Journal of Industrial Microbiology & Biotechnology (2002) 29, 214–220

www.nature.com/jim

Enhanced activity by poly(ethylene glycol) modification of *Coriolopsis gallica* laccase

HA Vandertol-Vanier[1], R Vazquez-Duhalt[2], R Tinoco[2] and MA Pickard[1]

[1]*Department of Biological Sciences, CW 405 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9;* [2]*Instituto de Biotecnologia, UNAM, Apdo Postal 510-3 Cuernavaca, Morelos 62271, Mexico*

We are studying the enzymatic modification of polycyclic aromatic hydrocarbons (PAHs) by the laccase from *Coriolopsis gallica* UAMH 8260. The enzyme was produced during growth in a stirred tank reactor to 15 units ml^{-1}, among the highest levels described for a wild-type fungus; the enzyme was the major protein produced under these conditions. After purification, it exhibited characteristics typical of a white rot fungal laccase. Fifteen azo and phenolic compounds at 1 mM concentration were tested as mediators in the laccase oxidation of anthracene. Higher anthracene oxidation was obtained with the mediator combination of ABTS and HBT, showing a correlation between the oxidation rate and the mediator concentration. Reactions with substituted phenols and anilines, conventional laccase substrates, and PAHs were compared using the native laccase and enzyme preparations chemically modified with 5000 MW-poly(ethylene glycol). Chemically modified laccase oxidized a similar range of substituted phenols as the native enzyme but with a higher catalytic efficiency. The k_{cat} increase by the chemical modification may be as great as 1300 times for syringaldazine oxidation. No effect was found of chemical modification on mediated PAH oxidation. Both unmodified and PEG-modified laccases increased PAH oxidation up to 1000 times in the presence of radical mediators. Thus, a change of the protein surface improves the mediator oxidation efficiency, but does not affect non-enzymatic PAH oxidation by oxidized mediators.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 214–220 doi:10.1038/sj.jim.7000308

Keywords: polycyclic aromatic hydrocarbons; *Coriolopsis gallica*; PEG-laccase; oxidation; white rot fungi

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants found in terrestrial and aquatic environments [39] and are a potential health risk because of their possible carcinogenic and mutagenic activities [13,39]. Those PAHs which fungi are able to metabolize are attacked by one of two systems, either by an intracellular cytochrome *P*-450 monooxygenase system or by extracellular ligninolytic enzymes such as laccase, manganese peroxidase or lignin peroxidase [3,13]. *In vitro* oxidation of PAHs has been demonstrated by crude and purified fungal enzymes from a variety of fungi, such as lignin peroxidase [23,43], manganese peroxidase [4,5] and laccases [6,15,26,29,33].

Laccases (EC 1.10.3.2) are copper-containing enzymes widespread in white rot fungi which catalyze the oxidation of a variety of aromatic phenols and anilines, reducing oxygen to water. Their characteristics have been reviewed comprehensively [21,40]. *Coriolopsis gallica*, a white rot fungus, degrades lignin and produces ligninolytic enzymes, mainly laccase and manganese peroxidase [12]. Culture conditions and medium composition can play a major role in the level of enzyme expression, and a medium consisting of bran flakes produces increased levels of laccase and manganese peroxidase in this and in other white-rot fungi [34]. *C. gallica* demonstrated enhanced metabolism of anthracene, pyrene and phenanthrene white growing in this medium, and we have studied the metabolism of the 10 PAHs using laccase purified from *C. gallica*. The purified laccase of *C. gallica* metabolized 7 of 10 PAHs examined, and the enzyme retained 90% of its activity in some reaction systems that contain organic solvents to enhance the solubility of PAHs [33]. Whereas the substrate range for laccase is normally limited to phenolic substrates, it can be extended to nonphenolic compounds with the addition of mediating substrates such as 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) [7–10,28,46]. The rate of oxidation of several PAHs is enhanced by the addition of the cooxidant ABTS [25,26,29,33].

Since PAHs are hydrophobic, another potential method to enhance PAH metabolism is to chemically modify the enzyme and then to assess the activity of the modified enzyme in organic solvents which aid PAH solubility. Chemical modification of cytochrome *c* and lignin peroxidase enhance the oxidation of PAHs, to protect from loss of activity in organic solvents, and to affect the kinetics of oxidation [41–44]. In this study, purified and chemically modified laccase from *C. gallica* UAMH 8260 were assayed for their activity toward selected PAHs, and the effect of different mediators was evaluated.

Materials and methods

Chemicals

Acenaphthene, anthracene, benzo(*a*)pyrene, biphenylene, 2-methyl anthracene, 9-methylanthracene, fluoranthene, phenanthrene, pyrene, 2,5-xylidine and mediators were from Aldrich (Oakville, Ontario, Canada). ABTS acid HBT were from Sigma (St. Louis, MO).

