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Soil Biology & Biochemistry 36 (2004) 359–369

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Biotransformation of soil humic acids by blue laccase of *Panus tigrinus* 8/18: an in vitro study

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Received 18 November 2002; received in revised form 11 August 2003; accepted 1 October 2003

Abstract

We describe here the role of the polyphenoloxidases in the oxidation of recalcitrant soil organic compounds and consider what changes occur in their structure during experiments on the biotransformation of soil and peat-derived humic acids (HA). These transformations were carried out by laccase (EC 1.10.3.2.) of the white-rot basidiomycete *Panus tigrinus* 8/18. It was shown that purified laccase alone is capable both of polymerizing and depolymerizing HA in vitro. The direction of transformations depends on the nature and properties of HA. Those fractions of HA are affected by laccase, which cause the lowest inhibitory effect on the enzyme. Contrary to previous studies depolymerization of HA was not necessarily accompanied by decolorization: chernozem-derived HA showed increase in absorbance of its aqueous solution in the region of 240–500 nm during depolymerization, while peat-derived HA showed decrease in absorbance throughout the entire spectrum during polymerization. All studied HA were competitive inhibitors of laccase towards oxidation of synthetic substrate 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS). When studying the nature of the inhibitory effect, it was shown that more 'hydrophobic' HA as well as more 'hydrophobic' HA fragments were stronger inhibitors of blue laccase.

Keywords: White rot fungus; Laccase; Humic acids; Biodegradation

1. Introduction

Humic substances are a recalcitrant dark-colored product of biotransformation of plant material, described as a complex mixture of polydisperse polyelectrolyte-like molecules, that have irregular structure and varying composition and consist of aromatic rings and aliphatic chains bearing O-, N- and S-containing functional groups (Orlov, 1990; Stevenson, 1994). Humic substances comprise the major part of stable organic matter in the diversity of the environments and their formation and decomposition processes regulate the global carbon cycling. Current rise of global mean temperatures may lead to increase in activity of soil biota towards degradation and mineralization of humus. Investigations into the mechanisms of humus biotransformation and the responsible organisms are particularly important under the changing environment and may promote better predictions of the dynamics of soil organic matter.

Humic substances are formed during transformation of organic residues of biotic origin and an important step in their synthesis is oxidative co-polymerization of the products of lignin breakdown with other transformed precursors such as proteins, tannins, carbohydrates etc. (Orlov, 1990; Stevenson, 1994). Phenoloxidases (peroxidase, laccase) are considered to catalyze this process. This was confirmed in laboratory experiments (Kononova, 1963; Burns, 1987). This led to substantial interest in organotrophic microorganisms, especially lignin-degrading fungi, with respect to their possible role in humus turnover. The ability to decolorize, depolymerize or mineralize humic substances in vivo was shown for a number of the white-rot fungi among which *Phanerochaete chrisosporium* (Blondeau, 1989; Ralph and Catcheside, 1994), *Trametes* (*Coriolus*) species (Dehorter and Blondeau, 1992; Yanagi et al., 2002), *Nematoloma frowardii* (Hofrichter and Fritsche, 1997a), *Pycnoporus cinnabarinus* and *polyporus ciliatus* (Temp et al., 1999)

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doi:10.1016/j.soilbio.2003.10.010

could be mentioned. Soil litter-colonizing fungus *Collybia dryophila* was also found to degrade humic acid from pine-forest litter with formation of lower-molecular mass compounds and carbon dioxide (Steffen et al., 2002). Like lignin decomposition, degradation of HA in vivo was a cometabolic event, i.e. occurred in the presence of easily metabolizable carbon source, e.g. glucose.

Efficiency of white-rot and soil-inhabiting fungi in modifying humic substances is considered to be associated with their extracellular non-specific enzyme system, consisting of lignin peroxidase, manganese-peroxidase (MnP), and laccase. In vitro studies showed that MnP was able to decolorize/depolymerize (Hofrichter and Fritsche, 1997; Hofrichter et al., 1998) or polymerize (Ralph and Catcheside, 1998) coal-derived humic substances. The decolorizing/depolymerizing effect was also shown for coal macromolecules in presence of purified lignin peroxidase (Ralph and Catcheside, 1999). These findings led to a conclusion about the crucial role of peroxidases in transformation of humic substances while the role of laccase remains unclear.

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2; *p*-diphenol oxidase) is a well-characterized extracellular, copper-containing glycoprotein, belonging to the group of so-called blue oxidases. It reduces dioxygen to water, coupled to the one-electron oxidation of a broad spectrum of phenolic and related substrates to the corresponding radicals (Reinhammer and Malmstrom, 1981). These radicals may subsequently couple with one another. The production of quinones that readily polymerize is seen to be the major function of laccase. Therefore it is still under discussion whether laccase is involved in the decay of lignin and phenolic polymers like humic substances, or whether it is essential for polymerization of low molecular weight breakdown products that may be toxic to the fungus.

Being produced by higher plants, wood- and litter-degrading fungi (Thurston, 1994; Steffen et al., 2000), laccase-like enzymes were found in soil litter and upper soil horizons in free and immobilized forms (Gul'ko and Khaziev, 1992; Gramss et al., 1998; Criquet et al., 1999) suggesting their involvement in humus formation and degradation processes. Despite these facts there is only one report on in vivo decolorization and depolymerization of humic acids (HA) by laccase of *Pycnoporus cinnabarinus* (Temp et al., 1999) and no reports exist as far as we know on in vitro laccase–humic substances interactions. Existing experimental data does suggest that laccase act in synergy with MnP rather than alone during degradation of macromolecular phenolic substrates (e.g. Galliano et al., 1991; Schlosser and Hofer, 2002).

