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Direct Spectrophotometric Assay of Laccase Using Diazo Derivatives of Guaiacol

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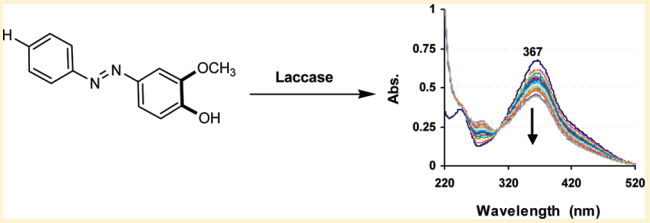
ABSTRACT: Laccase (EC 1.10.3.2) is a widespread cuproenzyme able to oxidize various types of phenols and similar aromatic compounds through a one-electron transfer mechanism. The enzyme has already found its way into the market as a biocatalyst. Because of its ability to be paired by electron mediators, the expectation for employing laccases in versatile processes is very high. There are a few spectrophotometric methods for assaying the laccase activity; however, all of them are based on the formation of product(s) resulting from the enzymatic and inevitable succeeding chemical reactions. Use of diazo derivatives of guaiacol (DdG) was developed as a new spectrophotometric method based on substrate depletion allowing direct assessment of enzyme activity has been introduced. This method allows accurate comprehensive kinetic studies of laccases and provides reliable information about the quality of docking of different substrates or one substrate to the active sites of different laccases. Using this method, the kinetic parameters of various DdG carrying different electron donating and withdrawing substituents were used to assay laccase from *Neurospora crassa*. 2-Methoxy-4-[(4-phenyl)azo]-phenol (K_m = 93.5 μM and V = 1.98 μM/min) was identified as an appropriate substrate for the accurate and routine spectrophotometric based assay of laccases.



Laccases (EC 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) are widespread glycoproteins that catalyze the oxidation of various phenolic compounds with the concomitant reduction of molecular oxygen to water. Although the mechanism of their catalysis is not well understood, they are categorized under the blue multicopper oxidase family.[1] Most of the fungal laccases are monomeric, but enzymes consisting of several subunits are also known.[2]

In comparison with the other well-known oxidases, laccase has gained a special place in the current topics of biotechnology as it is proving to be a more promising enzyme for use in oxidation of aromatic pollutants.[3] For bioremediation purposes, tyrosinase is limited to the phenols that allow ortho-hydroxylation. Peroxidase requires hydrogen peroxide to oxidize phenolic compounds, but laccase functions in the presence of molecular oxygen and possesses a broader range of specificity for aromatic compounds converting them into reactive species that react with other molecules in the medium.[3,4] In addition to the detoxification of water and bioremediation of waste waters, other potential applications of laccases include biodegradation of lignins, biobleaching of dyes, pharmaceutical synthesis, controlling xenobiotics, and fabrication of biosensors.[5−9] The oxidative reactions of laccases and tyrosinases can also be employed for cross-linking of biopolymers which has found applications in green organic synthesis and food industries.[10−13]

A survey of the literature revealed that there are a few spectrophotometric methods for measuring laccase activity.[14] Spectrophotometric methods have advantages but factors such as rapid inactivation in the presence of substrates, instability of the products, low extinction coefficients of the substrates or products, and interference of the intermediates or the products absorption with substrates severely limits accuracy and precision of the spectrophotometric methods. Review of the existing spectrophotometric assay methods for laccases revealed that few of them can be used for precise and accurate kinetic analysis mainly because of the indirect nature of these methods. Here, we propose the use of diazo derivatives of guaiacol (DdG) as a new spectrophotometric method for the direct assay of laccases.

