ARTICLE
Journal of Cellular Biochemistry 114:599–605 (2013)

Journal of Cellular Biochemistry

Recombinant Laccase: I. Enzyme Cloning and Characterization

Claudio Nicolini,[1,2,3*] Debora Bruzzese,[2] Maria Teresa Cambria,[2] Nicola Luigi Bragazzi,[1,4] and Eugenia Pechkova[1,2]

[1] *Biophysics and Nanobiotechnology Laboratories, DIMES, University of Genoa, Genoa, Italy*
[2] *Nanoworld Institute Fondazione EL.B.A Nicolini, Pradalunga, Bergamo, Italy*
[3] *Biodesign Institute, Arizona State University, Tempe, Arizona*
[4] *School of Public Health, Department of Health Sciences (DISSAL), University of Genoa, Genoa, Italy*

ABSTRACT

We obtained structural and functional characterization of a recombinant Laccase from *Rigidoporus lignosus* (formerly *Rigidoporus microporus*), a white-rot basidiomycete, by means of circular dichroism (CD) spectra, cyclic voltammetry (CV) and biochemical assays. Here we report the optimization of expression and purification procedures of a recombinant Laccase expressed in supercompetent *Escherichia coli* cells. We amplified the coding sequence of Laccase using PCR from cDNA and cloned into a bacterial expression system. The resulting expression plasmid, pET-28b, was under a strong T7/Lac promoter induced by IPTG (isopropyl-β-*d*-thiogalactoipyranoside). We obtained purification by fast protein liquid chromatography (FPLC) method. We recorded the variation of the current of a solution containing purified Laccase with increasing Syringaldazine (SGZ) concentration using a potentiometer as proof of principle, showing its compatibility with the development of a new enzymatic biosensor for medical purposes, as described in Part II. J. Cell. Biochem. 114: 599–605, 2013.

KEY WORDS: LACCASE; RECOMBINANT PROTEIN; EXPRESSION; FPLC PURIFICATION; VOLTAMMETRY; SYRINGALDAZINE

Laccases (benzenediol:oxygen oxidoreductase, *p*-diphenol oxidase EC 1.10.3.2) are blue multicopper oxidases containing glycoproteins, abundantly present in many plants and fungal species [Geiger et al., 1986; Mayer, 1987; Bourbonnais and Paice, 1990; Coll et al., 1993; Slomczynsky et al., 1995; Eggert et al., 1997].

Rigidoporus lignosus (formerly known as *Rigidoporus microporus*) is a basidiomycete causing a white-rot of roots in more than 100 species of trees. As reported in the literature, several fungal Laccases have been purified and characterized biochemically [Cole et al., 1990; Palmieri et al., 1993], and also some of the genes encoding Laccase have been cloned and investigated [Garavaglia et al., 2004].

The first interest in these enzymes was related to their production as a fungal taxonomic tool. The function and properties of these proteins have been extensively studied [Ragusa et al., 2002] with particular attention to their possible role in lignin biodegradation and cell detoxification [Mayer, 1987; Kojima et al., 1990; Ragusa et al., 2002].

These enzymes are able to catalyze the oxidation of diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group [Harrs and Huttermann, 1980; Wang and Chen, 1995; Yaver et al., 1996]. This biological function of Laccase is correlated to lignin biodegradation in combination with either manganese [Leonowicz et al., 1985] and/or lignin peroxidase [Bourbonnais and Paice, 1990; Thurston, 1994].

Being the by-products of large-scale production especially of man-made organic components, phenols can cause undesirable adverse ecological effects. Many of these compounds are highly resistant to biotic and abiotic degradation and, as a consequence, may remain in the environment at toxic levels. Different phenol biosensors have been implemented and those based on Tyrosinase have been proven to be the most sensitive ones [Toyota et al., 1985; Kulys and Schmid, 1990; Cosnier and Innocent, 1993; Ortega et al., 1993; Vianello et al., 2006].

