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Lag time spectrophotometric assay for laccase kinetics

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Lag time spectrophotometric assay for laccase kinetics

Abstract:

Kinetics of industrially important laccases is commonly determined by activity assays of 2,6-dimethoxyphenol (DMP), a widely used model substrate. DMP is oxidized in multiple steps with rate-limiting step of non-enzymatic radicals' formation that affects assay accuracy. This thesis comprehensively analyzes DMP oxidation and develops a more reliable lag time spectrophotometric assay for laccase kinetics.

Keywords:

Lag time, spectrophotometry, laccase, kinetics

CERCS: P310 Proteins, enzymology

Viiteajal põhinev meetod lakaaside kineetika määramiseks

Lühikokkuvõte:

Tööstuslikult oluliste lakaaside kineetika on tavapäraselt määratud 2,6-dimetoksüfenooli (DMP), laialdaselt kasutatava mudelsubstraadi, aktiivsuse analüüsiga. DMP on oksüdeeritud mitmes etapis kiirust limiteeriva mitteensümaatilise vaheradikaali vormistamisega, mis mõjutab ensüümi analüüsi täpsust. See väitekiri põhjalikult analüüsib DMPi oksüdeerumist ja arendab usaldusväärsema viiteajal põhineva meetodi lakaasi kineetika määramiseks.

Võtmesõnad:

Viiteaeg, spektrofotomeetria, lakaas, kineetika

CERCS: P310 Proteiinid, ensümoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ABTS 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

AscA L-ascorbic acid

CL coerulignone

DMP 2,6-dimethoxyphenol, syringol

E° redox potential

HCL hydrocoerulignone

$\mathbf{S_{(red)}}$ reduced substrate

$\mathbf{S_{(ox)}}$ oxidized substrate

SVLAC *Streptomyces viridosporu*s laccase

T1 type 1 copper ion

T2 type 2 copper ion

T3 type 3 copper ion

τ lag time

INTRODUCTION

Widely distributed across all kingdoms of life, laccases are the largest subclass of multicopper oxidases, capable of oxidation of a wide range of substrates in an environmentally friendly way (Yoshida, H., 1893; Bertrand, G., 1896; Yamazaki, H.I., 1969; Givaudan, A. et al., 1993; Laufer, Z., 2006; National Center for Biotechnology Information, 2022). Laccases have dual physiological functions to catalyze polymerization and depolymerization processes (Ranocha, A. et al., 2002; Eggert, C. et al., 1996). Due to synthetic and degradative ability, broad substrate specificity and eco-friendliness, laccases have become important industrially relevant enzymes. Laccases have a wide range of applications in environmental industry such as waste detoxification (Kalčíková, G. et al., 2014) and textile dye decolorization (Blánquez, A. et al., 2019), in the food industry such as beverage processing (Cantarelli, C. et al., 1989) as well as in the pulp and paper industry as delignification of lignocellulosic material and lignin biorefinery (Call H.P., 1993). Bacterial laccases have attracted attention because of their notable advantages over other laccases, including wide pH range, high thermostability, and tolerance to alkaline environments (Margot, J. et al., 2013). Moreover, bacterial laccases are advantageous because of the ease to be cloned and expressed in the host with suitable manipulation (Dubé, E., 2008). The most extensively studied bacterial laccases are from actinomycetes, particularly *Streptomyces* species because of their reported efficiency in lignin degradation, established genetic and molecular engineering tools and the availability of complete genome sequences (Crawford D. L., 1978; Bekker, V., 2014; Lee, N., 2020). Widely used laccase model substrate 2,6-dimethoxyphenol (DMP) is oxidized in multiple steps with formation of intermediate radicals that affect assay accuracy (Breslmayr, E. et al., 2018). This thesis is aimed to comprehensively analyze DMP oxidation and develop a more reliable DMP-based assay for laccases.

1 LITERATURE REVIEW

1.1 *Streptomyces viridosporus* laccase

Streptomyces viridosporus laccase (SVLAC) originates from a type of non-pathogenic actinobacteria that produces extracellular enzymes that degrade lignin, a class of complex organic polymers that form key structural materials in the support tissues of most plants (Majumdar et al.., 2014; Crawford, D. L., 1983; Payen, A., 1838). SVLAC (EC 1.10.3.2) is a multicopper-containing oxidoreductase that can oxidize a range of aromatic and non-aromatic compounds containing hydroxyl and amine groups in presence of atmospheric oxygen (Kanehisa M., 2017; Majumdar et al.., 2014). Laccases catalyze four-electron oxidation of a reducing substrate with concomitant four-electron reduction of O_2 to H_2O (Nakamura T., 1958). SVLAC (Figure 1) is a small laccase (Majumdar et al., 2014). SVLAC forms trimers with an approximate 3-fold symmetry and head-to-tail organization of the domains (Majumdar et al., 2014). Each SVLAC domain consists of two multicopper oxidase domains that have a β-sandwich architecture (Majumdar et al., 2014). SVLAC contains three types of Cu ions: type 1 (T1), type 2 (T2), and double type 3 (T3) (Majumdar et al., 2014). Each of the three SVLAC active centers is formed by a mononuclear T1 site and a trinuclear T2/T3 cluster (Majumdar et al., 2014).

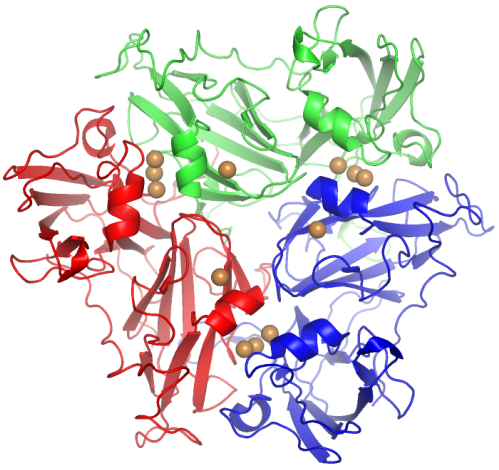

Figure 1. The structure of *Streptomyces viridosporus* laccase (SVLAC): trimers with 3-fold symmetry and head-to-tail organization of the domains (red, green, blue). Each of the three SVLAC active centers is formed by a mononuclear T1 site and a trinuclear T2/T3 cluster of copper ions (copper). Uniprot entry 3TBB (Majumdar et al., 2014) was visualized by using PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)

Laccase catalysis (Figure 2) involves three main steps: reduced substrate oxidation at T1, internal electron transfer from T1 to T3/T2 and O_2 reduction to H_2O at T3/T2. Four substrates are consecutively oxidized at T1, which is the primary acceptor of electrons. Three primary accepted electrons are transferred from T1 to T2/T3, which are the secondary acceptors of electrons. One electron attachment to all four Cu ions results in a reduced laccase that is able to reduce O_2 to H_2O at T3/T2 (Messerschmidt, 1993; Solomon et al., 1992; Thurston, 1994; Yaropolov et al., 1994).