Correspondence: Dr Michael A Pickard, Department of Biological Sciences, CW 405 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
Received 10 December 2001; accepted 20 July 2002

Fungus and culture conditions

C. gallica UAMH 8260 (NOF 138) ex *Populus tremuloides* was obtained from the University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Gardens, University of Alberta, Edmonton, Alberta, Canada. Inocula were grown in glucose–yeast extract–malt extract medium (GYM), containing (g/l) glucose: 10, yeast extract: 2.5, malt extract: 3.5, KH_2PO_4: 2.0 and $MgSO_4 \cdot 7H_2O$: 0.5 [34]. Inoculum was prepared by homogenizing 1 cm^2 of mycelium from a potato dextrose agar plate with a Sorvall Omnimixer containing 50 ml GYM for 5 s at full speed. The fungus was grown on a shaker for 3 days at 28°C, at 200 rpm, in a 500-ml shake flask containing 200 ml of GYM. After homogenizing, a 5% inoculum was used to inoculate the production medium, which consisted of 3% (w/v) ground cereal bran flakes (Kellogg's Bran Flakes; Kellogg Company, Battle Creek, MI) in 60 mM phosphate buffer, pH 6.0 [34]. Samples were assayed daily for reducing sugars, pH and laccase activity.

Purification of extracellular laccase

Eight-liter cultures of *C. gallica* were grown in a 14-l stirred tank reactor (Microferm, New Brunswick Scientific, New Brunswick, NJ) at 28°C in the production medium. Laccase activity reached its maximum on day 9 of cultivation (Figure 1) at which time the culture was harvested and the mycelium removed by filtration through cheesecloth. The filtrate was frozen for 3 days, thawed, and solids were removed by centrifugation at 20,000×*g* for 30 min. The following purification steps were then carried out at 4°C, essentially as described earlier [37]. The culture supernatant was concentrated about 10- to 20-fold and dialyzed against 20 mM potassium phosphate buffer, pH 6 (buffer) using an Amicon hollow fiber ultrafiltration system with a 10-kDa cutoff membrane. The centrifuged concentrate (40,000×*g*, 20 min) was applied to an anion exchange column (Whatman DE-52, Maidstone, England, UK) preequilibrated with buffer, washed with two column volumes of buffer and the enzyme was eluted with a linear gradient of 0–0.4 M NaCl in buffer. Fractions containing laccase activity were pooled, concentrated and dialyzed against buffer, and subjected to another DE-52 column, this time with a gradient from 0.05 to 0.25 M NaCl in buffer. The active fractions were again concentrated before gel exclusion chromatography using Sephadex G100 preequilibrated with 0.1 M NaCl in buffer. The final stage of purification used anion exchange chromatography (Mono Q; Pharmacia, Mississauga, Canada) in a Fast Protein Liquid Chromatography (FPLC) system and the enzyme was eluted with a gradient from 0 to 0.4 M NaCl in buffer. The enzyme was concentrated and stored at −20°C in buffer.

Laccase assay

Laccase activity was determined by ABTS oxidation [45] in a 1-ml reaction mixture containing 1 mM ABTS in 0.1 M sodium acetate pH 4.0 and 5–50 μl of enzyme. The oxidation was followed at 30°C and at 436 nm (ABTS, ε_{436}=29,300 M^{-1} cm^{-1}). One unit was defined as the amount of laccase that oxidized 1 μmol of ABTS/min.

Reducing sugar

Reducing sugar content of the growth medium was determined by the reduction of dinitrosalicylic acid (DNS) using glucose as the standard.

Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10% polyacrylamide gel containing 0.1% SDS according to Laemmli methodology. The protein was visualized by staining the gel with Coomassie Blue G-250 (Bio-Rad, Hercules, CA) and was compared to both low-range and high-range molecular weight markers (Sigma, St. Louis, MO).

Protein concentration

Protein was determined by the method of Bradford using the Bio-Rad protein reagent (Bio-Rad) with bovine serum albumin as a standard.

Carbohydrate content

The carbohydrate content of laccase was determined by the phenol method of Dubois *et al* [16] using glucose as a standard.

Substrate specificity and enzyme inhibition

Spectrophotometric measurement of substrate oxidation by laccase was carried out at 30°C in a 1-ml reaction volume containing the test substrates in 0.1 M sodium acetate, pH 4. Potential inhibitors were tested for their ability to inhibit the standard ABTS assay.

N-terminal amino acid sequencing

N-terminal amino acid sequencing was carried out using Edman degradation techniques at the Alberta Peptide Institute at the University of Alberta.

Molecular weight

Molecular mass determinations were carried out by SDS-PAGE using commercial molecular weight markers, and by matrix-assisted laser desorption ionization time-of-flight (Maldi-TOF) mass spectrometry recorded on an HPTOF instrument, courtesy of Dr Liang Li, Department of Chemistry, University of Alberta. Laccase samples were prepared at 2.5 mg protein ml^{-1} and

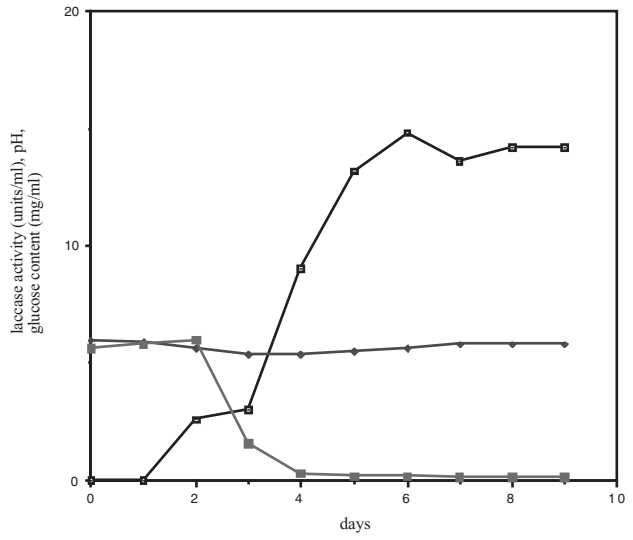

Figure 1 Time course of laccase production by submerged culture in a stirred tank reactor. Legend: (-□-) laccase activity (units/ml); (-■-) glucose content (mg/ml); and (-◆-) pH.