The present work was performed in order to define the role of laccase in transformation of humic substances and to study what changes may occur in their structure during interaction with laccase. Soil and peat-derived HA and blue laccase of the white-rot fungus *Panus tigrinus* 8/18 were chosen as a model.

2. Materials and methods

2.1. Microorganism and growth conditions

White-rot basidiomycete *P. tigrinus* 8/18 isolated from decaying wood near Dushanbe (Tadzhikistan) was used. The fungus was grown under conditions of submerged cultivation on 'high-nitrogen' liquid mineral medium (Kirk et al., 1978) with 0.1% Tween-80, 1% glucose as carbon source, 1 mmol l^{-1} 2,4-dimethylphenol and 1 mg l^{-1} $CuSO_4$ as laccase inducers.

2.2. Laccase assay

Laccase activity was determined by the rate of oxidation of 0.2 mmol l^{-1} ABTS (2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate) in 20 mmol l^{-1} sodium-acetate buffer, pH 5.0 (buffer A). The increase in absorbance at 436 nm was measured. Enzyme activity was expressed in arbitrary units (arb. U) as ΔA_{436} min^{-1} ml^{-1}.

2.3. Laccase purification

Submerged cultures of *P. tigrinus* were grown in 750-ml flasks with 200 ml of medium, at 29 °C, with agitation at 200 rpm. Laccase was isolated from the liquid medium of submerged cultures of *P. tigrinus* grown for 7 days when maximum enzyme activity was reached. Enzyme was purified as described earlier (Leontievsky et al., 1990) with some modifications. Briefly, purification scheme included (Table 1): (1) Separation of *P. tigrinus* mycelium from the culture liquid by filtration through nylon fiber. (2) Anion-exchange chromatography-1 on Servacel DEAE-52 (Serva, Germany) with elution by 0.3 mol l^{-1} NaCl in buffer A. (3) Anion-exchange chromatography-2 on a Mono-Q HR 5/5 column (Amersham-Pharmacia-Biotech, Sweden) with elution by linear gradient of 0–0.5 mol l^{-1} NaCl in buffer A. Prior to elution on a Mono-Q column enzyme preparation was diluted by buffer A to give NaCl concentration that does not exceed 0.05 mol l^{-1}. (4) Gel-filtration

Table 1
Purification scheme of blue laccase of *Panus tigrinus*

Purification step	Volume (ml)	Specific activity (arb.U)	Total activity (arb.U)	Yield (%)
1. Culture liquid	8740	0.9	7912	100
2. Anion-exchange chromatography-1 (Servacel DEAE-52)	113	53	5989	76
3. Anion-exchange chromatography-2 (Mono-Q)	4	544	2176	28
4. Gel-filtration (Sephadex G-100)	20	103	2050	26
5. Ultrafiltration	4	430	1720	22

on Sephadex G-100 (Amersham-Pharmacia-Biotech) in buffer A. The purified preparation was concentrated by ultrafiltration on PM 10 membranes (Amicon, The Netherlands). Protein concentration was determined by the Bradford's technique (Bradford, 1976).

2.4. Laccase characterization

Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gel (Laemmli, 1970) followed by staining with Coomassie R-250. Protein standards (Amersham-Pharmacia-Biotech) were (kDa): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), α-lactalbumin (14.4). The enzyme absorption spectrum was recorded on a Shimadzu UV-2501 PC spectrophotometer at 200–800 nm in buffer A and protein concentration of 0.48 mg ml^{-1}. Kinetic constant (K_m) of the enzyme was determined using 0.005–2 mmol l^{-1} ABTS and 0.14 μg ml^{-1} enzyme in buffer A.

2.5. Humic acids preparations

Humic acids (HA) from peat (HA_p, Merck preparation), soddy–podzolic soil (HA_s) and chernozem (HA_{ch}) were used. Extraction, purification and characterization procedures as well as the physicochemical properties of the HA were described in details earlier (Zavarzina and Demin, 1999; Zavarzina et al., 2002). HA were transferred to a water-soluble NH_4^+-form by dissolution of preparations in 10% NH_4OH followed by their evaporation on the water-bath at 40 °C.

2.6. Fractionation of humic acids by salting-out with ammonium sulfate

Salting-out of HA was performed at pH 7.0 using 0–20, 20–30, 30–40 and 40–60% of $(NH_4)_2SO_4$ saturation (HA_p and HA_{ch}) or 0–40, 40–50, 50–60 and 60–70% of $(NH_4)_2SO_4$ saturation (HA_s). HA fractions that coagulated at respective concentrations of ammonium sulfate, were separated from the supernatants by centrifugation at 4000 rpm for 20 min. Precipitates were washed on centrifuge (15 min at 4000 rpm) from excess of salt with distilled water until neutral pH of the supernatant was reached, transferred to NH_4^+-form and dried on the water bath. HA that remained in solution at 60 or 70% of saturation were precipitated by concentrated HCl, washed on the centrifuge until neutral pH, transferred to NH_4^+-form and dried. The relative hydrophobicity of HA fractions was confirmed by extraction in 5% polyethylene glycol (15 kDa)–7.5% Dextran T-70–H_2O system at pH 7.0 as described in (Zavarzina et al., 2002). Molecular weight distributions were obtained as described below. Prior to gel-filtration HA fractions were dissolved in the eluent buffer.

