Notes

Decolorization of soil fulvic acids by laccases from *Trametes versicolor* and *Trametes villosa*

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Abstract
Laccase is considered to be one of the humic substance-transforming enzymes. In this study, the decolorization of different soil fulvic acids (FAs) by 2 laccases derived from *Trametes versicolor* and *T. villosa* was investigated, and the relationship between the decolorization and the chemical properties of the FAs was examined. Eight FAs (4 from Inceptisols and 4 from Andisols) dissolved in phosphate buffer were incubated at 50˚C with both laccases. After 24 h, decolorization of the reaction mixtures was measured at 400 nm absorbance. The elemental composition and carbon distribution by ^{13}C nuclear magnetic resonance (NMR) spectroscopy of the FAs were determined. Six FAs were decolorized by both laccases, and the decolorization percentages ranged 7.7%–19.2%. Thus, the ability of laccases from *Trametes* species to transform soil FAs was shown for the first time. Andisol FAs exhibited a lower percentage of decolorization than Inceptisol FAs did, and this difference was significant at a 1% level. However, there was no significant difference in the elemental composition and carbon distribution between Inceptisol FAs and Andisol FAs. Therefore, decolorization of FAs by laccase may not be related to such basic structure of FAs but to the intra–molecular environment of the phenolic hydroxyl and methoxyl groups.

Keywords: Biotransformation, Chemical property, Degradation, Fulvic acid, Humic substance, Enzyme

Introduction

Humic substances are the most ubiquitous refractory-colored natural nonliving organic materials in terrestrial environments. These substances constitute the major fractions of soil organic matters and represent a significant proportion of organic carbon in the global carbon budget. Therefore, investigating the biotransformation of humic substances is important for understanding global carbon cycling in the biosphere.

A number of microorganisms have demonstrated the ability to transform (decolorize or degrade) humic substances (Kästner and Hofrichter, 2000; Grinhut et al., 2007). Most humic substance-transforming microorganisms are ligninolytic, and the process of transformation has therefore been attributed to their ligninolytic enzymes, namely, lignin peroxidase, manganese peroxidase, and laccase. However, the detailed mechanism of humic transformation by microorganisms is not clear. Analysis of microbial reactions is complicated because the microorganisms produce multiple enzymes and secondary metabolites. Nonetheless, the enzymatic reaction system is useful for analyzing humic degradation by microorganisms because it is simpler and produces fewer secondary metabolites, unlike microbial reactions. However, enzyme activity depends on reaction conditions. Hence, humus-transformation experiments using isolated enzymes may have to be carried out under laboratory-controlled conditions rather than in natural conditions. Several researchers have reported the transformation of humic substances by isolated ligninolytic enzymes (Claus and Filip, 1998; Gramss et al., 1999; Zavarzina et al., 2004) and have

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confirmed the contribution of these enzymes to humus transformation. However, the information on the mechanism of humus transformation by these enzymes is limited.

Here, we report our attempts to study the transformation of humic substances by laccase, a ligninolytic enzyme. We used fulvic acids (FAs) in the first step of the study because of their high solubility and convenience of handling. In this study, we examined the changes in the optical properties of FAs derived from different soils induced by laccases from 2 *Trametes* spp.

Materials and Methods

FA sample

Six FAs were extracted from 3 Inceptisol (Hanaore, HO; Keirozan, KR; Yamashiro, YS) and 3 Andisol (Gyoseizan, GS; Hachibuse, HB; Sugadaira, SG) samples according to the methods described by the International Humic Substances Society (Swift, 1996). Two FAs (Inogashira, IG, Andisol; Dando, DN, Inceptisol) were obtained from the Japanese Humic Substances Society. The soil samples used for FA extraction are described in Table 1.

Elemental analysis

The amount of carbon, nitrogen, and hydrogen in the FAs was determined using an elemental analyzer (2400 -C; Perkin Elmer Japan Co., Ltd., Yokohama, Japan). The oxygen content (%) was derived with the formula [100 − (C% + H% + N%)]. All results were expressed on a moisture and ash-free basis.

