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Oxidative cleavage of non-phenolic ß-0-4 lignin model dimers by an extracellular aromatic peroxygenase

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Abstract

The extracellular aromatic peroxygenase of the agaric fungus *Agrocybe aegerita* catalyzed the H_2O_2-dependent cleavage of non-phenolic arylgiycerol-ß-aryl ethers (ß-O-4 ethers). For instance 1-(3,4-dimethoxyphenyl)-2-(2-methoxy-phenoxy)propane-1,3-diol, a recalcitrant dimeric lignin model compound that represents the major non-phenolic substructure in lignin, was selectively *O*-demethylated at the *para*-methoxy group to give formaldehyde and 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol. The phenol moiety of the latter compound was then enzymatically oxidized into phenoxy radicals and a quinoid cation, which initiated the autocatalytic cleavage of the dimer and the formation of monomers such as 2-methoxy-1,4-benzoquinone and phenoxyl-substituted propionic acid. The introduction of 18O from $H_2{}^{18}O_2$ and $H_2{}^{18}O$ at different positions into the products provided information about the routes of ether cleavage. Studies with a ^{14}C-labeled lignin model dimer showed that more than 70% of the intermediates formed were further coupled to form polymers with molecular masses above 10 kDa. The results indicate that fungal aromatic peroxygenases may be involved in the bioconversion of methoxylated plant ingredients originating from lignin or other sources.

Keywords: *Agrocybe aegerita*; hydroxylation; lignin model compound; 0-dealkylation; peroxidase; peroxygenase.

Introduction

Six years ago, a new type of secreted peroxidative biocatalyst, nowadays designated as aromatic peroxygenase (APO), was discovered in the agaric *Agrocybe aegerira* (Black poplar mushroom), which inhabits hardwoods and mulch (Ullrich et al. 2004; Hofrichter et al. 2010). Later, similar enzymes have also been found in related fungi such as *Coprinellus* radians, *Coprinopsis cinerea* and *Marasmius rotula,* which are all biodegraders of lignocellulose-derived compounds in soil, leaf litter or dung (Anh et al. 2007; Pecyna et al. 2009; Hofrichter et al. 2010). Aromatic peroxygenases are heme-thiolate proteins that combine unique capabilities of cytochrome P450 monooxygenases and properties of classic peroxidases. However, the best-characterized aromatic peroxygenase, from Agrocybe aegerita (*Aae*APO), exhibits no significant sequence identity with the common peroxidases or P450s (Pecyna et al. 2009). Recent studies established that *Aae*APO is involved in the H_2O_2-dependent bromination and hydroxylation/epoxidation of aromatics (Ullrich et al. 2004; Anh et al. 2007; Aranda et al. 2008; Kinne et al. 2008, 2009a; Kluge et al. 2009), phenol oxidation (Kinne et al. 2008, 2009a,b), sulfoxidation of tricyclic heterocycles (Aranda et al. 2008), *N*-oxidation of pyridine derivatives (Ullrich et al. 2008), and cleavage of diverse ethers (Kinne et al. 2009b). The physiological function of these peroxygenases remains unclear, but their extracellular location and the versatile reactions catalyzed can be interpreted that they play an integral role in the bioconversion and detoxification of organic chemicals and materials encountered by the fungi in their natural habitats.

To reveal a possible physiological function of these peroxygenases, we studied the *Aae*APO-catalyzed oxidation of dimeric lignin model compounds (LMCs), which represent lignin substructures. Although such dimers do not occur in nature, they are well suited for studies of biochemical lignin degradation, as their ß-O-4 ether bonds represent the most abundant linkage in the lignin polymer (Kawai et al. 2002; Ortiz-Bermudez et al. 2003; Liers et al. 2009). For example, the first ligninolytic peroxidase (EC 1.11.1.14; LiP) was first recognized by adding LMCs to the extracellular culture fluid of the white-rot basidiomycete *Phanerochaete chrysosporium* (Tien and Kirk 1983). Moreover, methoxy groups in LMCs are also typical for lignins and lignin fragments (Zemek et al. 1979) and secondary metabolites from plants, e.g. lignans (Cutillo et al. 2006; Han et al. 2008) that show fungicidal activity (Gertsch et al. 2003). In this paper will be reported that *Aae*APO cleaves non-phenolic LMC dimers via an initial selective *O*-dealkylation of the para-substituted ring's methoxy group followed by oxidative ß-O-4-cleavage of the resulting phenolic dimers and repolymerization. On the basis of these results it will be suggested that peroxygenases may be involved in the degradation of methoxylated compounds deriving from lignin and other aromatic plant sources.

