International Biodeterioration & Biodegradation 127 (2018) 146–159

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Characterization of *Trametes versicolor* laccase-catalyzed degradation of estrogenic pollutants: Substrate limitation and product identification

Samuel Beck[1], Ellie Berry, Sarah Duke, Alex Milliken[1], Hannah Patterson[1], Deborah L. Prewett[1], T. Carter Rae, Varsha Sridhar, Nils Wendland, Brian W. Gregory**, Corey M. Johnson*

Samford University, Howard College of Arts and Sciences, Department of Chemistry and Biochemistry, 800 Lakeshore Drive, Birmingham, AL 35229, United States

ARTICLE INFO

Keywords:
Laccase
Biodegradation
Bioremediation
Wastewater
Estrogen
BPA
Endocrine-disrupting compounds

ABSTRACT

Laccase, the multi-copper oxidase from *Trametes versicolor (Tv*Lcc), is of interest in wastewater decontamination applications due to its ability to oxidize and remove the estrogenic activity associated with a number of pollutants. In this paper, the known estrogenic pollutants, 17α-ethinylestradiol (EE2), β-estradiol (E2), bisphenol-A (BPA) and bisphenol-S (BPS), were submitted to *in vitro* incubations with pure *Tv*Lcc. In the presence of the enzyme, EE2, E2 and BPA all formed a precipitate product, whereas no precipitate was observed in incubations with BPS. Electrospray ionization mass spectrometry identified the precipitate oxidation products as oligomers, consistent with a mechanism involving initial enzymatic production of phenoxy radicals and step-wise oligomer formation via radical coupling. Specifically, BPA, EE2 and E2 products reveal mass spectral evidence of oxyphenylene linkages and oligomer degradation. While the initial rate for BPA oxidation was faster than that for EE2 or E2, no significant rate was observed for BPS incubations. The comparable size and geometry of BPA and BPS indicate that geometric and steric complementarity are not the primary predictors of their reactivity with *Tv*Lcc. Reactivity differences for such species more likely reside in dissimilarities between their standard redox potentials compared to that for the T1 copper of *Tv*Lcc. Cyclic voltammetry supports this notion, indicating that BPS oxidation occurs at a potential nearly 300 mV more positive than that for BPA. Together, these data better define which estrogenic pollutants can be oxidized by *Tv*Lcc in bioremediation applications.

1. Introduction

Laccase (EC 1.10.3.2) is a multi-copper oxidase that catalyzes the one-electron oxidation of four reducing substrate molecules concomitant with the four-electron reduction of molecular oxygen to water (Reinhammar and Malstrom, 1981; Solomon et al., 1992). In fungi, laccase (along with lignin peroxidase and manganese peroxidase) plays a major role in the biodegradation of lignin, a prevalent, polyphenolic structural polymer in plants (Coll et al., 1993). Since laccase is the first lignolytic enzyme secreted by the fungus, its putative function is to rapidly produce low molecular weight radicals via one-electron oxidation of the lignin phenols (e.g., phenoxy radicals), which then act as redox mediators or co-factors in the delignification process (Kawai et al., 1999; Maciel and Silva, 2010). Compared to the non-phenolic subunits comprising most of the lignin structure, lignin phenols exhibit redox potentials that more closely coincide with those of the type 1 (T1) Cu site in fungal laccases and are therefore more readily oxidized than the non-phenolic subunits. The production of small, soluble radical species facilitates the delignification process, since they can penetrate the structurally complex, bulky lignin structure (which itself has difficulty interacting with the enzyme active site due to steric hindrance) and oxidize the non-phenolic subunits by H-atom abstraction (ten Have and Teunissen, 2001; Astolfi et al., 2005; d'Acunzo et al., 2006). While lignin is the natural substrate, a broad range of acceptable substrates (primarily phenolic lignin model compounds) have been demonstrated for laccase (Xu, 1996).