Table 1 Physical characteristics of *C. gallica* UAMH 8260 laccase

Single isoenzyme, p*I* 3.4
Molecular weight by SDS-PAGE=66,000
Molecular weight from MALDI-TOF mass spectrum=56,688
Carbohydrate content=21% (w/w, glucose equivalents)
Copper content=3.3 atoms/laccase molecule
Purity index (A_{280}:A_{606})=5

sinapinic acid was used as the matrix on the sample plate. Bovine serum albumin was used for external calibration.

Chemical modification of laccase

Chemical modification of the lysine free amino groups of laccase was carried out with a 5-fold excess of methoxypolyethylene glycol activated with cyanuric chloride (Sigma) in 40 mM borate buffer, pH 10 [42]. The reaction was performed at 25°C for 2 h, the mixture was then diluted 10-fold with 50 mM phosphate buffer (pH 6) at 4°C to stop the reaction, and concentrated by ultrafiltration (Amicon PM-10) to the original reaction volume. This procedure was repeated twice. Trinitrobenzene sulfonate was used to determine the number of amino groups modified [22].

Laccase-mediated oxidation of PAHs

Laccase-mediated oxidation of PAHs was determined by incubating a mixture of individual PAHs (20 μM) in 15% acetonitrile in 0.1 M acetate buffer (pH 4) with 5 units of enzyme in a 100-μl reaction volume. Mediating substrates, such as ABTS and HBT, were added to the mixture to a final concentration of 1 mM. The assay was started by adding enzyme and was terminated by adding acetonitrile to a final concentration of 60%. Reaction times were from 5 min to 1 h depending on the susceptibility of the substrate. Boiled enzyme controls showed no activity. After centrifugation (14,000×*g*, 3 min), 10-μl samples were analyzed by HPLC using a C_{18} reverse-phase column and isocratic elution with acetonitrile–water (60:40). PAH oxidation was calculated from peak areas using a standard curve, and the first order oxidation reaction rates were plotted by fitting the data to the equation $A_o=A_t e^{-kt}$ [33].

Table 2 Fungal laccase N-terminal amino acid sequences

Fungus	N-terminal amino acid sequence
Coriolopsis gallica UAMH 8260	**A I G P V A D L T I S N G A V S P D G F**
Trametes versicolor	**G I G P V A D L T I** - **N** A **A V S P D G F**
Pycnoporus cinnabarinus	**A I G P V A D L T** L T **N** A **A V S P D G F**
Panaeolus papilionaceus	G **I G P V A D L T I** T **N** A A **V S P D G F**
Polyporus pinsitis	G **I G P V A D L T I** T **N** A A **V S P D G F**
Coriolus hirsutus	**A I G P** T **A D L T I S N** A E **V S P D G F**
Phlebia radiata	S **I G P V** T **D** F H **I V N** A **A V S P D G F**
Coprinus friesii	**A I G P V A D L** Y **I** G **N** K V I A **P D G F**
Panus tigrinus	**A** V **G P V A D L T** V T **N** A N I **S P D G F**
Panaeolus sphinctrinus	**A I G P V A D L** Y **I** G **N** K V I A **P D G F**
Coriolopsis gallica A-241	S **I G P V A** - **L T I S N G** - **V** - **P**
Pleurotus ostreatus	**A I G P** A G N M Y **I V N** E D **V S P D G F**
Ceriporiopsis subvermispora	**A I G P V** T **D** I E **I** T D A F **V S P D G** P
Phlebia tremellosa	**A I G P V** T N F H **I V N** A I A A **P D G F**
Agaricus bisporus	**A** K T R T F **D** F D L V **N** T R L A **P D G F**
Neurospora crassa	G G **G** G G C N S P T N R O C W S **P**
Cryptococcus neoformans	X K T D E S P E A V S D N Y M P K
Chaetomium thermophilum	F N P D L L P S L E P

Except for *C. gallica* UAMH 8260 (this study), all sequences were from Blast, the NCBI sequence similarity search tool (http://www.ncbi.nlm.nih.gov/BLAST/index.html).

Results

Laccase production, purification and characterization

C. gallica was grown in submerged culture in 8l of 3% bran flakes medium in a stirred tank reactor. Using a 5% GMY medium inoculum, the fungus produced 15 units of laccase/ml in the medium after 9 days growth at 27°C (Figure 1): this was a higher yield than was generally observed in shake flask culture. The reducing sugar content of the medium, presumably produced from the starch of the bran flakes, decreased concomitantly with an increase in laccase activity, and the bran particles gradually disappeared later during the growth period. Biomass increased visibly for the first 4 days but attempts to quantify the biomass gravimetrically were frustrated by the high background of the particulate bran flakes. Similar problems were encountered when attempting to assay for biomass protein and RNA. The pH remained relatively constant throughout the fermentation, not varying by more than 0.5 pH unit, and the enzyme level in the medium remained stable for at least 5 days, a positive attribute for large-scale production.

