Field

The dominant ligninolytic peroxidase produced by the white rot fungus *Phellinus fastuosus* was identified as a manganese independent peroxidase (MIP), which efficiently oxidizes phenols but not veratryl alcohol nor Mn(II). Two MIP isozymes were purified and characterized for their ability to oxidize the natural *P. fastuosus* metabolite tetrachloro-4-methoxyphenol and tetrachloro-1,4-dimethoxybenzene. Both MIP isozymes oxidized chlorophenolic compounds, including the natural tetrachloro-4-methoxyphenol and pentachlorophenol. The oxidation of tetrachloro-4-methoxyphenol resulted in the formation of two octachlorinated dimers. MIP could not oxidize tetrachloro-1,4-dimethoxybenzene. The kinetics of the oxidation of 2,6-dimethoxyphenol, guaiacol and ABTS by the MIP isozymes showed that manganese did not change the catalytic efficiency of the reaction. The molecular weight of 43 kDa, the isoelectric point of 3.3 and the N-terminal amino acid sequence indicate that the MIP isozymes are somewhat homologous to LiP and MnP from other white rot fungi. Since the MIP isozymes are the dominant extracellular oxidative enzymes present in cultures of *P. fastuosus*, they might be important in its ligninolytic system.

Submitted for publication

Introduction

Many ligninolytic Basidiomycetes have been described that produce significant amounts of chloroaromatic metabolites (34, 89). The most commonly produced metabolites are chlorinated anisyl metabolites (CAM) and chlorinated hydroquinone metabolites (CHM) (89).

The biosynthesis of these chloroaromatics is purposeful. CAM and CHM have strong antibiotic properties (68, 138). Besides their antibiotic properties, both CAM and CHM have roles in the ligninolytic system of white rot fungi. De Jong et al. (87) showed that CAM serve as substrates for the aryl alcohol oxidase involved in the production of extracellular hydrogen peroxide. Furthermore, the CHM, 2-chloro-1,4-dimethoxybenzene, was shown to be a powerful redox mediator of lignin peroxidase, stimulating the oxidation of various compounds (164, 165).

The white rot fungus *Phellinus fastuosus* is a significant producer of the CHMs, tetrachloro-4-methoxyphenol (drosophilin A (DA)) and tetrachloro-1,4-dimethoxybenzene (drosophilin A methyl ether (DAME)) (150, 163). Previously, we showed that the CHM production in *P. fastuosus* coincided with the expression of ligninolytic enzymes in *P. fastuosus* (163). This observation might indicate that tetrachloro-4-methoxyphenol and tetrachloro-1,4-dimethoxybenzene have physiological roles in lignin degradation.

The dominant ligninolytic activity in *P. fastuosus* corresponds to a manganese independent peroxidase (MIP), which does not show any reactivity with veratryl alcohol in the standard LiP assay (168) and which is not dependent on Mn(II) for its activity. The objectives of this study were to characterize the MIP from *P. fastuosus* and to determine whether MIP can utilize the natural chlorinated metabolites from *P. fastuosus* as substrates.

Material and Methods

ORGANISM AND CULTURE CONDITIONS

Phellinus fastuosus ATCC 26.125 was maintained on malt agar plates at 4° C. Inoculum was prepared on malt extract plates containing, per liter, 5 g of glucose, 3.5 g of malt extract (Oxoid Ltd., Basingstoke, Hampshire,United Kingdom), and 15 g of agar. MIP production by *P. fastuosus* was investigated in the low nitrogen medium and high nitrogen medium described below. The low nitrogen medium contained, per liter, 10 g of glucose, 0.2 g of diammonium tartrate, 20 mmol of 2,2-dimethylsuccinate, 2 mg of thiamine, and 100 ml of manganese free or manganese containing BIII mineral medium (168). The high nitrogen medium was prepared according to Kimura et al. (99), containing, per liter, 20 g of glucose, 5 g of mycological peptone, 2 g of yeast extract (Gibco BRL, Paisley, United Kingdom), 1 g of KH_2PO_4 , 0.5 g of $MgSO_4.7H_2O$ at pH 5.5.

For cultivation of *P. fastuosus,* 300 ml flasks each containing 10 ml of medium were inoculated with one cylindrical agar plug (diameter 5 mm), which was taken from the outer periphery of a malt extract agar plate covered with mycelium of *P. fastuosus* incubated at 25°C for 3 weeks. The flasks were left to grow statically in the dark at 25°C.

PURIFICATION OF MIP

MIP was purified from the extracellular fluid of *P. fastuosus* cultures grown in high nitrogen medium. The culture fluid was filtered through cheesecloth to remove fungal mycelium. Ammonium sulfate was added to the filtrate to 85% saturation at 0°C. The precipitated crude MIP fraction was recovered by centrifugation (10000 × g, 0°C, 10 min.). The pellet was resuspended in demiwater and subsequently brought on a DEAE Sephadex column (20 ml). The enzyme fraction was eluted with 1 M sodium acetate pH 6.0. This step was applied to remove peptides, which interfered with the enzyme. Subsequently, the enzyme was dialyzed against 10 mM sodium acetate pH 6.0 for 8 h. The dialyzed fraction was further purified on a Source 15Q anion-exchange column (Pharmacia, Woerden, The Netherlands) with a gradient of 150 mM to 240 mM sodium acetate pH 6.0 (30 min., flow rate 1.0 ml/min). Two different peroxidase isozymes, MiP-B and MiP-C, present in this fraction were further separated using a Mono P chromatofocussing column (Pharmacia, Woerden, The Netherlands). The applied gradient was a linear gradient from 100 mM malonic acid pH 4.0 to 100 mM malonic acid pH 2.5 (30 min., flow rate 0.5 ml/min). The collected fractions were considered pure MIP isozymes since both fractions gave one single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% polyacrylamide).

ENZYME ASSAY

The H_2O_2-dependent oxidation of 2,6-dimethoxyphenol (DMP) to coerulignone (ε_{468nm} = 49,600 M^{-1} cm^{-1}) was used to measure MnP and MIP activity in a combined assay (87). MnP activity: The reaction mixture contained 50 mM sodium malonate pH 4.5 (187), 1 mM DMP, 1 mM $MnSO_4$, and up to 650 µl culture broth in a total volume of 1 ml. The reaction was started by the addition of 0.4 mM H_2O_2 and corrected for background laccase activity present. MIP activity was measured in the same manner but Mn was excluded. Instead 1 mM EDTA was added to chelate the possible Mn contamination. MnP activity was expressed as combined MnP/MIP activity minus MIP activity.

LiP activity in the culture broth was measured using the standard veratryl alcohol oxidation assay (168).

ENZYME CHARACTERIZATION

Protein concentration was determined using the bicinchroninic acid protein assay (Pierce Chemical Company, Rockford, Illinois, USA) according to the description with bovine serum albumin as standard. To determine the molecular weight of MIP-B and MIP-C, SDS/PAGE was performed in 12.5% polyacrylamide gels using low-molecular-mass standards (Biorad). Protein bands after SDS/PAGE were stained with Coomassie Brilliant Blue. Isoelectric point was estimated by chromatofocussing by measuring the pH of the collected fraction containing MIP isozyme. Enzyme absorbance spectrum was determined with 119 mg/liter MIP-B and 130 mg/liter MIP-C in the presence of 10 mM NaAc (pH 6.0) by scanning the absorbance of the enzyme at the wavelength range from 350 to 700 nm using a Perkin-Elmer lambda 2 UV/VIS spectrophotometer. The oxidized enzyme was scanned after addition of 0.4 mM H_2O_2.

DETERMINATION OF SUBSTRATE OXIDATION BY HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The oxidation of 2,4-dichlorophenol, 2,6-dichlorophenol, tetrachloro-4-methoxyphenol, pentachlorophenol, 1,4-dimethoxybenzene, 2-chloro-1,4-dimethoxybenzene, tetrachloro-1,4-dimethoxybenzene, 2,6-dimethoxyphenol, and veratryl alcohol was examined. 2 µg MIP-B or MIP-C was incubated with 0.3 mM compound in 50 mM malonate buffer (pH 4.5). The reaction was initiated with 0.1 mM H_2O_2. H_2O_2 was added once every hour reaching the final concentration of 0.4 mM. After 4 hours incubation time the reaction was stopped with 1 equivalent volume of acetonitrile. All the incubations were carried out in triplicate. The compound oxidation was based on the elimination of compound as compared to parallel incubation of abiotic controls lacking either enzyme or H_2O_2 or both. 50 µl of the incubation mixtures was analyzed for products by HPLC as described before (163) with the

column (200 mm x 3 mm) filled with ChromSpher C18-PAH (5 μm particles) (Chrompack, Middelburg, The Netherlands).

