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Characteristics of *Trametes villosa* laccase adsorbed on aluminum hydroxide

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Abstract

The interaction of metal oxides with extracellular enzymes may influence humus formation and bioremediation processes. In this study, short-range ordered aluminum hydroxide exhibited a strong affinity for laccase from *Trametes villosa* ($X_m = 6.18\ \mu g\ mg^{-1}$) as evidenced by an H-type adsorption isotherm. Based on oxygen uptake measurements, free laccase had only slightly greater kinetic parameters ($K_m = 0.18$ mM; $V_{max} = 14.35$ mmol O_2 min^{-1}) than those of adsorbed laccase ($K_m = 0.15$ mM; $V_{max} = 12.77$ mmol O_2 min^{-1}). Free and adsorbed laccase showed similar activities under various pH conditions (from 3.8 to 7.8) and also showed similar activity loss over time (0–30 days). Adsorbed laccase was less resistant to thermal (4–100 °C) and proteolytic degradation, but also less sensitive to inhibition by humic acid than free laccase. Attenuated total reflectance infrared (ATR-IR) spectroscopy did not show significant changes in the secondary structure of laccase due to adsorption on aluminum hydroxide. These results indicate that laccase in soil may be found predominantly complexed with short-range ordered aluminum hydroxide, and laccase activity and stability is little affected by adsorption to the mineral. Consequently, use of immobilized enzymes such as laccase on aluminum hydroxide can be considered a practical tool for soil remediation.

Keywords: Laccase; Aluminum hydroxide; Adsorption; Enzyme activity; Soil enzymes

1. Introduction

Extracellular enzymes participate in a variety of transformation reactions in soil, including humus synthesis and degradation of organic pollutants [1–3]. Phenoloxidases such as laccase, tyrosinase, or peroxidase, constitute an important group of enzymes involved in these reactions [4–7]. Extracellular laccases, including that from *Trametes villosa* (EC 1.10.3.2), can catalyze the oxidation of organic substrates with the concomitant reduction of molecular oxygen [8], and may play a role in the formation of humic polymers and transformation of soil and water organic pollutants [9,10].

Although laccases are well-studied enzymes in the laboratory, little is known of their behavior in terrestrial systems, where enzymatic activity may be modulated by adsorption on soil components [11–14]. According to recent findings [10,15], adsorption phenomena may considerably change enzyme properties, such as specific activity, substrate affinity, or thermal and pH stability. Previous studies, for instance, consistently demonstrated that immobilization on soil and soil components led to increased laccase stability [16–18]. However, in at least one case, immobilized laccase (*Rhizoctonia praticola* laccase complexed with celite) did not display increased stability compared to the free enzyme [19].

Regardless, enzymes immobilized on solid supports, such as porous glass beads, various clay minerals, or soil, have been considered for the removal of toxic xenobiotics from the environment [2,18,20–22]. For obvious reasons (low cost, availability), soil is an appealing option for enzyme immobilization. To assess this option properly, it is necessary to determine the distribution of enzyme molecules among various soil components, a difficult task, because of losses associated with extraction and purifica-

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tion of soil enzyme. If, however, enzyme affinity can be determined for each of the pure soil components, it should be possible to predict the overall effect of interaction with the soil matrix.

Major inorganic adsorbents in soil include clay minerals with well-ordered crystalline structures, and short-range ordered mineral oxides. The latter are ubiquitous in terrestrial systems. There, they exist as discrete colloidal phases, frequently coating clay surfaces and forming complexes with organic matter [23]. With their relatively high specific surface areas and charge densities, they represent the majority of reactive sites in many soils, and show great potential to adsorb extracellular enzymes [23].

The purpose of this study was to determine the extent of adsorption of laccase from *T. villosa* on short-range ordered aluminum hydroxide and to examine the effect of adsorption on: (1) laccase activity under different conditions, (2) laccase resistance to degradation by protease and exposure to humic acid, and (3) the secondary structure of the laccase molecule, using attenuated total reflectance infrared (ATR-IR) spectroscopy. In so doing, the potential of aluminum hydroxide-sorbed laccase for soil remediation may be assessed.