The extracellular enzyme was purified from the concentrated and dialyzed culture medium from the stirred tank reactor using traditional column chromatographic techniques. Although laccase was the major protein in the culture supernatant, there was considerable contamination by pigments, which were largely removed by anion-exchange chromatography [37]. The laccase fraction eluted at 0.12 M NaCl in the 0- to 0.4-M NaCl gradient. Further pigment removal was achieved by repeating this procedure. The enzyme preparation was green at this stage but residual pigment was removed by passage through a gel filtration column and the resulting effluent fractions were blue. Subsequent anion exchange chromatography resulted in a single peak with almost no

Table 3 Anthracene oxidation by laccase with different radical mediators[a]

Mediator compound	Anthracene oxidation rate (min^{-1})
No mediator	0.17
ABTS+HBT	18.86
ABTS	4.18
Di-*t*-butyl-*p*-cresol	1.64
Veratryl alcohol+ABTS	1.51
HBT	1.44
Tri-*t*-butyl phenol	0.96
Syringaldazine+ABTS	0.55
Coniferyl alcohol	0.36
Coniferyl alcohol+ABTS	0.14
Elagic acid+ABTS	0.13
Elagic acid+HBT	0.13
Gallic acid+ABTS	0.12

[a]The reaction system contained 1 mM mediator and 20 μM anthracene in 15% acetonitrile in 100 mM acetate buffer, pH 4.5.

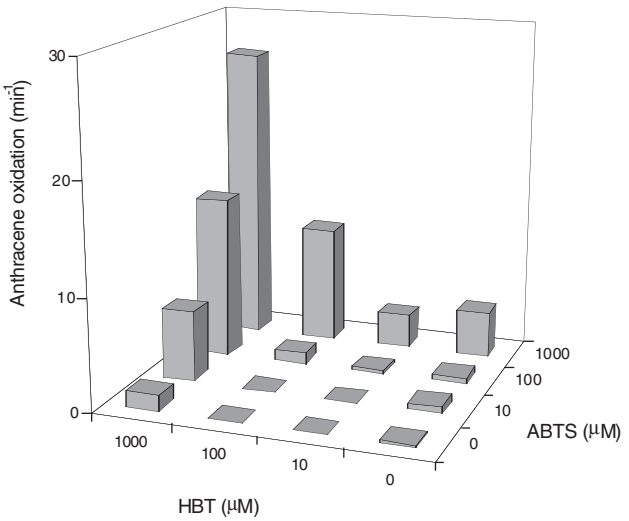

Figure 2 Effect of ABTS and HBT concentrations on the laccase-mediated anthrecene oxidation.

further increase in specific activity. The overall purification was 20-fold and enzyme recovery was 68%.

The pure laccase (Table 1) showed a single band on SDS-PAGE at a molecular mass of approximately 66 kDa and an isoelectric point of 3.4 on IEF gels. The molecular mass by MALDI-TOF mass spectrometry was 56,688, compared to 60,151 for laccase B from *Trametes versicolor* PRL 572 [32]. The carbohydrate content of this glycoprotein was about 21% by weight as glucose equivalents, and the copper content was estimated as 3.3 Cu atoms per enzyme molecule, about 20% lower than the theoretical 4 atoms per molecule (one type 1, one type 2 and two type 3 copper atoms), but not significantly different from other published values [40]. The UV–visible spectrum of the enzyme showed a shoulder at 320 nm indicative of the binuclear type 3 copper pair, and a peak at 606 nm typical of the type 1 cupric atom responsible for its blue color. The ratio of absorbance at 280–606 nm was 5, lower than published values for *Pleurotus eryngii* of 15 [30] and *Ceriporiopsis subvermispora* of 20 [19]. Low ratios are one indicator of enzyme purity but the absolute value depends on the aromatic amino acid content, mainly tryptophan and tyrosine, of the individual laccases. The N-terminal amino acid sequence of *C. gallica* laccase exhibited significant homology to other fungal laccases, as shown in Table 2, with one amino acid different from *T. versicolor*, and three different from *P. cinnabarinus*, *Coriolus hirsutus*, *Panaeolus papilionaceus* and *Polyporus pinsitis*. However, there were significant differences between the two *C. gallica* strains.

The temperature of highest activity for ABTS oxidation for *C. gallica* laccase was 65°C over a 30-s kinetic assay, and the enzyme was stable for 24 h at 60°C and 144 h at 40°C. The pH–activity profile for ABTS oxidation showed a peak of optimal activity at pH 3.8, well within the range of other fungal laccases, and it was stable from pH 5.5 to pH 9.0 for 5 days. ABTS oxidation was totally inhibited by 0.1 mM sodium azide, and dithiothreitol, thioglycolic acid and cysteine were all strongly inhibitory at 1 mM. However, neither EDTA nor phenanthroline were strongly inhibitory.