ANALYSIS OF OXIDATION PRODUCTS FROM TETRACHLORO-4-METHOXYPHENOL

70 μg MIP-B was incubated with 30 mM DA in 25 mM malonate buffer (pH 5.0). The reaction was initiated with the addition of 5 mM H_2O_2. The reaction mixture (150 ml) was incubated at 25°C for 15 min. In parallel an abiotic control measurement lacking MIP was run. The reaction mixture was extracted three times with 50 ml freshly distilled ethylacetate. The combined organic layers were concentrated under reduced pressure at ambient temperature. The remaining residue was subjected to a Preparative Layer Chromatography (PLC) plate (Silica gel 60 F254, 2 mm) and was separated using a mixture of ethylacetate/petroleum ether (1:9) as eluent. Two unknown spots were scraped of the PLC plate, and to each one 20 ml of ethylacetate was added and stirred for one hour. Each mixture was filtered, washed with ethylacetate and concentrated under reduced pressure at ambient temperature. Each residue was subjected to an accurate mass measurement by high resolution electron ionization mass spectroscopy (HREIMS), using a Finnigan EI-MAT95 spectrophotometer.

KINETIC STUDIES

Kinetic constants of MIP activities for H_2O_2, Mn(II), ABTS, guaiacol and 2,6-dimethoxyphenol were calculated in the presence and absence of 1 mM $MnSO_4$. Oxidation of Mn(II) was measured by the formation of a Mn(III)-malonate complex at 270 nm (ε_{270} = 11,590 $M^{-1}cm^{-1}$) (190). The oxidation of ABTS to $ABTS^{+\cdot}$ was measured at 420 nm (ε_{420}= 36,000 $M^{-1}cm^{-1}$) (191). The oxidation of 2,6-dimethoxyphenol to coerulignone (ε_{468}= 49,600 $M^{-1}cm^{-1}$) was measured, the kinetic constants of H_2O_2 were determined in the presence of 0.5 mM 2,6-dimethoxyphenol. The oxidation of guaiacol was measured at 465 nm (ε_{465} = 12,100 $M^{-1}cm^{-1}$) (132).

N-TERMINAL SEQUENCE DETERMINATION

The N-terminal amino acid sequence determination was carried out at the Rijksuniversiteit Leiden, Faculteit der Geneeskunde, Vakgroep Medische Biochemie, Leiden (The Netherlands). Sequence analysis was performed on a Hewlett-Packard G1005A protein sequencer system, which was equipped with a 1 x 250 mm microbore PTH amino acid column (LC Packings, Amsterdam, The Netherlands).

CHEMICALS

Tetrachloro-1,4-dimethoxybenzene and tetrachloro-4-methoxyphenol were synthesized according to Knuutinen et al. (102). All other chemicals were commercially available and used without further purification.

Results

MIP PRODUCTION IN PHELLINUS FASTUOSUS AND ENZYME PURIFICATION

The dominant ligninolytic activity in culture fluids of *P. fastuosus* corresponded to a manganese independent peroxidase (MIP), since DMP was oxidized without any dependence on manganese. LiP activity according to the veratryl alcohol oxidation assay and MnP activity were not detected. MIP activity was present in low and high nitrogen medium both lacking manganese (Figure 1). Even in medium containing Mn (33 µM) MIP was the dominant activity and MnP activity was negligible. The highest MIP activity was obtained in cultures grown on the high nitrogen medium, the maximum MIP activity was observed after 35 days (Figure 1).

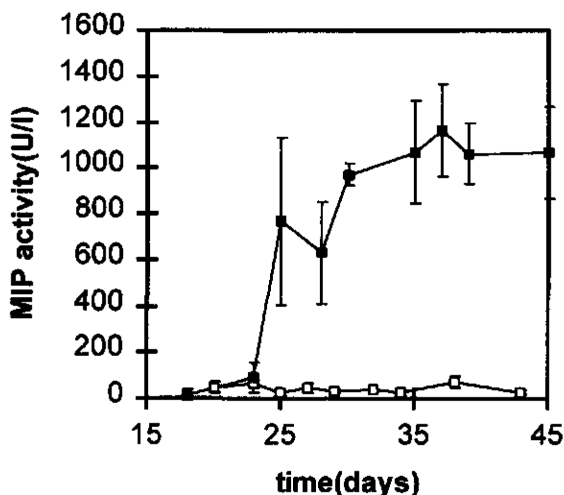


Figure 1. *Effect of manganese-free high nitrogen medium (■) and low nitrogen medium (□) on the production of MIP by Phellinus fastuosus.*

In order to characterize the *P. fastuosus* MIP, the enzyme was purified from the extracellular culture fluid. The purification results are described in Table 1. The culture fluid was highly contaminated with other peptides and proteins. Ammonium sulphate precipitation reduces the interfering peptides and proteins considerably. An anion exchange column was used to separate the different MIP isozymes. At least three

isozymes, showing MIP activity could be detected (Figure 2). However, the activity of MIP-A was too low for further characterization. Therefore, only MIP-B and MIP-C were purified further using a Mono-P chromatofocussing column. Finally, about 1 mg of each pure MIP isozyme was obtained which was sufficient for further characterization.

Table 1. *Purification of peroxidase isozymes from P. fastuosus. The enzymatic activities in the culture liquid and the different purification steps were estimated using the oxidation of 2,6-dimethoxyphenol as a substrate. Protein content was estimated using the BCA assay.*

Step	Protein	Activity		Purification	
		total	*specific*	*yield*	*factor*
	mg	U	U/mg	%	-fold
Culture fluid	951	508	0.53	100	1
Ammonium sulphate precipitation	20	514	25.7	101	48
ultrafiltration	10	219	21.9	43	41
Source 15Q MIP-B	3.6	88	24.4	17	46
Source 15Q MIP-C	3.4	59	17.4	12	33
Mono-P MIP-B	0.8	24	30	5	57
Mono-P MIP-C	0.97	16	17	3	32

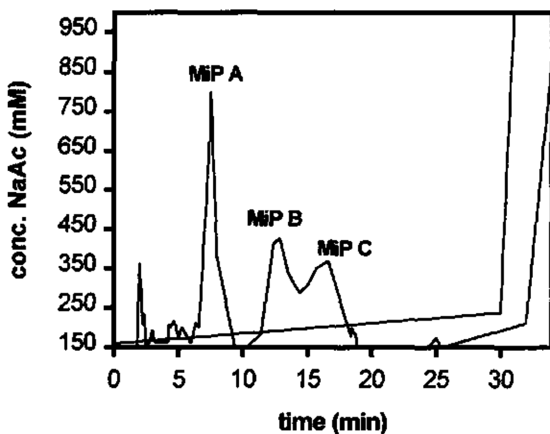
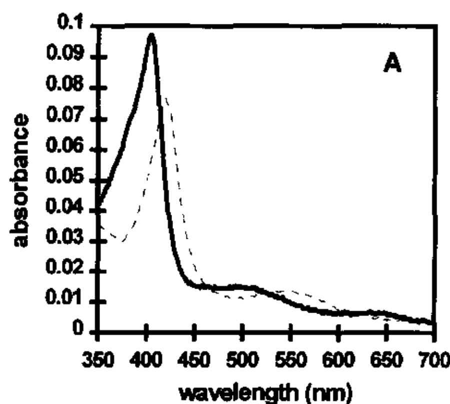


Figure 2. *The elution profile of MIP isozymes (A-C) produced by Phellinus fastuosus from a Source 15Q FPLC column.*

CHARACTERIZATION OF MIP-B AND MIP-C

Both isozymes have the same molecular weight of 43 kDa and isoelectric point of 3.3. The spectrum of both isozymes showed a typical peak for a native heme enzyme of 407 nm with a molar extinction coefficient of 34.6 mM^{-1} cm^{-1} for MIP-B and 30.6 mM^{-1} cm^{-1} for MIP-C. The addition of excess H_2O_2 resulted in a shift to 420 nm (Figure 3).



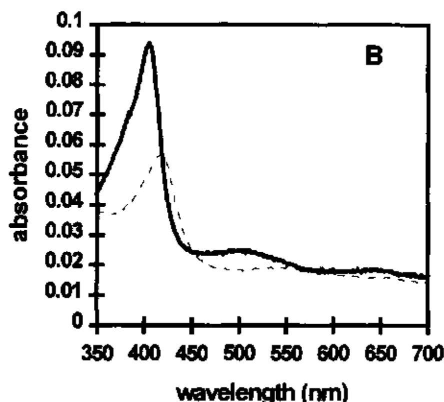


Figure 3. *Absorption spectra of the native and the hydrogen peroxide-oxidized Phellinus fastuosus MIP-B (A) and MIP-C (B) isozymes. Solid line, native enzyme; dotted line, hydrogen peroxide-oxidized enzyme.*

The N-terminal amino acid sequence of MIP-B and MIP-C was compared to other known N-terminal sequences of peroxidases (Table 2). The N-terminal sequences of MIP-B and MIP-C were identical, whereas the N-terminal sequences of *Trametes versicolor* LiP10 and the MnP/LiP isozyme from *Bjerkandera* sp.

