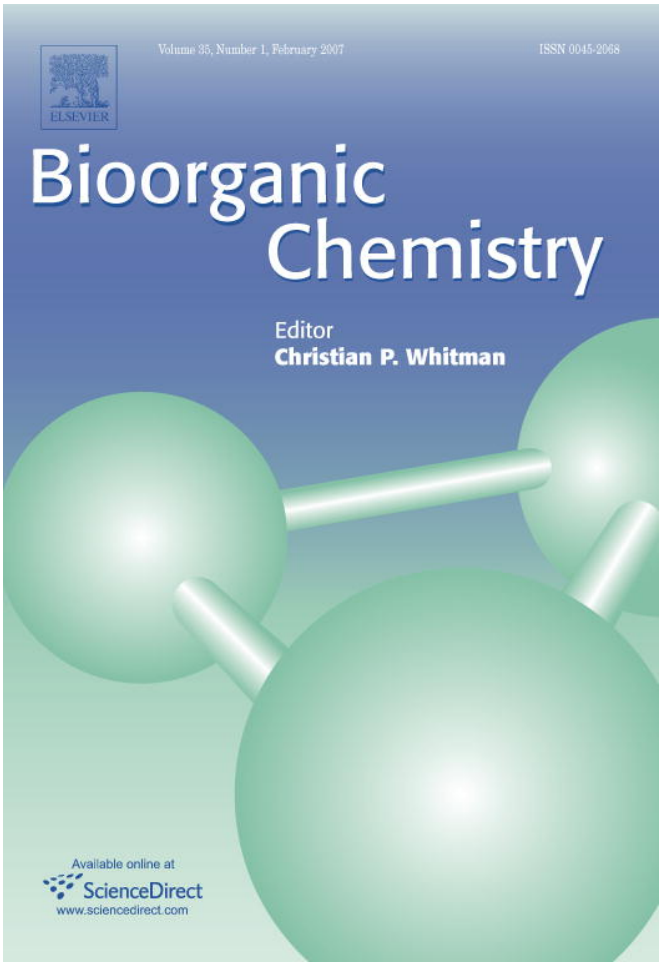

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Characterization of two new multiforms of *Trametes pubescens* laccase

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Abstract

Electrochemical properties of two multiforms of laccase from *Trametes pubescens* basidiomycete (LAC1 and LAC2) have been studied. The standard redox potentials of the T1 sites of the enzymes were found to be 746 and 738 mV *vs.* NHE for LAC1 and LAC2, respectively. Bioelectroreduction of oxygen based on direct electron transfer between each of the two forms of *Trametes pubescens* laccase and spectrographic graphite electrodes has been demonstrated and studied. It is concluded that the T1 site of laccase is the first electron acceptor, both in solution (homogeneous case) and when the enzymes are adsorbed on the surface of the graphite electrode (heterogeneous case). Thus, the previously proposed mechanism of oxygen bioelectroreduction by adsorbed fungal laccase was additionally confirmed using two forms of the enzyme. Moreover, the assumed need for extracellular laccase to communicate directly and electronically with a solid matrix (lignin) in the course of lignin degradation is discussed. In summary, the possible roles of multiforms of the enzyme based on their electrochemical, biochemical, spectral, and kinetic properties have been suggested to consist in broadening of the substrate specificity of the enzyme, in turn yielding the possibility to dynamically regulate the process of lignin degradation according to the real-time survival needs of the organism.

Keywords: *Trametes pubescens*; Laccase; Redox potential; T1 site; Lignin degradation

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1. Introduction

Laccase (benzenediol:oxygen oxidoreductrase; EC 1.10.3.2.), a multicopper enzyme belonging to the family of blue oxidases, catalyses the oxidation of a wide variety of organic and inorganic substrates with the concomitant four-electron reduction of oxygen to water [1]. Multicopper oxidases with laccase activity are found in plants, fungi, insects, and bacteria [1–4]. Laccases are attractive, industrially relevant enzymes that can be used for a number of diverse applications, e.g., biofuel cells [5], biosensors [6], green biodegradation of xenobiotics including pulp bleaching [7,8], labelling in immunoassays [9], bioremediation [10], green organic synthesis [11], and even design of laccase fungicidal and bactericidal preparations [12]. Broad possibilities of application stimulate new waves of fundamental research concerning this enzyme. Activities of current interest include screening of laccase sources, studying new laccases [2,13–17], investigating the structure of the enzyme [18–21], elucidating the mechanism of the intraprotein electron transfer as well as the mechanism of oxygen reduction to water [22,23], investigating the electrochemical properties of laccases [24], and much more.

Laccase contains four copper ions, which are historically classified into three types according to their spectroscopic characteristics, namely, the T1, T2, and T3 sites. One of the key characteristics of laccase is the standard redox potential of the T1 site. The values of the redox potential of this site have been determined using potentiometric titrations with redox mediators for a great number of different laccases and they are found to vary between 430 and 790 mV vs. NHE [13,25–29].

