LACASSE CATALYZED-SYNTHESIS OF 4,4'-BIPHENYLDIAMINE FROM *P*-CHLOROANILINE. EVALUATION OF ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

Laccase is a copper-containing oxidase that catalyzes reduction of molecular oxygen to water and the oxidation of a phenolic compound. In this paper, laccase was utilized to synthesize 4,4'-Biphenyldiamine using *p*-chloroaniline as substrate by means of a coupling reaction. The synthesized compound, 4,4'-Biphenyldiamine, presented low antifungal activity against the phytopathogenic fungus *Botrytis cinerea*, however the antioxidant ability, measurement by ORAC-PGR method, was higher than substrate.

This work corresponds to the first report of synthesis 4,4'-Biphenyldiamine, from *p*-chloroaniline in a lacasse-catalyzed reaction.

Keywords: *p*-chloroaniline, 4,4'-Biphenyldiamine, laccase, antioxidant activity, antifungal activity.

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidases that catalyze the single-electron oxidation of a wide range of phenolic compounds and reduction of molecular oxygen to water[1]. The produced radical can reacts non-enzymatic to produce covalent coupling to form dimers, oligomers and polymers through C–C, C–O and C–N bonds[2]. This enzyme has been used to synthesize hydroxybiphenyl dimers[3], isoeugenol dimers[4] and ferulic acid dimers[5].

Laccase is also able to couple a laccase substrate with a non-laccase substrate (known as a mediator) to form new molecules (heterodimers)[2,6]. Niedermeyer *et al*. reported the heterodimeric synthesis between *p*-hydroquinones and primary aromatic amines using laccase[7]. Another example is the coupling between *p*-hydroquinones with *p*-aminobenzoic acid[8]. Antibiotic modifications using laccase coupling reactions have shown products with lower activities[9]; however, using nitrogen compounds to modify the antibiotics, the synthesized products showed higher antibacterial activities[10,11]. The anticancer activity of naphtohydroquinones has been improved by nuclear monoamination with anilines using laccase[12]. These results indicate that the laccase-mediated amination of a phenolic compound could increase their biological activity.

On the other hand, these enzymes utilize as substrates anilines, hydroxyindoles, and benzenethiols and also some inorganic ions such as $[Mo(CN)_8]^{4-}$, $[Fe(CN)_6]^{4-}$, $[Os(CN)_6]^{4-}$, and $[W(CN)_8]^{4-}$[13]. It has been reported that laccase catalyses the *in vitro* polymerization of aniline. However, the polymerization rate is relatively low, therefore mediators must be used[14].

Likewise, this enzyme has been used to increase the antioxidant activity of several flavonoids by polymerization[15]. On the other hand, laccase has been used to couple antioxidant compounds to wood[16]. Also, due to their demethylation and dehalogenation ability it can be applied to control environmental pollution, for example, as a remediation of soil contaminated with chlorophenol residues[17]or TNT(2,4,6-trinitrotoluene)[18]. Furthermore, laccase has been used as stabilizing wine by removing unwanted wine phenolics[19] and the stabilization of black fruit juice grape is possible by action of this enzyme[20]. Supporting the improve of the antioxidant capacity of certain compound using laccase, it is possible to mention as an example, the functionalization of chitosan by laccase-catalyzed oxidation. Bozic *et al*. (2013) described that functionalization of chitosan by phenolic acids as a gallic acid and caffeic acid mediated by laccase, enhanced antioxidant and antimicrobial properties[21]. Reinforcing these results, recently Aljawish*et al*., (2014) reported that the chitosan derivatives presented improved antioxidant properties especially for ferulic acid-chitosan derivative when compared with chitosan alone. In addition, the thermal antioxidant stability as well as the preservation of initial antibacterial activity of chitosan was improved[22].

Botrytis cinerea, also known as "gray mould fungus", attacks a broad range of host resulting in great economic losses on Chilean export products. However, despite of many varieties of botrycides, the fungus has generated resistance against these chemical products[23]. Therefore, it is imperative to develop new molecules with antifungal activity.

The aim of this work was to analyze the product of laccase-catalyzed reaction utilizing *p*-chloroaniline as substrate and to evaluate the antioxidant and antifungal activity against *B. cinerea* of the obtained product.

2. EXPERIMENTAL

2.1 Chemicals

Malt extract was obtained from Cramer Co., Ltd. (Santiago, Chile). Yeast extract, Laccase from *Trametes versicolor* (EC 1.10.3.2), and *p*-chloroaniline were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Agar was obtained from Difco laboratories (Detroit, MI, USA). Organic solvents and salts were obtained from Merck Química Chilena (Santiago, Chile).