BOS55 had the closest similarity to MIP with 5 and 6 amino acids difference, respectively.

Table 2. *Comparison of the N-terminal amino acid sequence of MIP-B and MIP-C with those reported for other peroxidases (LiP, MnP) produced by other white-rot fungi.*

	N-terminal sequence
Phellinus fastuosus MIP-B[a]	V-T-C-P-D-G-V-N-T-A-S-N-E-A-C-C-S-W-F-A-V-R-D-D-L
Phellinus fastuosus MIP-C[a]	V-T-C-P-D-G-V-N-T-A-S-N-E-A-C-C-S-W-F-A-V-R-D-D-L
Trametes versicolor LiP10[b]	V-T-C-P-D-G-V-N-T-A- -N- -A-C-C- -F-A-V-R- -D-L
Bjerkandera sp. BOS55 MnP/LiP[c]	V- -C-P-D-G-V-N-T-A- -N- -A-C-C- -F-A-V-R-D-D-
Phanerochaete chrysosporium H1[d]	V- -C-P-? -G-V- -T-A-S-N- -A-C-C-
Phanerochaete chrysosporium H2[d]	V- -C-P-D-G-V- -T-A-S-N- -A-C-C-
Trametes versicolor MnP5[b]	V- -C-P-D-G-V-N-T-A-S-N- -A-C-C- F- -V- D-
Bjerkandera sp. BOS55 LiP-2[e]	V- -C-P-D-G- -T-A- -N- -A-C-C- -F- -V-R-D-D-
Bjerkandera sp.BOS55 LiP-5[e]	V- -C-P-D-G- -T-A- -N- -A-C-C- -F- -V-R-D-D-
Pleurotus eryngii MnP2[f]	-T- -D-G- -T-A-? - -A-C-C- -F- ?- ?-X
Pleurotus eryngii MnP1[f]	-T- -D-G- -T-A- ?-N- -A-C-C- -F- ?- ?-
Pleurotus pulmonarius MnP[g]	-T-C- -D-G- -T-A- ?-N- -A-C-C- -F- ?- ?-

[a] *This work*

[b] *Adapted from Johansson et al. (81)*

[c] *Adapted from Mester and Field (117)*

[d] *Adapted from Johnson et al. (82)*

[e] *Adapted from ten Have et al. (69)*

[f] *Adapted from Martínez et al. (114)*

[g] *Adapted from Camarero et al. (18)*

CHLOROPHENOLS AS SUBSTRATES FOR MIP-B AND MIP-C

The naturally produced tetrachloro-4-methoxyphenol and related chlorophenols were compared as substrates for the MIP isozymes. Both MIP-B and MIP-C completely oxidized the dichlorophenols. Tetrachloro-4-methoxyphenol and pentachlorophenol were also oxidized by both isozymes, although not completely (Table 3).

The tetrachloro-4-methoxyphenol oxidation by MIP was further investigated by identifying the oxidation products. Two spots were detected on a PLC plate which were subjected to an accurate mass measurement. Both compounds gave an accurate mass of 517.7774. The electron ionization spectra of these compounds showed $[M]^+$, $[M+2]^+$, $[M+4]^+$, $[M+6]^+$, $[M+8]^+$ and $[M+10]^+$ at m/z 518, 520, 522, 524, 526, and 528 in a ratio of 33 : 86 : 100 : 65 : 22 : 5 and 30 : 93 : 100 : 67 : 23 : 5, respectively,

suggesting octachlorinated compounds with the molecular formula $C_{14}H_6Cl_8O_4$. Three different structures are possible: coupling of the two phenolic groups, coupling of a phenolic group with the para methoxygroup, and coupling of the two para methoxygroups. Coupling of the two phenolic groups would form an unstable peroxide unlikely to survive the purification protocol and therefore the other two isomers, shown in Figure 4, were more likely.

Table 3. *Oxidation of various phenolic and nonphenolic compounds by the purified MIP-B and MIP-C. The reaction mixture contained 2 mg/liter enzyme, 0.3 mM compound, 0.4 mM H_2O_2 in 50 mM malonate buffer (pH 4.5).*

Compound	MIP-B or MIP-C	% of oxidation[a]
2,4-dichlorophenol	MIP-B	99 ± 0.1
	MIP-C	99 ± 0.1
2,6-dichlorophenol	MIP-B	100
	MIP-C	100
pentachlorophenol	MIP-B	50 ± 2.2
	MIP-C	23.5 ± 6.1
tetrachloro-4-methoxyphenol	MIP-B	80 ± 0.6
	MIP-C	78 ± 0.8
tetrachloro-1,4-dimethoxybenzene	MIP-B	0
	MIP-C	0
2,6-dimethoxyphenol	MIP-B	100
	MIP-C	100

[a] *Calculated as ($[substrate]_{initial}$ - $[substrate]_{final}$/$[substrate]_{initial}$) x 100*

NONPHENOLIC COMPOUNDS AS SUBSTRATES FOR MIP-B AND MIP-C

The nonphenolic compounds 1,4-dimethoxybenzene, 2-chloro-1,4-dimethoxybenzene, the *P. fastuosus* metabolite tetrachloro-1,4-dimethoxybenzene and veratryl alcohol were examined as substrates for the MIP isozymes. None of these nonphenolic compounds was oxidized by MIP-B or MIP-C.

A

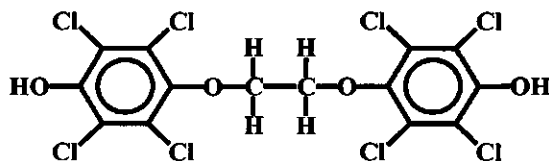
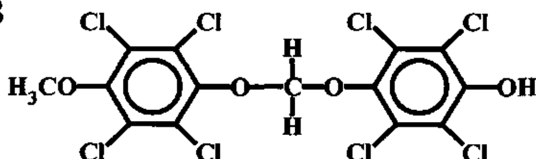


Figure 4. *Proposed structures of the two oxidation products of tetrachloro-4-methoxyphenol. (A) Coupling of two methoxygroups; (B) Coupling of a methoxygroup with a phenolic group.*

B



KINETIC PROPERTIES OF MIP-B AND MIP-C

The kinetics of the oxidizing substrate H_2O_2 and of various direct reducing substrates of MIP-B and MIP-C were compared in terms of K_m and turnover number (Table 4). Furthermore, the influence of Mn(II) on the kinetics was investigated. Based on assays monitoring for the formation of Mn(II)-malonate complex it was confirmed that Mn(II) was not directly oxidized by MIP-B nor MIP-C. At a physiological pH of 4.5, ABTS was the best reducing substrate for MIP-B as shown by the turnover number (TN). The presence of Mn(II) apparently decreased the TN and K_m of ABTS (Table 4A); whereas, the TN and K_m of guaiacol was apparently increased in the presence of Mn(II) (Table 4). However, when the kinetics of these substrates were measured in a N_2 atmosphere, the presence of Mn(II) did not affect the TN and K_m (results not shown). In terms of catalytic efficiency (TN/K_m), Mn(II) did not have a significant effect on either MIP-B and MIP-C for the various substrates tested (Table 4).

Table 4A. *Kinetic constants of MIP-B from Phellinus fastuosus.*

Substrate	+/- Mn(II)	K_m (μM)	V_{max} (U mg^{-1})	Turnover number (s^{-1})	Catalytic efficiency (TN/K_m) ($M^{-1}s^{-1}$)
DMP[a]	-	454	107.5[b]	77	1.7×10^5
	+	751	110.8	79	1.1×10^5
ABTS	-	2258	1277.5	916	4.1×10^5
	+	920	453.3	325	3.5×10^5
H_2O_2	-	47	37[b]	27	5.7×10^5
	+	45	43.6	31	6.9×10^5
guaiacol	-	265	79.6	57	2.2×10^5
	+	2353	381.5	273	1.2×10^5

Table 4B. *Kinetic constants of MIP-C from Phellinus fastuosus.*

Substrate	+/- Mn(II)	K_m (μM)	V_{max} (U mg^{-1})	Turnover number (s^{-1})	Catalytic efficiency (TN/K_m) ($M^{-1}s^{-1}$)
DMP	-	605	77	55	9.0×10^4
	+	2614	239.6	172	6.6×10^4
ABTS	-	nd	nd	-	-
	+	489	247.3	177	3.6×10^5
H_2O_2	-	49	37.1	27	5.5×10^5
	+	40	32.3	23	5.8×10^5
guaiacol	-	331	53.9	39	1.2×10^5
	+	1185	144.2	103	8.7×10^4

[a] *2,6-dimethoxyphenol is referred to as DMP*

[b] *An extinction coefficient of 24800 $M^{-1}cm^{-1}$ was used since each coeruglignone (ε = 49600 $M^{-1}cm^{-1}$) refers to two DMP molecules oxidized.*

Discussion

The white rot fungus, *Phellinus fastuosus* is a significant producer of the chloroaromatics, tetrachloro-4-methoxyphenol and tetrachloro-1,4-dimethoxybenzene (150, 163). However, it is not clear whether these chloroaromatics could be oxidized by ligninolytic enzymes present in *P. fastuosus*. In this report, the main ligninolytic enzyme was purified and tested for its ability to oxidize the chlorinated metabolites.