Fungal laccase is an extracellular glycosylated protein of mass 60–85 kDa of which 10–30% is carbohydrate [1,30,31]. In white-rot fungi, laccases are typically produced as multiple isoenzymes [32,33] encoded by gene families ([34,35]; Table 1). It has been suggested that genes encoding various isoenzymes are differentially regulated [36], with some being constitutively expressed and others being inducible [32,37]. Several biochemical studies of different forms of fungal laccase from different basidiomycetes,

Table 1
Some biochemical and electrochemical properties of different forms of *Trametes* laccase

Laccase	MW (kDa)	p*I*	pH-Optimum	Carbohydrate content (%)	$E^{0'}$, T1 (mV)	$E^{0'}$, O_2 (mV)
Trametes pubescens LAC1 [42]	67 ± 2	5.3 ± 0.1	4.0–4.5[1]	13 ± 1	746 ± 5	775 ± 20
Trametes pubescens LAC2 [42]	67 ± 2	5.1 ± 0.1	4.0–4.5[1]	13 ± 1	738 ± 5	770 ± 20
Trametes pubescens LAP2 [40]	65	2.6	3.0–4.5[2]	18	n.d.	n.d.
Trametes C30 LAC1 [63]	63	3.6	4.5–5.0[2]	12	730	n.d.
Trametes C30 LAC2 [13]	65	3.2	5.5–6.0[2]	12	560	n.d.
Trametes C30 LAC3 [41]	≈65	4.0	5.5–6.0[2]	n.d.	530	n.d.

Note: In the present study, the redox potentials of the T1 sites ($E^{0'}$, T1) as well as half-wave potentials of oxygen bioelectroreduction ($E^{0'}$, O_2) were determined; carbohydrate content: *N*-acetylglucosamine, mannose, and xylose; pH-optima were determined using the following substrates: 1, catechol; 2, syringaldazine; n.d., not determined.

such as *Trametes hirsuta*, *Coriolopsis rigida*, *Trametes* C30, and *Trametes pubescens* have been performed so far [13,16,38–42]. However, only a few publications describe complete characterization of several multiforms (isoforms) of laccase from identical sources [13,39,41,42], and only a few references present a comparison (albeit limited) of a variety of laccases from different sources (e.g., [43]). Usually, of each species, only one major laccase isoenzyme is described in detail [31,38,40]. In general, the data on the properties of multiforms of the enzyme are still very limited, present results are not fully understood, and the significance of laccase multiplicity in nature is absolutely not clear [1,38,44–46].

In our recent publication a method for purification of enzymes from the ligninolytic complex of the basidiomycete *Trametes pubescens* has been elaborated [42]. Two homogeneous preparations of laccase (LAC1 and LAC2) were isolated. Basic biochemical parameters of the enzymes were determined (Table 1). The pH dependences and thermal stabilities of the laccases were investigated and the kinetic parameters of the enzymatic reactions catalysed by the laccases were also determined using different substrates (Table 2). The structures of the active sites of both laccases were studied with EPR, CD, and UV–vis spectroscopies, as well as using fluorescence analysis. Our studies showed similarity of the biochemical and spectral characteristics of the two laccases, whereas their kinetic properties were found to be different. This earlier work could not shed any light on the reasons for such differences.

Indeed, the main objective of the work reported below was to further characterise these two forms of laccase from the basidiomycete *Trametes pubescens* using electrochemical, spectroelectrochemical, and mass spectrometric tools. The ultimate aim is to propose a possible role of laccase multiplicity in nature.

2. Materials and methods

2.1. Chemicals

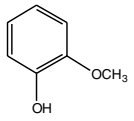
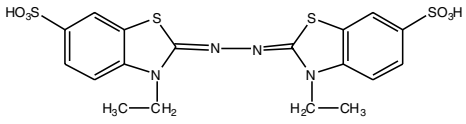
Catechol and ABTS[1] were from Sigma (St. Louis, MO, USA); NaOH, KCl, NaCl, Na_2HPO_4, and KH_2PO_4 were from Merck (Darmstadt, Germany); and buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, Milford, CT, USA). The mediator $K_4[Mo(CN)_8]$ was synthesized and purified according to a previously published method [47].

2.2. Isolation and purification of laccases

Basidiomycete *Trametes pubescens (Schumach.) Pilát* (Syn.: *Coriolus pubescens (Schum. ex Fr.) Quél.)* BCB 923-2 was obtained from the laboratory collection of the Moscow State University of Engineering Ecology (Russia). Basidiomycete was grown by submerged cultivation and two preparations of laccase were isolated and purified from the cultural liquid as described in [42]. The enzymes were homogeneous as judged by HPLC and SDS–PAGE. Homogeneous preparations of the two laccases were stored in 0.1 M phosphate buffer, pH 6.5, at −18 °C.

[1] *Abbreviations used:* ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); SPGE, spectrographic graphite electrode.

Table 2
Kinetic parameters of oxidation reactions of guaiacol and ABTS by multiforms of laccase from different *Trametes* basidiomycetes

Laccase	Guaiacol 			ABTS 		
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}/\mu M$)	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}/\mu M$)
Trametes pubescens LAC1 [42]	136	220	0.6	150	50	3.0
Trametes pubescens LAC2 [42]	55	120	0.5	400	60	6.7
Trametes pubescens LAP2 [40]	180	360	0.5	350	43	8.1
Trametes C30 LAC1 [13]	38	71	0.5	10	2.9	3.5
Trametes C30 LAC2 [13]	1261	1006	1.3	683	536	1.3
Trametes C30 LAC3 [41]	721	1600	0.5	944	280	3.4

Note. In the present study, the kinetic parameters of oxidation reactions of guaiacol by two multiforms of *Trametes pubescens* laccase were determined.