2.7. Inhibitory effect of the humic acids

The effect of HA as a laccase inhibitors and the type of inhibition were determined using 2 ml reaction mixtures contained in buffer A: 0.3–1 mmol l^{-1} ABTS, 0.002–0.2 mg ml^{-1} HA and 0.14 μg ml^{-1} enzyme. The inhibitory effect attributed to HA functional groups (total and carboxylic) was studied using 2 ml reaction mixtures contained in buffer A: 0.2 mmol l^{-1} ABTS, 0.02 mg ml^{-1} HA_p or 0.05 mg ml^{-1} HA_s and HA_{ch}, aliquots of 10 mmol l^{-1} $CuCl_2{\cdot}2H_2O$ stock solution added in such a way that a ratio of Cu ions to HA functional groups was 1:5, and 0.16 μg ml^{-1} enzyme. The inhibitory effect of HA fractions was studied using 0.2 mmol l^{-1} ABTS, 0.02 mg ml^{-1} HA_p or 0.05 mg ml^{-1} HA_s and HA_{ch}, and 0.16 μg ml^{-1} enzyme in 2 ml of buffer A. All reactions were initiated by adding the enzyme.

2.8. Transformation of humic acids by laccase

HA were dissolved in buffer A to give a concentration of 5 mg ml^{-1}. Incubation mixtures contained 400 μl of HA stock solution, corresponding to 2 mg of HA and 20 μl of laccase preparation (protein concentration 480 μg ml^{-1}, ΔA_{436} 190 min^{-1} ml^{-1}) in 1 ml of buffer A. Mixtures without the enzyme were used as controls. After 24, 48 and 96 h of incubation at 30 °C, aliquots were taken and diluted 1:1 and 1:200 in 0.025 mol l^{-1} Tris–HCl buffer (pH 8.2) with 0.05 mol l^{-1} NaCl, 0.1% SDS, and 0.02% NaN_3 for recording gel-filtration profiles and absorption spectra, respectively.

2.9. Analytical measurements

The molecular weight changes of HA were determined by gel-filtration on a K9 column (0.9 × 60 cm, Amersham-Pharmacia-Biotech) filled with Sephadex G-75 using 0.025 mol l^{-1} Tris–HCl buffer (pH 8.2) with 0.05 mol l^{-1} NaCl, 0.1% SDS, and 0.02% NaN_3, as an eluent. The elution rate was 8 ml h^{-1}. The elution profiles were recorded at 280 nm using a 2238 UVICORD SII detector (LKB, Sweden). The column was calibrated by Blue Dextran 2000 and $(NH_4)_2Cr_2O_7$ solutions in the eluent buffer to determine a column void volume and a total gel bed volume, respectively. The average molecular weights (MW) of humic acid fractions and the percentage of each fraction in HA were determined as described earlier (Zavarzina et al., 2002). Absorption spectra of HA before and after interaction with laccase were recorded on Specord M-40 spectrophotometer.

3. Results

3.1. Enzyme preparation

Laccase preparation had a specific activity of 430 arb.U ml^{-1}, corresponded to 900 arb.U mg^{-1} (Table 1). Purified enzyme was electrophoretically homogeneous with MW of 63 kDa (Fig. 1). Concentrated preparation was bluish and had a spectrum with a 'blue' maximum at 616 nm, absorption ratio at 278 and 616 nm was 25 (Fig. 2). The K_m value of laccase in oxidation of ABTS was 0.05 mmol l^{-1}.

3.2. Humic acids preparations

Nitrogen content in HA preparations followed the order $HA_p < HA_s < HA_{ch}$ (Table 2). The H/C ratio often used to assess a contribution of aliphatic chains bearing ≡CH, $=CH_2$, $-CH_3$ groups to the HA structure was lowest in HA_{ch}. That is in agreement with the absorption spectra data, showing the highest E values (absorbance of 0.001% HA solution at 465 or 650 nm) for this HA. HA_{ch} was also characterized by the largest total content of functional groups and by largest content of carboxylic groups.

An increase in average molecular weight and content of high molecular weight fractions of HA formed the order $HA_s > HA_p > HA_{ch}$ (Table 3). An increase in relative hydrophobicity (hydrophobic interaction chromatography data and extraction in the aqueous biphasic systems) of HA followed the order $HA_p \gg HA_s > HA_{ch}$ (Table 3), i.e. most low molecular weight humic acid from chernozem was less hydrophobic.

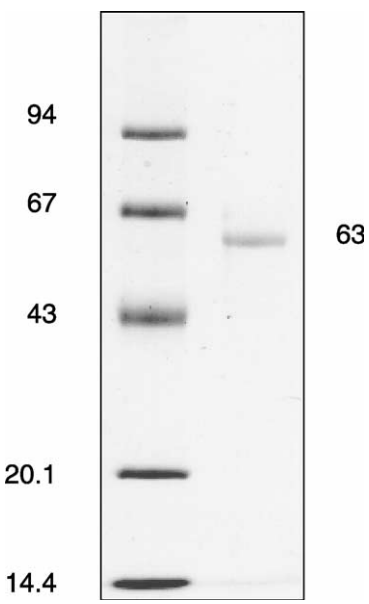

Fig. 1. SDS-PAG electrophoresis of blue laccase in 10% gel. Molecular weights (kDa) are according to low molecular weight markers kit (Amersham Pharmacia Biotech).

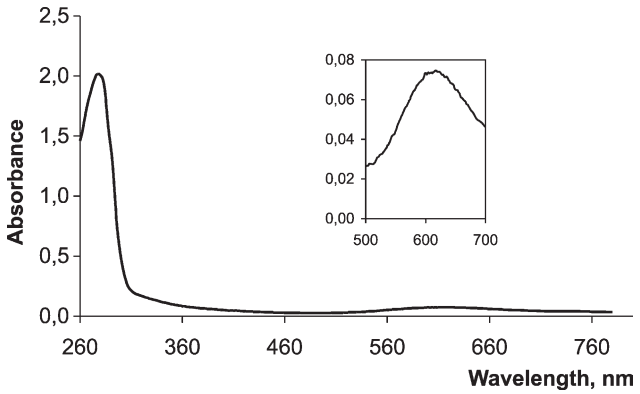

Fig. 2. Absorption spectra of 1 mg ml^{-1} preparation of blue laccase in buffer A; $A_{278}/A_{616} = 25$.