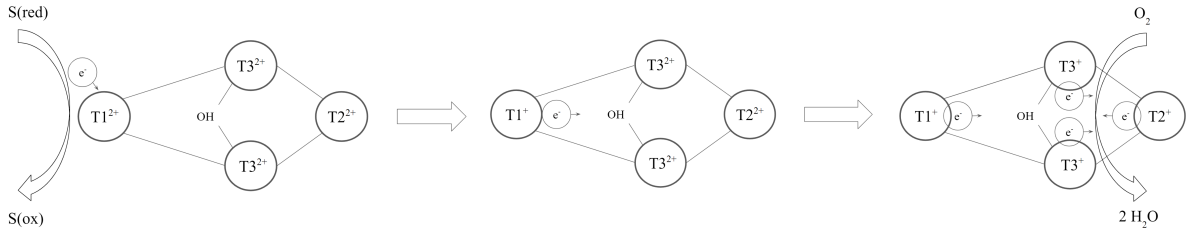

Figure 2. Laccase catalysis steps (from left to right): reduced substrate ($S_{(red)}$) oxidation ($S_{(ox)}$) at T1, internal electron transfer from T1 to T3/T2, O_2 reduction to H_2O at T3/T2 (Google Slides).

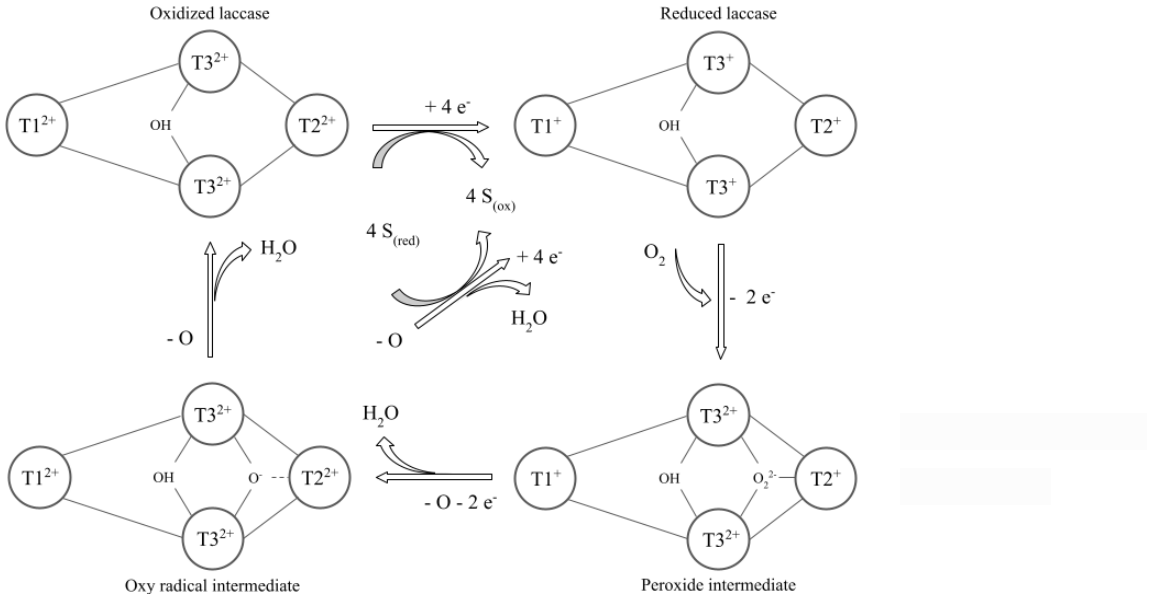

Figure 3. The catalytic cycle of laccase: oxidized laccase is reduced by attaching four electrons to Cu ions from four reduced substrates ($S_{(red)}$) that become oxidized ($S_{(ox)}$); reduced laccase is converted to peroxide intermediate by binding O_2 and transferring two electrons; the peroxide intermediate is converted to oxy radical intermediate by transferring two electrons to oxygen with the release of the first H_2O. In the presence of reducing substrates, the oxy radical intermediate can rapidly perform four substrates' oxidation to form reduced laccase with the second H_2O release. In the absence of reducing substrates, the oxy radical can slowly convert to oxidized laccase with the second H_2O release (Google Slides).

Oxygen reduction is performed by the formation of bound oxygen intermediates (Figure 3). Following laccase reduction, O_2 binding to the T2/T3 leads to two electrons' rapid transfer from the T3s to form peroxide intermediate. Subsequent rapid one electron transfer from T1 and slow one electron transfer from T2 forms an oxy radical intermediate by cleavage of O–O bond with the release of the first H_2O. (Palmer, A. E. et al., 2001; Lee, S., 2002)

In the presence of reducing substrates, the oxy radical intermediate can rapidly perform four substrates' oxidation to form reduced laccase with the second H_2O release. In the absence of

reducing substrates, the oxy radical can slowly convert to oxidized laccase with the second H_2O release. (Shleev, S. et al., 2006)

1.2 Laccase substrates

Substrate specificity of laccases is determined by the conformation and size of the substrate-binding pocket, the binding-site specific amino acid residues, and the redox potential (*E*°) difference between the T1 copper site and the substrate (Gupta, N. et al., 2010; Kallio, J.P. et al., 2011). The *E*° of the T1 copper site affects the substrate oxidation type and rate. Direct oxidation occurs in case of lower *E*° substrates, whereas mediated oxidation occurs in case of higher *E*° substrates (Zimbardi, A. L. et al., 2016). The oxidation rate is affected by the *E*° difference between the T1 copper site and substrate (Zimbardi, A. L. et al., 2016). Laccases oxidize a wide range of substrates that can be divided into phenolic monomers with relatively lower *E*° and non-phenolic ones with relatively higher *E*° (Hilgers R. et al., 2018). Lignin contains both phenolic and non-phenolic subunits (Lundquist, K., and Parkås, J., 2011).

Phenolic substrates of laccases mainly originate from lignin phenolic units (Figure 4) that are differentiated by the number of methoxyl substituents: p-hydroxyphenylpropane units lack methoxyl substituents, guaiacylpropane units have one methoxy group and syringylpropane units have two methoxy groups (Wacek, A.V., & Kratzl, K., 1948). Guaiacol, DMP and syringaldehyde (Figure 4) are commonly used phenolic substrates in laccase reactions (Niladevi, K. N., 2008).

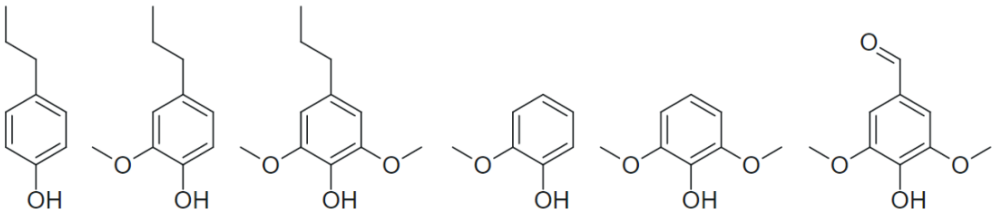

Figure 4. (from left to right) Phenolic substrates of laccases originate from lignin phenolic units, differentiated by the number of methoxy substituents: p-hydroxyphenylpropane, guaiacylpropane, syringylpropane. The most commonly used laccase phenolic substrates are guaiacol, 2,6-dimethoxyphenol, syringaldehyde (Marvin JS).