2. Materials and methods

2.1. Materials

Short-range ordered aluminum hydroxide was synthesized by gradual neutralization of a 0.5 M $AlCl_3$ solution to pH 7.0 using 0.5 M NaOH [24]. The suspension was aged for 48 h at room temperature and centrifuged at 10,300 × *g* for 20 min. The precipitate was washed with deionized water to completely remove Cl^- and then freeze-dried. Analysis of aluminum hydroxide by X-ray diffraction and infrared spectroscopy showed the presence of poorly crystalline structures, with characteristic peaks matching those reported in the literature [25]. The X-ray powder diffraction patterns were obtained using a PADV X-ray diffractometer with Co-Kα radiation source (Scintag, Inc., Cupertino, CA). The specific surface area of aluminum hydroxide was 297.5 $m^2\ g^{-1}$ as determined by N_2 sorptometry on a Micromeretics ASAP 2021 sorptometer (Norcross, GA) from the linear segment of the multi-point N_2 adsorption isotherms according to the Brunauer–Emmett–Teller theory (BET) [26].

Extracellular laccase (*T. villosa*, EC 1.10.3.2) was obtained from Novo Nordisk (Danbury, CT) and is likely of a single molecular form (evidence below). Protease was purchased from Sigma Chemical Co. (St. Louis, MO) and Bio-Rad reagent for protein assays was obtained from Bio-Rad Lab (Richmond, CA). Suwannee River humic acid was purchased from the International Humic Substances Society (IHSS; St. Paul, MN).

2.2. Adsorption of laccase on aluminum hydroxide

To determine laccase adsorption isotherms, 5 mg of aluminum hydroxide (1.49 m^2) was mixed with 10 mL volumes of 0.5% NaCl aqueous solution of the enzyme (0–371 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. Concentration of enzyme remaining in the supernatants was determined by colorimetric protein assay, using the Bio-Rad reagent to obtain colored protein complexes [27]. The calibration curve was prepared using bovine serum albumin as a standard, and 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 aluminum hydroxide was calculated as the difference between the amount of protein initially added and the amount remaining in the supernatants after equilibration.

Sorption data were fitted to the Langmuir model:

$$q = \frac{X_m Q K}{1 + Q K}$$

where q and Q are the sorbed and solution protein concentrations, respectively. The maximum sorbed concentration (X_m) and the adsorption equilibrium constant (K) were determined by non-linear regression.

Aluminum hydroxide–laccase complexes for activity and stability experiments were prepared by adding 5 g aluminum hydroxide to 50 mL laccase solution (0.791 mg mL^{-1}) in 0.5% NaCl. After an 18-h contact period (shaken at 50 rpm, 25 °C), mineral with adsorbed laccase was recovered by centrifugation, washed with 0.5% NaCl solution until no protein was detected in the washings, and resuspended in 0.5% NaCl before initiation of enzyme assays. Based on the protein assay, between 90% and 93% of the initially added laccase was adsorbed on aluminum hydroxide during the preparation procedure.

2.3. Laccase activity

The activity of free and adsorbed laccase was determined by measuring oxygen consumption during reaction with a substrate. This method was chosen over a spectrophotometric approach because it avoids problems associated with interference by mineral particles. Specific amounts of free or adsorbed *T. villosa* laccase (1.3–11.8 μg) were placed into a biological oxygen monitor (Model 5300, Yellow Spring Instruments Co., Yellow Springs, OH) equipped with a Clark oxygen electrode. The sealed sample chamber was under continuous agitation with a Teflon magnetic bar, thermostated (25 °C) and 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 in the same buffer (stored at 25 °C) through a side port next to the electrode. Oxygen consumption was monitored for 2 min. To determine the effect of pH on the activity of free laccase and adsorbed *T. villosa* laccase (440 unit mL^{-1}), citrate–phosphate buffer with pH ranging from 3.8 to 7.8 was used under the conditions described above. Laccase activity was defined as the amount of laccase required to consume 1 mmol min^{-1} of O_2.

The kinetics of free and adsorbed laccase (440 unit mL^{-1}) was evaluated using the Michaelis–Menten model: $V = V_{max} S/(K_m + S)$, where V is the reaction velocity (mmol O_2 min^{-1}) and S is the substrate concentration (0.089–1.071 mM). The kinetic parameters, Michaelis constant (K_m) and maximal velocity (V_{max}), represent the affinity of laccase for the substrate and the maximum reaction velocity, respectively, and were calculated by fitting kinetics data to the model.