3.3. Humic acids fractionation by salting-out with ammonium sulfate

Five fractions of each HA were obtained by salting-out with $(NH_4)_2SO_4$. The first fraction of HA_p precipitated at 0–20% of saturation (Fig. 3(a)). This fraction was most abundant in the preparation (40 wt%) and most polymeric: the relative content of components eluted in a void volume of the column was about 40%. By increasing the percentage of saturation the content of these high molecular weight components in HA_p fractions gradually decreased. When 60% saturation was reached, only low molecular weight components of HA_p remained in supernatant (Fig. 3(a)). They represented about 25 wt% of initial preparation.

The high molecular weight components of HA_s began to coagulate only when 40% of $(NH_4)_2SO_4$ saturation was reached (Fig. 3(b)). Until 60% of saturation the relative content of this fraction in the obtained precipitates had not substantially changed and consisted of approximately 40% of high molecular weight fraction. A two-fold decrease in high molecular weight fraction was observed in the precipitate only at 70% of saturation, while HA_s components that remained in the supernatant at this degree of saturation still contained about 15% of high molecular weight fraction. This last fraction was of the largest content (about 45 wt% of initial preparation), while the content of other fractions was 10–12 wt%.

The ratio of high molecular weight to low molecular weight components in each fraction of HA_{ch} (Fig. 3(c)), precipitated until 60% of saturation was almost the same (0.2–0.3), the content of high molecular weight fraction being only 20%. Fraction of HA_{ch} that remained in supernatant at 60% of saturation was the most abundant (50 wt% of initial preparation).

The relative hydrophobicity of the HA fractions was confirmed by their extraction in aqueous biphasic systems. 'Hydrophobicity' of HA_p fractions decreased with increasing degree of $(NH_4)_2SO_4$ saturation (Table 4) as more

Table 2
Elemental composition, absorption spectra data and functional groups content of humic acids

HA	Ash (%)	Content (at.%)				H/C	Spectral data		Functional groups (mmol g^{-1})	
		C	H	O[a]	N		E_{465}	E_{465}:E_{650}	Total	COOH
HA_p	1.9	35.9	40.7	21.7	1.7	1.13	0.051	3.9	6.48	2.10
HA_s	3.2	37.3	41.3	18.7	2.7	1.12	0.043	4.3	6.30	2.88
HA_{ch}	5.2	41.8	35.3	19.7	3.2	0.84	0.070	3.8	6.75	2.97

[a] O content was found from the difference.

'hydrophobic' compounds are extracted in aqueous biphasic systems with higher distribution coefficients (K_d). The K_d values of HA_s or HA_{ch} fractions were similar.

3.4. Oxidative transformation of humic acids by blue laccase

Interaction of laccase with HA led to rearrangement in their molecular weight distributions and changes in absorption spectra that were most evident after 96 h of incubation. The HA_{ch} exhibited depolymerization (Fig. 4(c)): the relative content of its low molecular weight fraction increased, apparently in expense of high molecular weight fraction. After 48 h of incubation the average molecular weight of low molecular weight fraction became 13 kDa instead of 15 kDa in initial preparation (Table 5). After 96 h, two low molecular weight peaks were observed on the gel-chromatogram with the average molecular weight of 13 and 7.5 kDa. Contrary to HA_{ch} preparation, the HA_p was polymerized: the content of its high molecular weight fraction increased as well as the average molecular weight of the low molecular weight fraction (Fig. 4(a), Table 5). The result of HA_s interaction with the enzyme was intermediate between two other HA. The content of high molecular weight fraction decreased, but the average molecular weight of low molecular weight fraction increased (Fig. 4(b), Table 5).

Changes in molecular weight distributions were accompanied by changes in absorption spectra of HA (Fig. 5 (a)–(c)). As is seen from the Figures, absorption spectra before the reaction with laccase were typical for HA. No maxima appeared in the spectra of HA after their incubation with laccase, except for HA_{ch} spectrum where a shoulder was observed in the region of 280–350 nm. After the reaction with laccase the absorption of HA_{ch} preparation increased, while that of two other HA decreased (Table 5).

3.5. Humic acids as laccase inhibitors

HA were competitive inhibitors of laccase (Fig. 6). Inhibitory effect had increased with increasing of HA concentration. The HA_p showed highest inhibitory effect, with a constant of inhibition (K_i) being 5–8 times higher than that of two other HA, which were similar in their effect on laccase activity. HA_{ch} was the weaker inhibitor among the studied HA (Fig. 6, Table 6).

To eliminate the effect of HA functional groups, the HA–copper complexes were prepared in such a way that ratio of all HA functional groups to copper ions or the ratio of carboxylic ones to copper ions was 5:1. This resulted in 'inactivation' of HA functional groups through complex and salt formation. However almost no difference was observed between inhibitory effect of 'pure' HA and that of their Cu complexes (data not shown) suggesting that the other properties of HA were responsible for the decrease of laccase activity in the presence of HA.

In order to study the inhibitory effect of HA fractions, the most 'contrast' ones were chosen, i.e. with precipitation at low (e.g. 0–20%) or high (e.g. >60%) degrees of ammonium sulfate saturation. As is seen from Table 6, the inhibitory effect of most high molecular weight and hydrophobic fraction of HA_p was 15% higher than that of low molecular weigh and 'hydrophilic'. It should be noted, that this effect would be more pronounced if molar concentrations of HA were used instead of weight per volume concentrations. As average molecular weight of hydrophobic fraction was higher (Fig. 3) it contained a smaller number of molecules per weight of HA than hydrophilic fraction. Thus on the molar basis, concentration of hydrophobic fraction in our experiments was lower than the concentration of hydrophilic fractions. As it was shown above (Fig. 6) inhibitory effect of HA increased with increasing HA concentration thus the inhibitory effect of

Table 3
Gel filtration and hydrophobic interaction chromatography data of humic acids

HA	Fraction 1		Fraction 2		S_{phob}:S_{phil}[a]
	MW (kDa)	Content (%)	MW (kDa)	Content (%)	
HA_p	>75	22	13.2	78	1.5
HA_s	>75	26	13.2	74	1.0
HA_{ch}	>75	15	15.4	85	0.7

[a] The ratio between the areas of hydrophobic and hydrophilic fractions on hydrophobic interaction chromatography profiles of HA (from Zavarzina et al., 2002).