^{13}C nuclear magnetic resonance (NMR) spectroscopy

The aromaticity of the FAs was examined by solution ^{13}C NMR. Solution ^{13}C NMR was recorded at 125.76 MHz on a spectrometer (Avance 500K; Bruker GmbH, Karlsruhe, Germany) by the inverse gated-decoupling technique using a pulse of 45˚, an acquisition time of 0.839 s, and a pulse delay time of 2.5 s. Chemical shifts were referenced with an internal standard of sodium 3-trimethylsilylpropionate-2,2,3,3,D_4 (Euriso-top, Saint Aubin, France). FA samples were dissolved in 0.2 mol L^{-1} NaOD at a ratio of approximately 100 mg mL^{-1}, and 4,000–20,000 scans were accumulated. Aromaticity was calculated by expressing the integral value from 105 to 165 ppm as a percentage of the integral value from 0 to 165 ppm (Hatcher et al., 1981).

Laccase samples and reaction conditions

Laccases (EC 1.10.3.2) from *T. villosa* (SP504; Novozymes Japan, Ltd., Chiba, Japan) and *T. versicolor* (Sigma 53739, Sigma-Aldrich Japan K.K., Tokyo, Japan) were used in this study. The laccase was dissolved in cold distilled water, and the concentration of laccase activity was adjusted to 672 U mL^{-1}. Laccase activity was measured by using 2,6-dimethoxyphenol (2.6-DMP) as a substrate. The activity assay mixture consisted of 3.4 mL of 1 mmol L^{-1} 2,6-DMP in 0.1 mol L^{-1} phosphate buffer (pH 5.5) and 200 μL of the laccase solution. One DMP unit is the amount of laccase that causes a change of 1 absorbance unit per minute at 468 nm (Shuttleworth et al., 1986). A 0.3 g L^{-1} FA solution was prepared in 67 mmol L^{-1} phosphate buffer (pH 5.5). A 2.5 mL aliquot of this solution was placed in a vial, to which 50 μL of laccase solution was added (33.6 U, Test), and the mixture was incubated at 50ºC in a shaking bath (100 rev min^{-1}). After 24 h incubation, 2 mL of the reaction mixture was collected and diluted to 4 mL with 0.2 mol L^{-1} NaOH to enable measurement of the absorbance at 400 nm (*A*400). Phosphate buffer with laccase (N-FA) and FA solution without laccase (N-LC) were used as controls and incubated under

Table 1. The descriptions of soil samples used for extraction of FAs.

Soil name	FA	Soil type	Land use	Location
Dando	DN[a]	Inceptisol	Forest	Dando, Aichi prefecture
Hanaore	HO	Inceptisol	Forest	Mt. Hanaore, Kobe, Hyogo prefecture
Keirozan	KR	Inceptisol	Forest	Mt. Keirozan, Ako, Hyogo prefecture
Yamashiro	YS	Inceptisol	Forest	Yamashiro, Kyoto, Kyoto prefecture
Hachibuse	HB	Andisol	Grassland	Mt. Hachibuse, Yabu, Hyogo prefecture
Inogashira	IG[a]	Andisol	Grassland	Inogashira, Shizuoka prefecture
Gyosei	GS	Andisol	Grassland	Mt. Gyosei, Kamikawa, Hyogo prefecture
Sugadaira	SG	Andisol	Grassland	Sugadaira highlands, Ueda, Nagano prefecture

[a] The standard samples of Japanese humic substances society.

the same conditions as described above. All treatments were performed in triplicate. The percentage of decolorization was determined as follows: percentage of decolorization (%) = *A*400 of Test/(*A*400 of N-LC + *A*400 of N-FA) × 100. GS and HB were examined using *T. versicolor* laccase. The UV-Vis spectra of the 8 FAs exhibited no specific peaks and no crossing between any of the FA spectra was observed. The percentages of decolorization calculated from the absorbance at all visible regions exhibited a similar tendency. Therefore, absorbance at 400nm was selected for this study.

