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Transformation of Soil Fulvic Acid by Immobilized Laccase from *Trametes villosa*

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Abstract

The clarification of the mechanisms associated with the enzymatic degradation of humic substances (HS) requires their structural analysis. However, in reaction systems involving free enzymes, HS analysis is typically impacted by the presence of coexisting enzymes. We here evaluated whether immobilized enzymes can be applied to the study of HS degradation. Laccase from *Trametes villosa*, an HS-degrading enzyme, was immobilized on a cross-linked cellulose support (Cellufine Amino gel), and the separation of and reaction between this enzyme and fulvic acid (FA) was investigated. No protein leakage from the immobilized laccase (IML) was detected during the incubation period. Although some of the FA was adsorbed onto the immobilized support, the maximum percentage decolorization of FA by IML was 20.1% after incubation for 24 h at 42°C. After treating with IML, ^{13}C NMR analysis of the residual FA was conducted to evaluate the interference in the analysis caused by laccase. The ^{13}C NMR spectra of FA samples treated with the IML did not exhibit the sharp peaks associated with laccase. This result indicates that the immobilized enzyme system was able to eliminate the interference of enzymes in the analysis of FA.

Keywords: Degradation, Decolorization, Fulvic acid, Humic substance, Immobilization, Laccase

Introduction

Humic substances (HS) are the most ubiquitous natural organic materials in the terrestrial environment. These substances are refractory and heterogeneous mixtures formed by chemical and biochemical reactions leading to the decay and transformation of plant and microbial residues (Stevenson, 1994). HS comprise the majority of fractions of soil organic matter and represent a large portion of the organic carbon budget, which is estimated to be approximately 1600×10^{15} g C worldwide in the surface soil layer (< 1m; Hedges and Keil, 1995). The C content of soil organic matter is approximately 3 times that of aboveground biomass and double that of the atmosphere (Clais et al., 2013). HS formation and decomposition processes thus play a crucial role in global carbon cycling, and investigation of the dynamics of these processes is of substantial interest.

Although HS have been regarded as resistant to microbial attack due to their peculiar molecular structure (Swaby and Ladd, 1962), several microorganisms capable of transforming (decolorizing or degrading) HS have been described in previous reports (Kästner and Hofrichter, 2000; Grinhut et al., 2007). Most of these microorganisms are ligninolytic. It has been suggested that the key enzymes involved in lignin degradation, such as lignin peroxidase, manganese peroxidase and laccase, are involved in the biodegradation of HS (Kästner and Hofrichter, 2000), and the transformation of HS by these enzymes has been reported (Hofrichter and Fritsche, 1997; Claus and Filip, 1998; Ziegenhagen and Hofrichter, 1998; Gramss et al., 1999; Zavarzina et al., 2004; Yanagi et al., 2011). However, the detailed mechanisms of these biodegradation processes are currently far from well understood. Clarification of these degradation mechanisms requires the analysis of the structure of

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HS transformed by enzymes. While in reaction systems using free enzymes, coexisting enzymes may interfere with this analysis, the use of an immobilized enzyme system is expected to suppress such interference. A large number of applications with immobilized laccase (IML) have been adopted in the food processing and bioremediation fields (Durán et al., 2002; Minussi et al., 2002). To our knowledge, there exists only a single report on the dynamics between HS and IML (Zavarzina, 2006), where it is suggested that laccase immobilized to soil minerals contributes to the humification process. The method has not yet been used to study the degradation of HS.

The aim of this paper was to evaluate whether IML is applicable for the study of HS degradation. Fulvic acid (FA), which is one of three main fractions in HS and soluble under both alkali and acid conditions, was used in the first step of the study because of its high solubility and convenience of handling. Since the minerals adsorb and abiotic oxidize the organic matter (Wang et al., 1978; McBride, 1987, Zavarzina, 2006), we immobilized laccase on a cross-linked cellulose support and investigated the separation and the reaction between this enzyme and FA.

Materials and Methods

FA sample preparation

FA was extracted and purified from the A horizon of an Inceptisol (Mt. Hanaore, Kobe, Hyogo Prefecture, Japan; Yanagi et al., 2011) following the NaOH extraction and Supelite DAX-8 resin (Sigma-Aldrich Japan, Tokyo, Japan) isolation methods reported by the International Humic Substances Society (Swift, 1996).