MATERIALS AND METHODS

Diazo derivatives of guaiacol (DdG) including 2-methoxy-4-[(4-methoxybenzo)azo]-phenol (Anis-G), 2-methoxy-4-[(4-methylphenyl)azo]-phenol (Tol-G), 2-methoxy-4-[(4-phenyl)azo]-phenol (Anil-G), 2-methoxy-4-[(4-sulfonamido)azo]-phenol (Sul-G), and 2-methoxy-4-[(4-chlorophenyl)azo]-phenol (Cph-G) were synthesized and purified.[15] Syringaldazine and ABTS were obtained from Sigma-Aldrich Co., and guaiacol was from Fluka Chemie GmbH. Commercial laccase and mushroom tyrosinase (MT) was purchased from Sigma-Aldrich Co. and used without further purification. Double distilled water was used for preparing buffers, mainly phosphate buffer solution (PBS). Spectrophotometric measurements were carried out using a UV/Visible

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spectrophotometer (Super Aquarius CECIL 9500 England) equipped with a water circulating temperature controlling accessory. The results were analyzed using the Sigmaplot (version 9.01) and Microsoft Office Excel. Extinction coefficients of the selected substrates were obtained at their λ_{max}(s) in PBS at pH 7 ± 0.1 and 20 °C.

***Neurospora crassa* (NC) and Growth Conditions.** The wild-type strain of NC (FGSC #321) was obtained from Fungal Genetic Stock Center (Kansas City, USA). Stocks were maintained on agar slants. To propagate the strain, 20 μL of a conidial suspension (absorbance at 600 nm ≈ 0.1) of FGSC #321 was added to 20 mL of Vogel minimal medium (2% sucrose) in a 100 mL Erlenmeyer flask and incubated for 4.5 to 5 days at 30 °C without shaking.[16] To increase the laccase production, the proliferated species was kept under the same conditions of the incubation for another 6 days at 35 °C.

Purification of Extracellular Laccase. The culture fluid was separated from the mycelia by conventional paper filtration and centrifuged (10 000*g*) for 30 min at room temperature. The filtrate was concentrated (≈20-fold) in an Amicon Stirred Ultrafiltration Cell using a 10 kDa membrane (Millipore). The concentrate was frozen at −70 °C and then lyophilized. The crude powder of the enzyme was further purified through the method described by Froehner and Eriksson.[1]

Preparation of the Enzyme and Substrates Solutions. The desired amounts of the enzyme were taken from the freshly prepared stock solutions of the laccases (5 mg/mL). To prepare stock solutions, the enzyme samples were weighed using a Mettler AG285 balance with a readability of 0.01 mg and dissolved in PBS (0.01 M, pH 7). All the working solutions of the laccases were maintained at 4 °C throughout the procedure. Stock solutions of ABTS and guaiacol (2 mM) were made in water. Syringaldazine stock solution was made by dissolving 2 mg of the compound in 10 mL of ethanol (96%). Due to the low solubility of the DdG in water, stock solutions of these compounds were made in 2-propanol. Each of these substances was diluted with PBS (0.01M, pH 7) to prepare the desired concentration. The stability of the prepared substrate solutions was examined by monitoring the UV−vis spectrum of each for 60 min at 20 °C. In all cases, no changes in the absorption spectra of the substrate solutions, due to precipitation or autoxidation, were observed.

To determine the impact of the cosolvent on the laccase activity, the enzymatic reaction was conducted in the media containing various amounts of 2-propanol. Results showed that the rate of the laccase reaction remained unchanged even in medium containing 15% 2-propanol. The amount of the cosolvent did not exceed 7% of the reaction mixture in these experiments.

Optimum pH and Temperature for the NC Laccase. To determine the optimum pH for the oxidase activity of the NC laccase, the enzymatic reactions were carried out in the presence of a constant amount of the enzyme (20 μL) and syringaldazine (20 μM) in three different buffer systems: PBS (0.01 M, pH 3.0−10.0), tris (0.01 M, pH 5.0−8.0), and acetate (0.01 M, pH 5.0−8.0) at 20 °C. All the reactions were carried out in a conventional quartz UV−vis cell containing 1 mL of the reaction mixture. Similar enzymatic reactions in PBS (0.01M, pH 7) were carried out at various temperatures ranging between 5 and 90 °C; therefore, 980 μL of PBS containing syringaldazine (20 μM) was added to a UV cell thermostatted at the desired temperature. The enzyme solution (20 μL) was then added to the cell, and the reaction was followed by monitoring the increase in the OD at

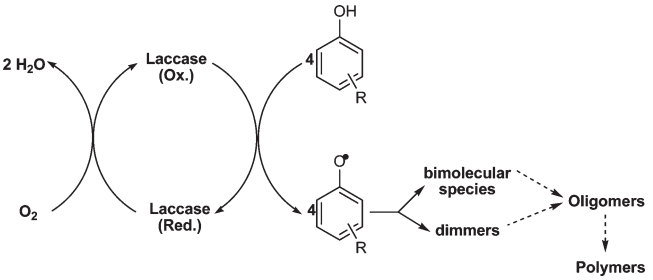

Figure 1. Laccase reactions from substrates to products.