Non-phenolic substrates of laccases mainly originate from lignin anisylpropane units (Figure 5) that are differentiated by the number of methoxyl substituents: anisyl propane units have one methoxyl substituent, veratryl propane units have two methoxy groups and 1,2,3-trimethoxybenzyl propane units have three methoxy groups (Wacek, A.V., & Kratzl, K., 1948). Anisyl alcohol, 1-(4-methoxyphenyl)ethanol and veratryl alcohol (Figure 5) are

commonly used non-phenolic substrates in laccase/mediator systems (Baiocco, P., et al. 2003).

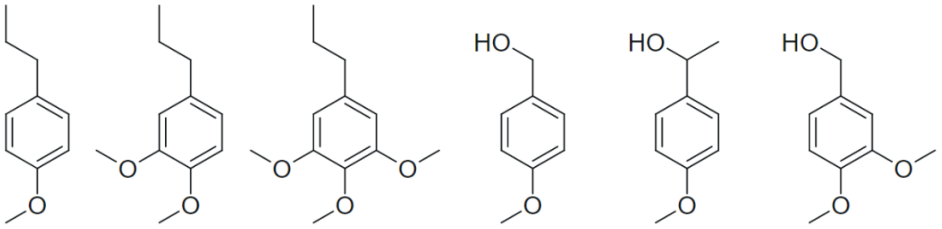

Figure 5. (from left to right) Non-phenolic substrates of laccases originate from lignin anisyl propane units, differentiated by the number of methoxy substituents: anisyl propane, veratryl propane, 1,2,3-trimethoxybenzyl propane. The most commonly used laccase non-phenolic substrates are anisyl alcohol, 1-(4-methoxyphenyl)ethanol, and veratryl alcohol (Marvin JS).

1.3 Laccase mediators

Products of laccase reaction can mediate the oxidation of different phenolic and non-phenolic compounds and, thus, extend the substrate specificity of laccases. Laccase catalysis mediation involves two main steps: mediator oxidation by laccase and substrate oxidation by oxidized mediator (Hilgers R. et al., 2018). Mediator oxidation mechanism is performed by the common laccase catalysis mechanism, but substrate oxidation by oxidized mediator can be achieved by three different mechanisms: electron transfer, hydrogen atom transfer and ionic mechanism (Fabbrini M. et al., 2002). In the electron transfer, only electron is transferred to the mediator radical; in the hydrogen atom transfer, besides an electron, a H^+ is transferred to the mediator radical; in the ionic mechanism, H^+ is transferred to the mediator ion (Fabbrini M. et al., 2002; Li K. et al., 1998; Tromp, S.A. et al., 2010).

Laccase mediator applicability is determined by the specificities of enzyme and substrate, stability of intermediates, continuity of cyclic redox conversion, reusability, non-toxicity, efficiency and cost-effectiveness (Li K et al., 1999; Johannes, C., & Majcherczyk, A., 2000; Jurado et al., 2009). The higher efficiency of synthetic mediators makes them commonly used in experimental conditions, but possible enzyme inactivation, toxicity and high cost limit their industrial applications. Naturally occuring mediators' eco-friendliness and low-cost makes them attractive at the industrial scale (González M.D., et al., 2009).

Natural mediators of laccases mainly originate from phenolic units of lignin: phenolic aldehydes, ketones and acids. Natural mediators of laccases (Figure 6) are among the most efficient mediators, including syringaldehyde, acetosyringone, vanillin, acetovanillone,

p-coumaric acid, ferulic acid, sinapic acid (Call H. P., 1994; Paice M. G., et al., 1997; Srebotnik, E., & Hammel, K. E., 2000; Xu, F. et al., 2000).

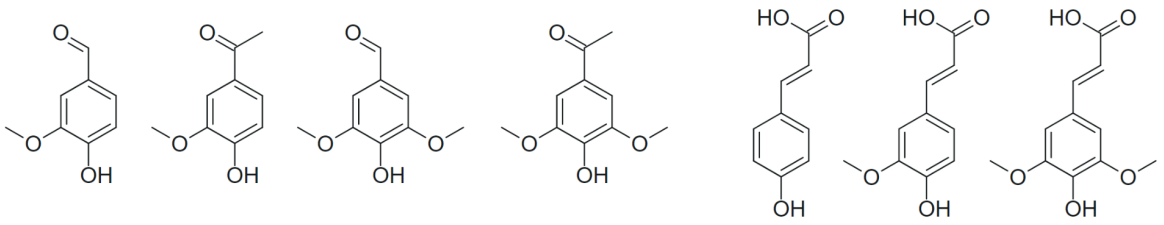

Figure 6. (from left to right) laccase natural mediators are among the most efficient mediators, including syringaldehyde, acetosyringone, vanillin, acetovanillone, p-coumaric acid, ferulic acid, sinapic acid (Marvin JS).

Artificial mediators of laccases (Figure 7) are mainly selected based on the presence of heterocyclic atoms, OH- and/or NH_2 groups and can be divided into >N–OH type, phenothiazine-type, pyrazolone-type and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Shleev, S. V., 2003). ABTS is the most commonly used artificial laccase mediator along with the >N-OH type mediators: the most efficient mediator tetramethylpiperidine N-Oxyl and highly efficient 1-hydroxybenzotriazole, n-hydroxyphthalimide and violuric acid (Fabbrini M. et al., 2002).

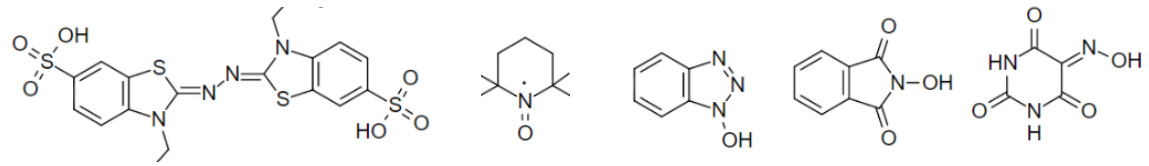

Figure 7. (from left to right) laccase artificial mediators: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), tetramethylpiperidine N-Oxyl and highly efficient 1-hydroxybenzotriazole, n-hydroxyphthalimide, and violuric acid (Marvin JS).