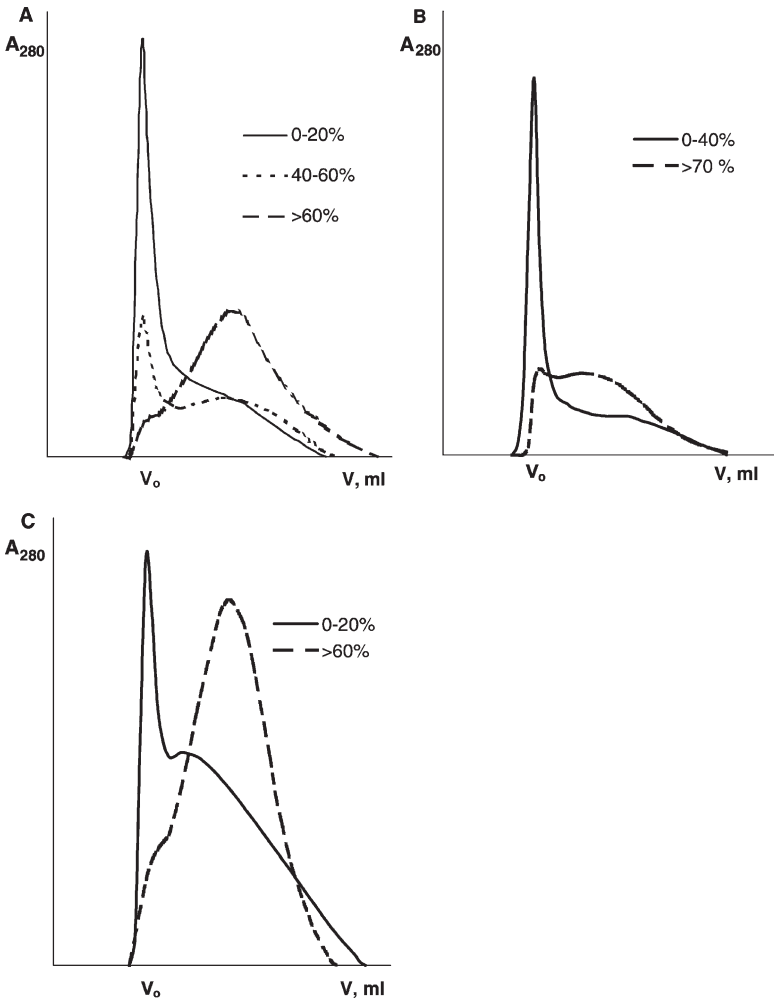

Fig. 3. Molecular weight distributions of humic acid fractions, obtained by salting-out with ammonium sulfate. Column 0.9 × 60 cm, Sephadex G-75 gel, elution by 0.025 mol l^{-1} Tris–HCl buffer (pH 8.2) + 0.05 mol l^{-1} NaCl + 0.1% SDS + 0.02% NaN_3 at a flow rate 8 ml h^{-1}; a, HA_p; b, HA_s; c, HA_{ch}.

hydrophobic HA fraction would be higher if equal molar concentrations of HA fractions were used. Almost no difference was observed in the inhibitory effects of HA_s and HA_{ch} fractions.

Table 4
Properties of HA fractions, obtained by salting-out with ammonium sulfate

HA	Saturation (%)	Relative content in initial HA (wt%)	Low MW fraction (kDa)	V_o/V_e [a]	K_d [b]
HA_p	0–20	43	18	0.61	2.7
	40–60	21	14	0.25	2.2
	>60	28	14	0	1.4
HA_s	0–40	18	13	0.89	0.5
	>70	40	34	0.18	0.6
HA_{ch}	0–20	8	32	0.20	0.4
	>60	50	19	0	0.5

[a] The ratio between the areas of high- and low- MW fractions in HA.
[b] K_d—distribution coefficient of humic acids in aqueous biphasic system of 5% PEG (15 kDa)–7.5% Dextran T-70-H_2O at pH 7.0. K_d is measured as a ratio of absorbances at 465 nm of the top phase (PEG-rich, more 'hydrophobic') and bottom phase.

4. Discussion

4.1. Laccase and humic acid preparations

Laccase, produced by *P. tigrinus* under conditions of submerged cultivation had the typical physico-chemical properties, described for laccases isolated from other wood- and litter-degrading fungi (Thurston, 1994). Absorption spectrum of the enzyme was typical for blue copper oxidases with absorption band near 600 nm (Reinhammer, 1984) and absorption ratio A_{280}/A_{610} within the range 17–25. This ratio reflects the balance between absorption of type 1 copper near 610 nm and the combined absorption of tryptophan and aromatic amino acid residues of the protein near 280 nm (Malkin et al., 1969).

The physico-chemical properties of HA were in accordance with properties of HA preparations extracted from respective soils (Orlov, 1990). HA from chernozem was the most aromatic as its H/C ratio was the lowest and its extinction coefficients were the highest (Table 2).