Materials and methods

Enzyme preparation

The extracellular peroxygenase of *A. aegerito* (isoform II, 46 kDa, see Suppl. 1) was produced in a stirred-tank bioreactor and purified by fast protein liquid chromatography (FPLC) as described previously (Ullrich et al. 2004). The enzyme preparation was homogeneous by SDS polyacrylamide gel electrophoresis and exhibited an A_{418}/A_{280} ratio of 1.75. The specific activity of the peroxygenase was 117 U mg^{-1}, where 1 U represents the oxidation of 1 µmol of 3,4-dimethoxybenzyl alcohol to 3,4dimethoxybenzaldehyde in 1 min at pH 7 and 23°C (Ullrich et al. 2004).

Reactants

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) except $H_2{}^{18}O_2$ [90% (atom%), 2% (wt/vol)] that was obtained from Icon Isotopes (Summit, NJ, USA). 2,4-Dinitrophenylhydrazine (DNPH) solution (0.1%) was prepared by adding 100 mg of DNPH to about 50 ml of boiling water and 5 ml of concentrated HCl (36%). After dissolving and cooling down, the solution was diluted with deionized water to give a final volume of 100 ml. The dimeric LMCs of the arylglycerol ß-aryl ether type (Figure 1, compounds I, II, III and IV) and a dehydrogenation polymer (DHP) of coniferyl alcohol [Mn 1.318; Mw 4.398; Mw/Mn (polydispersity) 3.337, ^{13}C-NMR spectra see Suppl. 2] were prepared according to methods described previously (Sipilä and Syrjänen 1995; Hofrichter et al. 1999). The radiolabeled compound II, *threo*-1-(4-ethoxy-3-methoxy-ring-[^{14}C]phenyl)-2-(2-methoxyphenoxy)-1,3dihydroxypropane (1.0 mCi $mmol^{-1}$), was purchased from the Forest Products Laboratory (Madison) were it was synthesized as reported in earlier publications (Landucci et al. 1981; Kawai et al. 1995; Kapich et al. 1999; Ortiz-Bermudez et al. 2003). The synthesized compounds were purified by silica chromatography and the purity was checked by HPLC. The identification of the compounds was done by NMR and ESI/MS. NMR-spectra were recorded using a Varian Inova 500 spectrometer and a Bruker 250 MHz Avance spectrometer in $CDCl_3$.

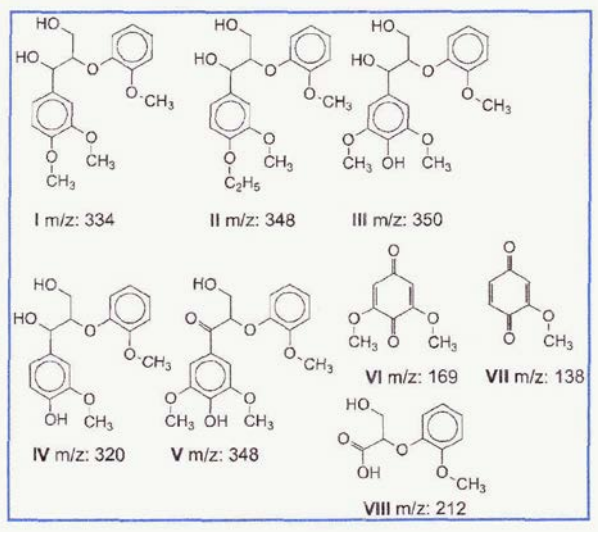

Figure 1 Structures of substrate models **I, II,** and **III** and of oxidized products produced by the aromatic peroxygenase from *Agrocybe aegerita* (*Aae*APO).