Due to its possible utilization as electrode for organic phase enzymatic assays [Ishihara, 1980] and since it can catalyze

Grant sponsor: Proteomics and Cell Cycle; Grant number: FIRB RBIN04RXHS; Grant sponsor: Nanobiosensors; Grant number: FIRB ITALNANONET RBPR05JH2P_003.
*Correspondence to: Prof. Claudio Nicolini, President Nanoworld Institute Fondazione ELBA Nicolini, Eminent Chair of Biophysics at University of Genova, Genova, Italy; Foreign Member Russian Academy of Sciences, Moscow, Russia. E-mail: cnicolini@ibf.unige.it
Manuscript Received: 15 March 2012; Manuscript Accepted: 7 September 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 18 September 2012
DOI 10.1002/jcb.24397 • © 2012 Wiley Periodicals, Inc.

regiospecific biotransformations and selective detoxifications [Galliano et al., 1991], Laccase may have an important role for biotechnology applications, especially for the development of amperometric biosensors for phenol determination and control [Dec and Bollag, 1990; Wang et al., 1993; Rogers, 1995]. An amperometric biosensor using an oxygen electrode (the Clark electrode) and a column with immobilized Laccase has been proposed for the determination of polyphenolic compounds [Xu et al., 1998] and the effects of Laccase immobilization procedures have been also analyzed [Kulys et al., 2000].

Despite such extensive biochemical studies and characterizations, the main draw-back is the fact that in most micro-organisms Laccases are produced at levels that are too low for commercial purposes. Cloning of Laccase gene followed by heterologous expression may provide higher enzyme yields and thus enable the production of Laccase with the desired properties. For this reason potentiometric characterization of Laccase in solution versus increasing concentrations of Syringaldazine represents a first step towards the development of recombinant Laccase-based medical biosensor. The data still required for the implementation of recombinant Laccase-based medical biosensor for sensing and analyzing drugs in real human blood samples—including also time stability test of the sensor—have been obtained from patients and are the object of a separate conclusive communication [Bragazzi et al., 2012], entirely based on what is here presented as Part I.

MATERIALS AND METHODS

MATERIALS

Chemicals were supplied by Sigma–Aldrich (Milano, Italy). Reagents for bacterial growth were obtained from Fluka (Milano, Italy). T4 DNA Ligase and Polymerase were supplied by QIAGEN, Inc. (Valencia, CA). Restriction enzymes were obtained from Boehringer-Mannheim (Monza, Italy). Primers were synthesized by TibMolBiol (Genova, Italy). *Escherichia coli* host strain BL21(DE3) and pQE30 vector were purchased from QIAGEN, Inc. The purification fast protein liquid chromatography (FPLC) column was obtained from Amersham. Deionized water (Milli-Q water purification system; Millipore, Bedford, MA, 18.2 MΩ/cm) was used in the preparation of all the solutions.

SUBCLONING OF THE CDNA ENCODING THE LACCASE GENE

The plasmid carrying the Laccase-encoding cDNA was kindly supplied by Professor Marina Garber from the Russian Academy of Sciences (RAS) at Pushchino. In order to modify the ends of the encoding fragment, the plasmid was used as a template for PCR. A 5′ primer was designed to introduce the start codon (ATG) within the *Nco*I site. A 3′ primer was designed to introduce the *Xho*I site and the stop codon. Laccase gene was amplified by PCR using the forward primer Lac2_for (5′-GGI ACI WII TGG TAY CAY WSI CA-3′), containing a *Nco*I restriction site, and the reverse primer Lac2_rev (5′-CC RTG IWK RTG IAW IGG RTG IGG-3′), containing a *Xho*I site, as described in Lyons et al. [2003]. Primers were designed using an opportune software and the high fidelity PfuTurbo™ Polymerase (Stratagene) was used for PCR reaction. The PCR product was purified with an extraction column (QIAGENE). The linearized vector pET28b, previously digested with the same restriction enzymes (*Nco*I and *Xho*I), was isolated and purified from agarose gel by gel extraction kit (QIAGENE).

CONSTRUCTION OF EXPRESSION PLASMID FOR LACCASE ENZYME

At the 5′ of the polylinker the pET28b vector contains a nucleotide sequence encoding the 6xHis affinity tag. The insertion of the modified cDNA inside the pET28b vector will result in a protein with 10 additional aminoacids with respect to the wild-type Laccase.

Starting from 10 μl of both insert and vector, the ligation with linearized pET28b vector was performed using T4 DNA ligase (2 U/ml) under standard experimental conditions.

The pET28b is widely used for expression of recombinant protein in *E. coli*. This vector allowed cloning Laccase gene under control of T7 promoter (strong promoter from T7 bacteriophage) in order to obtain the enzyme as fusion protein (with His-Tag).

The final recombinant plasmid pET28b was extracted, purified and then introduced, by transformation, in appropriate *E. coli* super competent cells strain B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ (DE3). Tests of expression have proven that BL21(DE3) cells were the best type cells among C41, M15, and BL21(DE3) itself. After transformation the new system was amplified and then the ligation product (Fig. 1) was appropriately checked for the sequence of the cDNA insert.