2.4. Laccase stability experiments

The effects of aging, temperature, protease, and humic acids on free versus sorbed laccase activity were conducted using 5-mL solutions of free laccase or laccase–aluminum hydroxide suspensions containing equivalent initial laccase activity (440 unit mL^{-1} in 0.5% NaCl, pH 7.0 ± 0.1). Aging treatments ranged from 0 to 30 days at 25 °C, and temperature treatments from 4 to 100 °C for 4 h. Resistance to proteolytic activity was studied by adding protease (1 mg mL^{-1}) to solutions or suspensions and shaking for 12 h at 25 °C before activity measurement. Humic acid (1.0 mg mL^{-1}) was added to solutions or suspensions and incubated for 0–72 h before activity measurement. Samples without protease or humic acid served as controls. After each time period, 25-μL aliquots were taken from samples, and the laccase activity was measured using the biological oxygen monitor as described above. There was negligible oxygen consumption in the presence of humic acid or protease in the absence of 2,6-dimethoxyphenol. All the experiments were triplicated and results were found to agree within 95% confidence limits. Paired and unpaired *t*-tests were used to compare the activities of free and immobilized laccase. Statistical significance limits were set at $p \leq 0.01$ or 0.05.

2.5. Attenuated total reflectance infrared (ATR-IR) spectroscopy

Infrared absorption spectra were obtained using a Nexus 670 Fourier Transform Infrared Spectrophotometer (Thermo Electro Co., Waltham, MA), with 1.0 mg samples of mineral dispersed in KBr (99.0 mg) pellets and scanned through a range of 4000–1000 cm^{-1}. Three microliters aliquots of aqueous solution or suspension, containing 0.222 μg of free laccase, 1.5 mg of aluminum hydroxide, or 0.222 μg of laccase complexed with 1.5 mg of aluminum hydroxide, were loaded on a 1-mm diameter single-bounce diamond-KRS5 ATR sensor

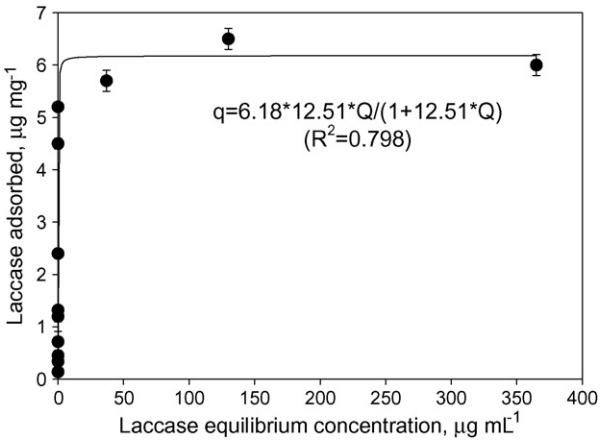

Fig. 1. Adsorption of *Trametes villosa* laccase on short-range ordered aluminum hydroxide during a 24-h shaking in 0.5% NaCl at 25 °C, as determined by UV–vis spectroscopy of colored protein complexes obtained with Bio-Rad reagent and calibrated using bovine serum albumin.

crystal (Pike Technologies, Madison, WI), and air-dried for 10 min before the initiation of spectral acquisition on a Bruker (Billerica, MA) Fourier transform infrared spectrometer. Each spectrum was the result of co-addition of 256 scans and the data were processed within the 1800–1400 cm^{-1} region of interest. The sensor background spectrum was a measurement without added specimens. The spectrum of aluminum hydroxide was subtracted (using Bruker software) from that of laccase/aluminum hydroxide complex, to obtain the differential ATR-IR spectrum of adsorbed laccase. The samples were prepared in triplicate and the reported peak position uncertainties are standard deviations.

3. Results and discussion

3.1. Laccase adsorption

The adsorption isotherm for laccase on aluminum hydroxide (Fig. 1) was well-described by the Langmuir model ($r^2 = 0.799$) with X_m and K values of 6.18 μg mg^{-1} and 12.51 mL $μg^{-1}$, respectively. The adsorption isotherm may be generalized as the extreme H-type isotherm [28], indicating that extracellular laccase is subject to strong affinity for short-range ordered aluminum hydroxide. The adsorption capacity of aluminum hydroxide is much greater than that of other non-crystalline clay minerals, such as ferrihydrite or birnessite ($X_m = 0.9$ or 0.5 μg mg^{-1}, respectively), which adsorbed about nine times less *T. villosa* laccase under the same experimental conditions [29]. The adsorption of enzymes by minerals is often observed in soil environments. The possible mechanisms of enzyme adsorption on clay minerals include cation exchange, electrostatic attraction, and hydrophobic binding [3,30,31]. According to Hsu [25], the value of the point of zero charge (PZC) – the pH at which number of positive and negative surface charges are equal [28] – for short-range ordered aluminum hydroxide is 8.0–9.2. For laccase, the PZC was reported to be 3.0–3.5 [12]. At the neutral pH that these adsorption experiments were carried out, laccase is likely strongly negatively charged due to its PZC, lower than 7, while aluminum hydroxide is positively charged due to its PZC, greater than 7. Therefore, electrostatic attraction is likely a dominant adsorption mechanism.

