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Characterization of catechol derivative removal by lignin peroxidase in aqueous mixture

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ABSTRACT

The use of lignin peroxidase (LIP) as an alternative method for the removal of four catechols (1,2-dihydroxybenzene): catechol (CAT), 4-chlorocatechol (4-CC), 4,5-dichlorocatechol (4,5-DCC) and 4-methylcatechol (4-MC) typical pollutants in wastewater derived from oil and paper industries, was evaluated. The removal of 2 mM catecholic substrates by 1 μM LIP after 1 h was in the following order: 4,5-DCC (95%) > 4-CC(90%) > CAT(55%) > 4-MC(43%). Except for 4-MC, all reactions were accompanied by the formation of insoluble products, leading to LIP precipitation. LIP was exposed to soluble or insoluble product-dependent inactivation, depending on the substrates tested, immediately at the start of the reactions. Despite immediate enzyme inactivation, removal of catecholic substrates continued, resulting in oligomeric product formation. Major oxidation products analyzed were compatible with dimeric, trimeric and tetrameric structures. Ether linkages and a benzoquinone structure were detected in two purified oligochlorocatechols.

Catechol derivatives removal initiated by LIP, seems to be different for each catecholic substrate in terms of substrate consumption and transformation, and of enzyme activity.

1. Introduction

Phenol-polluted wastewater is commonly produced by a number of industrial and agricultural activities. Phenolic compounds and their derivatives, e.g. catechols, are considered priority pollutants because they are harmful to living organisms even at low concentrations. In humans and mammals, catechols can occur as metabolites in the degradation of estrogens or in endogenous compounds, such as neurotransmitters and their precursors. In nature, catechols are formed as intermediary products of the degradation of aromatic compounds by microorganisms. Catechol is used in a variety of applications: as a reagent for photography, in rubber and plastic production and in the pharmaceutical industry. Substituted catechols, especially chlorinated and methylated ones, are by-products in pulp and oil mills (reviewed in Schweigert et al., 2001). If catechols are released into the environment, they can accumulate in the soil, groundwater, and surface water, and they have therefore become an issue of great environmental concern.

Removal of these compounds from wastewater can be addressed by conventional remediation methods (e.g., solvent extraction, chemical oxidation and adsorption to activated supports) (Freeman and Harry, 1995). Although effective, some of these methods present a number of disadvantages, such as high cost, time-consuming procedures and formation of toxic residues.

Biological technologies dealing with the use of oxidoreductive enzymes, e.g. laccases, tyrosinases and peroxidases, may offer an efficient alternative means of addressing the clean-up of phenol-polluted wastewater (Shuttleworth and Bollag, 1986; Adam et al., 1999; May, 1999; Regalado et al., 2004). Oxidoreductases can catalyze the transformation of several phenolic compounds through an oxidative-coupling reactions. This results in the formation of less soluble high-molecular-weight compounds that can be easily removed from water by sedimentation or filtration (Gianfreda et al., 2003).

The initial oxidation or removal of a wide range of toxic phenols by these enzymes has already been shown. For example, oxidation of phenol by tyrosinase (Ikehata and Nicell, 2000), horseradish peroxidase (HRP) (Wagner and Nicell, 2002) and soybean peroxidase (SBP) (Bassi et al., 2004; Caza et al., 1999; Kinsley and Nicell, 2000; Wilberg et al., 2002; Wright and Nicell, 1999) chlorophenols

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and catechols by laccase (Aktas et al., 2003; Bollag et al., 2003; Gianfreda et al., 2003; Park et al., 1999), HRP (Park et al., 1999; Wagner and Nicell, 2002; Ward et al., 2004), SBP (Bassi et al., 2004; Dubey et al., 1998; Wright and Nicell, 1999) and tyrosinase (Park et al., 1999; Wada et al, 1993); methylphenols by laccase (Ghosh et al., 2008), HRP (Wagner and Nicell, 2002) SBP (Caza et al., 1999; Wright and Nicell, 1999) and tyrosinase (Wada et al, 1993); methoxyphenols by laccase (Lante et al., 2000) and tyrosinase (Wada et al, 1993), and bromophenols by HRP (Levy et al., 2003).

Lignin peroxidase (LIP) is considered one of the most important enzymes of the extracellular lignin degradation system secreted by the white-rot fungus, *Phanerochaete chrysosporium* (Hatakka, 1994). LIP possesses a higher redox potential than any other peroxidase or oxidase (Hammel et al, 1986; Kersten et al., 1990) and has been reported to oxidize aromatic compounds with calculated ionization potential (IP) values of up to 9.0 eV (Have et al., 1998; Ward et al., 2003a). LIP is of interest in wastewater treatment processes and in catalyzing difficult chemical transformations. It is also of agricultural and environmental importance on account of its role in lignin biodegradation (Tien and Kirk, 1988). Phenol (Chung and Aust, 1995a), chloro- and bromophenols (Ward et al., 2002, 2003b), and guaiacol (Koduri and Tien, 1995) are some of the aromatic substrates that have been studied for their oxidation by LIP for bioremediation purposes.