BACTERIAL EXPRESSION OF RECOMBINANT LACCASE

For recombinant Laccase, *E. coli* BL21(DE3) cells were cultivated in 12.5 ml of Luria-Bertani medium (LB broth) and they were grown overnight at 37°C for about 16 h, under shaking conditions (at 3,000*g*). One litre of pre-warmed LB medium, containing ampicillin, was inoculated with 10 ml of the overnight cultured *E. coli* cells harbouring pET28b-laccase. It was grown under the same conditions and shaken until the optical density value at 600 nm reached about 0.6. The protein expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM and BL21(DE3) cells were grown for 2 h and they were harvested by centrifugation at 4,000*g* for 15 min and the pellet was stored at −80°C.

RECOMBINANT LACCASE PURIFICATION

One litre of biomass was defrosted for 15 min on ice. The biomass was resuspended in 6 ml of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8), after adding Lysozyme (1 mg/ml), and incubated on ice for 30 min. Serine protease inhibitor PMSF (phenylmethylsulfonyl fluoride) 1 mM was added to the suspension. When the suspension appeared homogeneous, it was sonicated, using repeated duty cycle at 30 s pulse for 5 min in dry ice. Then the obtained suspension was centrifuged at 10,000*g* for 25 min in order to obtain a limpid supernatant containing the recombinant Laccase protein. The supernatant was collected and applied to a nickel-nitriloacetic acid (Ni-NTA) metal affinity chromatography that selectively binds recombinant proteins tagged with six consecutive histidine residues. The chromatography apparatus was equipped with a HiTrap_Chelating_HP_5_ml column (Amersham), that had been previously equilibrated with five volumes of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8), until the absorbance value recorded at 280 nm was stable. Laccase was

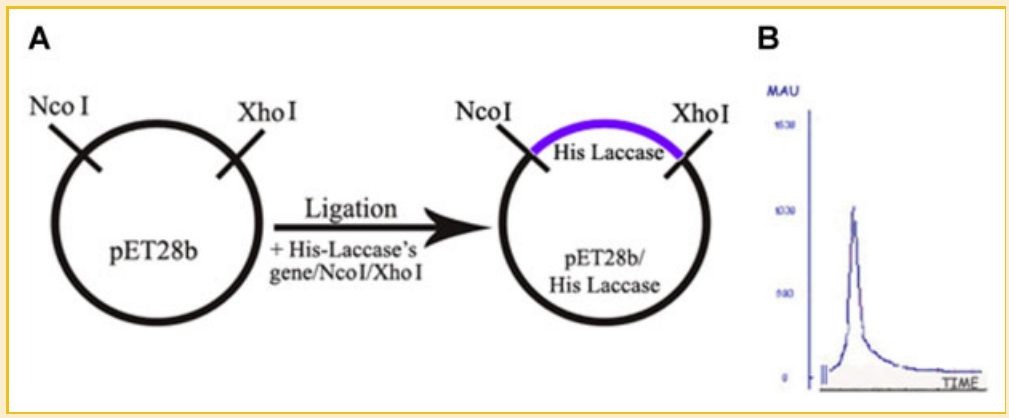

Fig. 1. cDNA ligation into the vector pET28b, under a strong T7/Lac promoter induced by IPTG (isopropyl-β-d-thiogalactoipyranoside), resulting in a protein with 10 additional aminoacids with respect to the wild type Laccase, (A) and FPLC elution (B).

then eluted from the column with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 60 mM imidazole, pH 8) under gradient elution, gradually increasing the concentration of imidazole up to 300 mM in the elution buffer (Fig. 2).

ENZYME ELECTROPHORESIS

After purification, all the fractions of eluted protein were analyzed by 10% SDS–PAGE performed according to the method described by Laemmli [Herman, 1989; Ghindilis et al., 1992; Freire et al., 2001]. Laccase was resolved on 10% polyacrylamide gel electrophoresis. Gels were stained with 0.1% Coomassie brilliant blue in 5% acetic acid and properly destained in destaining buffer with overnight agitation. The molecular weight of the enzyme was determined using the PageRuler™ Prestained Protein Ladder (Fermentas).