2.2 Laccase-mediated synthesis of 4,4'-Biphenyldiamine

A biphasic system containing sodium acetate buffer (50mM) at pH of 4.5 and ethyl acetate:acetone (1:0.1) was employed for the laccase reaction. The reaction mixture also contained *p*-chloroaniline (32.5mM) and laccase (3.79 U). The reactions were carried out for 15 h at 22°C with shaking, using an orbital shaker at 200 rpm. The reactions were monitored by thin layer chromatography (TLC) on aluminum–backed silica gel 60 F254 (Merck) plates using hexane:ethyl acetate (7:3 v/v) as the mobile phase.

After the incubation time, the solvent was evaporated using a rotary evaporator. The product was purified from the reaction mixture using semi-preparative thin layer chromatography (TLC silica gel 60 F_{254} glass plates 20x20cm) with hexane:ethyl acetate (8:2 v/v) as an eluent system. The purity of compound was analyzed by high performance liquid chromatography (HPLC) by using a Waters 600 HPLC chromatograph (Waters, Mildford, MA, USA) equipped with a Waters 2990 diode array detector, and a Symmetry C-18 (5 µm) (Waters, Milford, MA, USA) column (3.9 mm × 150 mm). Chromatography was conducted at 25 ◦C. Mobile phase was composed of 1.0% (v/v) acetic acid in distilled water (A) and acetonitrile (B). The system was run by 60 min with a gradient program as follows: linear gradient 10–20% B in 20 min and then 20–50% B in 25 min was applied. Finally, a gradient 50-100% B in 15 min was applied. The flow rate was 0.8 mL/min and it was recorded at 280 and 360 nm.

The purified product was characterized by nuclear magnetic resonance (NMR) analysis. The ^{1}H NMR and^{13}C NMR spectra were recorded using a Bruker Avance RW- 400 spectrometer operating at 400.13 MHz. Measurements were carried out at a probe temperature of 300 K, using deuterated chloroform containing tetramethylsilane (TMS) as an internal standard.

2.3 Fungal strain and culture conditions

The strain G29 of *B. cinerea* was used; it was isolated originally from grapes (*Vitis vinifera*) by the Instituto de Investigaciones Agropecuarias La Platina, Chile and is genetically characterized[24]. It was maintained on malt-yeast extract agar slants with (2% (w/v) malt extract, 0.2% (w/v) yeast extract and 1.5% (w/v) agar) at 4 °C.

2.4 Antifungal Assay

The effect of *p*-chloroaniline and 4,4'-Biphenyldiamine (bencidine) on mycelial growth of *B. cinerea* was assessed *in vitro* using the radial growth test on malt-yeast extract agar. Different concentrations of the synthesized

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compound and *p*-chloroaniline were dissolved in acetone and added to Petri dishes containing a malt-yeast extract agar medium (2% malt extract, 0.2% yeast extract and 1% agar). The final acetone concentration was identical in the control and treatment assays. Commercial fungicide iprodione was used as a control. After evaporation of the acetone in a laminar flow cabinet, the culture medium was inoculated with 0.5 cm agar disks from an actively growing culture of *B. cinerea*. Cultures were incubated in the dark at 22 °C. Mycelial growth diameters were measured daily. Results were expressed as IC_{50} (the concentration that reduced mycelial growth by 50%), determined by regressing the inhibition of radial growth values (percent control) against the values of compounds concentration.. Each experiment was done at least three times.

2.5 Evaluation of antioxidant activity from compound p-chlorononaniline and 4,4'-Biphenyldiamine.

To evaluate the antioxidant activity of the substrate and purified product, the oxygen radical absorbance capacity (ORAC) by the pyrogallol red (PGR) method by used [25]. The oxidation od PGR caused by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), monitored at 540 nm, was evaluated. 5µM PGR and 10mM AAPH in 75 mM phosphate buffer (pH 7.4) were used as control. The treatments included 30 µM *p*-chlorononaniline or 30 µM 4,4'-Biphenyldiamine. The assays were performed at 37ºC. The results were compared with the antioxidant activity of Trolox at 30 µM.