Compound I [1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol] ^{1}H 7.12-6.80 ArH, 4.97 α-H, 4.16 ß-H, 3.90 and 3.67 γ-H, 3.88 and 2×3.87 OCH_3 3.5 and 2.7 aliphatic-OH, ^{13}C: 151.7, 149.2, 148.7, 147.1, 132.8, 124.8, 124.3, 121.9, 121.8, 121.0, 119.8, 118.7, 112.4, 111.3, 109.5, 87.4 (ß-C), 72.94 (α-C),61.0(γ-C), 56.13, 56.09 and 56.07 (OCH_3). ESI/MS $[M+HCOOH-H]^-$ 379.

CompoundII [1-(4-Ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol] ^{1}H 7.16-6.83 ArH, 4.89 α-H, 4.28 and 4.18 ß-H, 4.00 OCH_2CH_3, 3.89 and 3.77 OCH_3, 3.66 and 3.47 γ-H, ^{13}C 151.6, 150.1, 148.5, 148.5, 123.5, 121.9, 120.1, 113.8, 113.4, 112.1, 88.5 and 86.8 (ß-C), 73.7 (α-C), 64.9 (OCH_2CH_3), 61.8 (γ-C), 61.8 and 56.1 (OCH3). ESI/MS $[M+HCOOH-H]^-$ 393.

Compound III [3-(4-Hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol] ^{1}H: 7.14-6.88 ArH, 6.62 ArH (2.6), 5.5 ArOH, 4.94 α-H, 4.14 ß-H, 3.89 and 3.66 γ-H, 3.88 and 3.86 OCH_3, 3.56 and 2.78 aliphatic-OH, ^{13}C: 151.8, 147.3, 147.1, 134.4, 131.2, 124.5, 121.9, 121.2, 112.4, 103.0, 87.6 (ß-C), 73.1 (α-C), 61.0 (γ-C), 56.6 and 56.1 (OCH_3). ESI/MS $[M-H]^-$ 349.

Compound IV [1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1.3-diol] 1H: 7.15-6.80 ArH, 5.73 ArOH, 4.97 α-H, 4.16 ß-H, 3.89 and 3.67 γ-H, 3.81 and 3.83 OCH_3, 2.90 and 1.81 aliphatic-OH, ^{13}C: 174.1, 146.9, 145.3, 132.1, 124.3, 121.9, 2×121.0, 119.3, 114.5, 112.4, 109.0, 87.4 (ß-C), 73.0 (α-C), 61.0 (γ-C), 56.13 and 56.11 (OCH_3). ESI/MS $[M-H]^-$ 319.

Reaction conditions

Typical reaction mixtures (0.2 ml) contained purified peroxygenase (2 U ml^{-1}) potassium phosphate buffer (100 mM, pH 7.0), acetonitrile (5% vol/vol) and the LMC (0.4 or 0.5 mM). When indicated, the reaction mixtures also contained the radical scavenger ascorbic acid (12 mM) to inhibit further oxidation of the phenolic products that were released (Kinne et al. 2008, 2009a,b). The reactions were started by the dosage of H_2O_2 via a syringe pump (5 mM h^{-1} = 83 µM min^{-1}) under continuously stirring at room temperature, and they were stopped when chromatographic analyses showed that product formation was completed. Aliquots of the reaction mixture were derivatized using acidic DNPH-solution (0.1%) to analyze the low-molecular mass aldehydes released during reaction.