2.3. Laccase assay and kinetic studies

Laccase activity was determined spectrophotometrically (Hitachi-557 spectrophotometer, Tokyo, Japan) in a reaction medium containing 10 mM catechol ($\lambda = 410$ nm; $\varepsilon = 740\ M^{-1}\ cm^{-1}$) in 100 mM citrate–phosphate buffer, pH 4.5, at room temperature. The volume of the reaction mixture was 2 ml.

The dependence of laccase activity on pH in homogeneous solution was determined by estimation of the initial rates of oxygen consumption by using a Clark oxygen electrode in a sealed cell at 25 °C with constant stirring. Appropriate concentrations of substrates were used in order to ensure a measurable linear rate for the first 40 s of the reaction, which was started by the addition of laccase. The concentration of oxygen was assumed to be 260 μM (from a calculation of the Henry coefficient: 773 A/mol/kg of water) [38].

2.4. Electrochemical studies

2.4.1. Redox titration of laccases

To determine the redox potentials of laccase T1 centres, the method of protein redox titration was employed with potassium octacyanomolybdate (IV) mediator [31,48]. Electrochemical potentiometric titrations were carried out using a spectroelectrochemical cell consisting of a gold capillary (with a volume of 0.7 μl). The design of the cell was described elsewhere [49]. The potential of the gold capillary of the cell was controlled by a three-electrode potentiostat BAS LC-3E (Bioanalytical Systems, West Lafayette, IN, USA). In these measurements, an $Ag|AgCl|KCl_{sat}$ reference electrode (200 mV vs. NHE) and a platinum counter electrode were used. The absorbance spectra were monitored with PC2000-UV–vis, a miniature fibre optic spectrometer from Ocean Optics (Dunedin, FL, USA) with an effective range between 200 and 1100 nm. The pre-treatment of the gold capillary working electrode of the spectroelectrochemical cell was carried out by washing the cell capillary with a peroxide–sulphuric acid mixture followed by rinsing with Millipore water.

2.4.2. Cyclic voltammetry measurements

The laccase-modified electrodes serving as working electrodes were fabricated from spectrographic graphite electrodes (type RW001, 3.05 mm in diameter, Ringsdorff Werke GmbH, Bonn, Germany). The surfaces of the electrodes were prepared by first polishing with fine emery paper (Tufback Durite, P1200), then thoroughly rinsing with Millipore water, and finally allowing drying. An aliquot of 10 μl of laccase solution (0.7 mg/ml) was placed on the electrode surface and after 20 min the electrode was rinsed again with water. Cyclic voltammograms (at sweep rates varying from 5 to 500 mV/s) of the laccase-modified electrodes were carried out using a conventional three-electrode system connected to a BAS CV-50 W potentiostat with BAS CV-50W software v. 2.1 and a one-compartment electrochemical cell (volume 10 ml). In these measurements an Ag|AgCl|3 M NaCl reference electrode (BAS, 210 mV vs. NHE) and a platinum counter electrode were used.

2.5. MS-related studies

2.5.1. Digestion of proteins

Protein solutions of 10 μM LAC1 and LAC2 from *T. pubescens* were diluted with 0.2 M ammonium bicarbonate buffer. To reduce any disulfide bridges in the proteins, dithiothreitol

was added to a concentration of 5 mM and the temperature was raised to 50 °C for half an hour. After cooling to room temperature, iodacetamide was added to a concentration of 10 mM and the samples were stored in darkness for half an hour. Finally, 1 μg of sequencing-grade trypsin was added and the samples were incubated at 37 °C for 4 h. The final concentration of digestion products was calculated to be about 2.5 μM. The chemicals were from Sigma–Aldrich except for trypsin, which was from Promega (Falkenberg, Sweden).

2.5.2. MALDI-MS

The matrix was 10 mg of 4-hydroxy-α-cyanocinnamic acid (HCCA, Sigma–Aldrich) dissolved in a 30:70 mixture of 0.5% TFA/acetonitrile, vortexed, and centrifuged. Aliquots of digest were combined with matrix (2 μl and 5–20 μl, respectively) and 0.5-μl droplets were spotted onto a target plate and dried (''dried-droplet'' deposition method) for matrix-assisted laser desorption–ionization (MALDI) mass spectrometry (MS) analysis. Peptide calibrants were deposited in the same fashion. The calibrants were from Bruker Daltonics (Part No. 206195; Bremen, Germany). An Applied Biosystems 4700 Proteomics Analyser MALDI time-of-flight (TOF) mass analyser (Foster City, California) was employed to characterize the protein digests. The m/z range used was 500–5000 and the focus m/z was 1400. Mass spectra were attained by randomly shooting the laser beam onto each target spot. Both external and internal calibrations were performed. To acquire sequence information, some peptide ions were subjected to tandem MS experiments involving a gate selection of (singly charged) ions of interest and then a second stage of TOF analysis of the fragment ions. In some experiments metastable decay of ions was probed and in other experiments collisions in a gas cell promoted fragmentation.

2.5.3. ESI-QIT-MS

The protein digests were also analysed on an Esquire quadrupole ion trap (QIT) equipped with a nanospray ionisation source (Bruker). Aliquots of digest were augmented with acetonitrile and acidified with formic acid (2.5%). Borosilicate pulled tips coated with platinum from Proxeon (Odense, Denmark) were used as sprayers. Selected doubly charged ions were isolated in the ion trap and subjected to fragmentation conditions after which fragment mass spectra were acquired.