Structural data indicate the reason for the lack of substrate binding specificity (Piontek et al., 2002). The shallow T1 copper ion, which oxidizes the reducing substrate, is only about 6.5 Å below the enzyme surface. In addition, the cavity containing the T1 copper is wide, and phenolic substrates do not appear to bind tightly to the site (Bertrand et al., 2002). Laccase is therefore a unique enzyme, in that the

* Corresponding author.
** Corresponding author.
E-mail addresses: becksam@tamu.edu (S. Beck), eberry1@samford.edu (E. Berry), sduke@samford.edu (S. Duke), amilliken623@gmail.com (A. Milliken), hannahepatterson@gmail.com (H. Patterson), deblprew@gmail.com (D.L. Prewett), trae@samford.edu (T.C. Rae), varsha_s@bellsouth.net (V. Sridhar), nwendlan@samford.edu (N. Wendland), bwgregor@samford.edu (B.W. Gregory), cjohnso9@samford.edu (C.M. Johnson).
[1] Participated in Samford University Research Experience for Undergraduates (REU), National Science Foundation.

https://doi.org/10.1016/j.ibiod.2017.11.020
Received 28 August 2017; Received in revised form 21 November 2017; Accepted 21 November 2017

geometric complementarity and chemical properties of the active site are not the primary determinants of an acceptable substrate. Prior studies indicate that suitability is principally determined by the redox potential difference between the substrate and the T1 copper of the enzyme (Xu, 1996; Solomon et al., 1996; Astolfi et al., 2005; d'Acunzo et al., 2006). The redox potentials of the T1 copper for fungal laccases (0.5–0.8 V vs. NHE) (Solomon et al., 1996; Jönsson et al., 1995; Xu et al., 1998) facilitate the one-electron oxidation of various phenol derivatives (and some non-phenolic compounds) so long as the electrochemical potential of the substrate is proximate. Numerous mono- and di-substituted phenol derivatives exhibit half-wave potentials (0.4–0.9 V vs NHE) that overlap well with the redox potential range of fungal laccases (Suatoni et al., 1961). The use of suitable mediators, such as 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), allows one to extend the selection of oxidizable substrates even to non-phenolic compounds outside this potential range (ten Have and Teunissen, 2001; Astolfi et al., 2005; d'Acunzo et al., 2006). In this regard, electrochemical methods may be used to determine which substrates can be oxidized by a given laccase, even in the absence of suitable mediators.

Due to its ability to oxidize a wide range of compounds, laccase is of interest in many biotechnological, environmental and industrial applications (Fisher and Fong, 2014; Hatakeyama and Hatakeyama, 2010; Lu et al., 2009; Feng et al., 2013; Luo et al., 2015; Tamagawa et al., 2006; Dong et al., 2016). One application currently in development is the removal of phenolic endocrine disrupting chemicals (EDCs) from effluents of wastewater treatment plants (DEPA, 2002; Auriol et al., 2006; Lloret et al., 2013; Mao et al., 2009; Chen et al., 2016). While laccase eliminates the estrogenic effects of some EDCs (Lloret et al., 2013), some more-recently identified pollutants have not been tested. For example, recent studies have identified bisphenol S (BPS) (Scheme 1), which is often used to replace bisphenol A (BPA) in "BPA free" products, as an estrogenic pollutant with a potency equal to or greater than BPA (Rochester and Bolden, 2015). Given the electrochemical nature of laccase activity, it would be instructive to characterize the redox behavior of BPS in relation to BPA and to test BPS as a laccase substrate.