3.2. Laccase activity

When the kinetics data were fitted to the Michaelis–Menten model, the K_m and V_{max} values for free laccase (0.18 mM and 14.35 mmol O_2 min^{-1}, respectively) did not greatly differ from those derived for adsorbed laccase (0.15 mM and 12.77 mmol O_2 min^{-1}, respectively) (Fig. 2A). Further, free and adsorbed laccase showed similar activities over a range of enzyme amounts (1.3–11.8 μg; $t = 2.74$, $p < 0.01$) (Fig. 2B). These results are consistent with other studies showing that adsorbed enzymes may remain nearly fully catalytically active

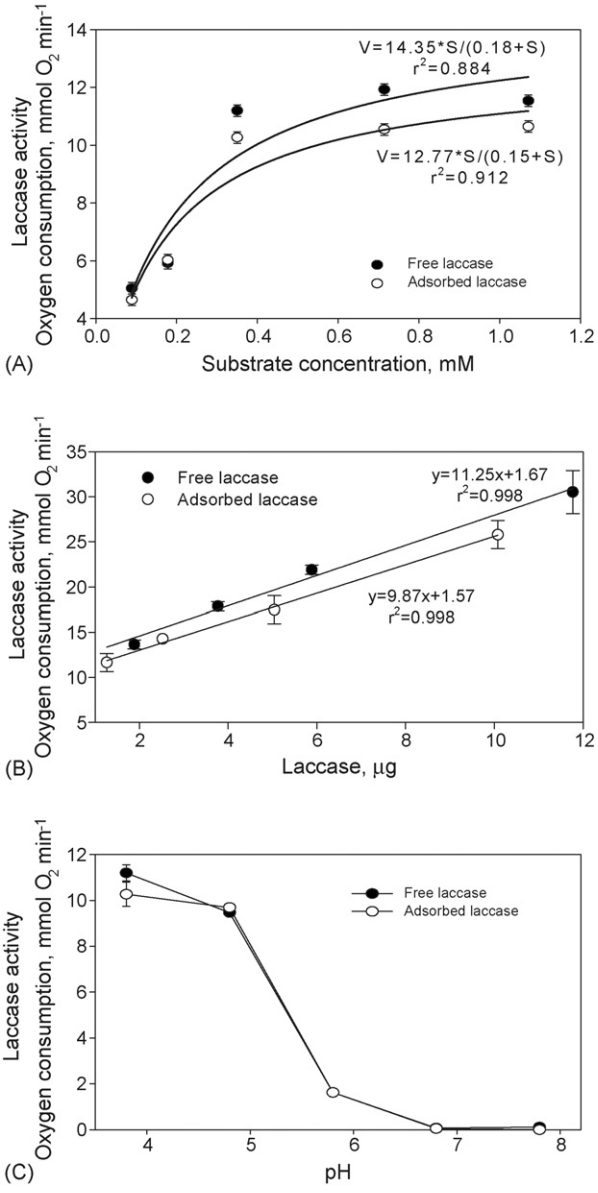

Fig. 2. Activity of free and adsorbed *T. villosa* laccase incubated at 25 °C and pH 3.8 in 2.7 mL of 0.1 M citrate–phosphate buffer (determined by a biological oxygen monitor) as a function of (A) substrate concentration (2,6-dimethoxyphenol) ranging from 0.089 to 1.071 mM (modeled by Michaelis–Menten equation) and (B) laccase concentration (1.3–11.8 μg). (C) The effect of pH on the activity of free laccase and adsorbed *T. villosa* laccase (440 unit mL^{-1}) under the same conditions. The pH was determined polarographically and ranged from 3.5 to 7.8.