2.5.4. Calculation tools

MALDI-TOF-MS results were processed with Data Explorer® Software Version 4.6 (Applied Biosystems). ESI-QIT-MS results were analysed with Data Analysis 3.0 (Bruker). Internet-based tools on the Expasy, ProteinProspector, and PROWL websites were employed as aids in interpreting mass spectra (http://www.expasy.org; http://prospector.ucsf.edu; http://prowl.rockefeller.edu).

3. Results

As was mentioned in the Introduction, in our recent publication [42] it was shown that basidiomycete *T. pubescens* produces two major forms of laccase under the specific growing conditions presented therein [42]. The presence of two forms of laccase in the cultural liquid of *T. pubescens* basidiomycete is in good agreement with previously published results [40], especially taking into account that two genes encoding *T. pubescens* laccase have been found so far (NCBI GenBank Website, http://www.ncbi.nlm.nih.gov/

Genbank/; AAM18408 and AAM18407). The purification procedure for the preparation of the two laccases was described, and the basic biochemical properties of the enzymes were studied [42] (Table 1).

The redox potential of the T1 copper, e.g., the midpoint potential of the titration curves (E_m at pH 6.5), was measured by direct redox titration using $K_4Mo(CN)_8$ as mediator. As an example, absorbance spectra recorded during the redox titration of LAC1 are shown in Fig. 1A. The reduction of the T1 copper was accompanied by the disappearance of the blue absorbance band at 610 nm. Redox titration curves for both laccases are presented in Fig. 1B. The redox potentials of the enzymes were found to be 746 ± 5 and 738 ± 5 mV vs. NHE for LAC1 and LAC2, respectively. The slope of the Nernst dependence during the course of laccase redox titrations in $\log[A/(A_0 - A)]$-E coordinates was calculated to be 61 and 65 mV for LAC1 and LAC2, respectively (Table 1; Fig. 1A, insert).

In the presence of the enzyme substrate (molecular oxygen), a reduction current was recorded at laccase-modified spectrographic graphite electrodes as a result of direct electron transfer between the electrode and the adsorbed enzymes. Cyclic voltammograms of bare and laccase-modified spectrographic graphite electrodes under aerobic conditions at pH 4.0 are shown in Fig. 2. When adsorbed on electrodes, both laccases largely decreased the overvoltage needed for the electroreduction of molecular oxygen. As can be seen from the cyclic voltammograms (Fig. 2), the electrocatalytic current at the electrodes modified with LAC1 and LAC2 started at a potential of about +870 mV with half-wave potentials for oxygen electroreduction of +745 and +735 mV vs. NHE (pH 4.0) for LAC1 and LAC2, respectively (Fig. 2; Table 1). Fig. 3A summarizes the dependence of the registered electrocatalytic currents on solution pH. Current densities are plotted vs. pH for values ranging from pH 2.5 to 6.0. The pH dependence experiment was initiated after stabilization of the laccase-modified electrodes as achieved by waiting for 10 min in order to obtain a reproducible signal as described in [50]. The small decay of the activity of the enzyme during the experiment was corrected by normalizing to the value at pH 5.0 recorded at regular intervals. As can be seen from Fig. 3, the pH dependences of both laccases were very similar whether the enzymes were adsorbed on the electrodes or dissolved in the solutions (cf. Figs. 3A and B).

As can be seen from all our data, biochemical, physico-chemical, and electrochemical properties of both enzymes were very close. Further MALDI analysis of digests of proteins LAC1 and LAC2 in the m/z range 500–5000 supported the suggestion that these proteins are highly similar (Fig. 4). Moreover, in both spectra, five peaks (at m/z 1514.8, 1705.8, 1727.0, 1976.0, and 2470.2) were found to be consistent with certain tryptic peptides from a laccase associated with *Trametes versicolor* (pdb 1GYCA), not *T. pubescens* (AAM18408 and AAM18407; Table 1). To check the correspondence in more detail, these peptides were subjected to tandem mass spectrometry experiments. Three of these peptides were found to fragment according to the hypothesized sequences, as now described.

SAGSTTYNYNDPIFR: this peptide has m/z 1705.7822 when singly charged and 853.3950 when doubly charged. In MALDI, a peak at 1705.8 was observed with an isotopic pattern in very good agreement with the expected one. Tandem MS with MALDI revealed a variety of fragment ions consistent with the hypothesized sequence, including a prominent y4 ion. ESI revealed the doubly charged peak at 853.4, with an equally strong singly charged interferent at 852.4. Thus, with ESI, it was not possible to obtain an unambiguous fragmentation spectrum of the precursor ion at 853.4. Nonetheless, the