λ_{max} = 530 nm for 5 min. The rates of the reactions were obtained from the corresponding velocity curves (absorbance vs time). The Lineweaver−Burk analyses of these results are illustrated in Figure 4C,D.

Velocity Curves. Velocity curves of the laccase activity were obtained from the reactions carried out at 20 °C in PBS (0.01 M, pH 7) containing a constant amount of the enzyme (20 μL) and at least 5 different concentrations of the substrates in a range of 10 to 100 μM. All data presented are the average of at least 5 repeated experiments. Michaelis−Menten constants for the substrates were calculated from the corresponding Lineweaver−Burk plots of the data.

RESULTS AND DISCUSSION

Current Assay Methods of Laccases. Laccase active site contains four copper ions in a special arrangement that allows the ions to play appropriate roles during the oxidation of the organic substrate by molecular oxygen. It is known that the first copper ion, a paramagnetic type I copper (designated T1), is distanced about 12.5 A from the other three and is accessible for the organic substrate.[17] Upon docking a phenolic or similar molecule onto the active site, this T1 accepts one electron from the organic substrate. The electron is transferred to the trinuclear cluster through a chain of residues where the reduction of molecular oxygen to water happens.[18] The details of formation of two water molecules in the active site of laccase are still ambiguous. Nonetheless, it is clear that four electrons, one by one, are absorbed from the organic substrates by T1 and transferred to the trinuclear cluster. Consequently, the organic radicals produced participate in intra/intermolecular reactions to form stable molecules, Figure 1.

It is possible to use different techniques to assay laccase activity; however, oxymetric and spectrophotometric methods are more desirable due to the resulting information. Oxymetry provides information regarding the consumption of the first substrate of laccase, O_2, during the enzymatic reaction, but the method is not specific to laccase and so cannot be used for detecting laccase activity.[19] In contrast, using a proper specific organic substrate, it should be possible to detect and follow laccase activity spectrophotometerically.

A literature survey revealed several different organic compounds have been used for assaying laccase activity. Except ABTS, Figure 2F, which does not appear to be capable of participating in an inner sphere electron transfer with T1 due to the lack of functionality for docking into the T1 coordination center, the other organic compounds depicted in Figure 2 can be considered as the suitable substrates for laccase. Interestingly, all of these compounds except DdG show an increase in the optical

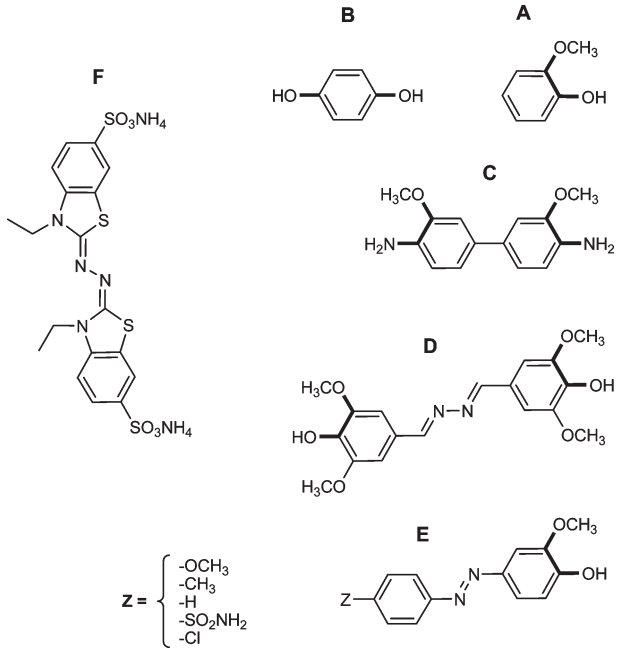

Figure 2. Chemical structures of some of the laccase substrates.