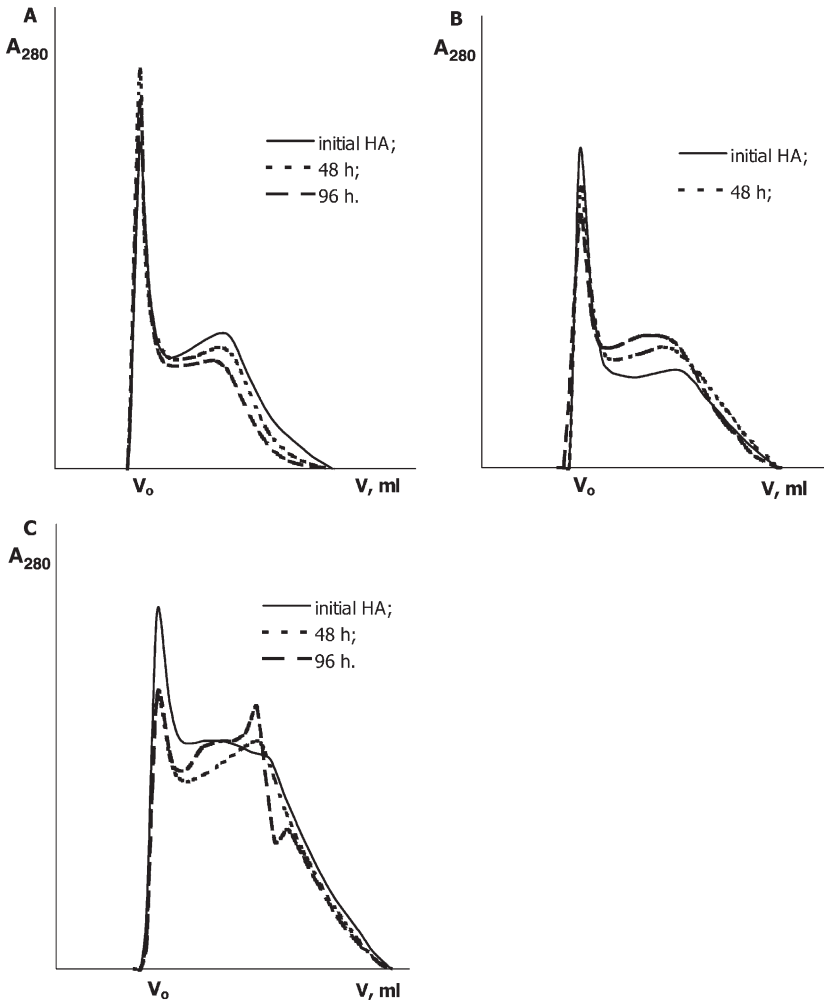

Fig. 4. Changes in molecular weight distributions of humic acids after interaction with laccase in vitro: a, HA_p; b, HA_s; c, HA_{ch}. Column 0.9 × 60 cm, Sephadex G-75 gel, elution by 0.025 mol l^{-1} Tris–HCl buffer (pH 8.2) + 0.05 mol l^{-1} NaCl + 0.1% SDS + 0.02% NaN_3 at a flow rate 8 ml h^{-1}.

The hydrophobic/hydrophilic properties of HA preparations were evaluated by three independent methods. The HA_p preparation was found to be the most hydrophobic. However it should be noted that in relation to HA the term 'hydrophobicity' is not quite proper and is used here for brevity. HA molecules contain both hydrophobic (aromatic rings, carbohydrate side chains) and hydrophilic fragments (e.g. those bearing polar functional groups). Thus the word hydrophobic in the context of this work means that the molecules of HA (HA fraction) in question contain more hydrophobic fragments than hydrophilic ones.

4.2. Transformation of humic acids by blue laccase

Our results show that laccase alone, without any mediators or other enzymes is capable of performing polymerization and depolymerization reactions of HA in vitro. These results are in contrary with in vivo systems where only depolymerization of polyphenols like lignin and humic substances occurred suggesting existence of agents that prevent polymerization (Galliano et al., 1991; Ralph and Catcheside, 1998).

The result of HA transformations depend on their initial molecular weight distribution, relative hydrophobicity and

Table 5
Humic acids gel-filtration and absorption spectra characteristics before and after incubation with laccase

Preparation	Gel-filtration data		Spectral data	
	LMW fraction (kDa)	V_o/V_e [a]	A_{280}	$E_{465}:E_{650}$ [b]
HA_p, initial	13.2	0.28	0.26	4.1
HA_p, 96 h	14.6	0.42	0.19	4.6
HA_s, initial	13.2	0.35	0.23	4.1
HA_s, 96 h	19.8	0.30	0.20	4.3
HA_{ch}, initial	15.4	0.18	0.27	3.6
HA_{ch}, 96 h	13.2 and 7.5	0.15	0.30	4.0

[a] The ratio between the areas of HMW and LMW fractions in HA.
[b] Absorption of 0.001% HA solution at 465 and 650 nm.