CIRCULAR DICHROISM

Circular dichroism (CD) spectra of the recombinant Laccase were recorded on a Jasco J-710 spectropolarimeter (Jasco, Japan) equipped with a Peltier thermostatic cell holder (Model PTC-343). All spectra were recorded under nitrogen atmosphere, at room temperature, using a 0.05-cm path length quartz cell. In order to reduce random error and noise, each spectrum was an average of 10 scans over 250–180 nm. The following parameters were used for the acquisition: time constant, 1 s; scanning speed, 50 nm/s; band width, 2 nm; sensitivity, 20 mdeg; step resolution, 0.2 nm; and PMT voltage

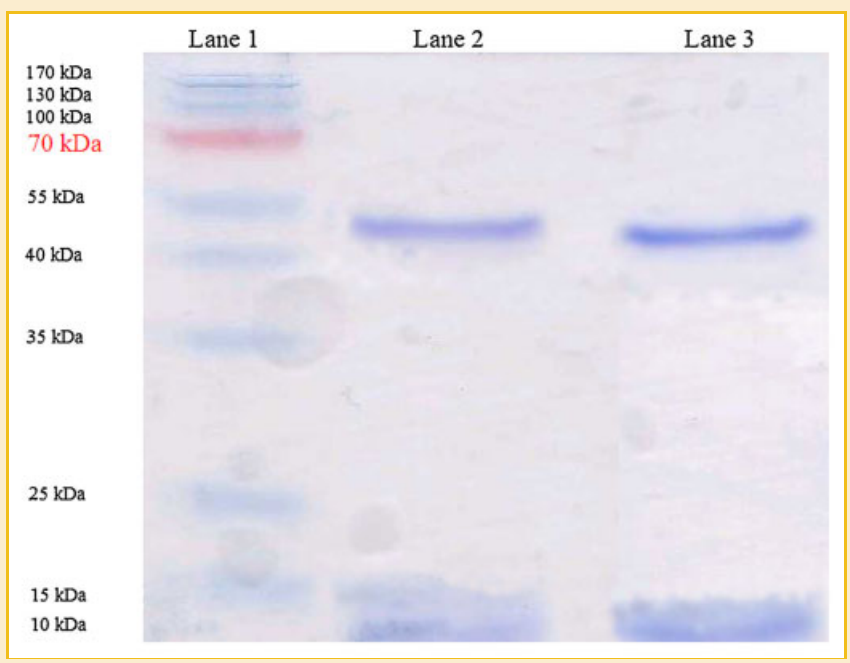

Fig. 2. SDS–PAGE of samples obtained by elution. Collected fractions were analyzed by SDS–PAGE on 10% gel and stained with Comassie Brilliant Blue. Lane 1 is the PageRuler™ Prestained Protein Ladder, Fermentas, Lane 2 represents fraction 38, while Lane 3 fraction 37.

TABLE I. Experimental data of the secondary structure of Laccase from *Rigidoporus lignosus* in comparison with theoretical data, fitting with the secondary structure of Laccase from the same *Rigidoporus lignosus* here reproduced with STRAP software from 1V10 PDB structure (Garavaglia S, Cambria MT, Miglio M, Ragusa S, Iacobazzi V, Palmieri F, D'Ambrosio C, Scaloni A, and Rizzi M) (Helices and beta sheets have a red and yellow background, residue annotations are underlined)

2D structure of recombinant Laccase (CD data)	%	2D structure of Laccase (theoretical data)	%
Helix	9.7	Helix	9.9
β	53	β	59.6
Random	37.3	Random	~30

below 600 W [Brenna and Bianchi, 1994]. No filtering for noise reduction was applied. Samples were acquired at protein concentration of 0.2 mg/ml. All the acquired spectra were corrected for the baseline.

SPECTROPHOTOMETRIC ASSAY

Laccase activity was measured spectrophotometrically at room temperature using as substrate Syringaldazine (SGZ), which is a non auto-oxidizable Laccase-specific compound [Malavalan and Johnson, 1985]. In order to obtain structural characterization of recombinant Laccase, UV–Vis spectrum was recorded (Spectrophotometer Jasco 7800), by using samples of recombinant Laccase at protein concentration of 0.4 mg/ml.

ENZYME ASSAY

Enzyme tests were performed in order to verify catalytic activity of recombinant enzyme from *R. lignosus*. The assay of Laccase activity was made according to the protocol suggested by Sigma–Aldrich. The reaction mixture was prepared with potassium phosphate buffer 100 mM; pH 6.5 containing 0.5 mg of purified enzyme. The solution was incubated in the cuvettes at 30°C under continuous stirring, and then 0.0648 mM of Syringaldazine was added.

The oxidation of Syringaldazine by Laccase was monitored for 1 h at 30°C using a spectrophotometer (Jasco 7800). The increase in absorption (Fig. 3) was recorded at 530 nm (specific value for oxidized Syringaldazine).