density at their λ_{max}(s) while being oxidized because the absorption of the product(s) overlap with the substrate spectrum at that region. As a result, laccase activity cannot be assayed through the depletion of the substrate, rather it can be monitored at the λ_{max} of the enzymatic reaction product(s). Figure 3B shows the spectra overlaid as recorded during the oxidation of guaiacol by NC laccase.

Drawbacks of the Existing Spectrophotometric Methods. There is a problem associated with the product(s) of the laccase reaction from an analytical point of view. As mentioned earlier, the real product of the laccase reaction with an aromatic compound is a radical.[4] As illustrated in Figure 1, this reactive molecule can attack another substrate molecule and forms a homomolecular dimer. It can attack another existing species in the medium and produces a hybrid dimer or looses another electron and forms a quinone. If any of these happen, there is no guarantee that the reaction terminates at that point. A radical reaction might continue to form oligomers or even proceed to produce a polymer.[20] In addition, an organic radical from the phenol family does not necessarily have only one site for the reaction. A phenolic radical can use its oxygen terminal or one of its carbons forming C−O or C−C bonds.[21] Although the details of these possibilities have been discussed in the literature from a synthetic perspective,[22] it is important to see the impact of these phenomena on the accuracy and precision of the selected assaying method.

If the product of the enzymatic reaction is involved in some chemical reactions and produces more than one product, following the enzymatic reaction at the λ_{max} of the product(s) will be an indirect observation. For instance, the λ_{max} of guaiacol is at 275 nm, but its reaction with laccase is monitored at 450 nm (Figure 3B) which belongs to a mixture of dimer and trimer products.[22,23] Some of the phenolic radicals involved in chain reactions proceed alongside the laccase reaction. A notorious example of this type is syringaldazine. As it is seen in Figure 3A, the reaction of NC laccase with syringaldazine has lost its linearity after a while so that the normal shape of the kinetic curve has been disturbed. Therefore, despite the fact that syringaldazine is a good substrate for detecting laccase activity, it does not seem to be a suitable substrate for kinetic studies.

Direct Assay of Laccase. For enzymes like laccase, which produce unstable product(s), following substrate depletion instead of product formation would result in direct assay of the activity provided the spectrophotometric behavior of the substrate is collaborating. An example of this application is tyrosinase. The product of the enzymatic reaction of tyrosinase is an *o*-quinone which starts chemical reactions right after its formation due to high instability.[24] Traditionally, the cresolase and catecholase activities of tyrosinase are assayed in the presence of tyrosine and L-DOPA, respectively, through formation of dopachrome which was formed by further chemical reactions of the product of the enzymatic reactions.[25] Introducing some synthetic diazo derivatives of phenol and catechol for the cresolase and catecholase reactions, respectively, made it possible to follow the tyrosinase activities directly through depletion of the substrates.[26] Therefore, some DdG with the general structure of "E" in Figure 2 were synthesized, and their reactions with NC laccase were studied thoroughly.

Diazo Derivatives of Guaiacol as Laccase Substrates. Since the medium can affect the electrochemistry of laccase, the activity of NC laccase was first studied at various pH(s) in three different well-known buffer systems. According to the results illustrated in Figure 4A, the pH for the optimum activity of NC laccase is 6, 7, and 8 in Tris Base, PBS, and acetate buffer, respectively. However, the enzyme shows its maximum activity in PBS at pH 7. Temperature studies (Figure 4B) also revealed that NC laccase reached its highest activity at 55 °C, yet it was preferred to run all the enzymatic reactions in PBS (pH 7) at 20 °C to keep the assay conditions simple.