Different mediator combinations for oxidation of anthracene

Fifteen azo and phenolic compounds at 1 mM concentration were tested as mediators for the laccase oxidation of anthracene. Catechin, coniferyl alcohol, congo red, di-*t*-butyl-*p*-cresol, elagic acid, eriochrome black, sudan black, gallic acid, propylgallate, syringaldazine, tri-*t*-butyl alcohol, vanillin, veratryl alcohol were tested alone or in combination with HBT or ABTS for the oxidation of anthracene. All the mediators proved to be substrates for laccase (data not presented), but only a few combinations were able to oxidize anthracene. In Table 3, only the successful combinations are shown. The combination of 1 mM ABTS and 1 mM HBT proved to be the best for anthracene oxidation, with an activity 110-times higher than that of laccase without mediator.

The effect of the concentration of both radical mediators, ABTS and HBT, was determined (Figure 2). The anthracene oxidation rate was higher as the mediator concentration was increased, specially when both mediators were present.

Chemical modification of laccase

In an attempt to improve the catalytic activity of laccase, and to improve the enzyme's stability in organic solvents, the hydrophobicity of the enzyme was increased by coupling polyethylene glycol (PEG) groups to the free amino groups of lysine at pH 10. The number of amino groups so modified was determined chemically by measuring the remaining free amino groups. We studied native laccase and PEG-laccase with 84% of its free amino groups modified. A number of substituted phenols were examined as laccase substrates as shown in Table 4. From the kinetic parameters determined, 2,6-dimethoxyphenol had the highest oxidation rate (k_{cat}) and catalytic efficiency (k_{cat}/K_M), in both unmodified and PEG-modified laccase preparations. Table 4 shows

Table 4 Kinetic constants for substrate oxidation by native and polyethylene glycol-modified laccase

Substrate	Native laccase			PEG-laccase			Catalytic efficiency increase
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} mM^{-1})	
2,6-Dimethoxyphenol	6.0×10^{7}	3.50	1.7×10^{7}	1.0×10^{8}	1.60	6.2×10^{7}	4
ABTS	3.7×10^{6}	0.43	8.6×10^{6}	8.3×10^{6}	0.25	3.3×10^{7}	4
Syringaldehyde	2.7×10^{4}	0.40	6.8×10^{4}	3.7×10^{7}	0.14	2.6×10^{8}	3820
HBT	7.0×10^{3}	0.55	1.3×10^{4}	1.5×10^{5}	3.92	3.8×10^{4}	3
4-Methoxyphenol	1.3×10^{4}	2.64	4.9×10^{3}	3.0×10^{6}	2.82	1.1×10^{6}	224
4-Aminophenol	2.8×10^{3}	0.62	4.5×10^{3}	4.7×10^{6}	1.36	3.4×10^{6}	740
Catechol	1.3×10^{4}	3.13	4.1×10^{3}	1.4×10^{5}	3.83	3.6×10^{4}	9
Vanillic acid	8.0×10^{3}	2.94	2.7×10^{3}	1.6×10^{5}	6.13	2.6×10^{4}	9
Vanillin	3.7×10^{3}	13.90	2.6×10^{2}	1.2×10^{5}	8.24	1.4×10^{4}	56

Table 5 Native- and PEG-laccase reaction rates on PAHs with and without mediating substrates

	Specific oxidation rate (min^{-1}) on					
	9-Methylanthracene	2-Methylanthracene	Anthracene	Acenaphthene	Pyrene	Chrysene
Native laccase						
No mediator	0.31 (±0.04)	13.3 (±1.9)	0.38 (±0.04)	NR	NR	NR
+ABTS	59.9 (±0.3)	30.8 (±1.6)	2.7 (±0.4)	3.4 (±0.2)	NR	NR
+HBT	502 (±24)	99.5 (±16.5)	1.9 (±0.2)	27.2 (±0.4)	0.13 (±0.01)	NR
+ABTS+HBT	363 (±25)	60.4 (±8.2)	20.5 (±0.5)	17.9 (±1.2)	0.11 (±0.01)	NR
PEG-modified laccase						
No mediator	0.29 (±0.03)	0.38 (±0.14)	7.9 (±0.4)	0.43 (±0.14)	NR	NR
+ABTS	19.6 (±0.1)	3.7 (±0.3)	6.3 (±0.3)	0.94 (±0.07)	NR	NR
+HBT	209 (±9)	16.2 (±0.4)	11.0 (±1.9)	3.4 (±0.3)	NR	NR
+ABTS+HBT	103 (±6)	15.3 (±2.9)	17.1 (±0.5)	3.2 (±0.5)	0.18 (±0.04)	NR

NR: No reaction detected.

that PEG-laccase increased both the oxidation rate and the catalytic efficiency of the enzyme in almost all cases studied, up to 3800-fold in the case of syringaldehyde and 740 times with 4-aminophenol as substrate. The effect on the affinity constant was variable, but the K_M values remain in the micromolar range.

The modified enzyme was also assessed for its ability to oxidize six PAHs: anthracene, chrysene, pyrene, acenaphthene, 9-methylanthracene and 2-methylanthracene. For each form of the enzyme, PAH oxidation was studied in the absence of mediators, with ABTS, with HBT and with ABTS plus HBT. The results show a dramatic increase of PAH oxidation when the mediators were present (Table 5). The oxidation rate of 9-methylanthracene increased up to 1600 times with the presence of 1 mM HBT with the unmodified laccase and 720 times with the PEG-laccase. However, no important activity enhancement on PAHs was found due to chemical modification of the enzyme, as was found in the case of phenols.