Enzyme activity was expressed as micromoles of substrate oxidized per milligram of protein per minute. The protein concentration was determined by the Lowry method [Lowry et al., 1951].

Further mathematical modeling of Laccase kinetics has been performed assuming a Michaelis–Menten like behavior (as discussed in Results and Discussion Section).

CYCLIC VOLTAMMETRY (CV) OF RECOMBINANT ENZYME

Cyclic voltammetric (CV) assays were performed using a potentiometer (EG & G PARC, model 263A) connected to PC (EG & G research electrochemical software model 270).

Current variation (μA) of a solution containing potassium phosphate buffer (100 mM, pH 7.4) and purified enzyme (1 mg/ml) was recorded. In order to verify whether the enzyme had catalytic activity, electric flow variation (μA) was recorded after addition of increasing concentrations of substrate (Syringaldazine).

RESULTS AND DISCUSSION

It is widely known that, like other ligninolytic enzymes, it is difficult to produce Laccases in large amounts as purified proteins. In fact only few examples of industrial uses of Laccases-based biosensors currently exist and one of the very few ones is DeniLite™ by Novozymes (launched in 1996). Our results indicated that it was possible to obtain high amounts of Laccase using *E. coli* as a vector (Fig. 1). We have obtained a purified recombinant Laccase of circa 70 kDa, as shown in Figure 2.

From computational analysis (Table I) we have predicted a predominant beta structure of the protein, a theoretical prediction that was confirmed by comparison with the experimental data obtained from X-ray resolved structure [Garavaglia et al., 2004] and from the CD study as shown in Figure 3A (it is easy to recognize the characteristic positive peak at 198 nm and the negative peak at around 216–218 nm).

Figures 3B and 4 show the good enzymatic activity of our Laccase, which was modeled assuming a Michaelis–Menten like behavior of the enzyme. In the presence of Syringaldazine as substrate, we found a V_{max} of 2 μM/min, an apparent K_M of 22.5 μM, a K_{CAT} of 17.14 s^{-1}, a ratio K_{CAT}/K_M of 0.76 $\mu M^{-1} s^{-1}$, and a turnover rate of 4.87×10^6 M/s. All these values confirmed the good catalytic activity of our enzyme.

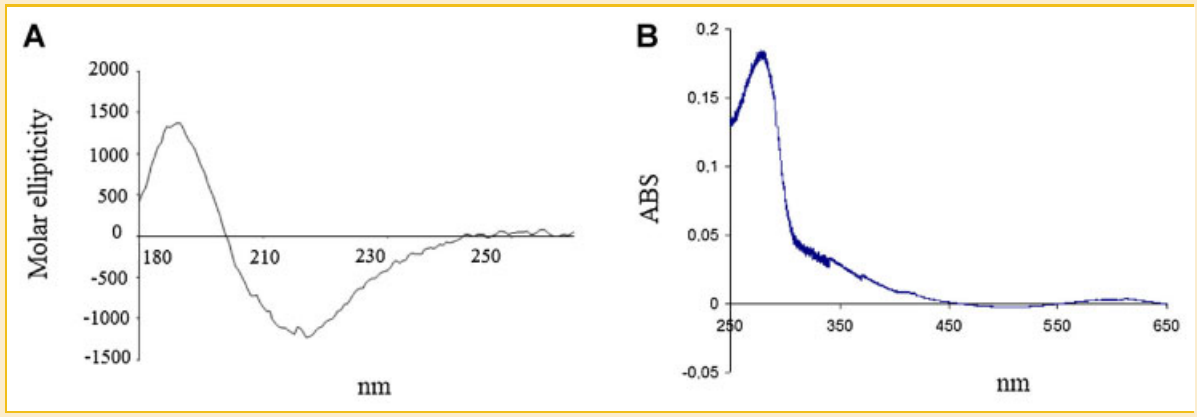

Fig. 3. Structure and function of obtained recombinant Laccase: In (A), we performed a CD spectrum of Laccase, showing a positive peak at 198 nm and a negative peak around 216–218 nm, thus confirming the predominant beta-structure of the enzyme, while in (B) it is shown the activity of Laccase in presence of Syringaldazine.

The current variation (μA) of a solution containing potassium phosphate buffer (100 mM, pH 7.4) and purified enzyme (1 mg/ml) allowed us to verify electronically that the enzyme had catalytic activity, with the current variation (μA) recorded after the addition of increasing concentrations of substrate (Syringaldazine). Results and data reduction (shown in Fig. 5) confirmed that recombinant Laccase had catalytic activity, which was consequently evaluated quantitatively. We began to study it through the CV assay and we found that the peak current ratio (i_{PA}/i_{PC}) confirmed the quasi-reversible nature of Laccase kinetics.

