Transformation of catechol in the presence of a laccase and birnessite

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Abstract

The transformation of naturally occurring phenols to humic polymers through oxidative coupling reactions may involve oxidoreductive enzymes and soil minerals as catalysts. There is limited information on the possible inhibitory or synergistic interactions between oxidoreductases and mineral catalysts as they participate in oxidative coupling of phenolic substrates. In this study, a ternary system was investigated, in which a fungal enzyme (Trametes villosa laccase), birnessite (d-MnO2), and a naturally occurring phenolic compound (catechol) were reacted together to model soil processes. Binary systems (catechol/laccase and catechol/birnessite) were included for comparison. In the absence of the mineral, T. villosa laccase (950 katal ml−1) transformed 31% of catechol, whereas birnessite (1 mg ml−1) in the absence of the enzyme showed a 24% catechol transformation. The percentages of catechol transformation in the binary systems did not accumulate in the ternary system; instead, birnessite and laccase tested together transformed only 36% of catechol. This suggested that birnessite had an inhibitory effect on substrate transformation by laccase catalysis. Enzyme assays indicated that inhibition was a result of enzyme deactivation by humic-like polymers produced by birnessite, and by Mn2+ ions released from the mineral. These observations underscore the importance of considering enzyme-soil mineral-organic matter interactions in studies of humus formation and contaminant removal.

Keywords: Birnessite; Catechol transformation; Laccase; Ternary systems

1. Introduction

Phenolic compounds occurring in soil environments are subject to oxidative coupling, a reaction that is important in humus formation (Bollag, 1992). The underlying mechanism of oxidative coupling is the oxidation of phenolic substrates to free radicals or quinones that subsequently undergo polymerization or binding to the existing humus (Dec and Bollag, 2000). Phenol oxidation may be mediated by oxidoreductive enzymes (e.g. laccase, peroxidase, tyrosinase) or abiotic catalysts (Bollag, 1992; Fillazzola et al., 1999; Naidja et al., 1999; Dec and Bollag, 2000). Birnessite is among the abiotic catalysts in soil that participate in oxidation, reduction, hydrolysis, polymerization, and complexation of many organic compounds, including catechol (McBride, 1987; Zhang and Huang, 2003). Previous studies demonstrated the formation of humus-like polymers in the presence of this mineral (Shindo and Huang, 1984). Substrates tested for birnessite-mediated oxidative coupling reactions include hydroquinone, resorcinol, catechol, or pyrogallol (Shindo and Huang, 1984; Kung and McBride, 1988; Naidja et al., 1999; Majcher et al., 2000; Wang and Huang, 2000a,b). Polyphenols involved in these reactions underwent partial ring cleavage with release of CO2, as they formed coordinate complexes with mineral surfaces via hydroxyl groups at the adjacent carbons (Shindo and Huang, 1984; Majcher et al., 2000; Wang and Huang, 2000a; Dec et al., 2001). Another effect of birnessite-mediated oxidative coupling reactions was substituent release (decarboxylation, demethoxylation and dehalogenation) from phenolic substrates (Dec et al., 2001). Similar substituent release was observed for reactions mediated by various oxidoreductases, including laccase (Dec et al., 2001).

To date, functional interrelationships of soil components have mostly been tested for binary systems (enzyme–clay mineral, enzyme–organic substrate, or clay mineral–organic

substrate). Ternary systems were studied by Claus and Filip (1988); Gianfreda and Bollag (1996), and Naidja et al. (1997), who investigated the activity of various oxidoreductases immobilized on clay surfaces and clay with hydroxylaluminum coatings.

The objective of this study was to investigate interactions of a fungal laccase (from Trametes villosa), birnessite, and a naturally occurring humic monomer (catechol) as interactive components of a model ternary soil system. Catechol (1,2-dihydroxybenzene) is a soil humic constituent, a plant polyphenol, and an intermediate of microbial metabolism of anthropogenic organic compounds (Majcher et al., 2000). Manganese oxides are common in soils and sediments, and birnessite is one of the most common forms (McKenzie, 1971; Sparks, 1995). The investigation of a model ternary system involving the above components was expected to reveal possible inhibitory or synergistic effects related to catalytic actions of birnessite and laccase, as they participate in humification processes.

2. Materials and methods

2.1. Reaction components

Birnessite was prepared according to the method of McKenzie (1971). Briefly, two moles of concentrated HCl was added dropwise to a boiling solution of 1.0 mol of KMnO₄ in 2.5 l of deionized water with vigorous stirring. After boiling for an additional 10 min, the precipitate was filtered and washed several times with deionized water, then freeze-dried. Analysis of birnessite, by X-ray diffraction and infrared spectroscopy showed the poorly crystalline structure, with characteristic peaks matching those reported in the literature (McKenzie, 1971; McBride, 1987; Dixon and Weed, 1989).