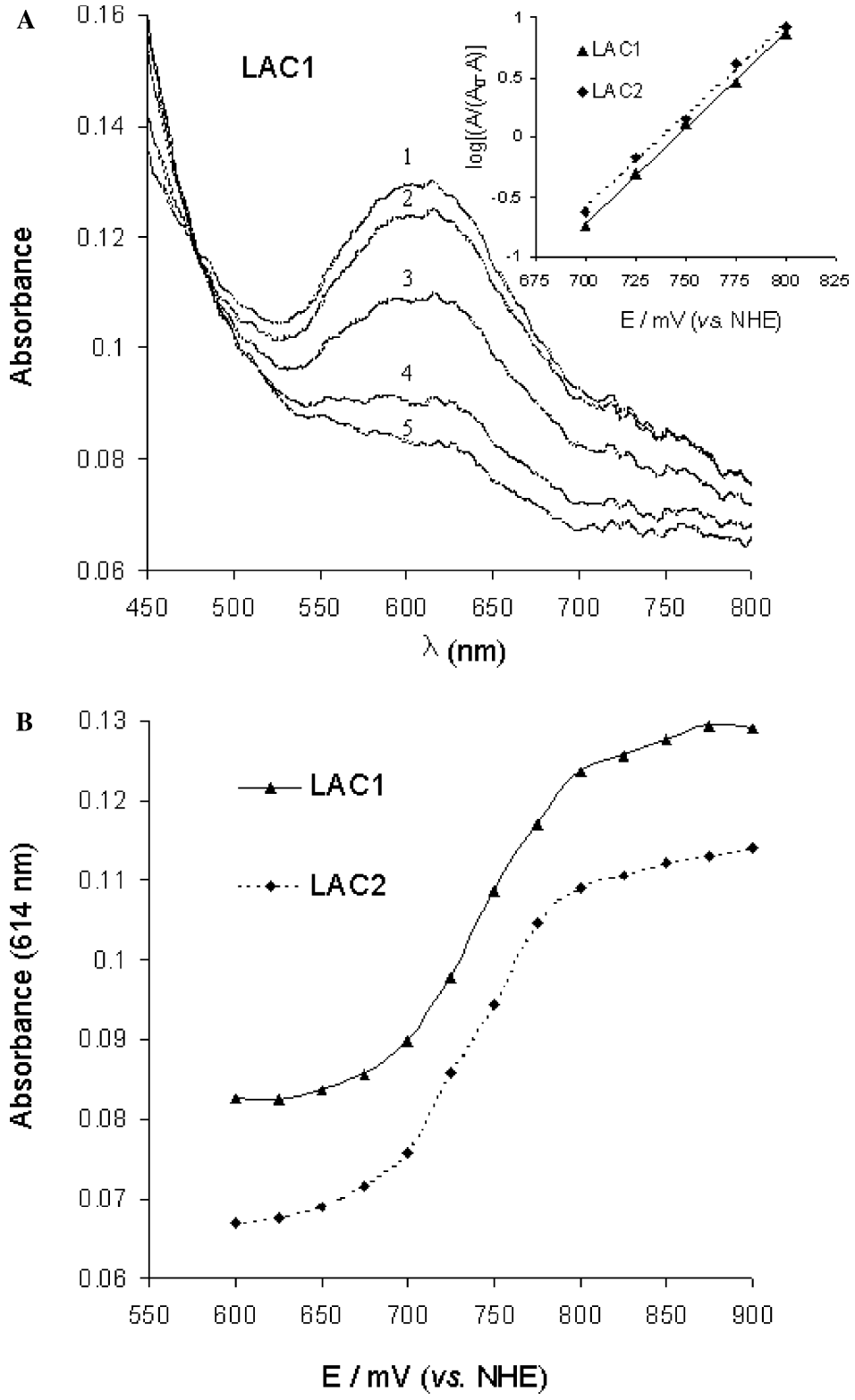


Fig. 1. Mediated spectroelectrochemical redox titration of two *Trametes pubescens* laccases using a gold capillary cell (22 μM LAC1 and 18 μM LAC2; 200 μM $K_4Mo(CN)_6$; 0.1 M phosphate buffer, pH 6.5). (A) Absorbance spectra recorded during the redox titration of LAC1. Curves 1–5 are absorbance spectra of the enzyme at applied potentials 900, 800, 750, 700, and 600 mV, respectively. Inset: Nernstian plots of the E dependences of the absorbance at 614 nm for both laccases. (B) Spectroelectrochemical titration curves reflecting the dependence of absorbance of the laccase solution at 610 nm vs. the applied potential.

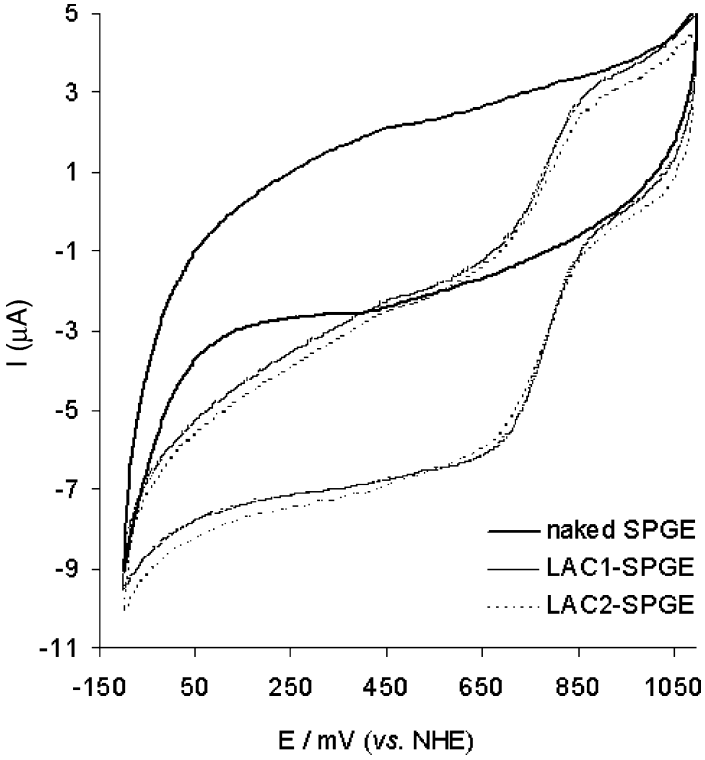


Fig. 2. Electroreduction of molecular oxygen on a naked spectrographic graphite electrode and on a spectrographic graphite electrode modified with adsorbed laccase. 0.1 M citrate–phosphate buffer, pH 4.0; scan rate 10 mV/s; start potential 1100 mV vs. NHE; second scan; 0.26 mM dioxygen.

obtained fragmentation spectrum clearly showed y-type ions associated with ...TTYNYND... along with prominent extra peaks, which we assume are associated with the interferent (data not shown).