The dominant ligninolytic enzyme produced by *P. fastuosus* was identified as a manganese independent peroxidase (MIP). Our results showed that MIP efficiently oxidizes phenols but cannot

oxidize veratryl alcohol nor Mn(II), which are the essential cofactors for lignin peroxidase (LiP) and manganese peroxidase (MnP) respectively (41, 103). Therefore, MIP has unique catalytic properties which cannot be compared to the well characterized ligninolytic peroxidases LiP and MnP.

Previously only three peroxidases have been isolated and characterized with catalytic properties distinct from LiP or MnP. These enzymes, referred to as manganese independent peroxidases, were purified from the white rot fungi *Bjerkandera* sp. BOS55, *Junghuhnia separabilima* and *Phellinus igniarius* (38, 83, 178). Aside from these species, manganese independent peroxidase activities were also detected in the culture fluids of other white rot fungi as well (122, 123, 183). Remarkably, only *P. igniarius* and *P. fastuosus* produce MIP as the dominant ligninolytic enzyme (38, this study); while the other fungi produce a whole battery of ligninolytic enzymes (83, 178).

To characterize the MIP from *P. fastuosus,* the enzyme was purified from the extracellular culture fluid of *P. fastuosus* cultures grown for 35 days in high nitrogen medium (Figure 1). Two MIP isozymes, MIP-B and MIP-C, could be purified to homogeneity, both with the same molecular weight (43 kDa) and isoelectric point (3.3). These physical characteristics are comparable to other ligninolytic peroxidases that have been described, including LiP and MnP isozymes (110, 114, 122, 171, 178). Also, the N-terminal amino acid sequences of *P. fastuosus* MIP-B and MIP-C have some homology with LiP and MnP isozymes from several white rot fungi (69, 81, 82). The closest homology was with *Trametes versicolor* LiP10 and the recently discovered LiP/MnP hybrid enzyme from *Bjerkandera* (81, 117). These similarities indicate that all white rot fungal ligninolytic peroxidases are highly conserved. However, the catalytic properties of MIP-B and MIP-C distinguish these enzymes from LiP and MnP.

The natural *P. fastuosus* metabolites tetrachloro-4-methoxyphenol and tetrachloro-1,4-dimethoxybenzene were examined as substrates for MIP-B and MIP-C and compared to related chlorophenols and chlorinated 1,4-dimethoxybenzenes. Both MIP isozymes oxidized chlorophenolic compounds, including the natural tetrachloro-4-methoxyphenol and even the environmental pollutant pentachlorophenol (Table 3). Tetrachloro-4-methoxyphenol was oxidized up to 80% by both MIP isozymes. The oxidation of tetrachloro-4-methoxyphenol resulted in the formation of two octachlorinated dimers with the molecular formula $C_{14}H_6Cl_8O_4$. Most coupling reactions described for chlorophenols result in direct coupling of aromatic rings through oxidative C-C and C-O bonds with reactions most likely occurring at the ortho and para positions of the phenolic oxygen, often resulting in dechlorination of the chlorophenol as well (27, 67). Since there was no net loss of chlorine, MIP did not dechlorinate the compound. Previously, peroxidases were shown to readily oxidatively dechlorinate chlorogroups in the para-position to phenol, as was shown for several chlorinated phenols (57, 90, 176). However, tetrachloro-4-methoxyphenol lacks a chlorogroup in the para-position, instead a methoxygroup is present.

Both MIP isozymes were not able to oxidize the natural *P. fastuosus* metabolite tetrachloro-1,4-dimethoxybenzene and related chlorinated 1,4-dimethoxybenzenes. Previously, Teunissen et al. (164) showed that LiP also did not oxidize tetrachloro-1,4-dimethoxybenzene, probably the ionization potential of this compound is too high.

Clearly, MIP-B and MIP-C oxidize a variety of chlorophenolic compounds. Also MIP isoenzymes from *P. fastuosus* can oxidize guaiacol, ABTS, and 2,6-DMP in the presence of H_2O_2. No oxidation of these compounds was observed in the absence of H_2O_2. This is a distinguishing characteristic compared to the MIPs isolated from *Junghuhnia separabilima* and *Bjerkandera* sp.

strain BOS55 (83, 178), which exhibit some oxidase activity for 2,6-DMP (83) and guaiacol (178).

Although the presence of Mn(II) appeared to influence the turnover number (TN) and K_m for ABTS, guaiacol and 2,6-dimethoxyphenol, we propose this is just an artifact. First Mn(II) itself is not a substrate of MIP. Secondly, catalytic efficiency (TN/K_m) was not affected by Mn(II). Ligninolytic enzymes can oxidize substrates to radicals that can react with O_2 to produce superoxide. Under aerobic conditions, the phenoxy radicals formed by MIP oxidation from guaiacol and 2,6-dimethoxyphenol can react with O_2 to form superoxide, as was shown for hydroquinones by Guillén et al (53). The superoxide can chemically oxidize Mn(II) to Mn(III) (4), which can subsequently oxidize the phenols (41, 154). Consequently, the apparent oxidation rate by the enzyme is accelerated, affecting the V_{max} and K_m proportionally. In an N_2 atmosphere, superoxide formation will be precluded and neutralize any effect of Mn(II) on TN. Indeed, in an N_2 atmosphere, no effect of Mn(II) on guaiacol oxidation was found (results not shown), confirming our hypothesis.

In case of ABTS, Collins et al. (23) already demonstrated that the ABTS cation radical can efficiently be reduced by superoxide formed indirectly from the oxidation of malonate. Furthermore, Mn(II) accelerated the ABTS cation radical reduction, which explains the decrease in V_{max} and K_m as observed in our results.

In conclusion, this work demonstrates the characterization of the dominant ligninolytic enzyme from *P. fastuosus* as a MIP, which can oxidize the natural *P. fastuosus* metabolite tetrachloro-4-methoxyphenol. Physical properties of MIP were comparable to those described for other peroxidases. The catalytic properties of MIP distinguish the enzyme from other ligninolytic peroxidases. Since MIP is the dominant extracellular oxidative enzyme produced by *P. fastuosus*, the

enzyme is probably important in the ligninolytic system of the fungus.

Acknowledgments

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7

Concluding Remarks

Evidence is accumulating that organohalogens are produced and discharged into our biosphere by plants, marine organisms, insects, bacteria, fungi and other natural processes (47). The number of natural organohalogen compounds identified has multiplied about 250 times in the past 40 years - from a dozen in 1954 to more than 2900 as of December 1997 (48). Not only are naturally occurring organohalogens ubiquitous in our environment, the concentration of some of them exceed anthropogenic levels.

Among the organohalogen producers, Basidiomycetes are a major source of organohalogens in terrestrial environments (89). Basidiomycetes are not only implicated in organohalogen biosynthesis, but are capable of degrading anthropogenic organohalogens as well. In many cases, lignin-degrading enzymes are responsible for the first steps in xenobiotic organohalogen degradation.

Chlorinated anisyl metabolites (CAM) and chlorinated hydroquinone metabolites (CHM) are the most common organohalogen metabolites described in Basidiomycetes (34, 89). Organohalogen production by Basidiomycetes is not just a biological accident. Several organohalogens have a role as antibiotics in the defense mechanism of fungi to protect them from other organisms, while other organohalogens have a more specific function (**Chapter 1**).

In our laboratory extensive research has been focused on CAM. *Bjerkandera* sp. strain BOS55 was the first white rot fungus in which the CAM, 3-chloro-anisaldehyde was detected (84). Further research showed the ubiquity of CAM among white rot fungi in the natural environment (88). CAM have an important physiological function in lignin degradation, serving as substrates for the aryl alcohol oxidase involved in extracellular H_2O_2 production (87). Furthermore, Mester et al. (116) elucidated a part of the biosynthetic pathway of CAM in *Bjerkandera* sp. strain BOS55, showing stimulation of CAM production by phenylalanine, benzoate, 4-hydroxybenzoate and cinnamate. Moreover it was demonstrated that labelled benzoate, 4-hydroxybenzoate and 3-chloro-4-hydroxybenzoate were incorporated in CAM indicating that they were precursors for the biosynthesis.

However, not much is known about CHM biosynthesis and function in white rot fungi. Therefore, the objectives of this thesis were to determine the extent of biosynthesis of CHM among white rot fungi and to elucidate their possible role in lignin degradation.