From the potentiometric measurement analysis, K_M was computed to be 20 μM, in good agreement with the value found in the spectrophotometric analysis. Solving Laviron's equation in order to investigate the ET (electron transfer), K_S resulted $3 \times 10^{-3}\,s^{-1}$ without electron mediator (i.e., Syringaldazine) and $1.08 \times 10^{-2}\,s^{-1}$ with Syringaldazine, thus confirming that the presence of an electron donor could significantly enhance the electron transfer process:

$$\log K_s = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \frac{\alpha n\Delta E_p(1-\alpha)}{2.3\,RT}$$

where K_S is the Laviron electron transfer constant, α is the charge transfer coefficient (assumed here reasonably 0.5), R the gas constant, T the temperature in Kelvin, F the Faraday constant, v the voltage scan, n the number of electrons (here at least one electron), ΔE_p the difference between the oxidizing potential and the reducing one.

The surface coverage area (Γ) is 1.15×10^{-10} as computed by solving this equation:

$$i_{peak} = \frac{n^2F^2vA\Gamma}{4RT}$$

We started our CV study testing the biosensor with immobilized Laccase at electrode potential of −200 mV, observing that the electrical response was nearly zero. So we continued our study applying different potential values and recording the electrical variations: we observed that a definite cathodic response was detectable at +180 mV potential and that there was a linear dose-dependent response in the millimolar range up to 2×10^{-4} M, as shown in Figure 5A,B. We found a linear relation between substrate concentration and current intensity, as shown in Figure 5B,

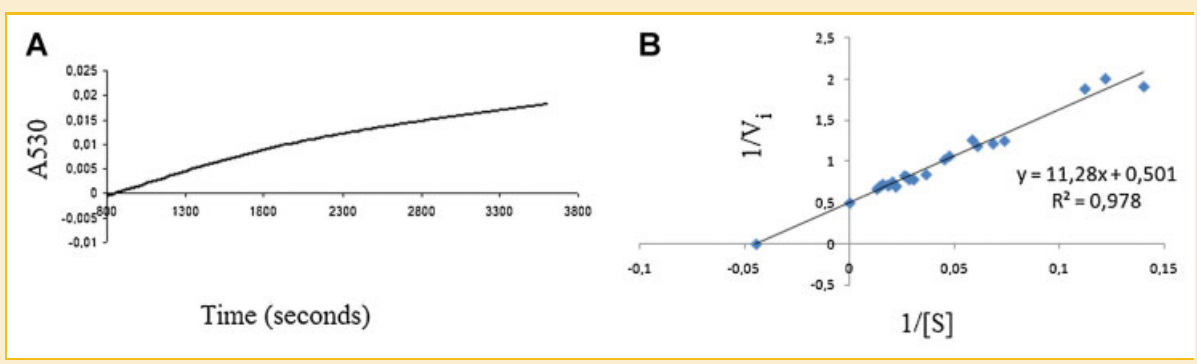

Fig. 4. Activity of Laccase in presence of Syringaldazine for 1 h at 30° (A) and (B) Lineweaver–Burke for the Laccase kinetic characterization, from which KM 22.5 μM has been computed.

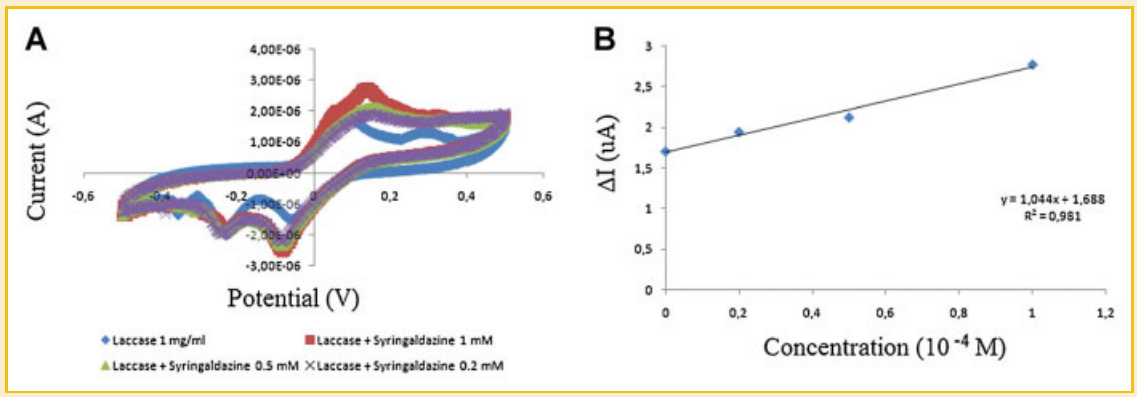

Fig. 5. Current variations after addition to Laccase of increasing Syringaldazine concentrations (A) and Laccase potentiometric response in the dynamic range (B).