Product identification

Portions of the completed reaction mixtures were analyzed by high performance liquid chromatography (HPLC). Instrument: Agilent Series 1100 equipped with a diode array detector and an electrospray ionization mass spectrometer (ESI/MS) on a Luna 5-µm-pore-size C18 column (Phenomenex, Aschaffenburg, Germany). The column was eluted at 40°C and 0.35 ml min^{-1} with an aqueous ammonium formate solution (0.1% vol/vol, pH 3.5)/acetonitrile, 95:5, for 5 min (10 min for ^{14}C-labeled LMC), followed by a 25-min (60 min for ^{14}C-labeled LMC) linear gradient to 100% (60% for ^{14}C-labeled LMC) acetonitrile. Products were identified relative to authentic standards, based on their retention times, UV absorption spectra, and $[M+H]^+$ or $[M-H]^-$ ions. The quantification of the reactants was performed by HPLC as described above based on a linear external standard curve (R>0.95) of the respective compound.

To detect and quantify radiolabeled products, fractions (0.5 ml) of HPLC-separated reaction mixtures were collected and assayed for ^{14}C by liquid scintillation counting (Tri-Carb 2900TR, Perkin-Elmer, Shelton, CT, USA) after mixing of the fractions with an

scintillation cocktail (Emulsifier-Safe, Perkin-Elmer, USA) to a total volume of 5 ml (Ortiz-Bermudez et al. 2003).

Results

*Aae*APO generated numerous products from LMCs in the presence of H_2O_2 (Figure 2). Typical chromatographic and mass spectrometric data of the *Aae*APO-catalyzed oxidation of a non-phenolic LMC are given in Figure 2a. The aldehydes with low molecular weight were identified by HPLC/MS as their 2,4-dintrophenylhydazones.

When ascorbic acid was added to the reaction mixtures, only dimeric reaction products were detected. For example, the total conversion of **I** [1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol; 500 µM] was 175 µM (35%) under a continuous formation of 110 µM of the major reaction product **IV** [1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane- 1,3-diol; 63% of total conversion] and 20 µM of formaldehyde-2,4-dinitrophenylhydrazone.

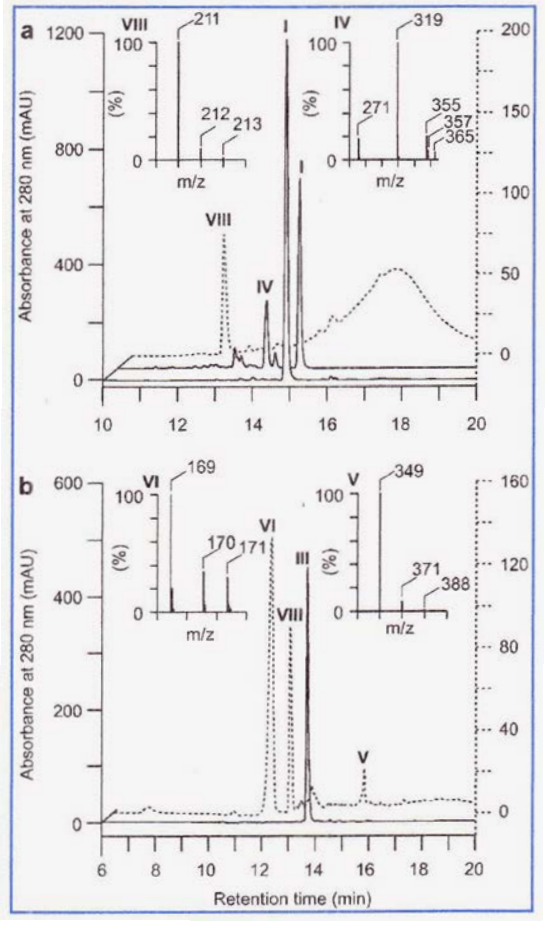

Figure 2 HPLC elution profiles of products obtained from the oxidation of model **I** (a) and model **III** (b) with *Aae*APO. Insets show the mass spectra of the respective products. Front solid lines: controls without enzyme. second solid line in (a): complete reactions in the presence of ascorbte. Dashed lines: complete reactions without ascorbate.