ANPNFGTVGFAGGINSAILR: this peptide has *m/z* 1976.0354 when singly charged and 988.5216 when doubly charged. In MALDI, a peak at 1976.0 was observed accompanied by an isotopic pattern (Fig. 4B) in good agreement with the theoretically expected one (Fig. 4C; second peak bigger than the first). No interferents were apparent. Corresponding doubly charged species were seen in ESI again with no interferents. Tandem MS with ESI showed an extensive y-type ion series consistent with ...GTVGFAGGINSAI... and a similar b-type ion series, from both proteins LAC1 and LAC2 (Fig. 4D). Tandem MS with MALDI showed a revealing y-type ion series also consistent with this sequence, again for both LAC1 (Fig. 4D) and LAC2 (data not shown).

YDVDNESTVITLTDWYHTAAR: this peptide has *m/z* 2470.1527 when singly charged and 1235.5803 when doubly charged. In MALDI, a peak was observed at 2470.2 characterized by the expected isotopic pattern (monoisotopic and third isotopic peaks of similar height). The peak at 1235.6 could be observed weakly with ESI. Tandem MS with ESI revealed for both LAC1 and LAC2 the sequence pattern ...STVITLTDWY... as a series of y-type ions (data not shown). Tandem MS with MALDI gave consistent results.

The signals at *m/z* 1514.8 and 1727.0 may correspond to the peptides FPL...LGR and DAI...TGK, respectively, but due to close interferents these correspondences could not be verified in the present study.

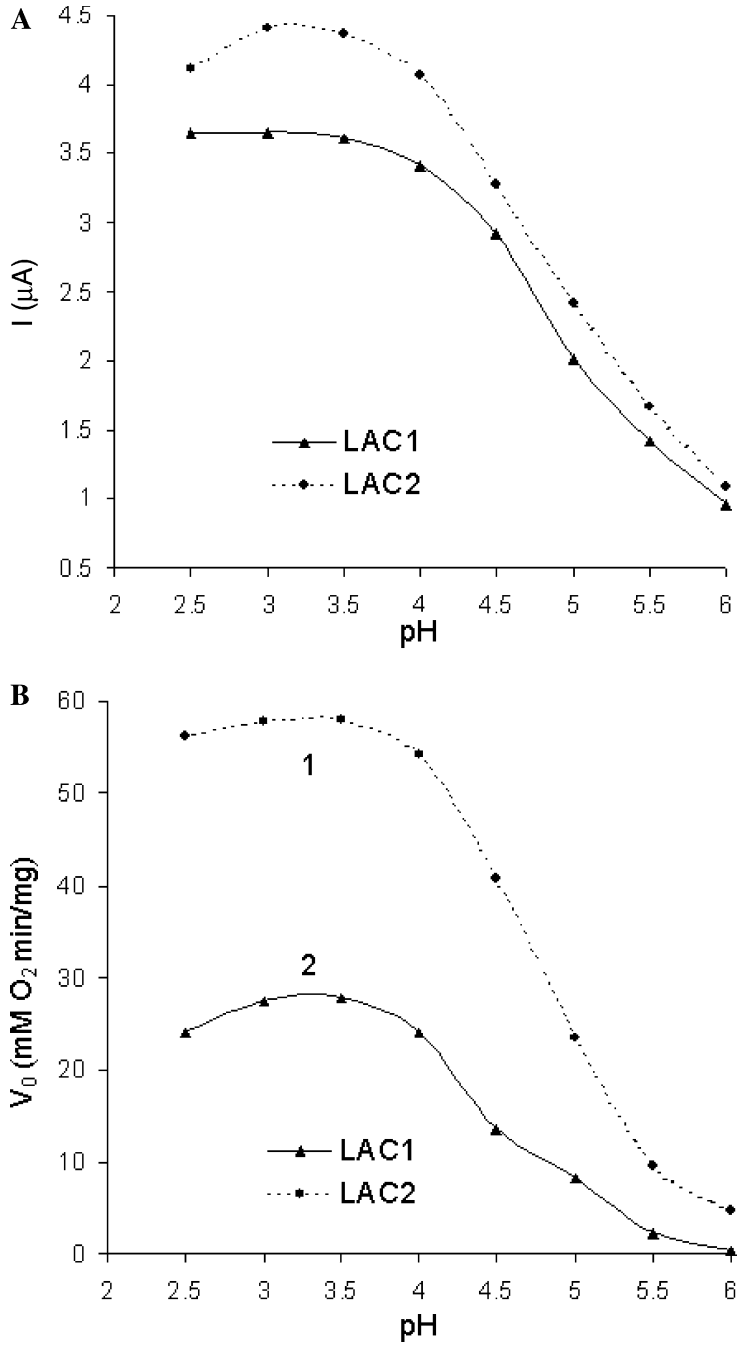


Fig. 3. Oxygen reduction activity vs. pH for *Trametes pubescens* laccases. (A) Bioelectrocatalytic activities were determined as the oxygen reduction current of the laccase-modified spectrographic graphite electrode at a potential of +350 mV vs. NHE electrode. 0.1 M citrate–phosphate buffer; ionic strength 200 mM Na_2SO_4; scan rate 25 mV/s; 0.26 mM dioxygen. (B) Activities of laccases in solution were determined by measuring the oxygen reduction at the Clark electrode in 0.1 M citrate–phosphate buffer using (1) $K_4Fe(CN)_6$ and (2) guaiacol as the substrates of the enzymes.