Results and Discussion

The elemental composition of each FA is listed in Table 2. The carbon content ranged from 48.8% for GS and HB to 52.7% for HO. The hydrogen, nitrogen, and oxygen contents ranged 3.24%–4.18%, 0.88%–2.03%, and 42.1%–46.4%, respectively. The atomic ratios of these FAs were similar to those reported previously (Steelink, 1985). Although the elemental compositions of the 8 FAs varied slightly, there were no obvious differences. This was in contrast to the wide distribution of the elemental composition of humic acids obtained from various types of soils (Kuwatsuka et al., 1978; Yonebayashi and Hattori, 1988). The distribution of carbon species and aromaticity of FAs calculated from ^{13}C NMR spectra are listed in Table 3. The concentration of alkyl carbons ranged from 9.9% for GS to 26.1% for HO; that of aromatic carbons ranged from 16.8% for HO to 34.0% for GS, and that of O-alkyl, phenolic, carboxylic, and carbonyl carbons ranged 17.3%–27.8%, 4.5%–8.4%, 18.8%–28.6%, and 2.8%–5.5%, respectively. GS exhibited the lowest alkyl, O-alkyl, and carbonyl peak values and the highest aromatic and carboxylic peak values; it also had the highest aromaticity. HO had the highest aliphatic peak value as well as the lowest aromatic peak value and aromaticity. However, no significant difference in carbon distribution of the FAs was observed in these soil types.

The decolorization percentages of the 8 FAs incubated with the 2 laccases are illustrated in Fig. 1. The percentages of decolorization by *T. villosa* laccase ranged from 8.9% for SG to 19.2% for YS and those by *T. versicolor* laccase ranged from 7.7%

Table 2. Elemental compositions of the eight FAs

FA	Element content (w %)				Atomic ratio				Saturation[a]
	C	H	N	O	H/C	O/H	N/C	O/C	
DN	49.4	3.80	0.89	45.9	0.94	0.69	0.02	0.73	54.5
HO	52.7	4.18	1.05	42.1	0.95	0.60	0.02	0.63	53.3
KR	49.7	3.52	0.88	45.9	0.85	0.69	0.02	0.82	58.3
YS	50.2	4.15	2.03	43.6	1.00	0.65	0.04	0.65	52.1
HB	48.8	3.24	1.59	46.4	0.80	0.90	0.03	0.72	61.6
IG	50.3	4.10	2.00	43.6	0.97	0.64	0.03	0.66	52.8
GS	48.8	3.91	0.99	45.3	0.96	0.72	0.02	0.70	52.8
SG	49.0	3.60	1.10	46.3	0.89	0.69	0.02	0.77	56.9

[a] Saturation was calculated using (C-6 × H + 0.429 × N) / C × 100 (Tsutsuki and Kumada, 1980).

Table 3. Distribution of carbon species of six FAs by ^{13}C NMR

FA	Chemical shift, δ (ppm)						Aromaticity[a]
	Alkyl C (5- 48)	O-alkyl C (48-110)	Aromatic C (110- 145)	Phenolic C (145- 165)	Carboxylic C (165- 190)	Carbonyl C (190- 230)	
DN	20.0	21.9	24.7	4.7	23.4	5.3	41.2
HO	26.1	27.1	16.8	5.6	19.7	4.7	29.5
KR	17.9	17.7	23.5	8.4	27.0	5.5	47.3
YS	24.3	24.5	21.0	5.0	20.0	5.1	34.8
HB	20.9	17.7	27.4	4.5	26.3	3.2	45.3
IG	19.2	24.9	24.2	5.7	22.7	3.4	40.4
GS	9.9	17.3	34.0	7.4	28.6	2.8	60.4
SG	18.9	27.8	23.8	5.5	18.8	5.1	38.6

[a] Aromaticity was calculated using (Aromatic C + Phenolic C)/(Alkyl C + O-alkyl C + Aromatic C + Phenolic C) × 100.

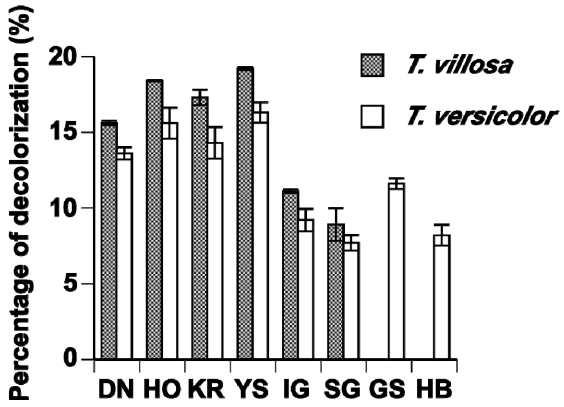

Fig.1 The percentages of decolorization of eight FAs by laccases from *T. versicolor* and *T. villosa*. Values are means and errors bars represent standard deviations.