3. RESULTS AND DISCUSSION

3.1 Synthesis of 4,4'-Biphenyldiamine.

The lacasse catalyzed-synthesis of 4,4'-Biphenyldiamine (yield 37%) was carried out using *p*-chloroaniline as substrate. As reaction product a less polar product than the substrate was formed. The coloration of the new compound was a stronger dish brown, while that *p*-chloroaniline was colorless under the used conditions. Based on that, it is possible to conclude that the *p*-chloroaniline was a substrate to laccase. Therefore, at the used reaction conditions, the fungal laccase from *T. versicolor* was able to mediate the cross-coupling of two molecules of *p*-chloroaniline to produce a main product.

After purification by semi-preparative thin layer chromatography, the purity was verified by HPLC retention times by p-chloroaniline and the product were 13.077 and 4.637 min, respectively. The synthesized product was analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy. The signals of this compound were compared with the ^{1}H NMR and ^{13}C NMR spectra of the *p*-chloroaniline (Table 1).

In ^{1}H-NMR spectrum signals corresponding to hydrogen associated to aromatic ring (6.6 and 7.1 ppm) and hydrogen associated to amine group (3.6 ppmn) were observed. No significant differences were observed between the signals of *p*-chloroaniline and synthesized compound; signals showed the same pattern, but they shifted to lower fields with the exception of corresponding signal to the amine protons (Ha in figure 1). The signal assigned to Hc (Figure 1) showed the clearest difference. This was observed at 7.11 ppm *p*-chloroaniline and in the product at 7.36 ppm. In the ^{13}C NMR spectra showed a notable different in the signals assigned to C_4 in the *p*-chloroaniline (123.6 ppm) and to C_1 and C_1' in the synthesized product, which shifted to lower field (131.9 ppm). By HSQC and HMBC analyses, it was possible to assign the protons to their respective carbons. Assigning signals, integrations and coupling constants are shown in Table 1, for both compounds, *p*-chloroaniline and synthesized compound.

The shift of the signals of the aromatic protons to a lower field and shift of the C4 signal of the *p*-chloroaniline suggests that the synthetized compound does not possess chlorine atom, and the structure shown in Figure 1 supports the new signals.

Table 1. ^{1}H NMR and ^{13}C NMR data of *p*-chloroaniline and the synthesized product. In the same row homolog carbons between *p*-chloroaniline and the new synthesized product are shown.

***p*-chloroaniline**						**Synthesized product**					
Carbon Atom	δ_C (ppm)	Proton	δ_H (ppm)	Integration peak	Coupling constant *J* (Hz)	**Carbon Atom**	δ_C (ppm)	Proton	δ_H (ppm)	Integration peak	Coupling constant *J* (Hz)
C_2 and C_6	116.6	H_b	6.60 (d)	2	8.8	**C_3, C_3', C_5 and C_5'**	115.6	H_b	6.74 (d)	4	8.4
C_3 and C_5	129.5	H_c	7.11 (d)	2	8.8	**C_2, C_2', C_6 and C_6'**	127.4	H_c	7.36 (d)	4	8.4
---	---	H_a	3.66 (s)	2	---	---	---	H_a	3.67 (s)	4	---
C_1	145.3					**C_4 and C_4'**	146.1				
C_4	123.6					**C_1 and C_1'**	131.9				

The ^{13}C-NMR spectrum of *p*-chloroaniline showed four signals assigned to four types carbons (Table 1, Figure 1). In addition, from synthesized compound it is possible to observe four signals corresponding to C4 and C4' (146.06 ppm); C3, C5, C3', and C5' (115.55 ppm); C2, C6, C2' ,C6' (127.38 ppm), and finally, C1' and C1' (131.8 ppm). It can be concluded that the synthesized compound corresponds to 4,4'-Biphenyldiamine [27] (Figure 1).

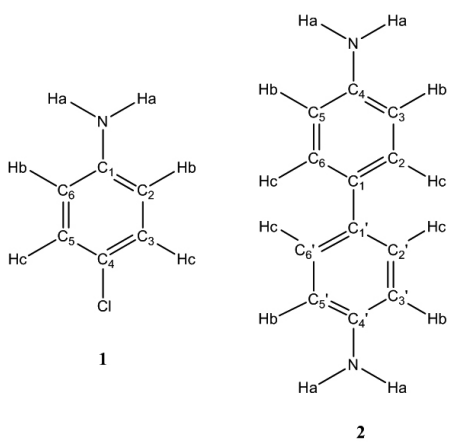

Figure 1.Structure of *p*-chloroaniline (**1**) and 4,4`- biphenyldiamine (**2**).