Production of chlorinated hydroquinone metabolites by basidiomycetes

To monitor the extent of biosynthesis of CHM among white rot fungi, 92 ligninolytic basidiomycetes were screened for their ability to produce tetrachloro-4-methoxyphenol (drosophilin A; DA) and tetrachloro-1,4-dimethoxybenzene (drosophilin A methyl ether; DAME) (**Chapter 2**). Five fungal strains produced DA and DAME in detectable amounts. The screening revealed two new genera capable of DA and DAME production, *Bjerkandera* and *Peniophora*. *Peniophora* was not known as a producer of CHMs at all; whereas *Bjerkandera* spp. produce different CHM such as 2-chloro-1,4-dimethoxybenzene and 2,6-dichloro-1,4-dimethoxybenzene (152, 156). To date, 9

CHM metabolites have been found in 11 genera of Basidiomycetes (**Chapter 1**; Table 1).

Table 1. *Ligninolytic Basidiomycete genera producing chlorinated hydroquinone metabolites (CHM).*

Genera [a]	CHM
Agaricus	*VIII* [b]
Bjerkandera	*VIII, IX, X, XII*
Coprinus	*IX*
Hypholoma	*VIII, IX, XV, 3,4,6-trichloro-2,5-dimethoxyphenol*
Lepista	*X, XII, XV*
Mycena	*IX*
Peniophora	*VIII, IX*
Phellinus	*VIII, IX, XIII, XIV*
Psathyrella	*IX*
Russula	*XI*
Schizophyllum	*IX*

[a] *Adapted from (34, 89); ref.* ***Chapter 1***

[b] *Roman numerals indicate metabolites mentioned in Figure 2,* ***Chapter 1.***

The genera belonging to the white rot fungi are *Bjerkandera, Hypholoma, Peniophora* and *Phellinus*. From these CHM producing genera, we selected *Bjerkandera* sp. strain BOS55 and *Phellinus fastuosus* ATCC26.125 to elucidate the role of CHM (**Chapter 2, Chapter 3, Chapter 4, Chapter 6**).

Selection of CHM producing strains

P. fastuosus ATCC26.125 was selected for further research, since this fungus had the highest and most reliable production of DA and DAME in the screening, respectively 15-60 μM and 4-40 μM in liquid peptone medium (**Chapter 2**). Furthermore, ligninolytic activity in *P. fastuosus* coincided with CHM production (**Chapter 2, Chapter 6**).

In addition, the screening showed that several *Bjerkandera* strains produce the CHMs DA and DAME, coinciding with the expression of ligninolytic activity (**Chapter 2**). *Bjerkandera* sp.

strain BOS55 synthesizes the CHM 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB) and several CAMs (86, 156). Furthermore, *Bjerkandera* sp. strain BOS55 is extensively studied in our laboratory with respect to the ligninolytic system and its secondary metabolites (116-118). Therefore, *Bjerkandera* sp. strain BOS55 was selected for detailed studies.

CHM as substrates for ligninolytic enzymes

Both CHM producing fungi, *Bjerkandera* sp. strain BOS55 and *P. fastuosus*, produce their own unique set of ligninolytic enzymes (83, 117, **Chapter 6**). The dominant ligninolytic enzyme present in *P. fastuosus* is manganese independent peroxidase (MIP). MIP is a unique extracellular peroxidase among white rot fungi, since it does not depend on veratryl alcohol (VA) or manganese for efficient catalysis. Therefore, MIP cannot be compared to LiP or MnP in terms of catalytic properties. MIP cannot oxidize nonphenolic aromatic compounds, however, MIP is capable of oxidizing a variety of (chlorinated) phenolic compounds (**Chapter 6**). Till now, only three MIP enzymes have been isolated and characterized from other white rot fungi (38, 83, 178). Since MIP is the dominant ligninolytic enzyme present in *P. fastuosus*, MIP might play an important role in the fungus's lignin degrading system.

Bjerkandera sp. strain BOS55 produces a whole machinery of ligninolytic enzymes, including LiP, MnP, MIP and a hybrid MnP/LiP (69, 83, 117). LiP is considered a unique enzyme, since it can oxidize nonphenolic aromatics with a higher ionization potential than other peroxidases (96). This capacity enables LiP to oxidize nonphenolic aromatic compounds such as the lignin model compound 1-(4-ethoxy-3-methoxyphenyl)-2-(2-2-methoxyphenoxy)-propane-1,3-diol, VA, and 1,4-dimethoxybenzene (55, 95, 153).

The CHMs 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB), 2,6-dichloro-1,4-dimethoxybenzene (26DCl-14DMB), DAME and DA were examined as substrates for the purified MIP from *P. fastuosus* and purified LiP from *Bjerkandera* sp. strain BOS55 (**Chapter 3, Chapter 6**). The results not only emphasized the different catalytic properties of the two enzymes, but also showed that some natural CHMs are indeed substrates for ligninolytic enzymes of the CHM-producing fungi (Table 2).

Table 2. *Types of CHM which are substrates for the ligninolytic enzymes MIP from* P. fastuosus *and LiP from* Bjerkandera *sp. BOS55.*

CHM	MIP	LiP
2-chloro-1,4-dimethoxybenzene	-	+
2,6-dichloro-1,4-dimethoxybenzene	nt[a]	-
tetrachloro-1,4-dimethoxybenzene (DAME)	-	-
tetrachloro-4-methoxyphenol (DA)	+	+

[a]*nt = not tested*

MIP only oxidized the phenolic compound DA (**Chapter 6**); whereas, LiP catalyzed the oxidation of DA as well as the oxidation of the nonphenolic compound 2Cl-14DMB (**Chapter 3**). 26DCl-14DMB and DAME were not significantly oxidized by LiP or MIP.

Since 26DCl-14DMB and DAME are not demonstrable substrates for the ligninolytic enzymes, we conclude these fungal metabolites do not have a physiological role in lignin degradation. However, antimicrobial as well as antifungal activity of DA has been reported (94, 138). We were able to demonstrate that DAME is produced in response to competition from other Basidiomycetes. Antagonizing a culture of *P. fastuosus* by adding filter-sterilized culture fluid of the competing fungus *Phlebia radiata,* resulted in a tenfold increase in DAME production by *P. fastuosus*. These observations suggest a physiological role of DAME as an antibiotic (**Chapter 2**).

2Cl-14DMB and DA are substrates for the ligninolytic enzymes, indicating a more active function in the wood decomposition process. In the research described in this thesis, we were able to demonstrate that 2Cl-14DMB has an important physiological role in lignin degradation as a substitute for the fungal metabolite veratryl alcohol in the catalytic cycle of LiP.

Role of 2Cl-14DMB in the catalytic cycle of LiP

The fungal secondary metabolite VA is considered a key component of LiP catalysis. Throughout the years, a variety of proposals have been made to account for the stimulation of LiP oxidations by VA. These include its role as a redox mediator, aiding in the turnover of the enzyme (cofactor role), and protecting the enzyme from H_2O_2 inactivation (63, 103, 175).

In this thesis 2Cl-14DMB was found to replace VA as a cofactor and redox mediator in the catalytic cycle of LiP (**Chapter 3, Chapter 4, Chapter 5**). However, 2Cl-14DMB is less effective in protecting the enzyme from H_2O_2 inactivation, compared to VA (**Chapter 3, Chapter 5**).

2Cl-14DMB is oxidized by LiP to the products 2-chloro-1,4-benzoquinone and two different dimers, although the percentage of oxidation was relatively low compared to VA and 14DMB (**Chapter 3, Chapter 5**). Furthermore, EPR results demonstrated that LiP oxidizes 2Cl-14DMB to an aryl alcohol cation radical (**Chapter 5**). The formation of the dimeric products suggests that the 2Cl-14DMB cation radical is sufficiently long-lived to diffuse away from the enzyme active site, enabling it to couple to a neutral molecule (**Chapter 5**, 92). In addition, a long-lived cation radical is suggested to diffuse away from the active site and act as a diffusible redox mediator.

The 2Cl-14DMB cation radical is indeed relatively long-lived; after 2 min, approximately 90% of the 2Cl-14DMB cation radical

is still present (Chapter 5). The half-life of the free VA cation radical has been reported to be only 59 ms (20) and 0.5 ms (97) in two different studies. The half-life of the free VA cation radical is too short to diffuse away from the enzyme, in contrast to 2Cl-14DMB and 14DMB (92, 97, **Chapter 5**).

Although the VA cation radical is not diffusible, VA acts as a localized redox mediator in the LiP-catalyzed oxidation of substrates with lower redox potential than the VA cation radical (1, 45, 104, 169). In **Chapter 4**, we demonstrated that 2Cl-14DMB can substitute VA as a redox mediator in the LiP-catalyzed oxidation of azo dyes and the polymeric dye Poly R-478 as well as 4-methoxymandelic acid and oxalate. With the exception of azo dyes, these substrates were not oxidized in the absence of 2Cl-14DMB or VA (**Chapter 4**, 130, 169). The substrates clearly inhibited the consumption of 2Cl-14DMB and the formation of the product 2-chloro-1,4-benzoquinone. The inhibition of 2Cl-14DMB consumption by these substrates indicate that the terminal substrates reduce the 2Cl-14DMB cation radical to regenerate 2Cl-14DMB with concomitant oxidation of the substrates (Figure 1).