When the reaction was conducted with **II** [1-(4-ethoxy-3-methoxyphenyl)-2–(2-methoxyphenoxy)propane-1,3-diol; 400 µM] instead of **I**, total conversion was 170 µM (43%) with formation of 150 µM of **IV** (88% of total conversion) and 30 µM acetaldehyde-2,4-dinitrophenylhydrazone.

The presence of other hydroxylated products in the reaction mixtures was detected, as indicated by the minor peaks in Figure 2a having mass-spectral changes (m/z + 16 and m/z + 32) attributable to single and double oxygen incorporation. Moreover, when $H_2{}^{18}O_2$ was added as oxidant instead of H_2O_2, the ion mass of the demethylated product **IV** remained constant as described previously (Kinne et al. 2009b), whereas the ion masses of several reaction product shifted (m/z + 2 and m/z + 4 relative to their natural abundant species). This is a consequence of the introduced $^{18}O_2$ into the substrate. The further identification of these mono- and dihydroxylated products was not possible because of the lack of authentic standards and their low yields. A formation of a-ketones from substrate **I** and **II** was not observed. In the absence of ascorbic acid, predominantly monomeric and polymerization products of **I** and **II** were obtained (Figure 2a). Models I and **II** were cleaved to give traces of 2-methoxy-1,4-benzoquinone **(VII)** and 3-hydroxy-2-(2-methoxy-phenoxy)propanoic acid **(VIII).** When the *Aae*APO-catalyzed oxidation of **I**, **II** and **III** was conducted with $H_2{}^{18}O_2$ in place of H_2O_2, mass spectral analysis of the resulting product **VIII** showed that the principal $[M-H]^-$ ion had shifted from the natural abundance of m/z 211 to m/z 213 (Figures 2b and 3a). Metabolite W could not be detected by mass spectrometry as it was

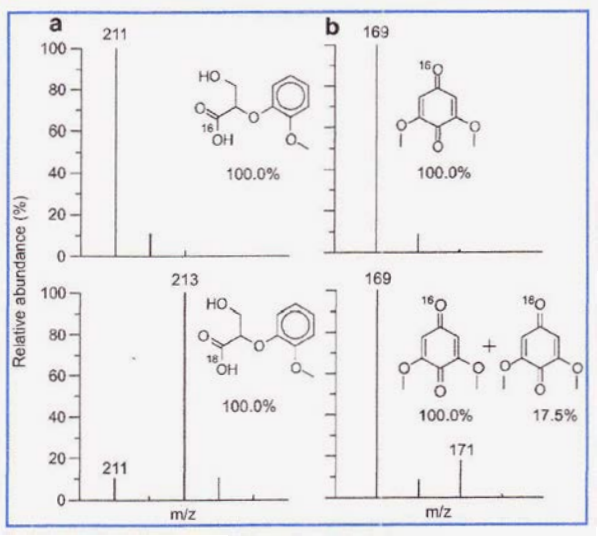

Figure 3 (a) Incorporation of ^{18}O via $H_2{}^{18}O_2$ into 3-hydroxy-2-(2-methoxyphenoxy)propanoic acid after hydroxylation of the arylglycerol aryl ether by AaeAPO Upper: MS of the product obtained with natural abundance H2O2. Lower: MS of the product obtained with 90% (atom%) $H_2{}^{18}O_2$. (b) Incorporation of ^{18}O via $H_2{}^{18}O$ into the quinone group of 3,5-dimethoxy-1,4-benzoquinone after cleavage of compound I by *Aae*APO. Upper: MS of the product obtained with natural abundance H_2O_2. Lower: MS of the product obtained with 20% of 99% (atom%) $H_2{}^{18}O$.

maybe rapidly polymerized by the peroxidative activity of Aae APO.