Discussion

Using a 14-l stirred tank reactor, we scaled up enzyme production by submerged pelleted cultures of *C. gallica*, and purified the enzyme from the culture supernatant to apparent homogeneity. Purification involved column separations using size exclusion (Sephadex G-100) and anion exchange (DEAE cellulose) chromatography. Although laccase was the major protein in the spent culture medium, there was considerable contamination by pigments released from the bran flakes during autoclaving. Often several passages through the ion exchange column were needed to remove the pigment.

C. gallica laccase is a typical white rot fungal laccase and its characteristics are summarized in Table 1. It is a blue glycoprotein with a copper content of 4 atoms/molecule, with our experimental value of 3.3 likely indicating copper depletion during purification. It has an isoelectric point of 3.4 based on focusing gels using other fungal glycoproteins of known p*I* as standards. The apparent molecular weight by SDS-gel electrophoresis was about 66,000, similar to that reported for other fungal laccases [40] but this is probably an overestimate due to the reduced migration of glycoproteins in acrylamide gels. A more reliable figure from MALDI-TOF mass spectrometry was closer to 57,000. The carbohydrate content of 21% is within the range reported for fungal laccases of 15–20% [40] and it exhibited a A_{280}:A_{606} ratio of 5 in its purest form. It has a unique N-terminal amino acid sequence which was similar to several other white rot fungi but significantly different from the reported sequence for *C. gallica* strain A-241.

Fungal laccases are usually active at low pH values so an optimum pH of about 4 was not unexpected. The enzyme was stable from pH 6.5 to pH 9 for up to 5 days which allowed the chemical modification to occur at the upper limit and little activity was lost during this procedure. Thermal characteristics of this laccase indicated that the optimal temperature range for its activity is 30–60°C [14]; it had a short-term temperature optimum of 65°C, higher than most laccases, and it was stable at temperatures up to 60°C for 24 h. The estimated carbohydrate content is within the range reported for the laccases of other basidiomycetes, including *T. versicolor*, *C. hirsutus* and *Phlebia radiata* [21]. The carbohydrate moiety of the laccase of *T. versicolor* imparts resistance to proteolytic attack and elevated temperatures [21].

C. gallica laccase activity was inhibited by known laccase inhibitors. Sodium azide, an inhibitor of metalloenzymes, produced 100% enzyme inhibition at 0.1 mM. Dithiothreitol, thioglycolic acid and L-cysteine also completely inhibited activity. EDTA, a divalent metal chelator did not significantly inhibit enzyme activity, nor did phenanthroline, a metalloenzyme inhibitor that chelates iron and other divalent metals. These results are consistent with inhibition of other laccases [14]. This laccase is inhibited by many anions that are able to interact with the copper sites. This includes chloride, used in this and other purification procedures without serious loss of enzyme recovery. The N-terminal sequence of *C. gallica* laccase displayed significant homology to other basidiomycete laccases including its close relative *T. versicolor* where 1 out of 10 amino acids are different. From these data, it can be concluded that the laccase from *C. gallica* is similar to laccases of other white rot fungi [17,19,24,27,30,31,47].

Radical mediators have been used successfully to enhance the oxidative capacity of laccases in a wide range of applications, such as pulp delignification [11,28], textile dye blanching [1,36], PAH degradation [25,33], pesticide degradation [2,38] and organic synthesis [18,35]. The oxidation by laccase-mediator-assisted reactions occurs starting with the direct oxidation of the mediator compound by the enzyme, producing a free radical. This free radical from the mediator compound should be stable enough to diffuse to the target substrate and oxidize it. Thus, a good mediator should: (i) be a substrate for the laccases, (ii) be stable enough to

reach the target substrate, and (iii) have ionization and redox potentials high enough to perform the oxidation of the target substrate. With the aim to find new mediator compounds, we have assayed reactions with several compounds of different chemical nature, but all of them substrates for laccase. Mediators that are natural, cheap, and with low environmental impact are desired. From our list of compounds and combinations of them, ABTS and HBT proved to be the best mediators, especially when they are used together (Table 3). However, di-*t*-butyl-*p*-cresol, tri-*t*-butyl phenol, and coniferyl alcohol proved to be good mediators for the oxidation of anthracene, while high anthracene oxidation rates were found with elagic and gallic acids in the presence of HBT or ABTS. The anthracene oxidation rate correlated with the mediator concentration in the reaction mixture, and a clear synergetic effect was found when two mediators were present, HBT and ABTS (Figure 2 and Ref. [12]). The mechanism of this synergistic interaction has yet to be elucidated.

The aqueous solubilities of PAHs are relatively low, which limits their *in vivo* bioavailability and their metabolism *in vitro*. Increasing the hydrophobicity of enzymes able to metabolize PAHs increases their rate constant and catalytic activity. Thus, benzylation and PEG modification of horseradish peroxidase increased enzyme activity in a variety of solvents [42] and methylated, PEG-modified cytochrome *c* was able to oxidize 17 of 20 aromatic compounds compared to the 8 compounds oxidized by the native protein [41]. Here we show that laccase was sufficiently stable at pH 10 in the short term to be chemically modified by polyethylene glycol and retain its activity. Enzyme modification with poly(ethylene glycol) increased the catalytic efficiency from 3 to more than 3000 times according the substrate (Table 4). This increase is mainly due to the increase in the k_{cat}, which may be higher by several orders of magnitude, while the affinity constants, K_M, were slightly modified. This effect of pegylation has been reported for other enzymes [20,41].