Laccase immobilization

Laccase (EC 1.10.3.2) from *Trametes villosa* SP504 was provided by Novozymes Japan (Chiba, Japan). The stock laccase solution showed 976 U mL^{-1} of laccase activity with 2,6-dimethoxyphenol as the substrate (Shuttleworth et al., 1986) and 37.5 g L^{-1} of protein concentration by the bicinchoninic acid (BCA) method, with bovine serum albumin as the standard (Smith et al., 1985). Covalent immobilization of laccase by the carbodiimide method was performed on the basis of the formation of a peptide bond by condensation between a free amino group of the support and a carboxylic group of the enzyme. A 1-mL portion (wet weight 769 mg) of Cellufine Amino gel (Active group: primary amine, 15–20 mmol L^{-1}; Seikagaku Kogyo, Tokyo, Japan) was mixed with the same volume of 1.3 mol L^{-1} 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Thermo Fisher Scientific, Yokohama, Japan) dissolved in 0.1 mol L^{-1} phosphate buffer (pH 5.5) and laccase solution. The gel suspension was shaken at 6°C for 8 h to immobilize the enzyme covalently onto the support. Subsequently, the IML was filtered and washed with phosphate buffer using membrane filtration (pore size 1.0 μm; Merck, Tokyo, Japan). The absorbance of the recovered filtrate and washings was measured at 280 nm (A_{280}) using a UV-Vis spectrometer (V-530; JASCO, Tokyo, Japan). The laccase immobilization yield on the support was calculated from the difference between the A_{280} of loaded laccase and that of the filtrate and the washings. One mL of EDC solution and 1.5 mL of 0.1 mol L^{-1} CH_3COONa solution was added to 1 mL of the resultant IML and shaken at 6°C for 8 h to block the remaining primary amine in the immobilized support. Then, the IML was washed with phosphate buffer (pH 5.5) by membrane filtration and suspended in 1 mL of the same buffer. To detect the protein leakage from IML during incubation, 250 μL of IML was added to 4.5 mL of phosphate buffer (pH 5.5) and incubated in a shaking bath at 50°C for 24 h. The incubation mixture was filtered through a membrane filter, and the protein content of the filtrate was determined using the BCA method. Inactivated IML (iIML) (which was prepared by boiling for 10 min) was used in the control assay, and its protein content was also determined under the same conditions.

Reaction of FA with IML

IML (50–350 μL) was added to 4.5 mL of 0.3–1.8 g L^{-1} FA solution dissolved in phosphate buffer (pH 5.5). The reaction mixture was incubated at 30–50°C in a shaking bath. Immediately after addition of IML to FA solution (0h) and after 24 h incubation, 2 mL of the reaction mixture was collected, filtered through a membrane filter, and diluted 2-fold with 0.2 mol L^{-1} of NaOH. The absorbance of the alkaline solution was measured at 600 nm (A_{600}). The iIML was used in the control assays to determine whether FA adsorbed onto the support, and A_{600} was measured after incubation under the conditions described above. All treatments were performed in triplicate.

The percentage colorimetric change was determined as follows: percentage colorimetric change (%) = (A_{600} of 0 h – A_{600} of 24 h)/ A_{600} of 0 h × 100. The percentage colorimetric change of iIML was represented as percentage adsorption. The percentage decolorization was calculated as the difference between the percentage colorimetric change of IML and the percentage adsorption. To determine the effect of the reaction conditions on the decolorization of FA by IML, the following parameters were varied: FA concentration (0.3–1.8 g L^{-1}), IML content (50–350 μL), and temperature (30–50°C). Tukey's HSD test was employed as a post-hoc mean separation test to determine changes in percentage adsorption and decolorization ($p < 0.05$). Calculations were carried out using StatPlus: mac 2009 (AnalystSoft, Vancouver, BC, Canada).