The X-ray powder diffraction patterns were obtained using a PADV X-ray diffractometer with a Co Kα radiation source (Scinitag, Inc., Cupertino, CA). Infrared absorption spectra were obtained using a Nexus 670 Fourier Transform Infrared Spectrophotometer (Thermo Electro Co., Waltham, MA), with 1.0 mg samples of the minerals dispersed in KBr (99.0 mg) pellets analyzed at a range of 4000–1000 cm⁻¹. The specific surface areas of birnessite was 27.7 m² g⁻¹, as determined by N₂ sorptometry on an ASAP 2021 sorptometer (Micrometrics, Londonberry, NH) from the linear segment of the multi-point N₂ adsorption isotherms according to the Brunauer–Emmett–Teller theory (BET; Brunauer et al., 1938).

Catechol was purchased from Aldrich Chemical Co. (Milwaukee, WI). Uniformly ring-¹⁴C-labeled catechol with specific activity of 3.4 mCi mM⁻¹ was purchased from Sigma Chemical Co. (St Louis, MO). Extracellular laccase (T. villosa, EC 1.10.3.2) was obtained from Novo Nordisk (Danbury, CT).

2.2. Laccase activity

The activity of laccase (in katals) was determined using a biological oxygen monitor (Model 5300, Yellow Spring Instruments Co., Yellow Springs, OH) equipped with a Clark oxygen electrode. A specific amount of enzyme was added under continuous agitation with a Teflon magnetic bar to the thermostatic (25°C) sample chamber filled with 0.1 M citrate–phosphate buffer (2.7 ml, pH 3.8). The enzymatic reaction was initiated by the addition of 0.1 ml of 10 mM 2,6-dimethoxyphenol (DMP) solution in the same buffer (stored previously at 25°C) through a side port next to the electrode. Oxygen consumption was monitored for two minutes. One katal of enzymatic activity was defined as the amount of laccase required to consume 0.017 mmol s⁻¹ of O₂. The effect of birnessite on laccase activity was tested by adding specific amounts of the mineral into the reaction mixture at a range from 0 (controls) to 37 mg ml⁻¹.

The inhibition of laccase by free Mn²⁺, was determined by amending the reaction mixture with MnCl₂. The concentration of metal ions ranged from 0 (control) to 5.0 mM. Negligible oxygen consumption was determined for metal ions in controls without laccase.

The kinetics of laccase in the absence and presence of MnCl₂ (0–16 mM MnCl₂) was evaluated using the Michaelis–Menten model: \( V = \frac{V_{\text{max}}S}{K_m + S} \), where \( V \) was the reaction velocity (absorbance change min⁻¹) and \( S \) was the substrate concentration (mM). Kinetic parameters (\( K_m \) and \( V_{\text{max}} \)) were calculated by fitting kinetics data to the Michaelis–Menten equation. The reaction velocity was measured colorimetrically on a Model UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) using DMP in 2.7 ml of 0.1 M citrate–phosphate buffer at pH 3.8, as a substrate at concentrations ranging from 0.023 to 0.375 mM (the respective \( S^{-1} \) values ranged from 0 to 50). The reaction velocity (\( V \)) was expressed as the increase in absorbance min⁻¹ at 468 nm.

2.3. Catechol transformation

To determine substrate transformation in the ternary model system, various combinations of birnessite (0–5 mg ml⁻¹) and/or laccase (0–3800 katal ml⁻¹) were incubated for 24 h at 25°C with a 0.1 M solution of uniformly ring-labeled ¹⁴C-catechol (2.1×10⁵ Bq kg⁻¹) in 0.5% NaCl (1 ml) and centrifuged. The supernatants were analyzed by high-performance liquid chromatography (HPLC) for remaining catechol, and radiocounting for soluble transformation products. The pellets (complexes of birnessite with catechol and/or its metabolites) were analyzed by radiocounting.