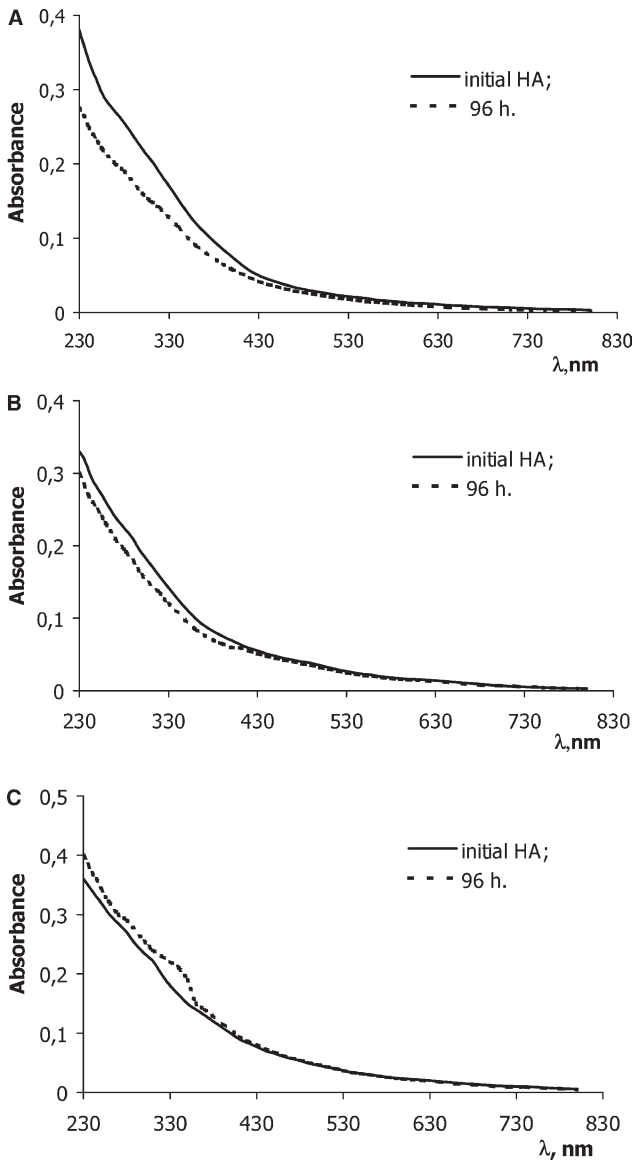

Fig. 5. Absorption spectra (pH 8.2) of humic acids before and after incubation with laccase in vitro; a, HA_p; b, HA_s; c, HA_{ch}

equilibrium between high- and low molecular weight fractions of HA. Alterations in HA molecular weight distributions observed in this study were similar to those that occurred in vitro with other polymeric phenolic compounds, i.e. lignin, in presence of laccase (Evans, 1985; Maltseva et al., 1991; Milstein et al., 1994). During lignin degradation by laccase two reactions can occur depending on initial molecular weight distribution in lignin preparations: polymer degradation with formation of low molecular weight products or condensation to form high molecular weight fractions indicative of existence of condensation–depolymerization equilibrium (Yaropolov et al., 1994). When degrading lignin, laccase is responsible for its demethylation, cleavage of C_α–C_β and alkyl–aryl bonds of phenolic substructures, and side-chain elimination. Condensation occurs due to spontaneous polymerization of free radicals, formed as a result of oxidation of hydroxyl groups in the presence of molecular oxygen (Reinhammer, 1984; Thurston, 1994). After interaction with laccase lignin is enriched with carboxylic groups due to oxidation of methoxy groups (Koroleva et al., 2002).

Depolymerization of HA in vitro was not necessarily accompanied by their decolorization: HA_{ch} showed increase in absorbance during depolymerization while HA_p that exhibited polymerization showed decrease in absorbance. This could be explained by hypo/hyperchromic effect accompanied polymerization/depolymerization reactions of HA. This effect is well known for biopolymers with regular structures. Despite the absence of such organization in HA structure, the transitions from monomer to polymer increases the order of structural complexity, this could be a reason for the hypochromic effect. The depolymerization of HA may be a reason for the hyperchromic effect. Similar transformations of absorption spectra were detected during the study of polymerization/depolymerization of milled wood lignin of birch by purified laccase and Mn-peroxidase of *P. tigrinus* (Leontievsky et al., unpublished data).

Failure to show any alterations in the structure of humic substances in the presence of purified laccase in previous works (Cohen et al., 1987) can be explained by the relatively strong inhibitory effect of humic compounds that were observed in the study. The inhibitory effect of HA was of the competitive type and increased with increasing HA concentration. That is in agreement with the results of Gianfreda and Bollag (1994) who reported almost a linear relationship between the organic matter content and its inhibitory effect on activities of free and immobilized laccase. Interestingly, the competitive nature of inhibition was shown for other enzymes incubated with humic substances, i.e. lignin peroxidase (Wondrack et al., 1989), pronase, trypsin, and carboxypeptidase (Ladd and Butler, 1971). Taking into consideration the differences in properties of HA in this study it has been suggested that their inhibitory effect may be due to: (1) HA functional groups, these may effect the laccase active site through complex formation with copper ions: carboxylic groups of HA seem to be effective chelators under the described experimental conditions as they are partially ionized at pH 5.0 (Zavarzina and Demin, 1999); (2) differences in molecular weight; (3) the different content of hydrophobic and hydrophilic fragments in HA. ‘Inactivation’ of HA complexing sites by copper ions did not cause any changes in the inhibitory effect. Earlier observations showed that chelating agents such as EDTA, were weak inhibitors of fungal laccases (Fukushima and Kirk, 1995; Kiiskinen et al., 2002). The comparison of HA as well as their fractions by their effect on laccase activity showed that most high molecular weight and hydrophobic HA_p and its respective fraction were the strongest inhibitors of the enzyme. Almost no difference was observed in the inhibitory effect of HA_s and HA_{ch} fractions that differed in molecular weight but had similar hydrophobic/hydrophilic properties. These results suggest