1.4 Laccase inhibitors

Laccase inhibitors can be differentiated by their binding specificity: inhibitors with specific binding to laccases and non-specific binding to many protein classes that result in a reversible or irreversible loss of catalytic activity (Dixon, H.B., & Cornish-Bowden, A., 1983). The most commonly studied laccase-specific inhibitors are halides that inhibit laccases at T2/T3, and non-specific laccase inhibitors are metal ions, reducing agents, chelating agents and chaotropic agents (Kumar, G.N., & Srikumar, K., 2012; Robles, A. et al., 2002). Laccase-specific inhibition depends on the accessibility of T2/T3 by halides that vary between laccases from different microorganisms (Abadulla, E. et al., 2000). Laccase non-specific

inhibition may appear to be inductive at specific conditions. The effect of metal ions can be dependent on concentration: at lower being inductive, at higher inhibitory (Li-yun, D., 2009).

In addition to inhibition, the product formation in the laccase-induced conversion can be hindered indirectly by conversion of intermediate product back to substrate by ascorbic acid (AscA). It is made possible by the ability of AscA to convert (Figure 8) between ascorbate, ascorbate radical and dehydroascorbic acid states (Lima, D.R. et al., 2016).

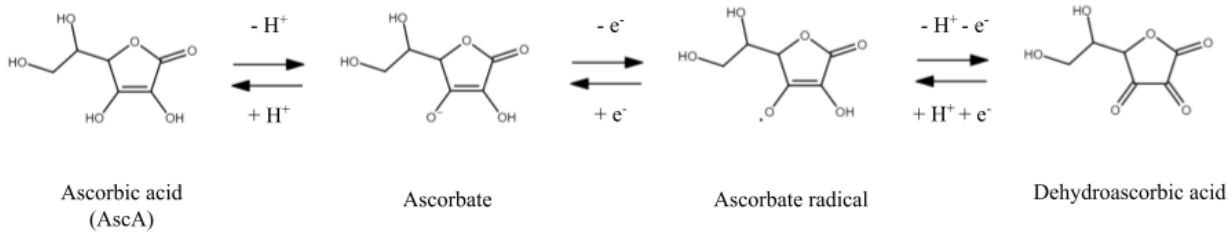

Figure 8. Ascorbic acid (AscA) conversion between ascorbate, ascorbate radical and dehydroascorbic acid states (Marvin JS, Google Slides).

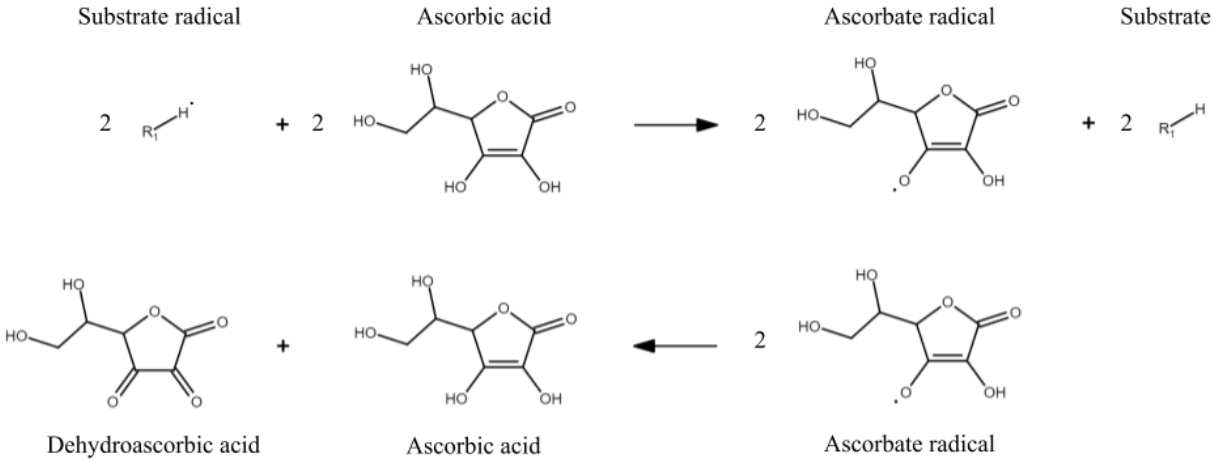

Figure 9. Ascorbic acid (AscA) reaction with a radical formed through laccase activity yields an ascorbate radical and the initial substrate of the laccase reaction. Two ascorbate radicals in turn can react to give one molecule of AscA and one dehydroascorbic acid. The overall stoichiometry of the reaction is one molecule of AscA per two radicals (Marvin JS, Google Slides).

AscA reacts with substrate radical (Figure 9) to form ascorbate radical and substrate, then ascorbate radical is reversely converted to AscA and dehydroascorbic acid that overall keeps substrate unconverted until AscA stays in solution (Gilabert, M. A. et al., 2006).

1.5 Laccase model substrate 2,6-dimethoxyphenol

2,6-dimethoxyphenol (DMP) is a widely used model substrate for oxidative enzymes because of the wide prevalence of dimethoxyphenol oxidase activity in the main ligninolytic enzymes (Solano, F. et al., 2001; Kumar, A., & Chandra, R., 2020). Moreover, the product of DMP

oxidation, coerulignone (CL), is convenient to detect due to a quite high absorption molar coefficient and relative stability (Solano, F. et al., 2001, Breslmayr, E. et al., 2018, Kallio, J. P. et al., 2009).

DMP conversion involves three main steps (Figure 10). In the first step, enzyme catalyzes the oxidation of DMP to the corresponding phenoxy radical. In the second step, the phenoxy radicals non-enzymatically dimerize to hydrocoerulignone (HCL). In the third step, enzyme catalyzes the conversion of HCL to chromogenic CL.

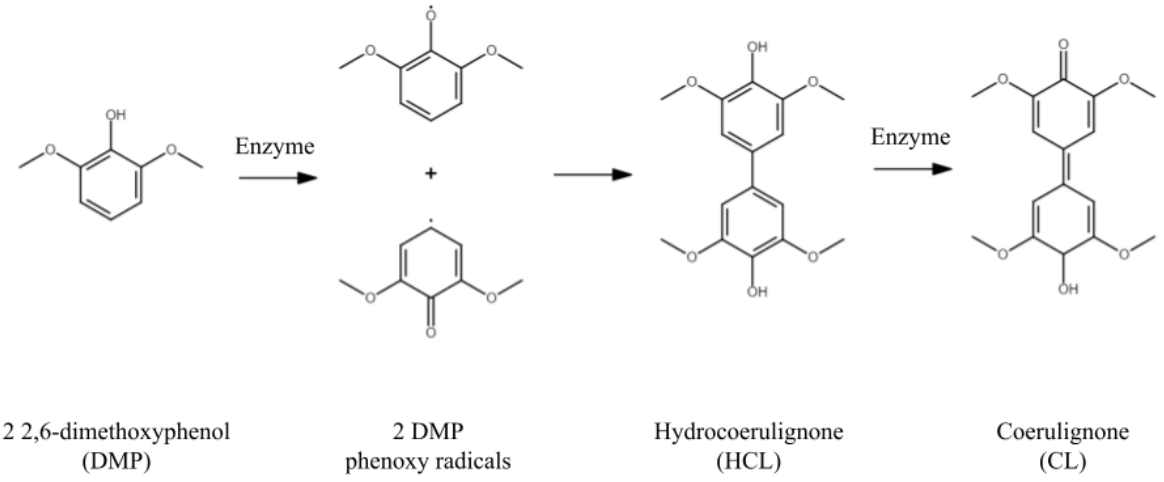

Figure 10. DMP conversion to CL: enzyme catalyzes the oxidation of DMP to the corresponding phenoxy radical; phenoxy radicals non-enzymatically dimerize to hydrocoerulignone (HCL); enzyme catalyzes the conversion of HCL to chromogenic CL (Marvin JS, Google Slides).