^{13}C nuclear magnetic resonance (NMR) analysis of FA that reacted with IML

To determine the ^{13}C NMR spectra of FA that reacted with IML, 4.5 ml of FA solution (0.6 g L^{-1}, pH 5.5) was incubated with 150 μL of IML at 42°C for 24 h. The incubation mixture (19 replicates, total of 85.5 mL) was then filtered through a membrane filter (pore size 0.2 μm; Merck) and dialyzed using an Amicon stirred cell system (model 8200; Merck) with an ultrafiltration membrane (nominal molecular weight cut-off 500 Da; Amicon YC05, Merck) against Milli-Q water. The ultrafiltration retentate was freeze-dried. The iIML-treated FA was prepared using the same procedure. In addition, 5 mL of FA solution (0.3 g L^{-1}, pH 5.5, 20 replicates) acidified immediately after the addition of laccase solution (183 U mL^{-1}, 50 μL) was neutralized, dialyzed with ultrafiltration using the systems described above, and freeze-dried. These treated FA samples, the laccase solution, and original FA were subjected to ^{13}C NMR analysis. The solution ^{13}C NMR spectra were obtained on a Bruker Avance 500K spectrometer (Bruker, Karlsruhe, Germany) using sample tubes 5 mm in diameter according to the method described by Yanagi et al. (2011). Chemical shifts were referenced to sodium 3-trimethylsilylpropionate-2,2,3,3-D4 (TMSPNa; Euriso-top, Saint Aubin, France). The assignments of the spectra were conducted according to previous studies (Preston and Blackwell, 1985; Schnitzer and Preston, 1986; Wilson, 1987; Thorn et al., 1989; Ricca and Severini, 1993).

Results and Discussion

In this study, laccase was immobilized onto a Cellufine Amino gel with an immobilization yield of 36.7% (13.8 g protein L^{-1} gel). Low ligand leakage due to exceptionally stable coupling chemistry and support matrix is one of the features of Cellufine Amino gels (JNC Corporation, 2014). As the protein could not be detected in the solution after incubation (24 h, 50°C) with both IML and iIML, it was concluded that the linkage between the laccase and the immobilization support did not break during the incubation period.

Decreases in A_{600} of FA under both iIML and IML incubation were detected under all conditions in this study. The effect of FA concentration on the percentage adsorption is shown in Fig. 1a. The maximum percentage adsorption (18.6%) was determined at 0.3 g L^{-1} (lowest FA concentration), followed by a rapid decrease above this level. A relative decrease in percentage adsorption likely occurred rapidly due to the saturation resulting from FA entrapment onto iIML. In contrast, when the FA was incubated with varying amounts of iIML, percentage adsorption increased linearly with iIML up to 350 μL (23.7%; Fig. 1b). The reaction temperature had no significant effects on the adsorption in the range of 30–50°C (Fig. 1c). We therefore conclude that the percentage adsorption did not represent the decolorization of FA by laccase activity remaining in iIML but rather the simple adsorption of FA to the support or enzyme protein. The percentages of colorimetric decrease caused by IML were higher than those caused by iIML. Consequently, net decolorization was observed. Thus, this is the first study to demonstrate that laccase immobilized on the support can decolorize soil FA. Yanagi et al. (2011) reported that the percentage decolorization of 6 soil FAs (0.3 g L^{-1}) by laccase from *T. villosa* ranged from 8.9% to 19.2% at pH 5.5 and 50°C. As we measured decolorization under conditions that differed from those used in their study, we could not easily compare the results; however, the decolorization values (maximum 20.1% at pH 5.5, 42°C) were similar. The effect of FA concentration on percentage decolorization showed high values up to an FA concentration of 0.6 g L^{-1} followed by a gradual decline when the concentration exceeded 0.6 g L^{-1} (Fig. 2a). Since this declining trend differed from that observed for the percentage adsorption with increasing