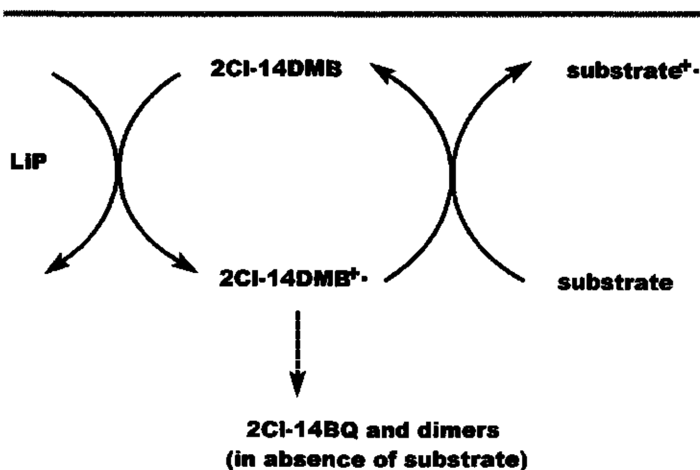

Figure 1. *The role of 2Cl-14DMB as a cation radical redox mediator. The formation of 2Cl-14BQ and other products is inhibited as long as the alternative substrate is not completely oxidized.*

To examine whether 2Cl-14DMB could replace VA as a cofactor as well, the oxidation of the methoxylated nonphenolic compound anisyl alcohol (AA) by LiP was investigated (**Chapter 3**). Koduri and Tien (103) showed that AA can only be oxidized by LiP compound I. VA is required as an essential cofactor for oxidation by LiP compound II in order to close the catalytic cycle of LiP.

In **Chapter 3**, we showed that 2Cl-14DMB enormously stimulated the LiP-catalyzed oxidation of AA, compared to VA and 14DMB. 2Cl-14DMB itself is poorly oxidized by LiP; whereas, VA and 14DMB are good LiP substrates (**Chapter 3**). Our results indicate an inverse relation between the ability to be oxidized by LiP and the ability to act as a cofactor for AA oxidation. A possible theory is that good LiP substrates, such as VA and 14DMB, compete with AA for oxidation by LiP compound I; whereas, a poor LiP substrate, such as 2Cl-14DMB, cannot compete with AA. In this case, 2Cl-14DMB would primarily be oxidized by LiP compound II closing the catalytic cycle, while AA is oxidized by LiP compound I. Koduri and Tien (103) showed that increasing VA concentrations indeed compete with AA for oxidation with compound I; whereas, increasing 2Cl-14DMB concentrations did not inhibit AA oxidation at all (**Chapter 3**). These data support the suggested theory. Furthermore, increasing AA concentrations did not inhibit 2Cl-14DMB oxidation, suggesting 2Cl-14DMB is not a direct redox mediator in AA oxidation but indeed aiding in the turnover of LiP (**Chapter 3**).

However, this theory does not explain why the molar ratio of anisaldehyde formed to 2Cl-14DMB consumed ranged from 3 to 13 (**Chapter 3**). This high molar ratio indicates that the 2Cl-14DMB cation radical, formed during oxidation by LiP (**Chapter 5**) must be recycled back to 2Cl-14DMB to support multiple turnovers of the cofactor for AA oxidation (**Chapter 3**). In **Chapter 5** we demonstrated that the 2Cl-14DMB cation

radical signal, formed after reaction with LiP, disappears rapidly when AA is added to the reaction mixture. This suggests that, although 2Cl-14DMB consumption was not apparently inhibited by AA at all, AA can directly reduce the 2Cl-14DMB cation radical back to 2Cl-14DMB.

Moreover, we showed that when AA is present to reduce the 2Cl-14DMB cation radical LiP is protected from H_2O_2-inactivation (**Chapter 5**). When AA is not present to reduce the cation radical or the concentration of 2Cl-14DMB is not sufficient to effectively remove the cation radical by forming a dimer product, LiP inactivation is observed (**Chapter 5**). This means that the stable 2Cl-14DMB cation radical probably interferes with the enzyme: (1) the cation radical might react with LiP compound II to form the inactive LiP compound III; or (2) the cation radical could couple with heme and/or protein.

Therefore, we propose the following mechanism for the behavior of 2Cl-14DMB in the catalytic cycle of LiP and its role in the stimulation of AA oxidation (Figure 2). Spectral studies showed that LiP compound I accumulated only when 2Cl-14DMB is present in the reaction mixture (unpublished results), which is probably caused by a reversal reaction of LiP compound II with the initially formed 2Cl-14DMB cation radical. The spectral studies also showed an incomplete recovery of native LiP in the presence of only low 2Cl-14DMB concentrations (unpublished results). These observations suggest that the 2Cl-14DMB cation radical interferes with the heme and/or protein in the LiP binding site. The interaction of the cation radical with heme could account for the proposed oxidation of LiP compound II to LiP compound I (Figure 2A), and is supported by the fact that kinetic studies showed a binding of 2Cl-14DMB with LiP compound II (unpublished results). The incomplete recovery of native LiP could be due to the oxidation of amino acid residues in the LiP binding site by the 2Cl-14DMB cation radical (Figure 2A). Addition of sufficient

reducing substrate, such as AA or excess 2Cl-14DMB, would lead to quenching of the 2Cl-14DMB cation radical and help prevent the interaction of the cation radical with the heme or protein and thereby lower inactivation (Figure 2B).

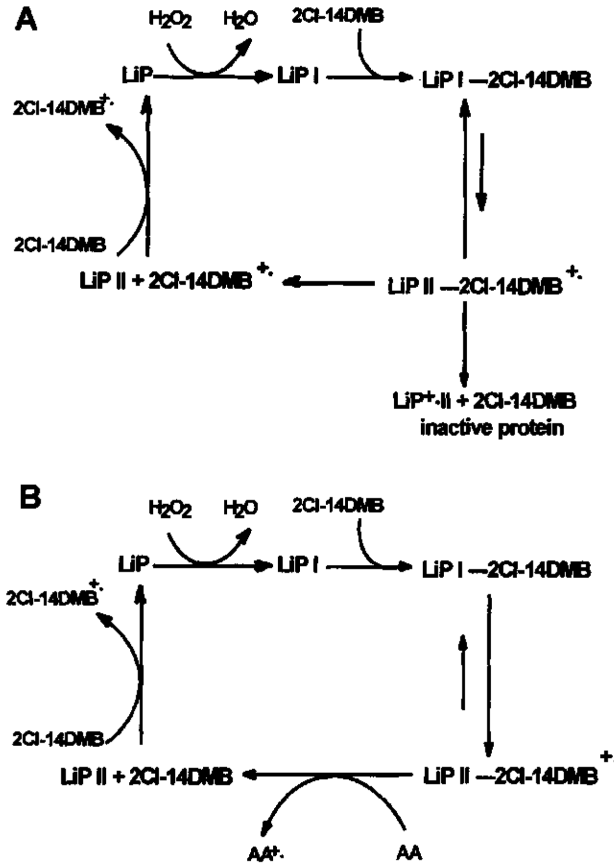

Figure 2. *Proposed reaction of 2Cl-14DMB with LiP compound I and LiP compound II.* (A) *In the absence of AA or only a low 2Cl-14DMB concentration in the reaction mixture, the initially formed 2Cl-14DMB cation radical can react with the heme and/or protein of LiP. The reaction with heme would lead to the oxidation of LiP II back to LiP I, whereas the reaction with protein would lead to inactive protein. (B) In the presence of AA the reverse reaction of LiP II to LiP I is much slower, because AA directly interacts with the 2Cl-14DMB cation radical. The 2Cl-14DMB cation radical is reduced back to 2Cl-14DMB, which can then reduce LiP II back to native.*

In conclusion, the mechanism as described in Figure 2B does seem to explain all the results we obtained throughout the research. AA is directly oxidized by the 2Cl-14DMB cation radical (**Chapter 4**), however, 2Cl-14DMB consumption is not inhibited (**Chapter 2**) because the 2Cl-14DMB which is released from the LiP II - 2Cl-14DMB+· is oxidized further by LiP

compound II. High 2Cl-14DMB concentrations prevent LiP from inactivation (**Chapter 2, Chapter 4**) by quenching the LiP II - 2Cl-14DMB$^{+\cdot}$ complex.

Role of DA in the ligninolytic system

Both LiP and MIP were able to oxidize DA (**Chapter 3, Chapter 6**). DA is oxidized by MIP, which was purified from *P. fastuosus,* to two octachlorinated dimers (**Chapter 6**). Most coupling reactions described for chlorophenols result in direct coupling of aromatic rings through oxidative C-C and C-O-C bonds with reactions most likely occurring at the *ortho-* and *para-*postion of the phenolic oxygen, often some dechlorination of the chlorophenols occurs as well (27, 67). Since there was no net loss of chlorine, MIP did not dechlorinate DA (**Chapter 6**). In most cases the chlorophenols are oxidatively dechlorinated at the *para-*position by ligninolytic enzymes to yield *para-*benzoquinone metabolites (57, 119). The fact that DA lacks a chlorogroup in the *para-*position to the phenolic oxygen might explain why the compound is not dechlorinated (**Chapter 6**).