To determine laccase inhibition by humic-like polymers produced by birnessite a two-stage experiment was carried out. In the first stage, ¹⁴C-catechol (25 mM) was incubated with birnessite (2 mg 1⁻¹) in 0.5% NaCl to generate catechol transformation products. In the second stage, 0–1 ml samples of the resulting supernatant, containing soluble transformation products (equivalents of 0–2.75 mg ml⁻¹, calculated on the basis of radioactivity remained in the solution) with no unaltered catechol, were added to a fresh binary reaction mixture consisting of ¹⁴C-catechol (0.1 M) and laccase (1800 katal ml⁻¹), and incubated for 24 h. After incubation, the samples were centrifuged, and the supernatants were analyzed for remaining catechol by HPLC and radiocounting.
2.4. Adsorption of laccase on birnessite surfaces

To determine laccase adsorption isotherms, 5 mg of birnessite was mixed with 10-ml volumes of 0.5% NaCl aqueous solutions of the enzyme (0–14.5 ± 0.5 μg ml⁻¹) and shaken for 24 h at 25 °C. At the end of the equilibration period, the suspensions were centrifuged at 17,000×g for 10 min and the supernatants were analyzed by a protein assay. The Langmuir model, $q = X_mQK/(1 + QK)$, where $q$ and $Q$ are the sorbed and solution concentrations, respectively, was used to fit sorption data. The maximum sorbed concentration ($X_m$) and adsorption equilibrium constant ($K$) were determined by non-linear regression.

2.5. HPLC, radiocounting and protein assays

Before HPLC analysis, the supernatants from catechol transformation and adsorption isotherm experiments were filtered through a 0.45-μm nylon membrane. The HPLC analysis was conducted on a Waters HPLC system (Milford, MA) consisting of a 2690 Alliance separation module, a Waters 2487 dual λ absorbance detector operating at 280 nm, and a Supelcosil 15 cm×4.6 mm LC-18 DB column of 5-μm particle size with a LC-18 DB guard column (Supelco, Bellefonte, PA). The mobile phase, delivered isocratically at a flow rate of 1.0 ml min⁻¹, was composed of an aqueous component A (Milli-Q water) and an organic component B (methanol) mixed at a 80:20 A to B ratio. The detection limit of catechol was 0.1 μg ml⁻¹. Radioactivity measurements were conducted on a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, IL). Aliquots of the supernatant (200 μl) were analyzed in 4.5 ml of Ecoscint (Manville, NJ). Birnessite pellets were combusted in a Biological Oxidizer OX 600 (R.J. Harvey Instruments, Hillsdale, NJ) to ¹⁴CO₂, which was trapped in Harvey Carbon-14 Cocktail for scintillation counting as above.

Protein assays were performed using Bio-Rad reagent to obtain colored protein complexes (Gogstad and Krutnes, 1982). The calibration curve was prepared using bovine serum albumin as the standard protein. The colorimetric measurements were carried out on a Model UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) at 595 nm. The amount of protein adsorbed on birnessite was calculated as the difference between the amount of protein initially added and the amount remaining in the supernatants after incubation.

3. Results

The effects of birnessite amount and laccase activity on catechol removal are presented in Fig. 1. Based on the HPLC analysis of the binary systems, the percentage of catechol removal increased from 3 to 100% as the amount of birnessite was increasing from 0 to 5 mg ml⁻¹ (Fig. 1A). With increasing laccase activity (from 0 to 3800 katal ml⁻¹), catechol removal increased from 3 to 94%. In the ternary system, birnessite (1 mg ml⁻¹) and laccase tested together removed 36% of catechol, as compared to a 24% removal for birnessite tested in the absence of laccase, and to a 31% removal for laccase tested in the absence of birnessite (Fig. 1C). Fig. 1D shows the recovery of radiolabeled carbon from ¹⁴C-catechol, as determined by scintillation counting analysis of both the binary and ternary systems. Less than 100% of radioactivity recovery may be due to CO₂ evolution during the catechol transformation. As determined by radiocounting and HPLC of the ternary system (Fig. 1D), 90% of the initial radioactivity was present in the supernatant, in the form of unaltered catechol (64%) and soluble reaction products (26%). Only 2%
was associated with the birnessite pellet, which indicated that adsorption of both catechol and its reaction products on birnessite was very limited.

Laccase activity (measured polarographically) decreased quickly (from 100 to 15%) with increasing amounts of birnessite (from 0 to 37 mg ml\(^{-1}\)) (Fig. 2A). As determined by protein assays, small amounts of laccase adsorbed to birnessite (\(X_m=0.52 \text{ mg ml}^{-1}\)) (Fig. 2B). The adsorption data were fit well by the Langmuir equation, showing a low adsorption equilibrium constant (\(K=0.3 \text{ mg ml}^{-1}\)).