When PAHs were used as substrates, the effect of the chemical modification on the activity was less dramatic (Table 5), but with both modified and unmodified laccases, the PAH oxidation rates were significantly increased by the presence of mediator compounds. This could be explained by the fact that PAHs are poor direct substrates for laccase, and that the reaction is dependent on mediator production. Thus, the change of enzyme surface does not affect the mediator-assisted reaction. Other workers have reported that phenanthrene oxidation is increased when reacted with HBT by a fungal laccase [6], and that anthracene oxidation by the laccase of *T. versicolor* was enhanced by the addition of ABTS [26]. Natural mediators may be as efficient as the synthetic compounds ABTS and HBT for oxidation of several PAHs [25]. In a previous study with *C. gallica* laccase, the addition of ABTS and HBT increased the oxidation rate of anthracene synergistically [33].

We conclude from this study that the pegylation of a fungal laccase can significantly affect the kinetic parameters for oxidation of more polar substrates commonly used for assay of enzymatic activity. However, for substrates that are oxidized by a mediator-assisted reaction, surface modification of the protein does not affect the oxidation rate, supporting a non-enzymatic reaction. Mediating substrates such as ABTS and HBT have been proposed to extend the substrate range of laccases to nonphenolic subunits of lignin, as well as accelerating the oxidation of several PAHs. However, these mediators are relatively expensive and alternatives have been described recently which might have potential use in large-scale applications.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (MAP) and by a grant of the National Science and Technology Council of Mexico (33611-U).

References

1 Abadulla E, T Tzanov, S Costa, KH Robra, A Cavaco-Paulo and GM Gubitz. 2001. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Appl Environ Microbiol* 66: 3357–3362.
2 Amitai G, R Adani, G Sod-Moriah, I Rabinovitz, A Vincze, H Leader, B Chafetz, L Leibovitz-Persky, D Friesem and Y Hadar. 1998. Oxidative biodegradation of phosphothiolates by laccase. *FEBS Lett* 12: 195–200.
3 Bezalel L, Y Hadar and CE Cerniglia. 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. *Appl Environ Microbiol* 63: 2495–2501.
4 Bogan BW and RT Lamar. 1995. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 61: 2631–2635.
5 Bogan BW, RT Lamar and KE Hammel. 1996. Fluorene oxidation *in vivo* by *Phanerochaete chrysosporium* and *in vitro* during manganese peroxidase-dependent lipid peroxidation. *Appl Environ Microbiol* 62: 1788–1792.
6 Bohmer S, K Messner and E Srebotnik. 1998. Oxidation of phenanthrene by a fungal laccase in the presence of 1-hydroxybenzotriazole and unsaturated lipids. *Biochem Biophys Res Commun* 244: 233–238.
7 Bourbonnais R and MG Paice. 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin degradation. *FEBS Lett* 267: 99–102.
8 Bourbonnais R, D Leech and MG Paice. 1998. Electrochemical analysis of the interaction of laccase mediators with lignin model compounds. *Biochim Biophys Acta* 1379: 381–390.
9 Bourbonnais R, MG Paice, B Freiermuth, E Bodie and S Borneman. 1997. Reactivities of various mediators and laccases with Kraft pulp and lignin model compounds. *Appl Environ Microbiol* 63: 4627–4632.
10 Bourbonnais R, MG Paice, ID Reid, P Lanthier and M Yamaguchi. 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and the role of the mediator 2,2′azinobis(3-ethylbenzthiazoline-6-sulfonate) in Kraft lignin depolymerization. *Appl Environ Microbiol* 61: 1867–1880.
11 Call HP and I Mucke. 1997. History, overview and applications of mediated ligninolytic systems, especially laccase-mediator systems (Ligno-zym-process). *J Biotechnol* 53: 163–202.
12 Calvo AM, JL Copa-Patino, O Alonso and AE Gonzalez. 1998. Studies of the production and characterization of laccase activity in the basidiomycete *Coriolopsis gallica*, an efficient decolorizer of alkaline effluents. *Arch Microbiol* 171: 31–36.
13 Cerniglia C. 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *J Ind Microbiol Biotechnol* 19: 324–333.
14 Chefetz B, Y Chen and Y Hadar. 1998. Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. *Appl Environ Microbiol* 64: 3175–3179.
15 Collins PJ, MJJ Kotterman, JA Field and ADW Dobson. 1996. Oxidation of anthracene and benzo[*a*]pyrene by laccases from *Trametes versicolor*. *Appl Environ Microbiol* 62: 4563–4567.
16 Dubois M, KA Gilles, JK Hamilton, PA Rebers and F Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350–356.
17 Eggert C, U Temp and K-EL Eriksson. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl Environ Microbiol* 62: 1151–1158.
18 Fritz-Langhals E and B Kunath. 1998. Synthesis of aromatic aldehydes by laccase-mediator assisted oxidation. *Tetrahedron Lett* 39: 5955–5956.
19 Fukushima Y and TK Kirk. 1995. Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. *Appl Environ Microbiol* 61: 872–876.