The synthesis of DdG completes by a one step coupling reaction,[15] so different derivatives carrying various electron donating and withdrawing substituents were synthesized. The reaction of each compound with NC laccase was studied spectrophotometrically. Results are presented in Figure 3C−G. The spectra in these figures clearly demonstrate two facts. First, all of the DdG examined show optical density decreases at their related λ_{max}(s) during oxidation by NC laccase. Second, the isosabestic points can be found distant from the λ_{max}(s) of the substrates. This means that the oxidation products of these compounds generate absorbance bands out of the λ_{max} regions of the substrates; hence, the enzymatic reactions of these dyes with laccase can be followed through the decrease in their λ_{max}(s) with little interference of the products.

Regarding the spectrophotometric behavior of the examined dyes, there are a few more important elements. If a spectrophotometric method is going to be based on the decrease in the absorbance of the substrate, there is no doubt that the higher the extinction coefficient, the more sensitive is the assay method. Comparing the extinction coefficients of these compounds (Table 1), it is clear that Anis-G has the highest ε. The isosbestic point of Anis-G (Figure 3C) is the nearest (38 nm) to the substrate λ_{max}, while the Anil-G one (Figure 3E) is the farthest (57 nm). In addition, Anil-G has the sharpest peak at its λ_{max} in comparison with the other examined guaiacol dyes. These two features enhance the precision of the assay method. Therefore, despite the lower extinction coefficient of Anil-G in regard with Anis-G, it is preferred to select Anil-G for a more reliable spectrophotometric assay.

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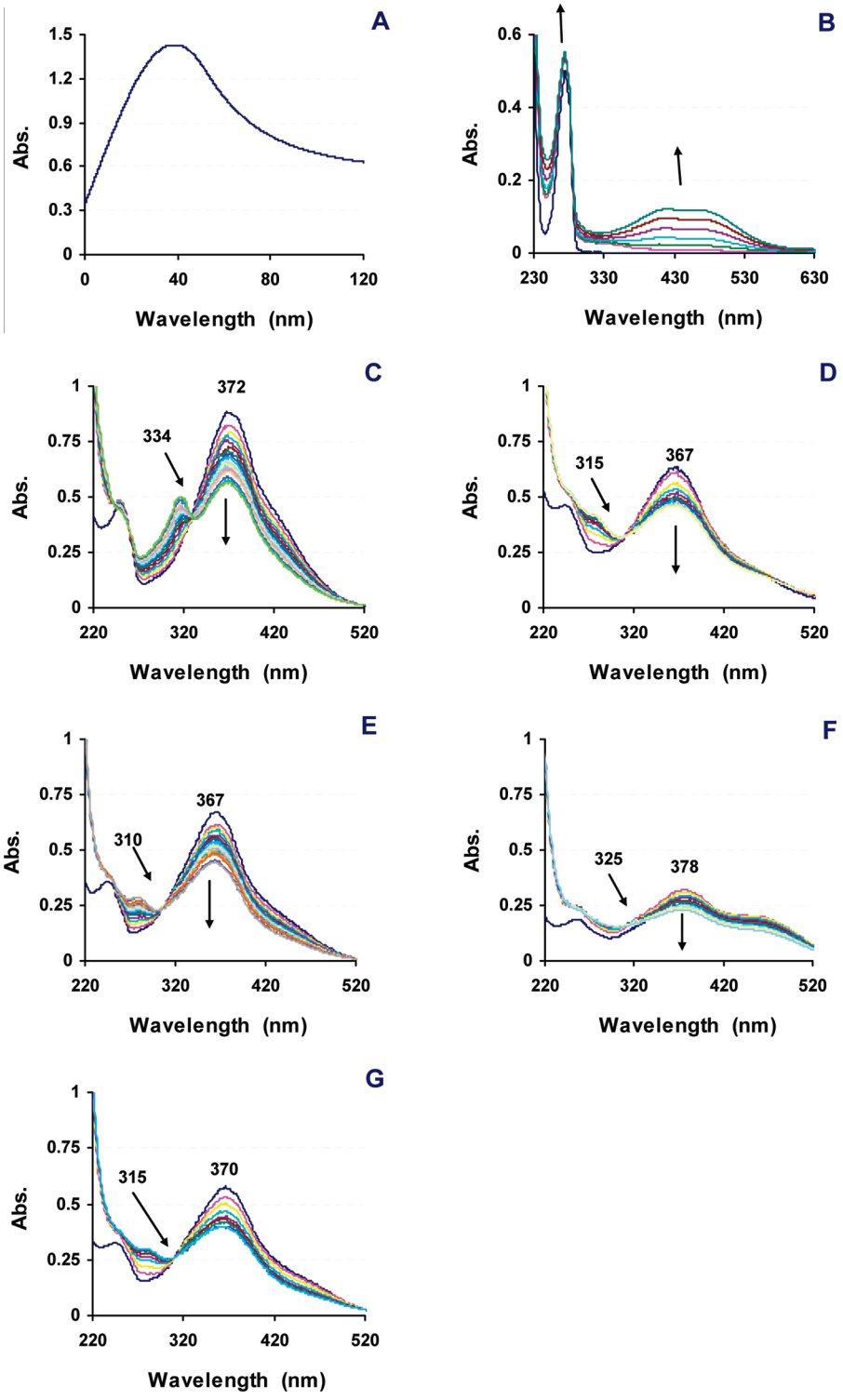