However to analyze quinone formation, compound **III** [1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxyl)propane-1,3-diol] was used, which gave the products 3,5-dimethoxy-1.4-benzoquinone **(VI),** 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one **(V)** and **VIII** during the *Aae*APO-catalyzed oxidation (Figure 2b). HPLC/MS analysis showed that the *Aae*APO-catalyzed cleavage of model **III** in the presence of 20% $H_2{}^{18}O$ resulted in 17.5% ^{18}O-incorporation into VI, as evidenced by the fractional shift of the principal molecular ion from m/z 169 to m/z 171 (Figure 3b). Compound (V) was identified by its mass spectrum (Figure 2b). Incorporation of ^{18}O into **V** from $H_2{}^{18}O$ was not observed.

Polymerization of the reaction products from the reaction of LMCs with *Aae*APO and H_2O_2 was observed in the absence of the radical scavenger (ascorbic acid). To quantify the polymerization products, *threo*-1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-2-(2-methoxy-phenoxy)propane-1,3-diol was incubated with *Aae*APO as well as H_2O_2, the reaction mixture filtrated through a filter membrane with a 10 kDa cut-off and analyzed by LSC (Figure 4). In a reaction, where a total activity of 97 600 dpm of the radiolabeled substrate was used, 26 000 dpm (27%) were found to pass the membrane and 71 630 dpm (73%) were retained. In the presence of ascorbic acid, polymerization was fully suppressed.

Discussion

*Aae*APO cleaves non-phenolic LMCs via selective *O*-dealkylation of the pura-alkyl group to give the corresponding aldehyde (formaldehyde and acetaldehyde) and phenolic dimer products, which were further oxidized by the enzyme yielding monomers and polymerization products. Figure 5 illustrates the hypothetical reaction mechanism. *Aae*APO first 0-dealkylates the non-phenolic LMC I via an unstable hemiacetal intermediate **I′,** whose hydrolyzation yields the phenolic LMC **IV** under release of the corresponding aldehyde (in this case, formaldehyde) with a reaction mechanism described previously (Kinne et al. 2009b). In the absence of a radical scavenger, compound **IV** is further oxidized to give a monomeric hydroxylated species **VIII** and polymeric coupling products. This reaction is inhibited in the presence of a radical scavenger such as ascorbic acid. This finding is interpreted that *Aae*APO oxidizes the phenolic moiety to form a phenoxy radical that can be re-reduced by ascorbic acid or otherwise couple oxidatively (Kinne et al. 2008, 2009a).

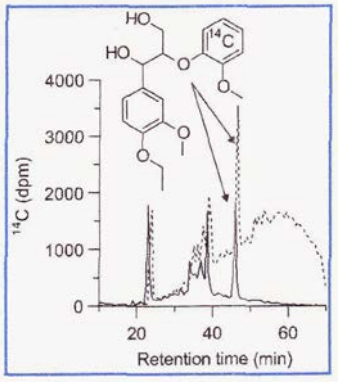

Figure 4 HPLC radiochromatogram of products obtained from the oxidation of radiolabeled model **II** catalyzed by *Aae*APO. Dashed he: completed reaction. Solid line: 10 kDa permeate of the completed reaction.