Laccase activity is commonly screened with DMP conversion assays (Wan, Y., et al., 2008). In addition to DMP, the reaction intermediate HCL can be used (Breslmayr, E. et al., 2018).

The laccase-catalyzed DMP activity assay (Figure 11) involves all the steps of DMP conversion to CL. The laccase-catalyzed HCL activity assay (Figure 11) involves a single step, where laccase catalyzes the conversion of HCL to chromogenic CL at the expense of O_2 (Breslmayr, E. et al., 2018).

In laccase-catalyzed DMP and HCL activity assays, CL formation is determined by the specific absorption at wavelength of 469 nm using the molar attenuation coefficient 53200 M^{-1} cm^{-1} (Breslmayr, E. et al., 2018).

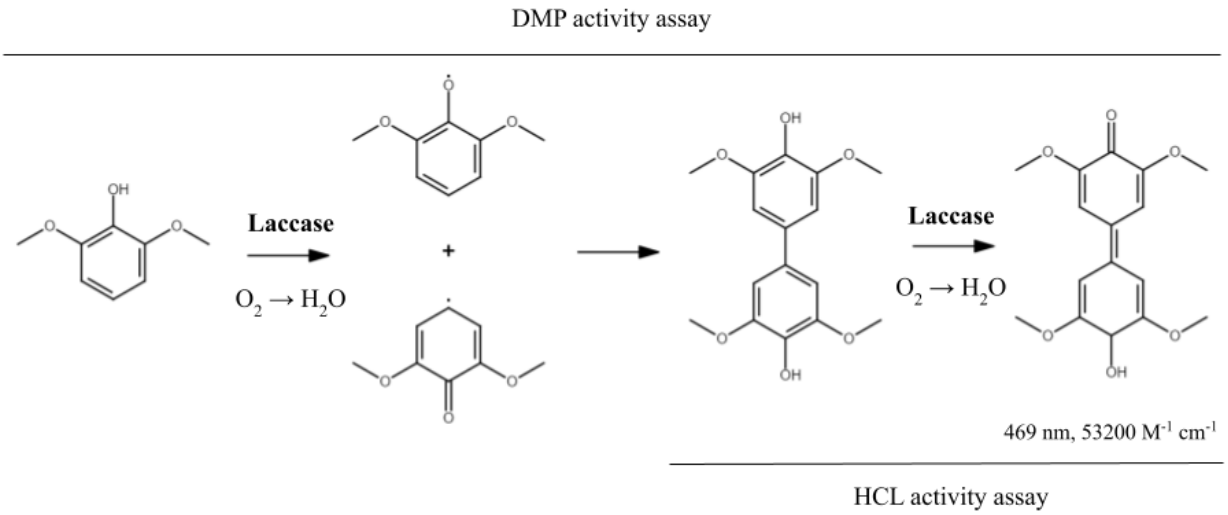

Figure 11. Laccase-catalyzed DMP activity assay involves all steps of DMP conversion to CL: laccase catalyzes the oxidation of DMP to the corresponding phenoxy radical at the expense of O_2, phenoxy radicals dimerize non-enzymatically to HCL, laccase catalyzes the conversion of HCL to chromogenic CL at the expense of O_2. Laccase-catalyzed DMP activity assay involves a single step, where laccase catalyzes the conversion of HCL to chromogenic CL at the expense of O_2 (Marvin JS, Google Slides).

All described steps are challenging to follow spectrophotometrically. The second enzymatic step can be analyzed separately from the rest of the reaction by using HCL as substrate. The final product of the catalyzed reaction is CL regardless whether DMP or HCL was used as substrate. Using HCL instead of DMP as the substrate in the assay increases the sensitivity, however, the lower solubility of HCL can pose a problem. The DMP phenoxy radical dimerization to HCL is a non-enzymatic step and cannot be easily observed directly via spectrophotometry. The first problem of the first enzymatic step is side products (Figure 12) in addition to HCL and CL. The second problem is the reaction of DMP radicals with CL, which decreases the absorbance of the assay. This effect is observed in both laccase and peroxidase assays. (Breslmayr, E. et al., 2018)

DMP radicals can form 2,6-dimethoxy-p-benzoquinone by oxidation; multiple DMP radicals can form oligomers through oxidative couplings: 4-(2,6-dimethoxyphenoxy)-2,6-dimethoxyphenol is formed by C-O dimerization and can be further polymerized; HCL is formed by C-C dimerization and can be further converted to CL; 4-(2,6-dimethoxyphenoxy)2-hydroxy-6-methoxyphenol is formed by intermediate oxidation to 2-hydroxy-6-methoxyphenol and subsequent C-O coupling (Breslmayr, E. et al., 2018, Kallio, J. P. et al., 2009).