In general, laccase uses as substrates phenolic substances rather than aromatic amines[28]. It has been reported than an amine containing two amino groups, *p*-phenylenediamine, can be used as substrate by some laccases[29]. Simmons *et al*., (1985) demonstrated that when *p*-chloroaniline was incubated with the *T. versicolor* laccase eight products were obtained[30]. These authors proposed that the substrate was enzymatically oxidized and an anilinium free radical was formed, then a free-radical coupling occurred, and three dimeric intermediates were produced through N-N, N-*para*, and N-*ortho* radical couplings[30]; however, the compounds structures were not reported. This is the first time that laccase catalyzed synthesis of 4,4'-Biphenyldiamine is reported.

The previously works would indicate that laccase catalyzed dimerization of *p*-chloroaniline would could occur by the radical formation in position *para,* after amino group oxidation generating a resonance in this position, causing the output of chlorine from the molecule. Supporting this observation, Longoria *et al* (2008) reported the free-radical generation in position *para* and *ortho* of aniline[31]. Based on these experiments, it is possible to conclude that the enzyme, lacasse, is able to produce oxidation in the molecule forming a radical which would give rise formed products. However, the specific mechanism is still unknown.

3.2 Evaluation of antifungal activity of the p-chloroaniline and 4,4'-Biphenyldiamine.

In order to determine the antifungal activity against *B. cinerea* of the compound formed, the effect on mycelial growth at different concentrations was evaluated in a solid medium and the IC_{50}value of *p*-chloroaniline and 4,4'-Biphenyldiamine were calculated (Table 2).

Table 2. Effect of *p*-chloroaniline and 4,4'-Biphenyldiamine on the mycelial growth of *B. cinerea* in solid medium.

Compound	IC_{50} (mM)
***p*-chloroaniline**	1.72 ± 0.11
4,4'-Biphenyldiamine	1.50 ± 0.19
Iprodione	0.0148

4,4'-Biphenyldiamine presented an IC_{50} value similar to that of p-chloroaniline and both compounds showed a much lower antifungal activity than the commercial fungicide, iprodione[32].In contrast, it has been described that some dimeric polyphenols are more toxic than monomers. For example resveratrol, a stilbene found in grape, wine and grape pomace show low antifungal activity against *B. cinerea*[33,34]. However, a specific laccase of this fungus converted resveratrol into a more fungitoxic dimer that caused it self-intoxication[35]. Thus, the utilization of the homomolecular dimeric compound to inhibit the hyphal growth of *B. cinerea* will be limited.

3.3 Evaluation of antioxidant activity from p-chloroaniline and 4,4'-Biphenyldiamine.

The antioxidant activity from both compounds using a ORAC-PGR method was performed. The Figure 2 shows the PGR oxidation and the antioxidant effect of 4,4'-Biphenyldiamine and *p*-chloroaniline. This result suggests that the antioxidant capacity of *p*-choloaniline was slightly increased by the dimerization. A possible explanation to this phenomenon is that in 4,4'-Biphenyldiamine structure there are two linked-phenyl groups in position *para* in relation to amine group producing a greater stability of radicals. However, the antioxidant ability of 4,4'-Biphenyldiamine was lesser than the antioxidant Trolox

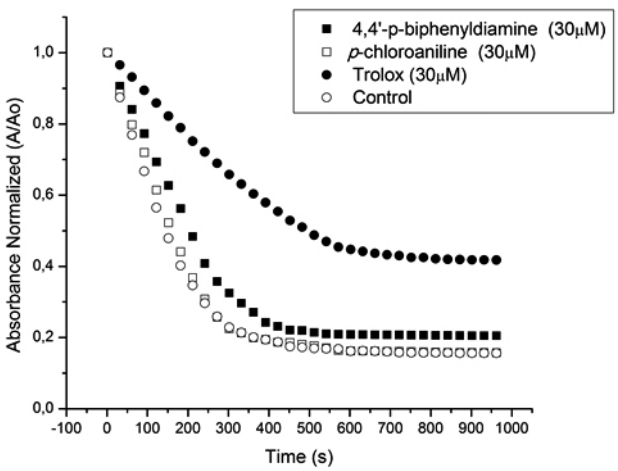

Figure 2. Antioxidant activity of *p*-chloroaniline and 4,4'-Biphenyldiamine obtained by ORAC-PGR method.

4. CONCLUSIONS

In conclusion, it was shown that *p*-chloroaniline can be used as substrate by the *T. versicolor* lacasse and 4,4'-Biphenyldiamine was obtained as product by a coupling reaction. The synthesis of this compound in a lacasse catalyzed reaction has not been reported previously. This compound presented a low antifungal activity; however, the antioxidant ability was slightly increased by the dimerization of *p*-chloroaniline.

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