Significance of CHM production

The fact that white rot fungi can use their own natural metabolites 2Cl-14DMB and DA as substrates for ligninolytic enzymes has a great impact on the understanding of lignin degradation. The recognition that these metabolites are potential physiological tools available for white rot fungi to mediate radical reactions that attack lignin is novel. We have not yet confirmed whether CHM are actually involved in lignin degradation. However, only catalytic concentrations of 2Cl-14DMB (10-50 µM) are necessary to mediate the oxidation of AA, Poly R-478, 4-methoxymandelic acid and oxalate (**Chapter 3, Chapter 4**). The low concentrations would make the involvement of 2Cl-14DMB in lignin degradation feasible. Furthermore, the knowledge of the existence of stable redox

mediators such as 2Cl-14DMB can be used to improve biotechnological applications.

In this thesis, it was shown that CHM are ubiquitous among Basidiomycetes, suggesting that such compounds are important sources of natural organohalogens. CHM have structures with striking structural similarity to black listed anthropogenic pollutants, such as the wood preservative pentachlorophenol. Recently it has been shown that many white rot fungi produce chlorinated anisyl metabolites in concentrations that exceed Dutch environmental legislation norms for chlorophenols (88). The question remains whether the natural production of CHM and other related organohalogens exceed the industrial output of chlorophenols.

Use of CHM in biotechnological applications

One of the most obvious applications of white rot fungi and their ligninolytic enzymes is in biobleaching and biopulping in the pulp and paper industry. Mester et al. (118) showed that *Bjerkandera* sp. BOS55 extensively degrades lignin and extractives in natural lignocellulosic substrates. LiP and MnP isoenzymes and their cofactors, VA and Mn(II), were present during degradation of lignocellulosic materials, indicating the involvement in ligninolysis. However, the presence of these enzymes and cofactors cannot fully explain wood degradation.

Ligninolytic peroxidases are larger than the maximum pore size of cell walls in wood (36), which indicates that the ligninolytic enzymes cannot be directly involved in lignin degradation localized deep in the cell wall. Consequently, diffusable redox mediators are needed which can operate at a distance of the active site of the enzyme.

Mn(III) formed by the oxidation of Mn(II) by MnP is a diffusable redox mediator, and Mn(III) can explain the oxidation of phenolic lignin moieties at a distance from the

enzyme (172). However, only 10% of lignin is truly phenolic, the vast majority of lignin has a nonphenolic character and is connected through both ether and carbon-carbon linkages (72).

The VA cation radical, formed by the oxidation of VA by LiP, is able to serve as a redox mediator for nonphenolic substrates; however, the cation radical is too short-lived to be considered as a true diffusible mediator (97). Lignin degradation by LiP can only be explained if the lignin is in the direct vicinity of the enzyme.

The results of this thesis show that CHM are natural redox mediators which have both the properties needed for efficient lignin degradation: CHM are stable enough to be diffusible and powerful enough to cause the oxidation of nonphenolic lignin moieties. CHM could really account for the oxidation of lignin at a distance of the enzyme active site and accelerate delignification of wood.

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Summary

Ligninolytic Basidiomycetes have been reported to produce a wide variety of chloroaromatic compounds as secondary metabolites, which are structurally similar to environmental pollutants. Among these are chlorinated hydroquinone metabolites (CHM), such as 2-chloro-1,4-dimethoxybenzene (2Cl-14DMB), 2,6-dichloro-1,4-dimethoxybenzene (26DCl-14DMB), tetrachloro-1,4-dimethoxybenzene and tetrachloro-4-methoxyphenol, which are synthesized by 11 genera of Basidiomycetes.

The biosynthesis of these chlorinated metabolites is not just a biological accident. An important physiological role of chloroaromatics, including CHM, is their antibiotic function protecting fungi from other organisms. However, since many ligninolytic white rot fungi synthesize CHM, these metabolites might also be involved in lignin degradation.

The lignin degrading system of white rot fungi is composed of peroxidases, oxidases and secondary metabolites, such as veratryl alcohol (VA) and anisyl alcohol (AA). VA is an important fungal metabolite in the catalytic cycle of lignin peroxidase (LiP), one of the main ligninolytic enzymes of white rot fungi. Three functions of VA have been described: (1) VA is a redox mediator for LiP-catalyzed oxidations via intermediate formation of a cation radical (2) VA is a cofactor necessary to close the catalytic cycle of LiP (3) VA prevents the inactivation of LiP by H_2O_2.

1,4-dimethoxybenzene (14DMB) can replace VA as a cofactor and/or redox mediator in the catalytic cycle of LiP; however, 14DMB is not a fungal metabolite. Nevertheless, 14DMB is structurally related to CHMs, which are fungal metabolites. This structural relationship between 14DMB and CHM implies that CHM may also replace VA in the catalytic cycle of LiP.

The objectives of this PhD study were to determine the extent of biosynthesis of CHM among white rot fungi and to elucidate their possible physiological role in lignin degradation.

For this purpose, 92 ligninolytic Basidiomycetes were screened for their ability to produce the CHMs tetrachloro-4-methoxyphenol (drosophilin A, DA) and tetrachloro-1,4-dimethoxybenzene (drosophilin A methyl ether, DAME). Five fungal strains (two strains of *Bjerkandera adusta, Agaricus arvensis, Peniophora pseudopini* and *Phellinus fastuosus*) produced these metabolites in detectable amounts. Furthermore, the CHM production coincided with the expression of ligninolytic activity.

P. fastuosus ATCC26.125 was used for further studies to elucidate if the ligninolytic enzymes from this fungus were able to use DA and DAME as substrates. The dominant ligninolytic peroxidase produced by *P. fastuosus* was a manganese independent peroxidase (MIP), which efficiently oxidizes phenols but not veratryl alcohol nor Mn(II). Purified MIP isozymes were able to oxidize chlorophenolic compounds, including the natural DA and the anthropogenic pentachlorophenol. MIP could not oxidize DAME or other (chlorinated) 1,4-dimethoxybenzenes.

The CHMs, 2Cl-14DMB, 26DCl-14DMB, DAME and DA were examined as substrates for LiP, which is an important ligninolytic enzyme from *Bjerkandera*. Our results indicated that 14DMB, 2Cl-14DMB, and DA were substrates for LiP; however, 26DCl-14DMB and DAME were not. According to these results, we concluded that 26DCl-14DMB and DAME do not have a physiological role in lignin degradation, but primarily function as antibiotic agents.

2Cl-14DMB, which is a LiP substrate, is synthesized by several fungi including the well studied fungus *Bjerkandera* sp. BOS55. We showed that 2Cl-14DMB, like 14DMB, can replace VA as a cofactor and redox mediator in the catalytic cycle of LiP.

We demonstrated that LiP oxidized 2Cl-14DMB to 2-chloro-1,4-benzoquinone and two different dimers. Furthermore, EPR results showed the formation of a long-lived 2Cl-14DMB cation radical. The dimer formation and EPR results suggest that the 2Cl-14DMB cation radical is stable enough enabling it to function as a diffussible redox mediator, which can oxidize substrates at a distance of the enzyme. Our results indeed showed that 2Cl-14DMB acts as a redox mediator in the LiP-catalyzed oxidation of the polymeric dye Poly R-478, 4-methoxymandelic acid and oxalate.

We examined whether 2Cl-14DMB could also replace VA as a cofactor in the LiP-catalyzed oxidation of AA. 2Cl-14DMB enormously stimulated the oxidation of AA, compared to VA. Although our results supported the theory that 2Cl-14DMB closed the catalytic cycle of LiP, we could not directly explain the high molar ratio of p-anisaldehyde formed to 2Cl-14DMB consumed. This high molar ratio indicated that the 2Cl-14DMB cation radical must be recycled back to 2Cl-14DMB. In support of this observation, AA was found to reduce the 2Cl-14DMB cation radical back to 2Cl-14DMB.

Furthermore, the presence of AA to reduce the 2Cl-14DMB cation radical prevents LiP from H_2O_2-inactivation. According to our observations, a mechanism for the behavior of 2Cl-14DMB in the catalytic cycle of LiP and its role in AA oxidation was proposed. In the absence of AA or at low 2Cl-14DMB concentrations, a 2Cl-14DMB cation radical - LiP compound II complex is formed which ultimately results in LiP inactivation. Addition of enough reducing substrate, AA or excess 2Cl-14DMB, reduces the 2Cl-14DMB cation radical - LiP II complex releasing LiP compound II and thereby preventing the inactivation of LiP.