Some differences between LAC1 and LAC2 digestion products: The digest spectrum for LAC2 displayed seven peaks not present in the digest spectrum for LAC1, namely *m*/*z*: 1078.6, 2769.5, 2807.3, 2931.5, 2969.4, 3870, and 4023 (Fig. 4A). These peaks may represent peptides with different sequences, peptides with different glycosylation patterns, peptides with other modifications, or other unspecified impurities.

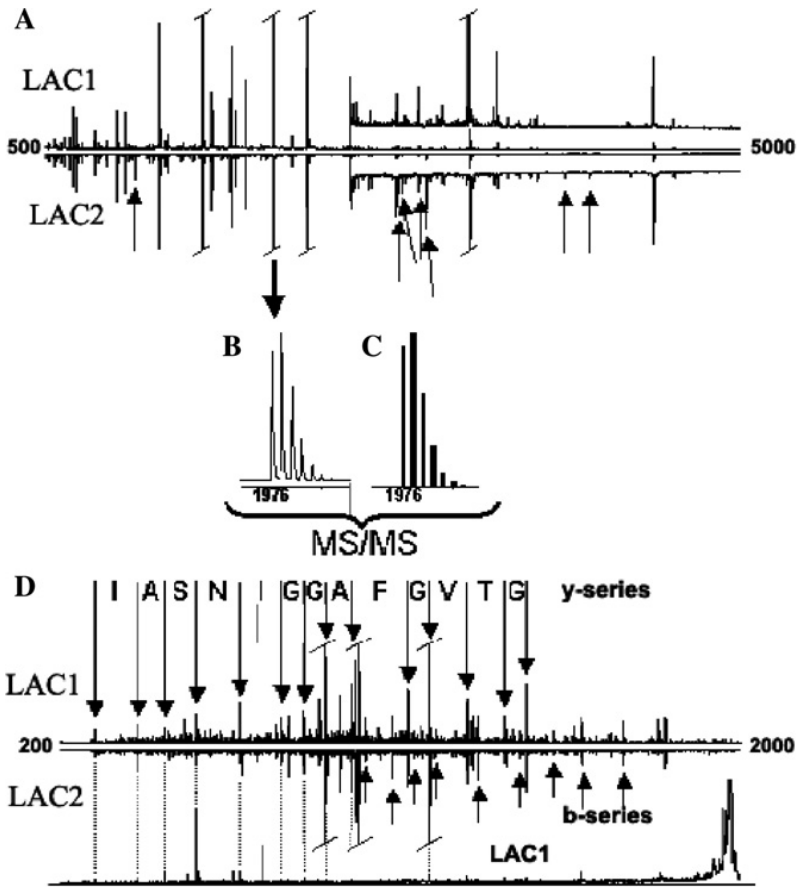


Fig. 4. Summary of mass spectrometry (MS) analysis of tryptic digestion products of LAC1 and LAC2. (A) Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) MS analysis of digestion products of LAC1 and LAC2. The spectra are very similar except for seven peaks present in the spectrum of digest products of LAC2. Insets are expanded vertically for extra visibility. (B) Isotopic pattern of the peak at m/z 1976.0 obtained from (A). (C) Expected isotopic pattern for the peptide ANP...ILR, in good agreement with (B). (D) Tandem MS analysis of the doubly charged peak at m/z 988.5 acquired with nanoelectrospray ionisation (nanoESI) MS. The parent ion m/z and fragment spacings are consistent with the sequence ANP...ILR; moreover this sequence is obtained both from LAC1 and LAC2. The bottom tandem MS spectrum is acquired with MALDI from the singly charged peak at m/z 1976.0. It is in good agreement with the tandem MS spectrum acquired with nanoESI.

4. Discussion

Laccase is a mandatory enzyme for lignin conversion, and laccase-deficient mutants completely lose their ability to degrade lignin [33,51]. However, laccase appears to play two "opposite" roles. On the one hand, laccase is responsible for lignin degradation processes exploiting natural redox mediators [33,52], as well being able to oxidize natural lignin by a long-range electron transfer mechanism [53]. On the other hand, laccase mediates a building process involving condensation and polymerisation of the products of lignin degradation by fungal laccases, which are well known reactions; the role of this condensation and polymerisation is the protection of the fungus mycelium from toxic substances [54,55]. Unfortunately, the mechanism of lignin transformation in nature is very complex, and even the role of laccase in this process is not fully understood.