in agreement with our hypothesis. The biosensor sensitivity is 0.014 mA/M and these data taken together with all the previous biophysical and bioelectrochemical data render feasible the use of Laccase in biosensor development for phenols [Gomes and Rebelo, 2003; Vianello et al., 2004; Vianello et al., 2006] and drugs (Part II) detection.

CONCLUSIONS

A well-known prerequisite for developing and implementing commercial biosensors is the availability of large amounts of a purified, stable, functionally and catalitically working enzyme substrate at a relatively low cost. Classical purification methods and procedures are time-consuming and expensive. To overcome time and cost problems, Laccase from *R. lignosus* has been cloned in *E. coli* vector system.

Purification and expression of recombinant Laccase and its biochemical characterization have been functional for the further development of a medical biosensor as discussed in Bragazzi et al. [2012] (Part II). Indeed potentiometric measurements appeared to be useful in order to determine the proper oxidation/reduction potential to be utilized for implementing the Laccase-based biosensor, first in solution using Syringaldazine as proof of principle (here) and then in real human blood samples obtained from healthy volunteers and psychiatric patients using a widely administered drug in psychiatry (namely Clomipramine, Anafranil™) as substrate [Bragazzi et al., 2012]. A time stability test of the biosensor was performed, showing that after a month it was still working. For further details and characterization of the biosensor, the reader is referred to Bragazzi et al. [2012].

ACKNOWLEDGMENTS

This work was supported by two grants—namely Proteomics and Cell Cycle (FIRB RBIN04RXHS), and Nanobiosensors (FIRB ITALNANONET RBPR05JH2P_003), by MIUR (Ministero dell'Istruzione, Università e Ricerca)—both to Claudio Nicolini University of Genova, and by a grant Funzionamento by MIUR (Ministero dell'Istruzione, Università e Ricerca) to Fondazione El.B.A.Nicolini.

We are grateful to Professor Marina Garber from Russian Academy of Sciences at Pushchino, to doctoral student Dora Scudieri and to technician Paola Ghisellini at University of Genova for their valuable cooperation.

We thank Dr. Julie Ivy Trundley for the English revision of the manuscript.

REFERENCES

Bourbonnais R, Paice MG. 1990. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. FEBS Lett 267: 99–102.

Bragazzi NL, Pechkova E, Scudieri D, Terencio TBC, Adami M, Nicolini C. 2012. Recombinant Laccase: Part II. Medical biosensor. Crit Rev Eukaryot Gene Expr (in press).

Brenna O, Bianchi E. 1994. Immobilised laccase for phenolic removal in must and wine. Biotechnol Lett 16:35.

Cole JL, Patrick PA, Solomon EI. 1990. Spectroscopic and chemical studies of the laccase trinuclear copper active site: Geometric and electronic structure. J Am Chem Soc 112:9534–9548.

Coll PM, Fernandez-Abalos JM, Villanueva JR, Santamaria R, Perez P. 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59:2607–2613.

Cosnier S, Innocent Ch. 1993. A new strategy for the construction of a tyrosinase-based amperometric phenol and o-diphenol sensor. Bioelectrochem Bioenerg 31:147–160.

Dec J, Bollag JM. 1990. Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. Arch Environ Contam Toxicol 19:543–550.

Eggert C, Temp U, Eriksson K-EL. 1997. Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. FEBS Lett 407:89–92.

Freire RS, Duran N, Kubota LT. 2001. Effects of fungal Laccase immobilization procedures for the development of a biosensor for phenol compounds. Talanta 54:681–686.

Galliano H, Gas G, Seris JL, Boudet AM. 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. Enzyme Microbiol Technol 13:478–482.

Garavaglia S, Cambria MT, Miglio M, Ragusa S, Iacobazzi V, Palmieri F, D'Ambrosio C, Scaloni A, Rizz M. 2004. The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair, J Mol Biol 342(5):1519–1531.

Geiger JP, Rio B, Nandris D, Nicole M. 1986. Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. I. Purification and some physico-chemical properties. Appl Biochem Biotechnol 12:121–133.