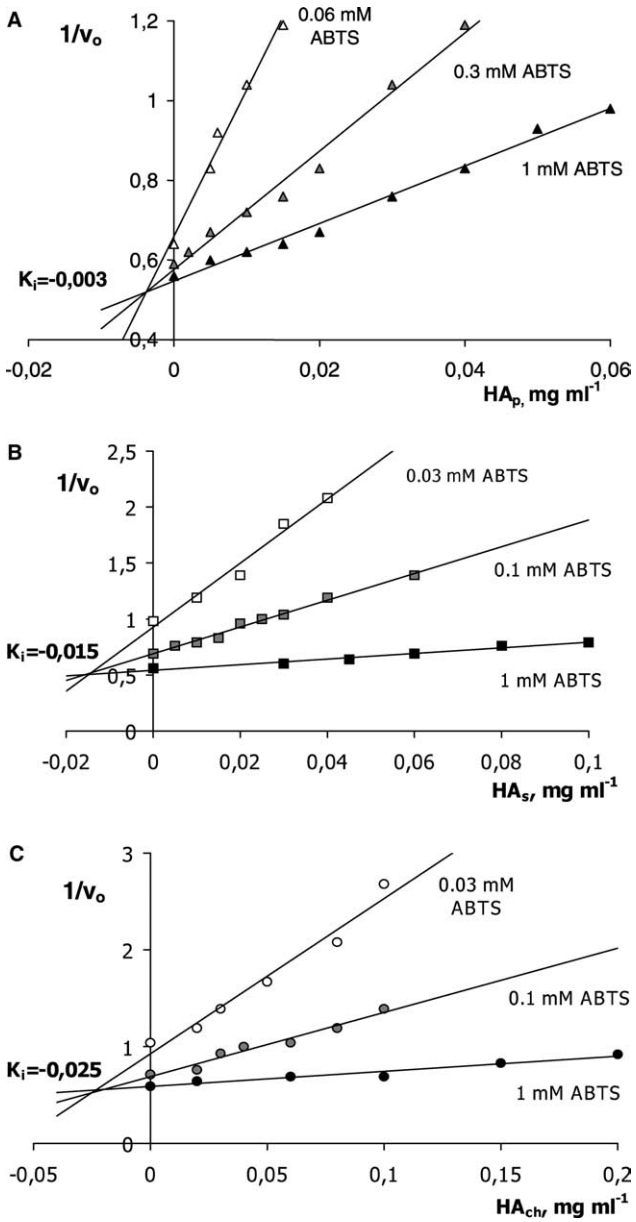

Fig. 6. Inhibitory effect of humic acids on laccase: a, HA_p; b, HA_s; c, HA_{ch}. 2 ml reaction mixtures contained in buffer A: 0.3–1 mmol l^{-1} ABTS, 0.002–0.2 mg ml^{-1} HA and 0.14 μg ml^{-1} enzyme.

that inhibitory effect of HA was related to their relative hydrophobicity rather than to molecular weight. More 'hydrophobic' humus constituents were also shown to be stronger inhibitors of horseradish peroxidase (Cozzolino and Piccolo, 2002) and proteolytic enzymes (Butler and Ladd, 1971; Rostovshchikova et al., 1998), although the mechanism of inhibition could be different. Cozzolino and Piccolo (2002) suggested that the larger the strength of humic hydrophobic associations, then the lower the reactivity in oxidative coupling reactions due to steric limitations. With this respect it is interesting to note that in this study HA fractions that were affected by laccase caused the lowest inhibitory effect on the enzyme. For example in HA_p the content of high molecular weight fraction was

Table 6
Inhibitory effect of humic acids and their fractions on blue laccase

Inhibitor[a]	Activity (%)[b]
HA_p	56
HA_p, 0–20% $(NH_4)_2SO_4$	55
HA_p, 40–60% $(NH_4)_2SO_4$	65
HA_p, >60% $(NH_4)_2SO_4$	70
HA_s	65
HA_s, 0–40% $(NH_4)_2SO_4$	74
HA_s >70% $(NH_4)_2SO_4$	71
HA_{ch}	75
HA_{ch}, 0–20% $(NH_4)_2SO_4$	85
HA_{ch} >60% $(NH_4)_2SO_4$	84

[a] Concentration of HA_p and its fractions—0.02 mg ml^{-1}; concentration of HA_s, HA_{ch} and their fractions—0.05 mg ml^{-1}.
[b] Activity of blue laccase in oxidation of ABTS is 100%.

higher than that of low molecular weight fraction and depolymerization reaction could be expected. However polymerization of low molecular weight fraction occurred, as more hydrophilic components of this fraction were weaker inhibitors of the laccase.

5. Conclusions

It was shown that purified blue laccase was capable of both polymerization and depolymerization of HA in vitro. Polymerization of HA_p was accompanied by its decolorization, while depolymerization of HA_{ch} resulted in increase of absorption. Those HA fractions were affected by laccase that were weaker inhibitors of the enzyme (more hydrophilic). HA were competitive inhibitors of blue laccase towards oxidation of ABTS. Hydrophobic fragments of HA were responsible for the inhibitory effect.

Our finding that laccase can be responsible for degradation and polymerization of HA clarifies and extends its role in natural processes as not only an important agent of lignin degradation but also biocatalyst in oxidative transformations of humic substances—the most abundant and stable form of organic carbon in soils and aquatic systems. Along with other phenoloxidases, laccase can participate not only in oxidative coupling of low molecular weight phenols—precursors of HA during humus biosynthesis, but also change the structure of already formed humic substances. Catalyzing degradation of HA to low molecular weight products laccase may contribute to the formation of fulvic-acid-like compounds—water-soluble recalcitrant components of soils and natural waters. Moreover by oxidizing humic substances, laccase may be important in regulation of soil redox potential. However due to competitive inhibition of laccase by HA, enzyme concentration and activity in soils should be rather high. That may be achieved by laccase immobilization on soil organic and inorganic components, protecting the enzyme at least

partially from denaturation and inactivation in soil environment.

Acknowledgements

The authors are grateful to Dr O.V. Moiseeva (IBPM RAS) for SDS-PAG electrophoresis, Dr A.A. Stepanov (MSU) for giving access to GPC equipment and M. Dolotina for the revising of English text. Authors are also grateful to grants RFBR 02-04-49900, INTAS 01-0512 and NATO SfP 972294 for financial support of this work.

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