for SG to 16.3% for YS. Since both *Trametes* spp. laccases were able to decolorize all 8 soil FAs used in this study, our results demonstrated for the first time that laccases from *Trametes* spp. can transform soil FAs. Claus and Filip (1998) reported the decolorization (22.2%, calculated from their data) of aquatic humic substances by laccase from *T. versicolor*. They conducted the decolorization experiment in 900 μL of humic solution (100 mmol L^{-1} phosphate buffer with 0.5 mg L^{-1} of humic substance at pH 5.9) with 100 μL of laccase at 30˚C for 7 d. Gramss et al. (1999) demonstrated decolorization (3.4%) of soil humic extract by laccase from *Pyricularia oryzae* in 4 mL of humic solution (100 mmol L^{-1} citrate-phosphate buffer with 1 g L^{-1} of humic extract at pH 4.8) with 1 mL of laccase at 24˚C for 24 h. Decolorization was demonstrated in both reports as well as in this study. Transformation of soil humic acids by laccase from *Panus tigrinus* has been reported to accompany either colorization or decolorization in 980 μL of humic solution (20 mmol L^{-1} sodium-acetate buffer with 5 mg L^{-1} of humic acid at pH 5.0) with 20 μL of laccase at 30˚C for 96 h (Zavarzina et al., 2004), indicating that chromatic transition depends on the properties of the humic substances and the laccase producer. As we measured decolorization under conditions different from those in previous studies, we could not compare the results easily; however, the decolorization values obtained in our study were by no means inferior to those of the previous studies. The decolorization percentages of the 6 FAs were rated in the same order for both laccases: YS > HO > KR > DN > IG > SG (Fig. 1). Moreover, a high positive correlation (r = 0.997, p < 0.01) was observed between the percentages of decolorization for these 2 laccases. It is considered that the reason for high positive correlation between the 2 laccases is that they exhibit similar primary structures and redox/enzymatic properties (Xu et al., 1999). Although these laccases demonstrated the same activity (33.6 U) with 2,6-DMP as the substrate, however, the percentage of decolorization by *T. villosa* laccase was slightly higher than that from *T. versicolor*. These results suggest that the mechanism of FA decolorization for both laccases is similar, but that their reactivity to FAs is different. Andisol FAs exhibited a lower percentage of decolorization than Inceptisol FAs did, and this difference was significant at a 1% level. Yanagi et al. (2002, 2003) reported that humic acid decolorization by the laccase-producing fungus *Coriolus consors* was strongly correlated with the elemental composition and aromaticity of the humic acids. However, in our study, there were no distinct differences in the chemical structures of the FAs from the 2 soil types. Consequently, no correlation was found between the percentage of decolorization and the elemental composition and carbon distribution of the FAs. Therefore, decolorization of FAs by laccases is not considered to be related to such basic structures of FAs. Watanabe and Kuwatsuka (1992) reported no clear difference in the chemical characteristics of poly 1-vinyl-2-pyrrolidone (PVP)-adsorbed, and PVP non-adsorbed fractions of FAs, and non-fractionated FAs between various types of soil; these observations agreed with the chemical properties of the FAs detected in this study. They also reported that different spectra of FAs between pH 12 and pH 7 are distinguishable by soil type (Watanabe and Kuwatsuka, 1991). These spectra were attributed to ionization of the phenolic hydroxyl group. Specifically, the variation in these spectra represents the difference in the intra–molecular environment of the phenolic groups. Laccases catalyze the removal of an electron and proton from phenolic hydroxyl groups to yield free phenoxy radicals. A group of copper–containing laccases with 4 copper atoms, all in the 2+ oxidation state in the active site, oxidizes and decarboxylates phenolic and methoxyphenolic acids (Agematsu et al., 1993) and also attacks their methoxyl groups through demethylation (Leonowicz et al., 1984). Thus, decolorization of FAs by laccases

may be related to the intra–molecular environment of the phenolic hydroxyl and methoxyl groups.

Conclusions

This study demonstrates for the first time the ability of laccase to decolorize and thus transform soil FAs. The decolorization of Andisol FAs exhibited a lower percentage of decolorization than Inceptisol FAs did. The difference in decolorization among soil types may be related to the intra–molecular environment of the phenolic hydroxyl and methoxyl groups. Further investigation of the mechanism of FA transformation by laccases and detailed structural analysis of FAs are required to understand the biotransformation process of FAs.

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