As shown above, compound **III** bearing an additional methoxy group was helpful to establish the reaction mechanism of *Aae*APO-catalyzed oxidation and cleavage of phenolic LMCs. If a phenolic moiety is present right from the beginning or if it is formed in the course of the reaction, AaeAPO may abstract an electron from the phenolic group (as in case of **III**) yielding the cyclohexadienyl radical **III′,** which is subsequently oxidized by the enzyme to give the corresponding cation **III″** (Kawai et al. 1988). Loss of an α-proton will result in the formation of an uncharged quinone methide intermediate that rearranges to yield the phenolic ketone V. In support of this mechanism, no oxygen from molecular oxygen or $H_2{}^{18}O$ was incorporated into V. Moreover, ketone formation from the non-phenolic substrate I was not observed and this finding indicates that a phenolic group is required for the cleavage of such LMCs, Alternatively, the cation intermediate **III′** can be attacked by water yielding a hydroxy-substituted cyclohexadienone intermediate, which may undergo alkyl-phenyl bond cleavage to yield the 3,5-dimethoxyhydroquinone **VI′** and the phenoxyl-substituted propanal **VIII′**. Under these reaction conditions, *Aae*APO may oxidize VI' to give **VI**, and **VIII′** to **VIII.** As predicted by this pathway, one atom of ^{18}O from $H_2{}^{18}O$ is incorporated into VI, whereas no incorporation of ^{18}O from $H_2{}^{18}O$ but from $H_2{}^{18}O_2$ occurs during the formation of VIII. The different mesomeric forms of the released radical intermediate **III′** and the charged benzoquinone intermediate **III″** may undergo further oxidation to form polymeric coupling products. The oxidation mechanism of **III** is identical to that proposed previously for the α-C oxidation of phenolic LMCs by manganese peroxidase (MnP) (Tuor et al. 1992). The structure of reaction product **VIII** was proposed on the basis of following observations: 1) incorporation of ^{18}O into **VIII** was detected when $H_2{}^{18}O_2$ served as co-substrate (Kinne et al. 2010), 2) **VIII** accumulated in the reaction mixture also in the absence of ascorbic acid and did not polymerize, and 3) this reaction product **(VIII)** was detected both when compound **I, II** and **III** were converted by *Aae*APO indicating the same reaction mechanism.

At first glance, *Aae*APO seems to combine activities of LiP and MnP, namely the oxidation and cleavage of non-phenolic **(I, II)** and phenolic **(III)** LMCs (Hammel et al. 1985; Tuor et al. 1992). A closer look on the mechanisms, however, reveals considerable differences. Whereas LiP acts via abstraction of an electron from the para-substituted ring of **I** or **II** (one-electron oxidation) resulting in the formation of unstable aryl cation intermediates (Hammel et al. 1985), *Aae*APO attacks the *para*-alkoxy group via a two-electron

