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Phenolic mediators enhance the manganese peroxidase catalyzed oxidation of recalcitrant lignin model compounds and synthetic lignin

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ABSTRACT

Fungal oxidative enzymes, such as peroxidases and laccases, are the key catalysts in lignin biodegradation *in vivo*, and consequently provide an important source for industrial ligninolytic biocatalysts. Recently, it has been shown that some syringyl-type phenolics have potential as industrial co-oxidants or mediators, in laccase-catalyzed modification of lignocellulosic material. We have now studied the effect of such mediators with ligninolytic peroxidases on oxidation of the most recalcitrant lignin model compounds. We found that they are able to enhance the manganese peroxidase (MnP) catalyzed oxidation reactions of small non-phenolic compounds, veratryl alcohol and veratrylglycerol β-guaiacyl ether (adlerol), which are not usually oxidized by manganese peroxidases alone. In these experiments we compared two peroxidases from white-rot fungi, MnP from *Phlebia* sp. *Nf* b19 and versatile peroxidase (VP) from *Bjerkandera adusta* under two oxidation conditions: (i) the Mn(III) initiated mediated oxidation by syringyl compounds and (ii) the system involving MnP-dependent lipid peroxidation, both with production of (hydrogen) peroxides *in situ* to maintain the peroxidase catalytic cycle. It was found that both peroxidases produced α-carbonyl oxidation product of veratryl alcohol in clearly higher yields in reactions mediated by phenoxy radicals than in lipid-peroxyl radical system. The oxidation of adlerol, on the other hand, was more efficient in lipid-peroxidation-system. VP was more efficient than MnP in the oxidation of veratryl alcohol and showed its lignin peroxidase type activity in the reaction conditions indicated by some cleavage of Cα–Cβ-bond of adlerol. Finally, the mediator assisted oxidation conditions were applied in the oxidation of synthetic lignin (DHP) and the structural analysis of the oxidized polymers showed clear modifications in the polymer outcome, e.g. the oxidation resulted in reduced amount of aliphatic hydroxyls indicated by ^{31}P NMR.

1. Introduction

Lignin is a heterogenic aromatic biopolymer comprising 18–35% of the dry weight of the woody tissues in higher plants and the most important renewable source of aromatic compounds on earth. Lignin provides mechanical strength for plant cell wall binding the fibers together through an intermolecular network with carbohydrates (hemicelluloses and cellulose) hereby also providing resistance against degrading microbes, enzymes and chemicals. By chemical structure, lignin is a complex three-dimensional heteropolymer composed of phenylpropane units interconnected by various stable carbon–carbon and ether linkages (Ralph et al., 2004; Vanholme et al., 2010). Generally, lignin macromolecules constitute 90% of recalcitrant nonphenolic structures and 10% of reactive phenolic structures (Martínez et al., 2005). The predominant structural pattern of the lignin intermonomer linkages is the so-called arylglycerol-β-aryl ether (β-O-4 ether) that reaches around 35–50% in coniferous gymnosperm lignin and up to 60–85% in angiosperm lignin. The cleavage of this substructure is considered as the crucial step in lignin degradation and release from biomass. Besides these ether bond interunit structures, lignins contain numerous condensed type-linkages e.g. resinol-type (β–β with aliphatic C—C linkages, <5–9%), phenylcoumaran-type (β5 with aliphatic–aromatic C—C linkages, 5–17%) and biphenyl-type structures (5-5′ with aromatic C—C linkages, 10–25%), including dibenzodioxocines (5-5′-4-O-5, 5–8%) with varying abundance according to origin of the plant species and isolation and analysis method (Adler, 1977; Capanema et al., 2005, 2004; Du et al., 2014). In most industrial processes, especially pulp and paper industry and modern biorefineries, lignin is considered as an undesirable component causing extra costs in removal during

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processing (e.g. Sarkanen, 1962; Chakar and Ragauskas, 2004; Rahikainen et al., 2011). Catalytic oxidation processes, also including oxidative lignin modifying enzymes, are considered as the most promising techniques besides gasification and thermochemical processes for both functionalization and depolymerization of lignin (Lange et al., 2013; Zakzeski et al., 2010).