Figure 3. (A) Velocity curve of the enzymatic oxidation of syringaldazine by NC laccases in PBS (0.01 M, pH 7) at 20 °C. Overlaid spectra recorded during the enzymatic oxidation of (B) guaiacol, (C) Anis-G, (D) Tol-G, (E) Anil-G, (F) Sul-G, and (G) Cph-G by NC laccase in PBS (0.01 M, pH 7) at 20 °C.

Diazotization extends the conjugation of the guaiacol to a second aromatic ring and increases the number of the functional groups. Consequently, the new compounds might be susceptible to oxidation by some other oxidases. For instance, indole-3-acetic acid carries no hydroxyl functional group, but it has been shown that peroxidase is able to oxidize this molecule even in the absence of H_2O_2.[27] Therefore, the reactions of DdG with hydrogen peroxide were examined in the absence and presence of peroxidase. Experiments proved that H_2O_2 had no effect on the dyes in the absence of peroxidase but adding peroxidase into the reaction mixture initiated rapid oxidations producing overlaid spectra similar to those illustrated in Figure 3C−G.

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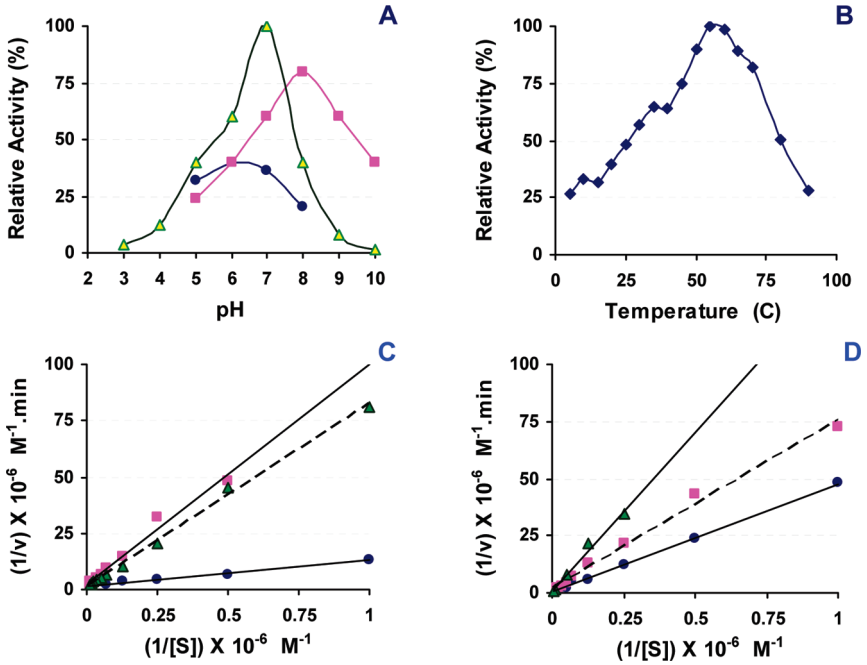