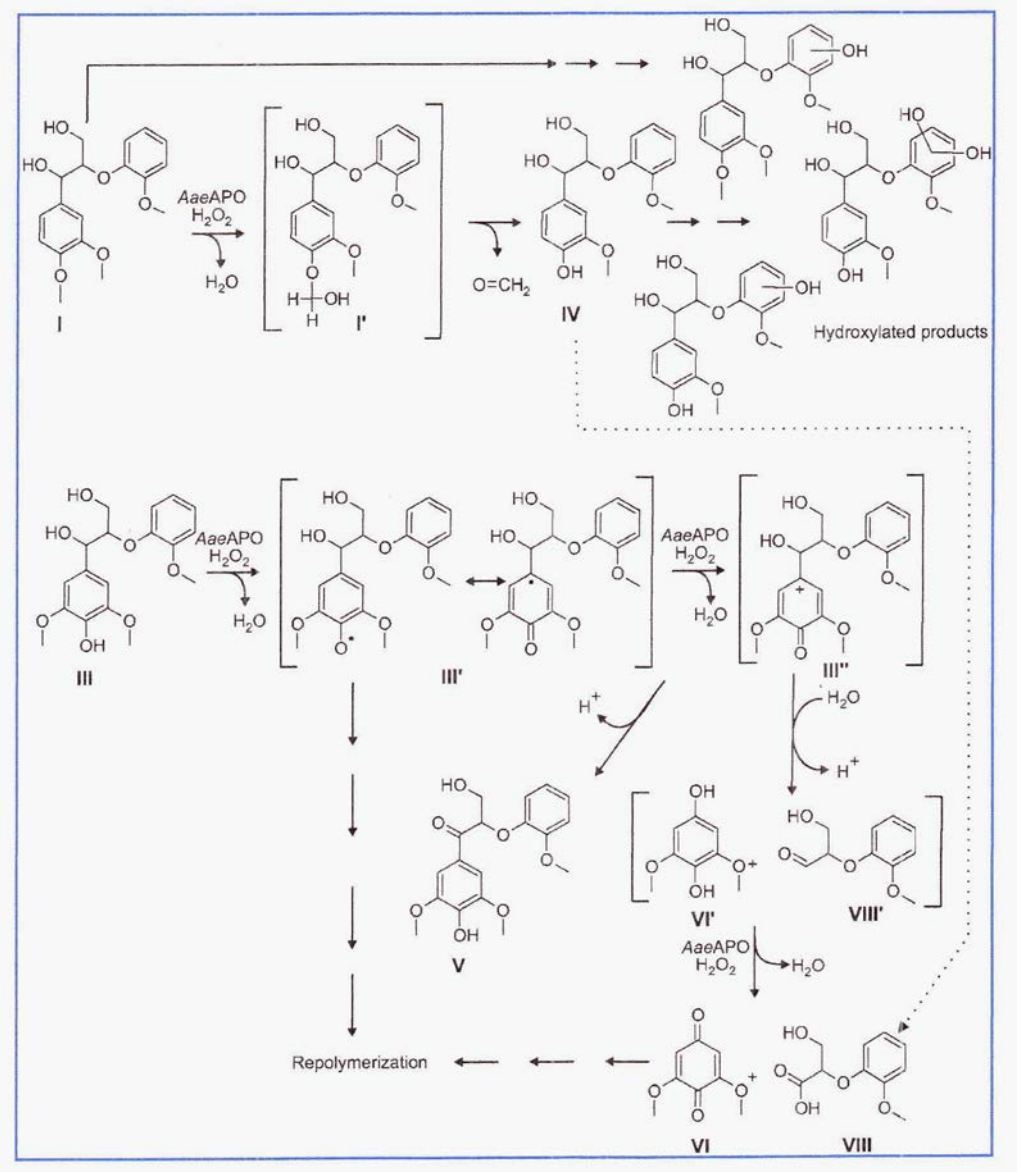

Figure 5 Hypothetical reaction mechanism of *Aae*APO-catalyzed cleavage of LMCs.

oxidation and oxygen transfer leading to an unstable hemiacetal intermediate. MnP, on the other hand, indirectly takes effect via primarily formed Mn^{3+} that oxidizes as redox mediator the phenolic group of **III** (Tuor et al. 1992), while AaeAPO directly oxidizes **III** into the corresponding phenoxy radical.

Recent studies have indicated that *Aae*APO is seemingly lacking "true" polymer-oxidizing activities, e.g. towards polyethylene glycol (Kinne et al 2009b). This is consistent with current studies on synthetic lignin (dehydrogenase polymer of coniferyl alcohol) or milled beech wood, which have not significantly been altered when treated with AaeAPO (data not shown). The HPLC-MS method applied in the present work to assess O-dealkylation via detection of DNPH-carboxyl adducts provides a sensitive tool to detect aldehyde release, but no significant amounts of such reaction products (e.g. formaldehyde) have been found in response to DHP/wood-treatment. The apparent inability of *Aae*APO to oxidize polymeric lignin and the moderate lignin degradation rates observed for the whole fungus *A. aegerita* (Isikhuemhen et al. 2009) (merely 15% mineralization of ^{14}C-DHP within seven weeks; Liers et al. 2010, unpublished result), indicate that *Aae*APO may rather have a physiological function in the biodegradation of methoxylated compounds with low-molecular masses, such as lignin fragments (e.g. formed by MnP) or lignans with fungicidal activity or phytoalexins.

Conclusions

*Aae*APO cleaves dimeric LMCs via initial *O*-dealkylation (monooxygenase activity) and subsequent oxidation of the

introduced phenolic moieties (peroxidase activity). To our best knowledge, AaeAPO is the first extracellular enzyme that combines catalytical features of intracellular phase-I enzymes (chytochrome P450s) and peroxidative biocatalysts (MnPs or LiPs), which are all involved in the biodegradation and detoxification of recalcitrant compounds. The results can be interpreted in a way that fungal peroxygenase may have a physiological role in the bioconversion detoxification) of methoxylated compounds originating from lignin or extractives with aromatic structure from plants.

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