[17,32]. On the other hand, adsorption onto mineral surfaces has, in some cases, been found to alter the active site structure of an enzyme, causing a decrease in V_{max} values and an increase in K_m values [3]. However, in this study, the small changes in V_{max} and K_m values for adsorbed versus free laccase over a range in laccase concentrations, suggest that the active site was essentially unaffected by mineral adsorption.

Decreased laccase activity was observed at higher pH's (pH range in 3.8–7.8, Fig. 2C), and laccase was completely deactivated at <pH 3.0 (data not shown). For example, the activity of both free and adsorbed laccase decreased from a maximum of about 11 mmol O_2 min^{-1} at pH 3.8 to about 0.1 mmol O_2 min^{-1} at pH 7.8 (Fig. 2C). However, differences between the pH–activity relationship of free versus aluminum hydroxide-sorbed laccase were minor or insignificant ($t = 0.85$, $p < 0.05$).

According to previous studies [33–35], *T. villosa* laccase displays maximal activity between pH 3.8 and 7.4, depending on the substrate and type of buffer. In this study, both free and adsorbed laccase showed maximal activity (9.5–11.2 mmol O_2 min^{-1}) between pH 3.8 and 4.8 (Fig. 2C). A commonly observed effect of enzyme adsorption on mineral surfaces is the shift of the optimal catalytic activity to either higher or lower pH values [16,36,37]. Changes in the pH of soil solution can affect the ionization state of the amino acids composing an enzyme, and the corresponding changes in the distribution of ionic bonds may alter an enzyme's structure. Consequently, the substrate recognition system may be disrupted, making the enzyme inactive [38,39]. The lack of difference in optimal pH between free and adsorbed laccase found in this study supports the conclusion that the active site of laccase is not proximal to the enzyme's bond with aluminum hydroxide.

3.3. Effects of environmental parameters on laccase stability

Decreased laccase activity was observed with increased storage times and higher temperatures (Fig. 3A and B). Aging experiments demonstrated that enzyme deactivation over 30 days followed an exponential decay model. Deactivation half-lives were 7.9 days ($r^2 = 0.970$) and 10.0 days ($r^2 = 0.966$) for free and adsorbed laccase, respectively (Fig. 3A). However, free and adsorbed laccase had similar temporal stabilities over a 30-day period at 25 °C. Some studies have indicated that mineral-adsorbed enzymes are more stable than free enzymes, losing less enzymatic activity during storage at ambient temperatures [16,17,29]. Here, we observe a lowered stability of adsorbed laccase versus free laccase (loss of 30% versus 16%, respectively) in the short term (3 days), and similar losses in stability (89 and 93% loss, respectively) over long term (30 days) storage of aluminum hydroxide-sorbed versus free laccase (Fig. 3A). Overall, the minor differences in temporal stability observed are consistent with the conclusion that the active site of *T. villosa* laccase was unaffected by laccase adsorption on short-range ordered aluminum hydroxide.

For both free and mineral-sorbed laccase, little activity remained at temperatures above 75 °C (Fig. 3B). However, significant differences in the thermal stability structure of free versus adsorbed laccase were observed ($t = 2.99$, $p < 0.05$). Free laccase showed a broad thermal stability range of 0–55 °C, while that of sorbed laccase was only 0–28 °C. The adsorbed laccase showed losses of enzymatic activity from 10.3 mmol O_2 min^{-1} at 28 °C to 5.4 mmol O_2 min^{-1} at 55 °C (52%). The adsorbed laccase showed a linear tendency in the loss of enzymatic activity from 28 °C to more than 60 °C. The deactivation energy was calculated from the deactivation rate constant values in Table 1 using the Arrhenius equation: $K_d = A_d \cdot e^{-\Delta E_d/RT}$ where K_d, ΔE_d, R, and T are the deactivation rate constant, deactivation energy, the gas constant (1.987 cal mol^{-1} K^{-1}), and temperature (K), respectively [40]. The deactivation energy for free and immobilized laccase was 25.0 kcal mol^{-1} ($r^2 = 0.953$) and 11.8 kcal mol^{-1} ($r^2 = 0.958$), respectively. Free laccase, therefore, displayed greater thermal stability than immobilized laccase.