Figure 4. Relative rates of the syringaldazine oxidation by NC laccase in (A) PBS 0.01 M, pH 3.0−10.0 (▲), Tris Base 0.01 M, pH 5.0−8.0 (●), and acetate 0.01 M, pH 5.0−8.0 (■) at 20 °C; (B) PBS (0.01 M, pH 7) at various temperatures. Lineweaver−Burk analysis of the kinetic studies on the laccases activity of NC laccase in the presence of various concentrations (10−100 mM) of (C) Anis-G (●), Tol-G (■), and Cph-G (▲) and (D) Anil-G (●), Sul-G (■), and guaiacol (▲) of guaiacol in PBS (0.01 M, pH 7) at 20 °C.

Table 1. Extinction Coefficients, Kinetic Rates, and Michaelis−Menten Parameters of DdG Oxidation by NC Laccase Compared with Guaiacol

substrate	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)[a]	dA/min	K_m (μM)	V_{max} (μM min^{-1})	specificity constant
Guaiacol	275	4717	0.006	139	1	0.0072
Anis-G	372	39 599	0.024	11.5	1	0.087
Tol-G	367	23 270	0.012	49	0.5	0.01
Anil-G	367	28 770	0.012	93.5	1.98	0.021
Sul-G	378	13 050	0.0042	36	0.5	0.014
Cph-G	370	24 380	0.006	81	1	0.012

Another widespread oxidase in nature is tyrosinase. The reactions of DdG with MT were also examined. There was no reaction between the guaiacol derivatives and MT. DdG were also employed in the inhibition studies on the MT reactions. Interestingly, these compounds had no inhibitory or activatory effect on MT (data is not shown). Apparently, the methoxy group at ortho position to the phenolic terminal causes a steric hindrance that hampers the guaiacol derivatives from entering into the MT active site.

Kinetic Parameters of DdG. The main advantage of a direct assay method originates from the fact that it allows accurate comprehensive kinetic studies of an enzymatic reaction. Such studies usually result in important kinetic constants that can be meaningful from a mechanistic point of view and also compare fundamental differences in the behavior of several isozymes. The kinetic parameters of each diazo substrate (Table 1) were extracted from the corresponding Lineweaver−Burk plots of the kinetic studies shown in Figure 4C,D. Comparing the K_m values of the DdG with the K_m value of guaiacol indicates that NC laccase has a higher affinity for the diazo derivatives. This could be a result of the extended conjugation in the dye molecules which stabilize the transition state of the enzymatic reaction.

From the rate data (dA/min), it appears that the electron donating groups increase the instant rate of the reaction while the electron withdrawing groups work in the opposite. Nonetheless, the limiting rate (V) for the enzymatic reactions of all the examined DdG are close together. In other words, despite the fact that an electron donating group facilitates the instant electron transfer to the copper T1, the limiting rates for all the DdG compounds carrying various types of substituents do not seem very different. This could be a result of the similarity in the nature of all these reactions as all of them proceed through an inner sphere electron transfer and the small discrepancies in the V values could be derived from the different affinity of copper T1 for different DdG. The effect of the substituents is also observed on the K_m values. Anil-G, which carries no substituents, shows the highest K_m value, but the other dye molecules carry a substituent which is able to participate in extending the resonance of the radical electron having smaller K_m values. Increasing the stability of the radical through resonance is assumingly the main reason for the observed discrepancy in the K_m values, yet the positive effect of the electron donating groups (induction effect) on the electron transfer process has to be considered. This evidence supports the radical nature of the activated complex of laccases substrate at the transition state.

■ CONCLUSION

DdG compounds can be used as substrate for direct spectrophotometric kinetic studies of various laccases. Comparing the kinetic parameters of these compounds obtained from their reactions with NC laccase, Anil-G seems to be a reliable substrate

for the routine assay and detection of laccases. This compound is easily synthesized, and its stock solution can be prepared in 2-propanol which can be used for making the assay solutions in a range of 1 to 100 μM without showing any sign of precipitation or auto oxidation. In this concentration range, Anil-G shows linear spectrophotometric responses at 367 nm during oxidation by laccase.

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