In conclusion, CHMs were observed to be substrates for ligninolytic enzymes suggesting purposeful roles of these chlorinated metabolites. 2Cl-14DMB was identified as a stable diffussible redox mediator which could have potential application in the improvement of biobleaching and biopulping.

Samenvatting

Ligninolytische Basidiomyceten produceren verscheidene gechloreerde aromatische verbindingen als secundaire metabolieten, waarvan velen een overeenkomstige structuur hebben met milieuvreemde afvalstoffen. Hiertoe behoren onder andere gechloreerde hydroquinone metabolieten (CHM), zoals 2-chloor-1,4-dimethoxybenzeen (2Cl-14DMB), 2,6-dichloor-1,4-dimethoxybenzeen (26DCl-14DMB), tetrachloor-1,4-dimethoxybenzeen en tetrachloor-4-methoxyfenol, welke gesynthetiseerd worden door 11 verschillende soorten Basidiomyceten.

De biosynthese van deze verbindingen is niet zonder reden. Een belangrijke fysiologische rol van chlooraromaten, inclusief CHM, is een antibiotische werking om schimmels te beschermen tegen andere micro-organismen. Aangezien veel ligninolytische schimmels CHM synthetiseren zouden deze verbindingen betrokken kunnen zijn bij de afbraak van lignine.

Het lignine-afbrekend systeem van witrot schimmels bestaat uit peroxidasen, oxidasen en secundaire metabolieten, zoals veratryl alcohol (VA) en anisyl alcohol. VA speelt een belangrijke fysiologische rol in de katalytische cyclus van lignine peroxidase (LiP), één van de voornaamste ligninolytische enzymen in wit rot schimmels. Drie functies zijn beschreven voor VA: (1) VA is een redox mediator voor oxidatie-reacties die door LiP worden gekatalyseerd via de formatie van een kation radicaal (2) VA is een fysiologische cofactor die nodig is om de katalytische cyclus van LiP te sluiten (3) VA voorkomt dat LiP wordt geïnactiveerd door H_2O_2.

1,4-dimethoxybenzeen (14DMB) kan dezelfde functie vervullen als VA in de katalytische cyclus van LiP, maar is geen natuurlijke metaboliet. Echter, 14DMB heeft een overeenkomstige structuur met CHMs, welke wel schimmelmetabolieten zijn. Deze structurele overeenkomst tussen 14DMB en CHM impliceert dat CHM dezelfde rol kunnen vervullen als 14DMB en ook VA kunnen vervangen in de katalytische cyclus van LiP.

De bedoeling van dit promotie-onderzoek is om te achterhalen in welke mate CHMs worden gesynthetiseerd door wit rot schimmels en of CHMs een fysiologische functie hebben bij de afbraak van lignine.

Daarom werden 92 basidiomyceet-stammen onderzocht of ze de CHMs tetrachloor-4-methoxyfenol (drosophilin A, DA) en tetrachloor-1,4-dimethoxybenzeen (drosophilin A methyl ether, DAME) kunnen synthetiseren. Vijf stammen (twee *Bjerkandera adusta* stammen, *Agaricus arvensis, Peniophora pseudopini* en *Phellinus fastuosus*) produceerden aantoonbare hoeveelheden van de metabolieten en de produktie was gelijktijdig met de expressie van ligninolytische activiteit.

P. fastuosus ATCC26.125 werd gebruikt in verder onderzoek om te achterhalen of de ligninolytische enzymen van deze schimmel DA en DAME als substraat kunnen gebruiken. Het mangaan onafhankelijke peroxidase (MIP) is het ligninolytische enzym dat overwegend geproduceerd wordt door *P. fastuosus*. MIP is in staat efficiënt fenolen om te zetten, maar niet veratryl alcohol of Mn(II). Gezuiverde MIP isozymen kunnen chloorfenolen oxideren, waaronder de natuurlijke metaboliet DA en het milieuvervuilende pentachloorfenol. MIP kan niet DAME of andere (gechloreerde) 1,4-dimethoxybenzenen oxideren.

De CHMs 2Cl-14DMB, 26DCl-14DMB, DAME en DA werden getest als substraten voor LiP, een belangrijk ligninolytisch enzym in *Bjerkandera*. Onze resultaten toonden aan dat 14DMB, 2Cl-14DMB en DA substraten waren voor LiP, maar 26DCl-14DMB en DAME niet. Uit deze resultaten werd geconcludeerd dat 26DCl-14DMB en DAME waarschijnlijk alleen een antibiotische werking hebben en verder geen fysiologische rol spelen in de lignine afbraak.

2Cl-14DMB, een LiP substraat, wordt gesynthetiseerd door verscheidene schimmels, waaronder *Bjerkandera* sp. BOS55. In dit proefschrift hebben we aangetoond dat 2Cl-14DMB, net als 14DMB, VA kan vervangen als cofactor en redox mediator in de katalytische cyclus van LiP.

Uit onze experimenten bleek dat LiP 2Cl-14DMB omzette in 2-chloor-1,4-benzochinon en twee verschillende dimeren. Verder toonden EPR resultaten aan dat 2Cl-14DMB wordt geoxideerd tot een langdurig bestaand kation radicaal. Zowel de vorming van de dimeren als de EPR resultaten suggereren dat het 2Cl-14DMB kation radicaal stabiel genoeg is om als een diffunderend redox mediator te functioneren. Dit betekent dat 2Cl-14DMB substraten zou kunnen oxideren op afstand van het enzym. Inderdaad konden we aantonen dat 2Cl-14DMB een redox mediator is in de LiP-gekatalyseerde oxidatie van de kleurstofpolymeer Poly R-478, 4-methoxymandelic acid en oxalaat.

Verder hebben we onderzocht of 2Cl-14DMB VA kan vervangen als cofactor in de oxidatie van AA, die gekatalyseerd wordt door LiP. Het bleek dat 2Cl-14DMB de oxidatie van AA enorm stimuleerde, vergeleken met VA. Alhoewel onze resultaten leken aan te tonen dat 2Cl-14DMB de katalytische cyclus van LiP sloot, konden we niet direct de hoge molaire ratio van gevormd p-anisaldehyde ten opzichte van geconsumeerd 2Cl-14DMB verklaren. Deze hoge molaire ratio gaf aan dat het 2Cl-14DMB kation radicaal moest worden gereduceerd tot 2Cl-14DMB. Inderdaad bleek dat AA in staat was om het 2Cl-14DMB kation radicaal terug te reduceren tot 2Cl-14DMB.

Ook bleek dat de aanwezigheid van AA om het 2Cl-14DMB kation radicaal te reduceren LiP beschermde tegen inactivatie door H_2O_2. Aan de hand van onze resultaten werd een mechanisme opgesteld om het gedrag van 2Cl-14DMB in de katalytische cyclus van LiP en de rol van 2Cl-14DMB in AA oxidatie te verklaren. Zonder AA of bij lage 2Cl-14DMB concentraties wordt een 2Cl-14DMB kation radicaal - LiP II complex gevormd wat uiteindelijk resulteert in inactivatie van het enzym. De toevoeging van genoeg reducerend substraat, zoals AA of een overmaat 2Cl-14DMB, reduceert het 2Cl-14DMB kation radicaal - LiP II complex en voorkomt zo de inactivatie van LiP.

Concluderend kan gezegd worden dat CHMs substraten zijn voor ligninolytische enzymen wat suggereert dat deze gechloreerde verbindingen een doel kunnen hebben in lignine afbraak. 2Cl-14DMB bleek een stabiele redox mediator, die mogelijk op afstand van het enzym kan functioneren. 2Cl-14DMB zou vanwege deze eigenschap mogelijk toegepast kunnen worden in de verbetering van de biobleaching en biopulping in de pulp- en papierindustrie.

Nawoord

Het nawoord. Dit is dan het stuk tekst, wat de meeste mensen wel zullen lezen. In dit stuk tekst kan ik na vier (en driekwart) jaar eindelijk zeggen dat het af is. En ik ben blij dat ik niet nog eens een proefschrift hoef te schrijven. Ik ben ervan overtuigd dat ook Michel zich daar in kan vinden.

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Curriculum vitae

Paula Johanna Maria (Pauline) Teunissen werd op 22 augustus 1970 te Gouda geboren. In 1988 werd het Atheneum diploma behaald aan het Dr. Schaepmancollege te Dongen, waarna in hetzelfde jaar werd begonnen met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. De doctoraalfase bestond uit afstudeervakken bij Biochemie, Toxicologie en Industriële Microbiologie. Een deel van het afstudeervak bij Industriële Microbiologie werd uitgevoerd bij het Unilever Research Laboratorium in Vlaardingen.

In 1994 startte de auteur haar promotie-onderzoek bij de vakgroep Levensmiddelentechnologie en Voedingswetenschappen, leerstoelgroep Industriële Microbiologie van de Landbouwuniversiteit Wageningen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf maart 1999 werkt de auteur als post-doc onderzoeker bij Genencor International in Leiden.

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