Ghindilis AL, Gavrilova VP, Yaropolov AI. 1992. Laccase-based biosensor for determination of polyphenols: Determination of catechols in tea. Biosens Bioelectron 7:127–131.

Gomes SASS, Rebelo MJF. 2003. A new laccase biosensor for polyphenols determination, Sensor 3:166–175.

Harrs A, Huttermann A. 1980. Function of laccase in the white-rot fungus *Fomes annosus*. Arch Microbiol 125:233–237.

Herman EM. 1989. Colloidal labeling of acrylic resin-embedded plant tissues. In: Hayat MA, editor. Colloidal gold: Principles, methods, and applications. New York: Academic Press, Inc. pp 303–322.

Ishihara T. 1980. The role of laccase in lignin biodegradation. In: Kirk TK, Higuchi T, Chang HM, editors. Lignin degradation: Microbiology, chemistry and potential application, Vol. 2. Boca Raton, FL: CRC Press. pp 17–31.

Kojima Y, Tsukuda Y, Kawai Y, Tsukamoto A, Sugiura J, Sakaino M, Kita A. 1990. Cloning, sequence analysis phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 265:15224–15230.

Kulys J, Schmid RD. 1990. A sensitive enzyme electrode for phenol monitoring. Anal Lett 23(4):589–597.

Kulys J, Krikstopaitis K, Ziemys A. 2000. Kinetics and thermodynamics of peroxidase- and laccase-catalyzed oxidation of Nsubstituted phenothiazines and phenoxazines. J Biol Inorg Chem 5:333–340.

Leonowicz A, Szklarz G, Wojtas-Wasilewska M. 1985. The effect of fungal laccase on fractionated lignosulphonates (peritan Na). Phytochemistry 24:393–396.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Lyons JI, Newell SY, Buchan A, Moran MA. 2003. Diversity of ascomycete laccase gene sequences in a Southeastern US salt marsh. Microb Ecol 45:270–281.

Malavalan P, Johnson WC, Jr. 1985. Protein secondary structure from circular dichroism spectra. Suppl J Biosci 8:141–149.

Mayer AM. 1987. Polyphenol oxidases in plants: Recent progress. Phytochemistry 26:11–20.

Ortega F, Dominguez E, Jönsson-Pettersson G, Gorton L. 1993. Amperometric biosensor for the determination of phenolic compounds using a tyrosinase graphite electrode in a flow injection system. J Biotechnol 31:289–300.

Palmieri G, Giardina P, Marzullo L, Desiderio B, Nitti G, Cannio R, Sannia G. 1993. Stability and activity of phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. Appl Microbiol Biotechnol 39:632–636.

Ragusa S, Cambria MT, Pierfederici F, Scirè A, Bertoli E, Tanfani F, Cambria A. 2002. Structure–activity relationship on fungal laccase from *Rigidoporus lignosus*: A Fourier-transform infrared spectroscopic study. Biochim Biophys Acta 1601(2):155–162.

Rogers KR. 1995. Biosensors for environmental applications. Biosens Bioelectron 10:533–541.

Slomczynsky D, Nakas JP, Tanenbaum SW. 1995. Production and characterization of laccase from *Botrytis cinerea*. Appl Environ Microbiol 61: 907–912.

Thurston CF. 1994. The structure and function of fungal laccases. Microbiology 140:19–26.

Toyota T, Kuan SS, Guilbault GG. 1985. Determination of total protein in serum using a tyrosinase enzyme electrode. Anal Chem 57:1925–1928.

Vianello F, Cambria A, Ragusa S, Cambria MT, Zennaro L, Rigo A. 2004. A high sensitivity amperometric biosensor using a monomolecular layer of laccase as biorecognition element, Biosens Bioelectron 20(2):315–321.

Vianello F, Ragusa S, Cambria MT, Rigo A. 2006. A high sensitivity amperometric biosensor using laccase as biorecognition element. Biosens Bioelectron 21(11):2155–2160.

Wang J, Chen Q. 1995. Highly sensitive biosensing of phenolic compounds using bioaccumulation/chronoamperometry at a tyrosinase electrode. Electroanalysis 8:746–749.

Wang J, Lin Y, Eremenko AV, Ghindilis AL, Kurachkin IN. 1993. A laccase electrode for organic-phase enzymatic assay. Anal Lett 26:197–207.

Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE, Solomon EI. 1998. Site-directed mutations in fungal laccase: Effect on redox potential, activity and pH profile. Biochem J 334:63–70.

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalbøge H. 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62: 834–841.