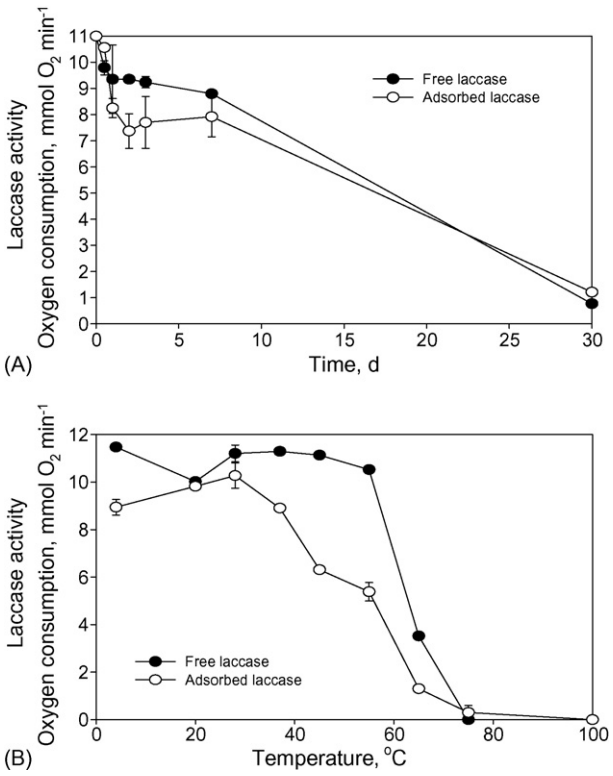

Fig. 3. The effect of (A) storage at 25 °C and (B) temperature after a 4-h incubation, on the activity of free and adsorbed *T. villosa* laccase, determined on 5-mL samples of laccase solution or suspension (440 unit mL^{-1} in 0.5% NaCl, pH 7.0 ± 0.1).

Table 1
Kinetic and thermodynamic parameters for irreversible thermal inactivation of free and adsorbed laccase from *Trametes villosa*

Temperature (K)	Free laccase		Adsorbed laccase	
	K_d (h^{-1})[a]	$t_{1/2}$ (h)	K_d (h^{-1})	$t_{1/2}$ (h)
310	0.045	127	0.343	15
328	0.238	24	1.223	4
338	1.988	3	2.243	2
348	2.867	2	2.493	2

[a] The first order rate constant for inactivation was determined by linear regression of laccase activity vs. time incubated (h).

Temperature is often claimed to negatively affect both free and immobilized enzyme activity and stability [16,32,41]. While some have found the negative effects to be more pronounced in free versus mineral-sorbed enzymes [15], other workers have suggested the opposite [3,42], while still others found no difference [43]. While enzyme immobilization may stabilize a protein against temperature-induced unfolding, it is difficult to explain why mineral-sorption would negatively affect the thermal stability of an enzyme versus that of a free enzyme. More research is required in this area.

3.4. Effects of protease and humic acid on laccase activity

Enzymes do not act as isolated molecules in the soil environment, but rather are present along with a wide range of organic compounds and compound classes originating as biotic exudations and decay products. Two such compound classes that are likely to influence soil enzyme activity and stability are proteases, enzymes that hydrolyse the peptide bonds in proteins, derived from soil microbes [44], and humic substances, the degradation products of plants. Incubations were conducted with representative members of these two classes of compounds, followed by measurement of activity of free and aluminum hydroxide-sorbed laccase to further test the efficacy of mineral-sorbed enzymes for the bioremediation of contaminants in soils.

Following a 12-h period of exposure to protease, the activity of free and adsorbed laccase decreased by 8 and 30%, respectively, relative to that of a control sample with no protease (Fig. 4A). The significantly lower stability of sorbed versus free laccase under protease attack ($t = 30.62$, $p < 0.05$) contrasts with other studies in which adsorbed enzymes were found to be significantly more stable [45,46]. While some enzymes may be protected from protease hydrolysis by occlusion within a pore on a mineral's surface [47], or repulsion of proteases by a mineral's surface charge, our experimental data suggest that the active site of aluminum hydroxide-adsorbed laccase is distal from the mineral's surface, and would thus be exposed to attack. Possible reasons for enhanced deactivation of mineral-sorbed laccase by protease include the attraction of protease to the surface of aluminum hydroxide, enhanced preservation of mineral-sorbed protease, and enhanced accessibility of the active site of sorbed laccase to protease because of steric considerations.