Wood-rotting basidiomyceteous fungi are very efficient at breaking down all components of woody biomass (Hatakka and Hammel, 2010). They are the most efficient lignin degraders in nature and have a crucial role in carbon recycling of the biosphere (Hatakka, 2001). These fungi and their enzymes have the ability to degrade and even mineralize lignin to carbon dioxide and water via formation and exploitation of diffusible oxidative mediator radicals of different origin (Hatakka and Hammel, 2010; Kirk and Farrell, 1987; Tuor et al., 1995; Valderrama et al., 2002; Wong, 2009). Wood decaying fungi have been extensively studied since 1980s as source of industrial biocatalysts with potential applications e.g. in chemical, pulp and paper, textile and food industry as well as in bioremediation and degradation of organopollutants from different industrial processes (Asgher et al., 2008; Martínez et al., 2009; Silva Coelho-Moreira et al., 2013; Tsukihara et al., 2008; Xu, 2005). White-rot fungi secrete low-specificity extracellular ligninolytic enzymes (lignin-degrading oxidative enzymes) in different combinations, including lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC 1.11.1.16) and laccases (EC 1.10.3.2) as well as other auxiliary oxidases like extracellular glucose oxidases (EC 1.1.3.4) contributing to the hydrogen peroxide supply for peroxidases (Hofrichter et al., 2010; Lundell et al., 2010). All these enzymes are classified in CAZy database in Auxiliary Activities families (Levasseur et al., 2013). Most white-rot fungi secrete MnP and laccase while the production of LiP is less common (Kirk and Farrell, 1987; Hatakka and Hammel, 2010). VPs were first found from *Pleurotus* and *Bjerkandera* spp. and have properties of both MnP and LiP (Martinez, 2002).

Lignin peroxidase-type enzymatic activity, catalyzed by LiPs and VPs, is responsible for the direct cleavage and degradation of lignin (Ruiz-Dueñas and Martínez, 2009). These enzymes catalyze the oxidation of non-phenolic lignin moieties directly to form highly reactive aryl cation radicals, which subsequently undergo a variety of nonenzymatic reactions like cleavage of Cα–Cβ-bonds, ring cleavages and α-oxidations as well as re-polymerization reactions. In contrast, MnPs are able to oxidize only the phenolic substructures in lignin with formation of phenoxy radicals to react further through non-enzymatic transformations like bond cleavages, α-oxidations, radical couplings, re-polymerizations, etc. (Wong, 2009). As the peroxidases with MnP-activity predominate in white-rot fungi, the generation of various small radical species as diffusible mediator molecules can have significant importance in lignin degradation. The complexity of lignocellulosic biomass creates challenges in revealing ligninolytic degradation mechanisms that remain still unrecognized. Rapidly increasing genome studies reveal even more variability among different fungi (Ohm et al., 2014; Riley et al., 2014).