20 Gaertner HF and AJ Puigserver. 1992. Increased activity and stability of poly(ethylene glycol)-modified trypsin. *Enzyme Microb Technol* 14: 150–155.
21 Gianfreda L, F Xu and J-M Bollag. 1999. Laccases: a useful group of oxidoreductive enzymes. *Bioremed J* 3: 1–25.
22 Habeeb AFSA. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem* 14: 328–336.
23 Hammel KE, B Kayanaraman and TK Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo(*p*)dioxins by *Phanerochaete chrysosporium* ligninase. *J Biol Chem* 36: 16948–16952.
24 Heinzkill M, L Bech, T Halkier, P Scneider and T Anke. 1998. Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae*). *Appl Environ Microbiol* 64: 1601–1606.
25 Johannes C and A Majcherczyk. 2000. Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator system. *Appl Environ Microb* 66: 524–528.
26 Johannes C, A Majcherczyk and A Huttermann. 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediating substrate compounds. *Appl Microbiol Biotechnol* 46: 313–317.
27 Koroljova OV, EV Stepanova, VP Gavrilova, VI Biniukov, AI Jaropolov, SD Varfolomeyev, F Scheller, A Makower and A Otto. 1999. Laccase of *Coriolus zonatus*: isolation, purification and some physicochemical properties. *Appl Biochem Biotechnol* 76: 115–127.
28 Li K, F Xu and K-EL Eriksson. 1999. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Appl Environ Microbiol* 65: 2654–2660.
29 Majcherczyk A, C Johannes and A Huttermann. 1998. Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb Technol* 22: 335–341.
30 Munoz C, F Guillen, AT Martinez and MJ Martinez. 1997. Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl Environ Microbiol* 63: 2166–2174.
31 Perez P, J Martinez and T de la Rubia. 1996. Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba*. *Appl Environ Microbiol* 62: 4263–4267.
32 Pickard MA and DWS Westlake. 1970. Fungal metabolism of flavonoids. Purification, properties and substrate specificity of an inducible laccase from *Polyporus versicolor* PRL 572. *Can J Biochem* 48: 1351–1358.
33 Pickard MA, R Roman, R Tinoco and R Vazquez-Duhalt. 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl Environ Microbiol* 65: 3805–3809.
34 Pickard MA, H Vandertol, R Roman and R Vazquez-Duhalt. 1999. High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. *Can J Microbiol* 45: 627–631.
35 Potthast A, T Rosenau, CL Chen and JS Gratzl. 1996. A novel method for conversion of benzyl alcohols to benzaldehydes by laccase-catalyzed oxidation. *J Mol Catal A* 108: 5–9.
36 Reyes P, MA Pickard and R Vazquez-Duhalt. 1999. Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. *Biotechnol Lett* 21: 875–880.
37 Rodriguez E, MA Pickard and R Vazquez-Duhalt. 1999. Industrial dye decolorization by laccases from ligninolytic fungi. *Curr Microbiol* 38: 27–32.
38 Sariaslani FS, JM Beale and P Rosazza. 1984. Oxidation of rotenone by *Polyporus anceps laccase*. *J Nat Prod* 47: 692–697.
39 Sutherland JB, F Rafii, AA Khan and CE Cerniglia. 1995. Mechanisms of polycyclic aromatic hydrocarbon degradation. In: Young LY and CE Cerniglia (Eds), Microbial Transformation and Degradation of Toxic Organic Chemicals. Wiley-Liss, New York, NY, pp. 269–306.
40 Thurston TF. 1994. The structure and function of fungal laccases. *Microbiology* 140: 19–26.
41 Tinoco R and R Vazquez-Duhalt. 1998. Chemical modification of cytochrome *c* improves the catalytic properties of polycyclic aromatic hydrocarbons. *Enzyme Microb Technol* 22: 8–12.
42 Vazquez-Duhalt R, P Fedorak and DWS Westlake. 1992. The role of enzyme hydrophobicity in biocatalysis in organic solvents. *Enzyme Microb Technol* 14: 837–841.
43 Vazquez-Duhalt R, DWS Westlake and PM Fedorak. 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing aromatic solvents. *Appl Environ Microbiol* 60: 459–466.
44 Vazquez-Duhalt R, DWS Westlake and PM Fedorak. 1995. Kinetics of chemically modified lignin peroxidase and enzymatic oxidation of aromatic nitrogen-containing compounds. *Appl Microbiol Biotechnol* 42: 675–681.
45 Wolfenden BS and RL Wilson. 1982. Radical cations as reference chromogens in studies of one-electron transfer reactions: pulse radiolysis studies of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate). *J Chem Soc Perkin Trans* II: 805–812.
46 Xu F, JJ Kulys, K Duke, K Li, K Krikstopaitis, HJW Deussen, E Abbate, V Galinyte and P Schneider. 2000. Redox chemistry in laccase-catalyzed oxidation of *N*-hydroxy compounds. *Appl Environ Microbiol* 66: 2052–2056.
47 Yaver DB, F Xu, EJ Golightly, T Halkier, K Mondorf and H Dalboge. 1996. Purification, characterization, molecular cloning and expression of two laccase genes from the white rot fungus *Trametes villosa*. *Appl Environ Microbiol* 62: 834–841.