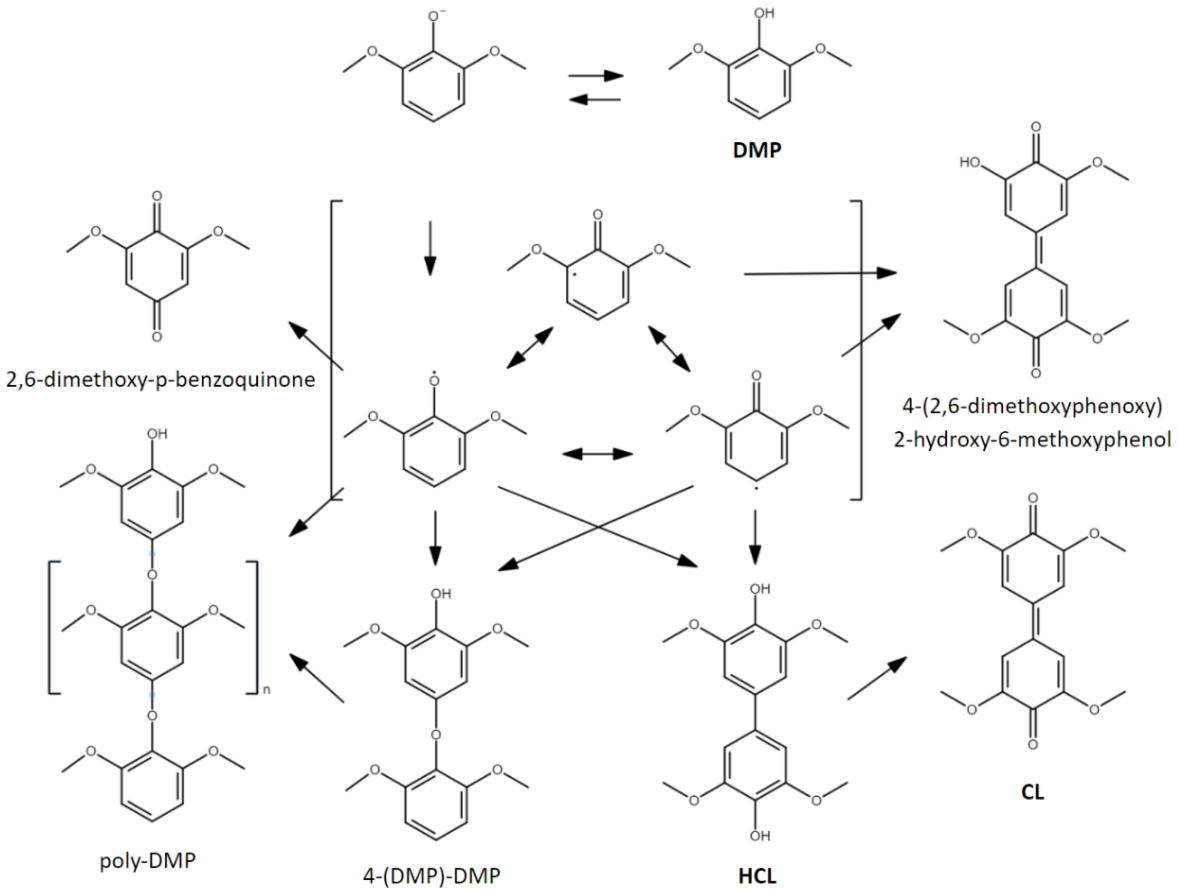

Figure 12. Possible oxidation products of DMP: monomeric 2,6-dimethoxy-p-benzoquinone by oxidation and oligomers through two oxidative couplings: 4-(2,6-dimethoxyphenoxy)-2,6-dimethoxyphenol is formed by C-O dimerization and can be further polymerized; HCL is formed by C-C dimerization and can be further converted to CL; 4-(2,6-dimethoxyphenoxy)-2-hydroxy-6-methoxyphenol is formed by intermediate oxidation to 2-hydroxy-6-methoxyphenol and subsequent C-O coupling (Marvin JS, Google Slides).

The reaction of DMP radicals with CL can reduce the signal registered in the assay. This effect can be reduced by using lower DMP concentrations. It is supposed that a high concentration of the formed DMP radical intermediates is responsible for the subsequent decolorization of CL possibly by a polymerization reaction. (Breslmayr, E. et al., 2018)

2 **AIM OF THE THESIS**

The aim of this thesis is to assess the suitability of the commonly used assay for determining the kinetic properties of laccases and to develop a more reliable method for this purpose by:

1) determining and evaluating kinetic parameters for DMP conversion to CL as a whole and the kinetic parameters for its two enzymatic steps separately;
2) determining the rate-limiting step of laccase catalyzed DMP conversion to CL;
3) determining the prevalence of possible side reactions in DMP and HCL conversion.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Disodium hydrogen phosphate and sodium dihydrogen phosphate were from Applichem. DMP and AscA were from Sigma-Aldrich. HCL was from MP biomedicals. Chelex 100 resin (50-100 mesh, sodium form) was from Bio-Rad. The water was Milli-Q ultrapure water that has further been purified with a column of Chelex® 100 resin to remove any residual heavy metal ions.

The stock solutions of DMP and HCL were kept in DMSO at 4 °C and diluted in water before use. AscA (50 mM in water) and phosphate buffer (500 mM in water) were kept as frozen aliquots at −18 °C and aliquots were melted directly before use.

SVLAC was provided by Tiit Lukk from TalTech. SVLAC concentration was determined from absorbance at a wavelength of 280 nm using the theoretical molar attenuation coefficient of $\varepsilon = 44015\ M^{-1}cm^{-1}$ (Pace, C. N. et al. 1995). The stock and diluted solutions were stored as aqueous solutions at 4 °C.

Experiments were performed under atmospheric air saturation conditions, meaning that the concentration of O_2 was in the range of 0.2 ~ 0.25 mM.

3.1.2 SVLAC activity assay with DMP as the substrate

SVLAC activity assays with DMP as the substrate were performed at room temperature in 50 mM sodium phosphate buffer with pH 8. The reactions were initiated by the addition of SVLAC to the reaction mixture.

CL formation was followed in real time with UV-Vis absorption spectrophotometry (UV-1601PC spectrophotometer, Shimadzu) and determined in a 10-mm quartz cuvette by the specific absorption at wavelength of 469 nm using the molar attenuation coefficient 53200 $M^{-1}\ cm^{-1}$.

Experiments were carried out as triplicates with DMP was 10 nM. A single repeat was used for the negative control experiments, where SVLAC was omitted. The steady state rates were

determined from the slopes of the 2-minute time courses and the rates of the negative controls were subtracted from the results. The rates are defined as the formation of CL in unit time.

3.1.3 SVLAC activity assay with HCL as the substrate

SVLAC activity assays with HCL as the substrate were done at similar conditions as the experiments with DMP as the substrate. CL formation was followed in real time by the specific absorption at the wavelength of 469 nm.

Experiments were carried out as triplicates with HCL concentrations ranging from 0.01 to 1 mM and SVLAC concentration was 10 nM. A single repeat was used for the negative control experiments, where SVLAC was omitted. The steady state rates were determined from the slopes of the 1-minute time courses and the rates of the negative controls were subtracted from the results. The rates are defined as the formation of CL in unit time.

3.1.4 SVLAC lag time assay with DMP as the substrate

SVLAC lag time assay with DMP as the substrate (Figure 14) was constructed to involve four main steps: (1) SVLAC-catalyzed oxidation of DMP to the corresponding phenoxy radical at the expense of O_2; (2) rapid reaction of AscA and free DMP phenoxy radicals that results in the conversion of DMP phenoxy radicals back to DMP; (3) non-enzymatic dimerisation of phenoxy radicals into HCL, once AscA has been depleted; (4) SVLAC-catalyzed conversion of HCL to chromogenic CL at the expense of O_2.

SVLAC lag time assays were performed at similar conditions as SVLAC activity assays with DMP and HCL as the substrates. CL formation was followed in real time and determined by its specific absorbance.

Experiments were carried out as triplicates with DMP concentrations in the range of 0.1 to 10 mM and SVLAC concentration was 10 nM. AscA concentration in experiments was in the range of 1 to 8 μM. SVLAC and AscA were mixed and the reactions were initiated with the addition of DMP. The dead time of the experiment was 20 seconds. Lag times are determined as the time required before CL formation started (Figure 14), indicated by an increase of absorbance compared to the control experiment performed in the absence of enzyme.