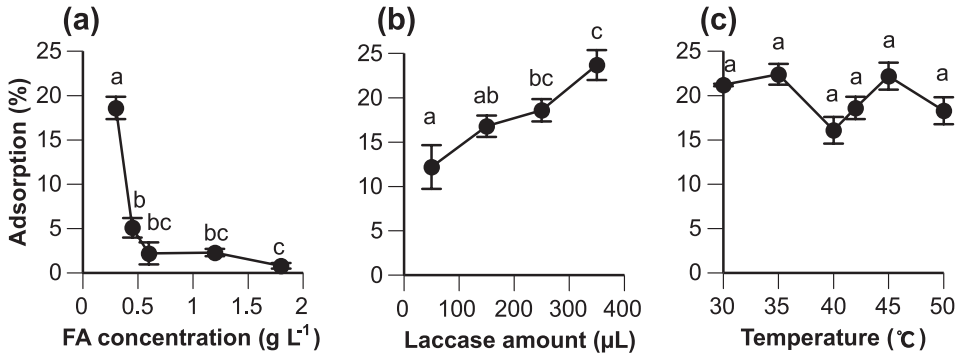

Fig. 1. The percentage adsorption of FA by laccase immobilized on Cellufine Amino gel. (a) FA concentration profile assay performed using 250 μL of laccase at 42°C. (b) Laccase profile assay performed using 0.3 g L^{-1} of FA at 42°C. (c) Temperature profile assay performed using 0.3 g L^{-1} of FA and 250 μL of laccase. Vertical bars indicate the standard error (n=3). Letters above bars indicate significant differences (p<0.05).

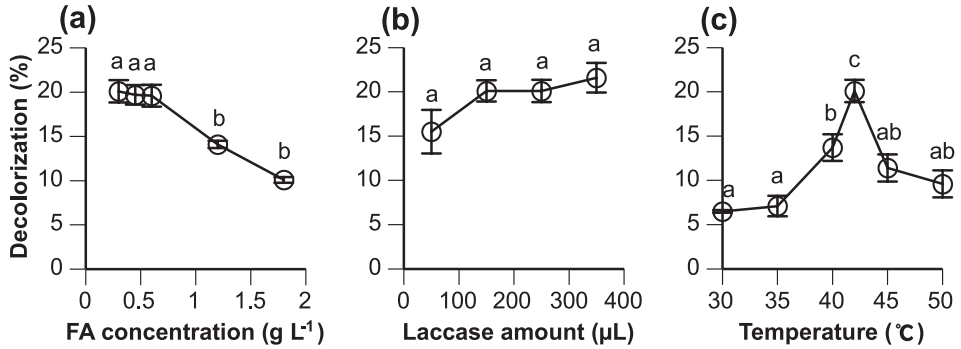

Fig. 2. The percentage decolorization of FA by laccase immobilized on Cellufine Amino gel. (a) FA profile assay performed using 250 μL of laccase at 42℃. (b) Laccase profile assay performed using 0.3 g L^{-1} of FA at 42℃. (c) Temperature profile assay performed using 0.3 g L^{-1} of FA and 250 μL of laccase. Vertical bars indicate the standard error (n=3). Letters above bars indicate significant differences (p<0.05).

FA concentration described above, it does not appear that percentage decolorization was decreased by the saturation of enzyme activity. It was reported that FAs are competitive inhibitors of laccase and that their inhibitory effect increases with increasing FA concentration (Sarkar and Bollag, 1987; Eichlerová et al., 2012; Shi et al., 2016). The decline in percentage decolorization at higher FA concentration can therefore be attributed to the inhibition of laccase activity by FA. There was no appreciable variation in percentage decolorization with an increase in the amount of IML, while percentage adsorption significantly increased with amount of iIML (Fig. 2b). Instead, percentage decolorization depended on reaction temperature and changed sharply, with a maximum value of 20.1% at 42°C (Fig. 2c). While it has been reported that in general, immobilization broadens the temperature range of laccase activity (Leonowicz et al., 1988; Rogalski et al., 1995; D'Annibale et al., 1999; Arica et al., 2009; Nicolucci et al., 2011), our results do not agree with these observations. On the other hand, the viscosity of HS in solution (and consequently the size and shape of HS molecules) was found to be influenced by temperature (Adhikari et al., 1980; Stevenson, 1994; Ray and Machado, 2000). It is therefore suggested that the conformational change in FA structure influences the interaction with the active site of the enzyme. Furthermore, the reaction time in this study was 24 h, which is longer than in the above previous studies. Thus, the stability of laccase must have an influence on the decolorization of FA.