The fact that all constitutive forms of *Trametes* laccase, which have been characterized so far, including the laccases studied in the present work, are high-redox potential enzymes

with similar biochemical, spectral, and electrochemical parameters [13,31,39], suggests a distinct physiological role for their synthesis. First of all, high-redox-potential laccases are able to oxidize both high- and low-redox-potential substrates, which significantly broadens the degradation ability of the fungi at the beginning of their growth. Secondly, for all high-redox-potential laccases, bioelectroreduction of oxygen on the carbon electrode based on direct electron transfer reactions between the electrode (solid substrate) and the enzymes has been shown [50,56,57], including the two laccases studied in the present work (Fig. 2; Table 1). Indeed, not only laccase, but also all ligninolytic enzymes from white rot fungi (lignin and manganese peroxidases, laccase, and cellobiose dehydrogenase) display the phenomenon of direct electron transfer [57–59]. This could reflect a need for extracellular redox enzymes to communicate directly and electronically with a solid matrix (lignin) in the course of lignin degradation [58,59]. In spite of some doubts regarding the possibility for laccase to initially attack wood [60], one still can suggest a possible enhancement of the efficiency of lignin degradation using the phenomenon of direct electron transfer shown for all ligninolytic enzymes, perhaps giving the fungus an important competitive edge in establishing a foothold in its chosen environment.

Our studies have shown that LAC1 and LAC2 are two bio- and electrochemically similar high-redox-potential enzymes. In addition, our electrochemical data are in good agreement with previously found pH-dependent behaviour of other fungal laccases in solution and in the adsorbed state [50]. Moreover, the redox potentials of the T1 sites are very close to the half-wave potential of oxygen reduction by LAC1 and LAC2 (Table 1). Indeed, the T1 site of the laccases is the first electron acceptor, both in solution (homogenous case) and when the enzymes are adsorbed on the surface of the graphite electrode (heterogeneous case). Thus, electrochemical experiments presented here additionally confirm the earlier proposed mechanism of laccase function on carbon electrodes [50,57]. However, inspection of the kinetic characteristics of LAC1 and LAC2 suggests that a finer distinction can be made among high-redox-potential laccases. As was already shown in our recent studies [42], LAC2 possessed higher catalytic constants with respect to non-phenolic compounds (e.g., ABTS and $K_4Fe(CN)_6$), whereas LAC1 showed the higher oxidation rate (k_{cat}) for many phenolic substrates (e.g., hydroquinone and guaiacol; [42] and Table 2). At the same time, the affinity of LAC1 towards hydroquinone and guaiacol is lower compared to that of LAC2 ([42] and Table 2). In most cases, the efficiency (k_{cat}/K_M) of the oxidation of phenolic substrates is higher for LAC1 and the efficiency of the oxidation of non-phenolic substrates is higher for LAC2 ([42] and Table 2). Almost the same situation can be found for the three isoenzymes of *Trametes* C30 laccase recently discovered and characterized [13,41], e.g., significant differences in affinity and efficiency of oxidation of different substrates are observed for different multiforms of the enzyme, even the ones in the same redox-potential family.

Obviously, great differences in the kinetic properties of the multiforms of laccases suggest a distinct physiological role of laccase multiplicity in lignin conversion. Different forms of the enzyme possess different efficiency and affinity towards phenolic and non-phenolic compounds. On the one hand, this broadens the substrate specificity of the enzyme family; on the other hand, this also enables a more sophisticated regulation of the process of lignin degradation. For instance, a low capacity/high specificity system (constitutive forms of laccase, like LAC1 and LAC2 for *T. pubescens* and LAC1 for *Trametes* C30) is needed when the substrate level is low, while a high capacity/low specificity system (inducible forms of laccase, like LAC1 and LAC3 for *Trametes* C30) is needed when

substrates are abundant [13]. Thus, laccase multiplicity has most likely arisen as an evolutionary response to the need for white rot fungi to be able to degrade a hard solid substrate (lignin) under a variety of environmental conditions which dynamically change during the fungal life-cycle.

Mass spectral analysis of protein tryptic digestion products provides some identification information which is quite independent of identification through details of origin and physico-chemical properties [61]. It is important to note that the "peptide mass mapping" approach most often results in the identification of a gene-product, as opposed to identification of the gene itself [62]. The mass patterns of the tryptic digest products of LAC1 and LAC2, both from *T. pubescens*, suggest a broad similarity between these two proteins. Unfortunately, the mass spectrometric data are not sufficiently comprehensive to enable deciding whether or not a single gene codes for both of these proteins. However, it is clear that more than one gene encodes laccase in *T. pubescens*, and that our characterization of LAC1 and LAC2 is consistent with the existence at least one new possible gene encoding laccase. Indeed, both LAC1 and LAC2 contained sequences consistent with at least three subsequences which are also displayed by a known laccase from *Trametes versicolor*, but not by laccase-encoding gene sequences known to be associated with *T. pubescens*—AAM18408 and AAM18407 (associated with the two genes, Q8TG94_TRAPU and Q8TG93_TRAPU, respectively). *T. pubescens* is therefore proposed to produce other variants of laccase (e.g., LAC1 and LAC2), the corresponding gene (or genes) for which has not yet been delineated. It is well known that *T. versicolor*, one of the well-studied basidiomycetes, contains numerous genes encoding different laccases (at present more than thirty are known; see the NCBI GenBank Website). Possibly, the same situation could be found for *T. pubescens*, if the complete genome of the basidiomycete were to be known.

It remains a subject for future investigations to determine whether LAC1 and LAC2 might be constitutive multiforms of the enzyme simultaneously produced by *T. pubescens* basidiomycete, which could be formed during post-translational modification, e.g., through glycosylation and/or partial degradation of the polypeptide chain. In such an event, one gene could possibly code for several multienzymes under certain conditions, and therefore the total amount of all possible forms of laccases for one basidiomycete would be a very high but unknown value.

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