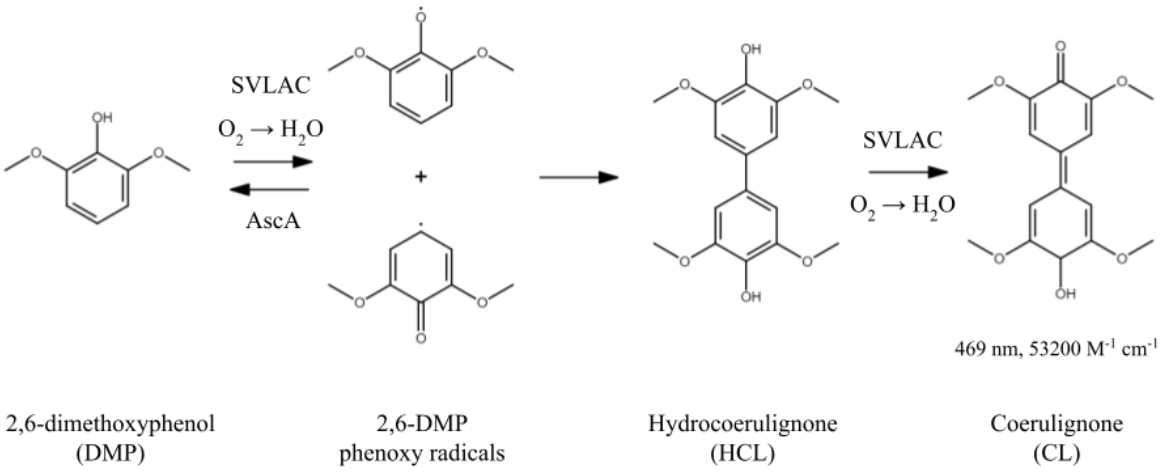

Figure 14. DMP lag time assay for *Streptomyces viridosporus* laccase: SVLAC catalyzes the oxidation of DMP to the corresponding phenoxy radical at the expense of O_2; AscA reacts rapidly with the free DMP phenoxy radicals and converts it back to DMP; phenoxy radicals dimerize non-enzymatically to HCL; SVLAC catalyzes the conversion of HCL to chromogenic CL at the expense of O_2. CL absorbance was measured at the wavelength of 469 nm with molar attenuation coefficient of 53200 $M^{-1}cm^{-1}$ (Marvin JS, Google Slides).

For the determination of the lag time (Figure 15) the linear part of the CL formation curve was extrapolated and the intersection point of this line and x-axis was defined as the starting point of CL formation. The time period from initiation of the reaction to the starting point of CL formation was defined as the lag time.

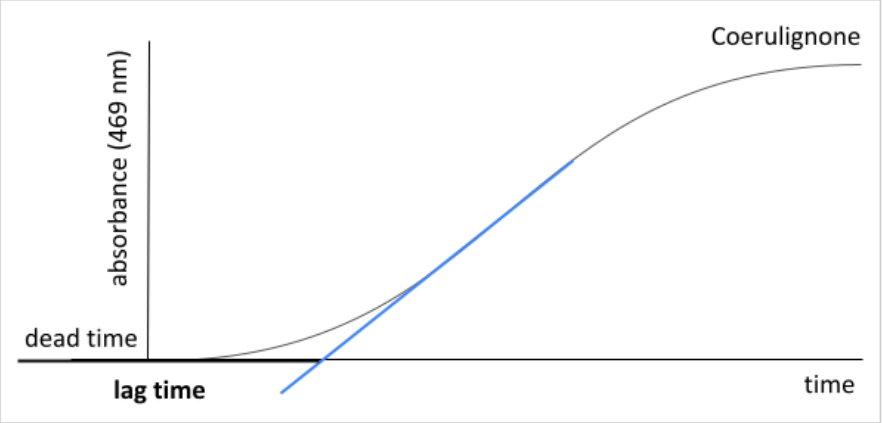

Figure 15. SVLAC lag time determination: the CL formation curve was extrapolated and the intersection point of this line and x-axis was defined as the starting point of CL formation. The time period from initiation of the reaction to the starting point of CL formation was defined as the lag time.

Essentially, the lag time was calculated as the sum of the dead time and the negative intercept divided by the slope of the linear part of the CL formation curve (1). The initial rates of the DMP radicals' formation were calculated according to the equation (2) (Gilabert, M. A. et al., 2006).

$$\tau = (-\ intercept)/(slope)\ +\ dead\ time \quad (1)$$

$$v_o = \frac{2[AscA]}{\tau} \quad (2)$$

3.1.5 Statistical analysis of DMP conversion kinetics

Statistica software was used to predict the kinetic parameters (K_M^{app} and k_{cat}^{app}) and the standard errors by fitting the data to the Michaelis-Menten equation (3). The Levenberg-Marquardt algorithm was used and the loss function was least squares.

$$v\ =\ k_{cat}[E][S]/(K_M\ +\ [S]) \quad (3)$$

3.1.6 UV-Vis spectral analysis of SVLAC action

DMP and HCL conversion with SVLAC was analyzed with UV-Vis absorption spectrophotometry (UV-1900i spectrophotometer, Shimadzu) to reveal the extent of possible side reactions. The range of the spectra (270 - 500 nm) was chosen to include the absorbance maxima of the known compounds in the reaction path: DMP (ε_{280nm} = 14800 M^{-1} cm^{-1}), HCL (ε_{280nm} = 16260 M^{-1} cm^{-1}), and CL (ε_{469nm} = 53200 M^{-1} cm^{-1}) (Galai et al., 2012, Breslmayr, E. et al., 2018, Breslmayr, E. et al., 2019). The duration of the spectral assay was longer than that of the activity experiments to assess the stability of the product. Substrate concentrations were chosen to yield absorbance values around 1. Enzyme concentrations were chosen to produce significant conversion of the substrate in the observed time frame.

SVLAC spectral assay with DMP was carried out using 2 mM DMP and 10 nM SVLAC. DMP absorbance, specific to wavelength of 280 nm and CL absorbance, specific to 469 nm wavelength were measured within the spectrum from 270 to 500 nm wavelength during a 25-minute time course with measurement interval of 5 minutes.

SVLAC spectral assay with HCL was carried out using 60 µM HCL and 5 nM SVLAC. HCL absorbance, specific to 280 nm wavelength and CL absorbance, specific to 469 nm wavelength were measured within the spectrum from 270 to 500 nm wavelength during a 55-minute time course with a measurement interval of 5 minutes.

3.2 RESULTS

3.2.1 Determination of kinetic parameters for DMP conversion to CL and its two enzymatic steps separately

In order to determine the rate limiting step in SVLAC-catalyzed DMP oxidation, different methods were used, so that different enzymatic steps of the overall reaction could be distinguished. Experimental results of SVLAC activity assay with DMP as the substrate, SVLAC activity assay with HCL as the substrate and SVLAC lag time assay with DMP as the substrate were measured and analyzed in relation to each other (Figure 16).