Experiments following the effects of humic acid addition on free and mineral-sorbed laccase activity yielded unexpected results (Fig. 4B). During the initial 24 h of the incubation, the presence of humic acid had a stabilizing effect on laccase activity, whether free of sorbed. Assuming exponential decay, deactivation half-lives for free and adsorbed laccase were calculated as 148 h ($r^2 = 0.903$) and 204 h ($r^2 = 0.924$) in the presence of humic acid, respectively, and 330 h ($r^2 = 0.741$) and 120 h ($r^2 = 0.852$) in the absence of humic acid, respectively. In the absence of humic acid, free and sorbed laccase lost activity during the initial 24 h (15 and 25%, respectively), as expected from previous experiments (Fig. 3A). After 48 h, the presence of humic acid produced an inhibitory effect on free laccase activity (by 10% relative to humic acid-absent incubations), but there was no inhibitory effect of humic acid on the adsorbed laccase,

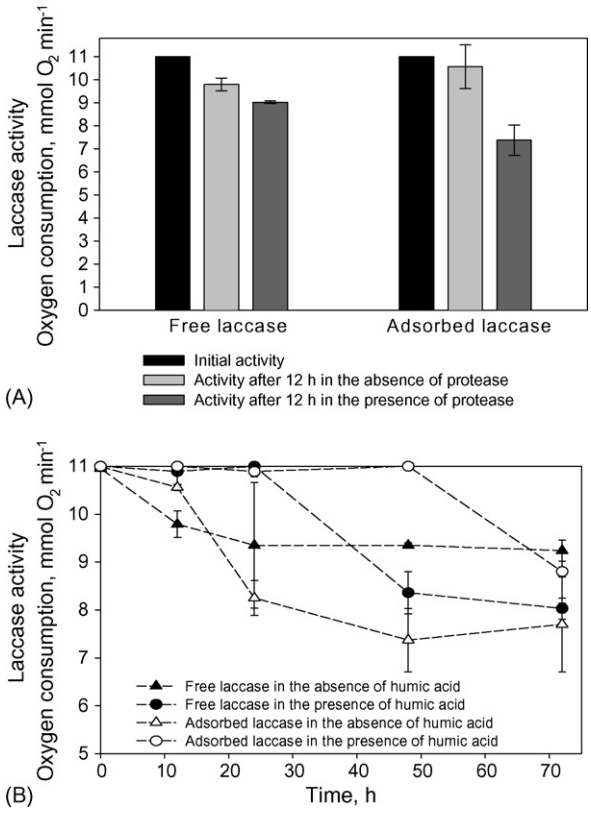

Fig. 4. The activity of free and adsorbed *T. villosa* laccase (440 unit mL^{-1} in 5 mL of 0.5% NaCl solution or suspension, respectively, at 25 °C, pH 7.0 ± 0.1) after (A) 12-h exposure to protease (1.0 mg mL^{-1}) or (B) 0–3 days exposure to IHHS Suwannee River humic acid (1.0 mg mL^{-1}).

which retained 100% of its initial activity. Only after 72 h did sorbed laccase activity in the presence of humic acid decrease to levels that were not significantly different than that of humic acid-absent incubations ($t = 1.15$, $p < 0.05$).

Although these experiments were characterized by greater deviation among replicate incubations, the stabilization of laccase by humic acid, particularly sorbed laccase, is apparent. These results contrast with other studies that have shown humic acid to inhibit enzyme activity in soil [2,17,48]. Enzymes are believed to be inhibited by conformational changes in the enzyme structure due to interaction with humic acid moieties [49–53]. It is possible that the greater apparent stability of adsorbed versus free laccase is due to mineral adsorption of the humic acid.

3.5. Spectroscopic effects of laccase adsorption

ATR-IR spectra of air-dried deposits of free laccase, aluminum hydroxide, and laccase adsorbed on aluminum hydroxide are presented in Fig. 5. The IR spectra of pure aluminum hydroxide (Fig. 5b) was subtracted from the laccase–aluminum hydroxide complex (Fig. 5a) in order to examine the secondary structure of laccase adsorbed to aluminum hydroxide (Fig. 5c). Major peaks in the IR spectra of both free and mineral adsorbed laccase were observed at wave numbers 1555 ± 0.5 and 1640 ± 2.5 cm^{-1} (Fig. 5d) and at 1557 ± 1.4 and 1640 ± 1.9 cm^{-1}, respectively (Fig. 5c). The bands between