Birnessite-generated products of catechol transformation had a mixed effect on laccase-mediated catechol transformation (Fig. 2C). At low concentrations (0–0.08 mg ml\(^{-1}\)), catechol transformation products showed no inhibitory effect. At intermediate concentrations of 0.14 and 0.28 mg ml\(^{-1}\), the products had a stimulatory effect, causing an increase in catechol transformation from 72% for the unamended reaction mixture to 85 and 90%, respectively (Fig. 2C). Increasing the concentration of the products to 0.55 and 1.38 mg ml\(^{-1}\) resulted in a return to the non-effect level (about 70% catechol transformation), and further increase in the product concentration (to 2.75 mg ml\(^{-1}\)) caused a 20% reduction of catechol transformation (to 50%) as compared to the non-effect level.

Laccase activity was severely inhibited by the presence of Mn\(^{2+}\) ions, decreasing from 100% in the absence of Mn\(^{2+}\) to 30%, as metal ion concentrations were increased to 5 mM (Fig. 2D). The Michaelis–Menten analysis of kinetics data (Table 1) for laccase-mediated substrate transformation showed increasing \(K_m\) values (from 0.07 to 1.70 mM) and decreasing \(V_{\text{max}}\) values (from 3.41 to 1.90 min\(^{-1}\)) with increasing concentrations of Mn\(^{2+}\) ions (from 0 to 16 mM).

### 4. Discussion

Since, both metal oxides and enzymes coexist in soils (Stotzky and Burns, 1982; Burns, 1990; Huang, 2000), one can expect the occurrence of synergistic or inhibitory effects on the rate and extent of catalytic reactions leading to humification. This research indicated that birnessite may have an inhibitory effect on soil enzymes. The total catechol transformation in our model ternary system of catechol, laccase, and birnessite, was less than the sum of catechol transformed by laccase and birnessite separately (Fig. 1C).

The inhibition of laccase activity could have occurred through one or more of the following three
mechanisms: (1) enzyme inactivation by birnessite-generated humic-like polymers, (2) enzyme immobilization (adsorption) on the mineral surfaces, and (3) enzyme inactivation by Mn$^{2+}$ ions released from the mineral as the reaction proceeded. Birnessite is very effective in oxidizing phenolic compounds, and it forms dark humic-like polymers with a high degree of humification (Shindo and Huang, 1984; Wang and Huang, 2000b) that may efficiently deactivate enzymes. Birnessite can also mediate ring cleavage in catechol and other polyphenols (Majcher et al., 2000). Therefore, humic-like polymers produced by birnessite tend to have more aliphatic groups than those produced by other minerals. The increased aliphaticity of the reaction products may be a factor in inhibiting enzyme activity. It is well known that soil organic matter can inhibit enzymes (Vuorinen and Saharinen, 1996), and that enzyme activity may be reduced by adsorption on humic polymers (Gianfreda et al., 1998). In this study, relatively low concentrations of humic-like polymers produced by birnessite stimulated laccase-mediated transformation of catechol (Fig. 2C). The stimulation probably resulted from binding of catechol to humic-like polymers formed during the birnessite-mediated reaction. However, at higher concentrations, humic-like polymers inhibited catechol transformation, probably due to complexation of the polymer products with the copper atom present in the active center of laccase, or from conformational changes in enzyme molecules adsorbed on the humic-like polymers. Similar patterns of initially increasing and then inhibited enzyme molecules adsorbed on the humic-like polymers. 

\[ V_{\text{max}} = \frac{V_{\text{max}}}{K_m} \]

The V$_{\text{max}}$ value increased with increasing Mn$^{2+}$ concentration, indicating that the affinity of laccase for the substrate decreased in the presence of Mn$^{2+}$. At the same time, the V$_{\text{max}}$ value decreased with increasing Mn$^{2+}$ concentration, resulting in drastically reduced V$_{\text{max}}$/K$_m$ ratios (from 48.7 to 1.1), which is a measure of diminished enzyme performance. According to Rawn (1989); Huang and Shindo (2001); Bisswanger (2002), such kinetic behavior of an enzyme indicates a mixed type of inhibition, which in the case of this study might involve a combination of a non-competitive inactivation of the enzyme by birnessite reaction products (mechanism 1), and a competitive interference of Mn$^{2+}$ ions in the formation of the substrate/enzyme complex (mechanism 3).

To summarize, birnessite tested in a model ternary system had an inhibitory effect on the transformation of catechol by laccase. As one of the most common manganese minerals, it may also affect laccase activity during humification processes in soils. The research using model ternary systems is continued to examine the effects of other common minerals, such as ferrihydrite and aluminum hydroxide, on enzymatic transformation of naturally occurring phenols.

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