As catechols are widely distributed, and considering the advantages of LIP relative to other peroxidases and oxidases, we set out to perform a study of the *in vitro* LIP-catalyzed removal of toxic catechol and halogenated and methylated catechols, in order to assess LIP's potential for the treatment of catechol-polluted wastewater.

2. Methods

2.1. Materials

Catecholic substrates: catechol (CAT), 4-methylcatechol (4-MC), 4-chlorocatechol (4-CC) and 4,5-dichlorocatechol (4,5-DCC) and hydrogen peroxide (H_2O_2) (30% solution) were all obtained from Sigma–Aldrich (Rehovot, Israel). The concentration of stock solutions of H_2O_2 was determined at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

2.2. LIP purification

LIP isoenzyme H1 was produced from low nitrogen cultures of *P. chrysosporium* Burds BKM-F-1767 (ATCC 24725) as previously described (Rothschild et al., 1997). The enzyme was purified by MonoQ (Dosoretz et al., 1990), using a 0.01–1 M sodium acetate gradient at pH 6.0. The purified enzyme had an RZ (A409/A280) value >4. LIP concentration was determined at 409 nm using an extinction coefficient of 169 mM^{-1} cm^{-1}.

2.3. Substrate consumption studies

All reactions were carried out in 1 ml 50 mM sodium tartrate buffer, pH 3.5 at 25 °C. Substrate consumption studies were conducted using 1 μM LIP, 2 mM substrate and 0–4 mM H_2O_2. To prevent H_2O_2-dependent enzyme inactivation, optimal H_2O_2 concentration was added stepwise in aliquots of 250 μM at 1.5 min intervals in the absence or presence of 0.04% gelatin (Ward et al., 2002), known to suppress product-dependent inactivation (Nakamoto and Machida, 1992). One hour after the reactions were initiated an equal volume of acetonitrile (ACN) was added in order to stop the reactions (Ward et al., 2002). To determine the level of oxidation, the remaining substrate was determined by reverse phase high performance liquid chromatography (RP-HPLC).

HPLC analyses were conducted in a Hewlet Packard HPLC (HP 1100, Waldbronn, Germany) provided with a diode array detector.

A Lichrospher 100 RP-18 column (25 cm × 5 mm i.d., 5 μm; Merck, Darmstadt, Germany) was employed. All solvents were of far UV quality HPLC grade purity where available. Elution was performed using a gradient system modified from a previously described method (Waldron et al., 1996). The gradient profile consisted of a solvent A (10% v/v aqueous ACN and 0.1% formic acid) and solvent B (40% v/v aqueous methanol, 40% v/v aqueous ACN and 0.1% formic acid) in the following program: initially, 100% A; linear gradient over 15 min to 100% B; held isocratically at 100% B for a further 5 min, and then linear gradient over 7 min to 100% A. The flow rate was maintained at 1 mL/min. Peak detection was at 210, 280, 400 and 500 nm. Catechols were quantified by integration of peak-areas at 280 nm, with reference to calibration, which were made using known amounts of catechol and derivatives.

2.4. LIP follow-up during substrate consumption reactions

Follow up of LIP was done by measuring residual activity and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

2.4.1. Residual activity

Residual LIP activity was tested on 100 μL samples taken from reactions that were incubated for 5 min at 25 °C. Gelatin, when added was at a concentration of 0.04% (Ward et al., 2002). Residual LIP activity was assayed by monitoring the oxidation of veratryl alcohol (VA) at 310 nm in 1 mL mixtures consisting of 2 mM VA, 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5 (Tien and Kirk, 1988). A 500 μL aliquot was also withdrawn from reactions and immediately added to an equal volume of ACN to quench the reaction. Remaining substrate was then determined by RP-HPLC.

2.4.2. SDS–PAGE

One and twenty four hours catechols reaction mixtures (1.5 ml) were centrifuged for 5 min at 7000 rpm. Supernatants and sediments were separated, frozen at 80 °C, freeze-dried and evaporated to dryness. The dried samples were redissolved in double distillated water to reach 4 μg protein, and loaded. SDS–PAGE was performed in gels containing 12% acrylamide according to Laemmli (1970), using Bio-Rad Mini Protean II gel electrophoresis system. Pre-stained protein marker (BioLabs®, Petach-Tikva, Israel) was used as molecular weight marker. LIP was stained with Coomassie brilliant blue (Sigma-Aldrich, Rehovot, Israel).

2.5. Analysis of polymerization products from catechol derivative

Reactions containing 6 mM catecholic substrates, 1 μM LIP and 4 mM H_2O_2 (added in aliquots of 250 μM at 1.5 min intervals) were carried out for gel-permeation and mass spectrometry (MS) (3 ml) and fourier transform infrared (FTIR) analyses (25 ml). The reactions mixtures were incubated for 24 h and then harvested as detailed below.

2.5.1. Molecular weights determination by gel-permeation chromatography

Reaction mixtures were centrifuged for 10 min at 12,000 rpm. Supernatants and sediments were separated, frozen at 80 °C, freeze-dried and evaporated to dryness. The dried fractions were 3-fold concentrated in tetrahydrofuran (THF), filtered through 0.45 μm nylon filter and injected to gel-permeation column.