Ligninolytic extracellular peroxidases perform catalytic reactions using hydrogen peroxide as the electron acceptor with concomitant release of water, and they function via one-electron transfer oxidations of their substrates which can be small phenolic compounds, transition metals, etc. (Camarero et al., 1999; Ertan et al., 2012; Ruiz-Dueñas et al., 1999). Although the heme active sites of these enzymes are structurally very similar and all contain a heme prostethic group (iron(III) protoporphyrin IX) at their active sites to bind hydrogen peroxide and also less reactive organic hydroperoxides, their reaction mechanisms are significantly different. Specifically, MnPs have in the vicinity of heme a manganese(II)-binding site, where Mn(II) is oxidized to reactive Mn(III), and Mn(II) is a default redox coupler in order to complete the enzymes' catalytic cycle and regenerate the native MnP enzyme from compound-II (Valderrama et al., 2002; Wong, 2009; Hofrichter et al., 2010). The released chelated Mn(III) serves then as secondary electron carrier, a mediator, to reach the substrates. The LiP-type activity for VP (and LiP) on the other hand, has been shown to involve Trp171 at the surface of the enzyme via long-range electron transfer (LRET) to the heme of compound-I, possessing high-redox potential (1.2 V in pH 3) to oxidize the non-phenolic substrates, e.g. veratryl alcohol and also other high-redox potential substrates, such as Reactive Black 5 (Tsukihara et al., 2008). The heme passageway of VP (and LiP) is too narrow for large molecules for direct interaction with the enzyme's active center, as shown by crystal structures of some LiP and VP (Martinez, 2002; Wong, 2009). Instead, the accessibility of small phenolic substrates in contact with LiP heme active center has been reported to inactivate the enzyme, especially when no higher redox-potential substrates are readily available. VPs have been reported not to have such inactivation effect (Busse et al., 2013; Ruiz-Dueñas et al., 2009; Tinoco et al., 2007), which makes these enzymes attractive for many applications e.g. in pulp and bleaching technologies.

MnP activity is also affected by the requirement of chelating agents such as oxalic or malonic acids to stabilize the highly reactive Mn(III) and stimulate the rate of Mn(III) release from the enzyme. Wood-rotting fungi produce these organic acids as metabolites in significant amounts accompanied by secretion of MnPs (Galkin et al., 1998; Kishi et al., 1994; Wariishi et al., 1992). The chelated Mn(III) then serves as a freely diffusible oxidant, a mediator, in solution. The complex formation stabilizes Mn(III) so that bidentate ligated Mn(III) usually have redox potentials of around 0.7–0.9 V (Armstrong, 2008; Cui and Dolphin, 1990) and, consequently significantly lower oxidation capacities when compared to non-chelated Mn(III). The degradation of recalcitrant non-phenolic compounds has been limited with MnP generated Mn(III)chelates alone due to this lower oxidation power, but some mediators or co-oxidants, such as thiols, unsaturated fatty acids and their derivatives and some common laccase mediators e.g. 1-hydroxybenzotriazole (HBT), have been reported effective in the oxidation reactions of more recalcitrant compounds (Bermek et al., 2002; Hofrichter, 2002; Kapich et al., 2005; Michizoe et al., 2004).

Recently it has been found that some syringyl-type phenols form stable phenoxyradicals in laccase catalyzed oxidation systems and have efficiency in modification of lignocellulosic material (Camarero et al., 2005; Canas and Camarero, 2010; Nousiainen et al., 2009). Several fungi are able to produce methoxylated aromatic alcohols and aldehydes as metabolites that have an important physiological function for a fungal ligninolytic system. It is possible that fungi in themselves are able to produce de novo phenolic compounds (Rogalski et al., 1996; Kirk and Farrell, 1987) or they can be available from the decaying wood as intermediates of hardwood lignin biodegradation. Various syringyl compounds can be isolated from pulp and paper wastewaters for industrial applications (Canas and Camarero, 2010). Previously we have reported the oxidation of nonphenolic lignin related compounds using 'natural' phenolic mediators with high-redox potential laccases and in this context shown that phenolic mediators are also applicable in reactions catalyzed by MnPs (Nousiainen et al., 2012). In this work, we have studied further the effect and suitability of various phenolic compounds as mediators in peroxidase catalyzed oxidation reactions. Two peroxidases from white-rot fungi, MnP from *Phlebia* sp. b19 and VP from *Bjerkandera adusta* were used in MnP reaction conditions (in the presence of Mn(II) at pH 4.5–5.5) with phenolic syringyl compounds as mediators and monomeric veratryl alcohol and dimeric adlerol resembling the predominating non-phenolic β-O-4-ether units in lignin were selected as final targets. We also developed a new type of reaction

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