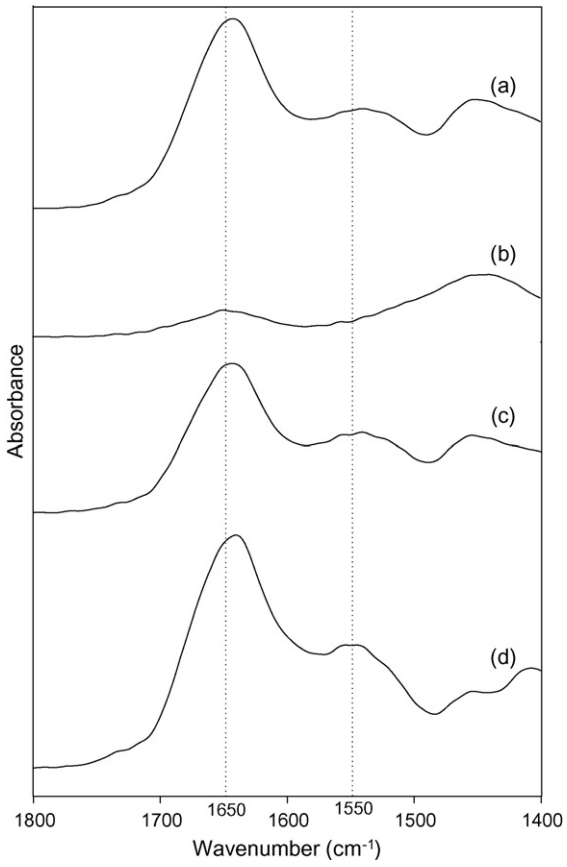

Fig. 5. Attenuated total reflection infrared spectra of air-dried deposits (loaded on a 1-mm diameter single-bounce diamond sensor crystal for spectral acquisition) of (a) laccase–aluminum hydroxide complex (0.222 μg/1.5 mg), (b) aluminum hydroxide (1.5 mg), (c) differential spectrum of adsorbed laccase obtained by subtracting the aluminum hydroxide spectrum from the laccase–aluminum hydroxide complex spectrum, and (d) free *T. villosa* laccase (0.222 μg).

∼1610 and 1690, and between ∼1550 and 1559 cm^{-1} in protein molecules have been identified as mode I and II amide groups, respectively [54–56]. The amide I mode is primarily a C=O stretching band, and the amide II is a combination of mainly N–H in-plane bending and C–N stretching [55].

These amide modes have proved useful in estimating the secondary structure of the polypeptide backbone chain –CO–NH– in enzymes [57–59] and shifts in these absorption bands can be interpreted as alteration to protein secondary structure [56,58]. For example, a shift of up to 11 cm^{-1} in tyrosinase IR absorption band wave number upon adsorption to birnessite was interpreted as alteration of enzyme conformation following adsorption [59]. In this study, the shift in both the amide I and II bonds upon adsorption were found to be smaller than the error associated with these measurements. The similarity between the amide I and II bands of free and adsorbed laccase observed here suggest that negligible alteration of the enzyme's secondary structure occurs during laccase adsorption to aluminum hydroxide.

4. Conclusions and implications

The results presented consistently show the laccase–aluminum hydroxide complex to be both strong and stable in regards to maintenance of enzymatic activity. Short-range ordered aluminum hydroxide showed greater affinity for laccase than all other inorganic soil constituents tested thus far. Since soil clay minerals are often covered with aluminum/iron (hydr)oxides [60], it is reasonable to expect that the predominant form of laccase in soils will be as a complex with aluminum hydroxide.

This study demonstrated the close similarity between free laccase and laccase adsorbed on short-range ordered aluminum hydroxide with respect to several measured properties including enzyme kinetics, aging, response to pH, and secondary structure. Aluminum hydroxide-adsorbed laccase showed somewhat decreased resistance to inactivation by heating (within the 40–60 °C range) and protease, but an enhanced resistance to inhibition by humic acids, further increasing the likelihood that the aluminum hydroxide–laccase complex will be the predominant form of laccase in soils. In retrospect, these findings might be considered unsurprising given that this laccase is produced by fungi that have evolved enzymes that can remain operational in a soil mineral environment often dominated by oxyhydroxide mineral surfaces.

A further implication of this work is that aluminum hydroxide, with no chemical or physical adhesive added, is a likely candidate for a cost effective support for immobilized enzyme contaminant remediation. Further research is needed to examine aluminum hydroxide-immobilized laccase in soil environments along with the efficacy of this complex with various substrates, and aluminum hydroxide as a support for other enzymes.

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