After treatment with iIML or IML under the optimal conditions described above (FA concentration of 0.6 g L^{-1}, 150 μL of IML, 42°C; percentage decolorization

19.8%), ^{13}C NMR analysis of the residual FA was conducted to evaluate the laccase-caused interference in the analysis. The ^{13}C NMR spectra of the laccase, original FA, FA treated with laccase, and FAs treated with iIML or IML are shown in Fig. 3. The laccase exhibited distinct and intense peaks at 60–90 ppm and 100–110 ppm. These signals are probably caused by the oligosaccharide side chain of laccase, based on their similarity to carbohydrate resonance in glucose oxidase (Sanner et al., 1991). In addition, weak and broad peaks at 20–60 ppm and around 175 ppm were observed, which can presumably be assigned to the aliphatic C and carboxylic C of protein, respectively. The original FA exhibited broad major bands at 5–58 ppm, 63–95 ppm and 110–160 ppm, and a narrow band associated with carboxyl carbons at 165–185 ppm. These four major bands have commonly been found in previously analyzed HS (Steelink et al., 1989). The FA treated with laccase had sharp and distinct peaks at 60–90 ppm and 100–110 ppm, indicating strong overlap in the FA signals with the laccase signals. In contrast, neither of the FA samples treated with iIML or IML exhibited the sharp peaks associated with laccase. Thus it appears that the separation of FA from laccase was succeeded by the immobilization of laccase, and the results indicate that the immobilized enzyme system was able to eliminate the interference of enzymes in the analysis of FA. Despite 20% decolorization, the FA spectra treated with IML showed no substantial changes except for the slight deformation in the shape of the peak at around 80 ppm as compared with the FA treated with iIML. There are two possible reasons for this. First, some of the molecular units of FA were depolymerized to low molecular mass products, which were cut off by the ultrafiltration or completely disappeared as a result of IML. Therefore, a reduction in absolute FA quantity may express as decolorization. Second, there is a possibility that partial structural changes, e.g., of chromophoric groups, were not detected by the ^{13}C NMR analysis.

In this study, we demonstrated for the first time the application of an enzyme immobilization method to investigate the degradation of HS and soil FA by immobilized laccase. This method enables the analysis of structural changes in HS during degradation by laccase. However, as adsorption of FA to the immobilized support was also observed, further improvement of this procedure may contribute to the investigation of the HS-degrading mechanism and process.

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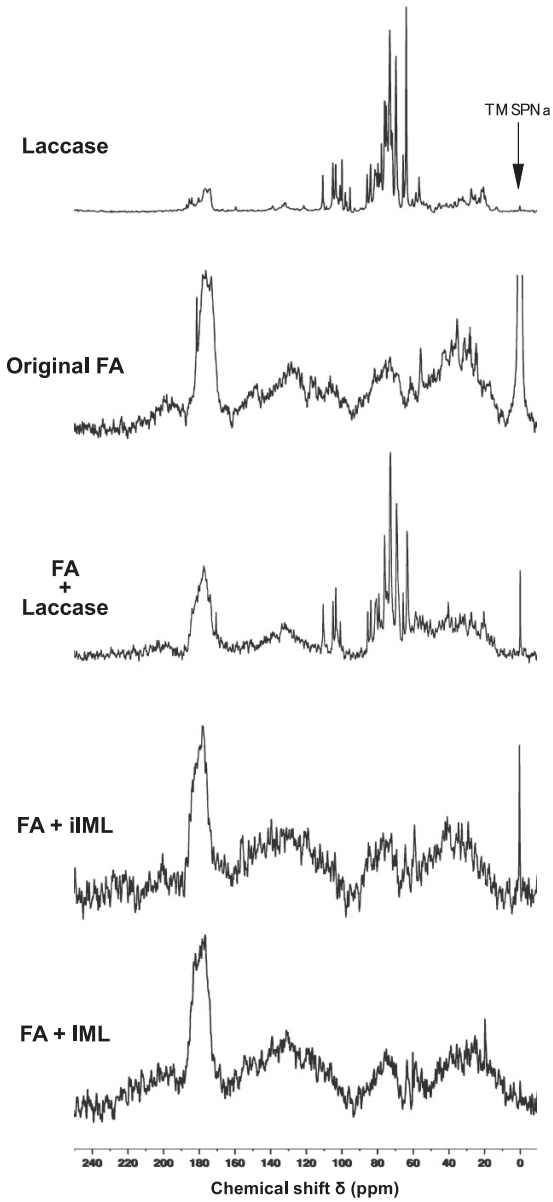

Fig. 3. The ^{13}C NMR spectra of laccase, original FA, FA treated with laccase (FA + laccase), and FAs treated with iIML (FA + iIML) or IML (FA + IML).

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