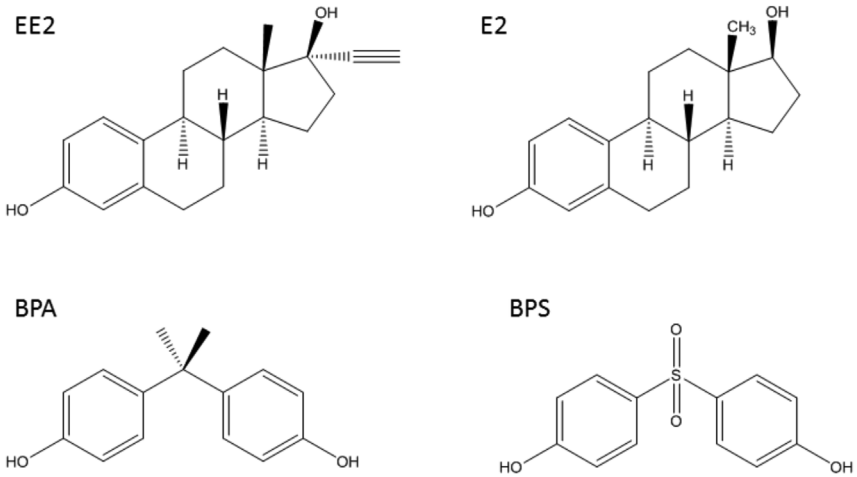
Developing methods to eliminate the estrogenic activity for these pollutants is of primary importance. While defining substrate limitations is beneficial, it is also instructive to identify the oxidized products of these reactions. Insoluble products can be separated by simple filtration and are less of a risk to be converted back to an active hormone. On the other hand, some soluble products may themselves be estrogenic (Cajthaml et al., 2009), or be converted to an active estrogen in the presence of bacteria or other microorganisms (Johnson and Sumpter, 2001). Previous studies have identified several degradation products of EE2 and BPA in the presence of a laccase (Daâssi et al., 2016; Rozalska et al., 2015). Mass spectral data for 17α-ethinylestradiol (EE2) and β-estradiol (E2) oxidation products (catalyzed by *Myceliophthora thermophila* laccase) were reported to form dimers and trimers of the oxidized monomer (Lloret et al., 2013). BPA oxidation products (catalyzed by *Trametes villosa* laccase) have also been reported as oligomers (as large as a heptamer) in an insoluble fraction of the reaction mixture (Uchida et al., 2001). Degradation of the phenolic oligomers to yield 4-isopropenylphenol was also observed in this study.

In this work, the four common phenolic EDCs shown in Scheme 1 were examined as reducing substrates for *Trametes versicolor* laccase (*Tv*Lcc). To the authors' knowledge, one of these EDCs (BPS) has never been tested as a laccase substrate. Electrospray ionization mass spectrometry (ESI-MS) was used to identify the oxidation products of each reaction. Interestingly, the initial rate of *Tv*Lcc-catalyzed oxidation was highest for BPA, while no reaction was observed for BPS. It has been demonstrated for lignin peroxidase that binding distance between the substrate's phenolic proton and the active site catalytic residue plays an important role in substrate reactivity (Mao et al., 2011). However, in this instance such dissimilarities in reactivity do not seem to be attributable to cofactor proximity, as both species are structurally alike in both size and shape. To better define the redox characteristics of these two substrates, cyclic voltammetry was used to examine their electrochemical behavior. In short, voltammetric data indicates BPS oxidation occurs at a potential that is nearly 300 mV more positive than that for BPA; this difference appears to be large enough to prohibit its oxidation by *Tv*Lcc. These findings are consistent with the prior notion that substrates for laccase-catalyzed reactions are primarily determined by the redox potential difference between the substrate and the T1 copper of the enzyme, so long as steric considerations are unimportant (Xu, 1996; Solomon et al., 1996; Astolfi et al., 2005; d'Acunzo et al., 2006). Together, the kinetic, mass spectral, and electrochemical data provide more detail on the products generated by *Tv*Lcc and better define which estrogenic pollutants can be oxidized by the enzyme.

2. Materials and methods

2.1. Chemicals

Trametes versicolor laccase, ≥ 0.5 U/mg (*Tv*Lcc, Sigma-Aldrich), 17α-ethynylestradiol (EE2, Sigma-Aldrich, ≥98%), β-estradiol (E2, Sigma-Aldrich, ≥98%), bisphenol A (BPA, Sigma-Aldrich, ≥99%), bisphenol-S (BPS, TCI, > 98%), 2,6-dimethoxyphenol (DMP, Sigma-Aldrich, 99%), ammonium acetate (Sigma-Aldrich, ≥98%), and ethanol (Sigma-Aldrich, absolute, > 99.5%) were used as received. All solutions were prepared in ultrahigh purity water (resistivity = 18.2 MΩ-cm) provided by a Millipore Synergy water purification system, or mixtures of ultrahigh purity water with ethanol.



Scheme 1. Structures of the estrogenic pollutants tested.