SVLAC activity assay for DMP as the substrate resulted in the lowest k_{cat}^{app} value of 2.34 ± 0.24 min^{-1}, while the standard errors were the highest. The K_M^{app} value was 0.345 ± 0.151 mM. SVLAC activity assay for HCL as the substrate resulted in the intermediate k_{cat}^{app} value of 21.5 ± 3.4 min^{-1} with intermediate standard errors and K_M^{app} value of 0.321 ± 0.122 mM for HCL conversion to CL. SVLAC lag time assay resulted in the highest k_{cat}^{app} value of 90.9 ± 5.6 min^{-1} with the lowest standard errors and K_M^{app} value of 3.85 ± 0.75 mM for DMP conversion to DMP radicals (Table 1).

Table 1. DMP activity, HCL activity and DMP lag time assays' predicted kinetic parameters of k_{cat}^{app} and K_M^{app} with standard errors.

	k_{cat}^{app} min^{-1}	**K_M^{app} mM**
DMP activity assay	2.34 ± 0.24	0.345 ± 0.151
HCL activity assay	21.5 ± 3.4	0.321 ± 0.122
DMP lag time assay	90.9 ± 5.6	3.85 ± 0.75

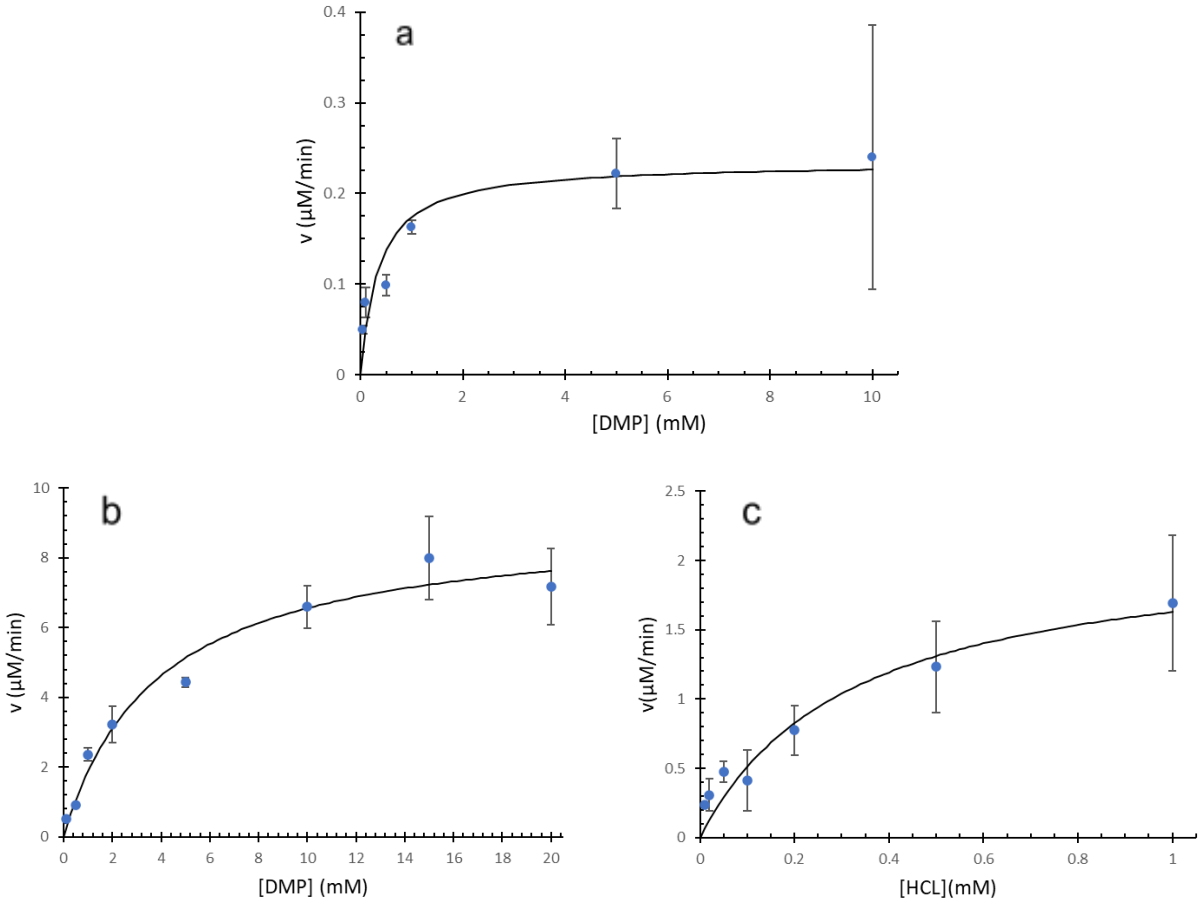

Figure 16. Michaelis-Menten graphs of (a) DMP activity assay, (b) DMP lag time assay, and (c) HCL activity assay. The experiments were performed at room temperature in 50 mM sodium phosphate buffer with pH 8. For every assay, the data points are the averages of the three independent experiments and error bars represent standard errors. The solid lines are according to the Michaelis-Menten equation: (a) experiments of SVLAC activity assay with DMP for the whole DMP conversion to CL were carried out as triplicates with DMP concentrations ranging from 0.1 to 10 mM and SVLAC concentration was 10 nM; (b) experiments of SVLAC lag time assay with DMP for DMP conversion to DMP radicals were carried out as triplicates with DMP concentrations in the range of 0.1 to 10 mM and SVLAC concentration was 10 nM, also AscA concentration was in the range of 1 to 8 µM; (c) experiments of SVLAC activity assay with HCL as the substrate for HCL conversion to CL were carried out as triplicates with HCL concentrations ranging from 0.01 to 1 mM and SVLAC concentration was 10 nM.

3.2.2 Determination of possible side products in DMP and HCL conversion to CL

SVLAC spectral assay of DMP conversion to CL (Figure 17) did not detect side products, detected CL absorbance at 469 nm wavelength constant increased over the time and the DMP absorbance at 280 nm wavelength decreased during the first 5 minutes with following increase to the end of the experiment.

SVLAC spectral assay of HCL conversion to CL (Figure 18) detected at least one side product, which absorbed approximately between 350 to 430 nm wavelengths; the CL absorbance at 469 nm wavelength increased during the first 5 minutes with following decrease to the end of the experiment and the HCL absorbance at 280 nm wavelength constantly decreased over the time.

DMP and HCL overlapping absorbance at 280 nm wavelength and time-limiting step prior to conversion of DMP to HCL may explain substrate absorbance increase in DMP conversion to CL by SVLAC spectral assay. In SVLAC spectral assay of DMP conversion to CL, absence of side products may indicate that there is no formation of other chromogenic side products at the spectrum between wavelengths, significant to the monitoring of DMP and CL absorbance. In SVLAC spectral assay of HCL conversion to CL, HCL values do not correlate with CL values, whereas the increase of side product absorbance correlates timely with CL absorbance decrease that may indicate that there is formation of at least one chromogenic side product with CL involvement.

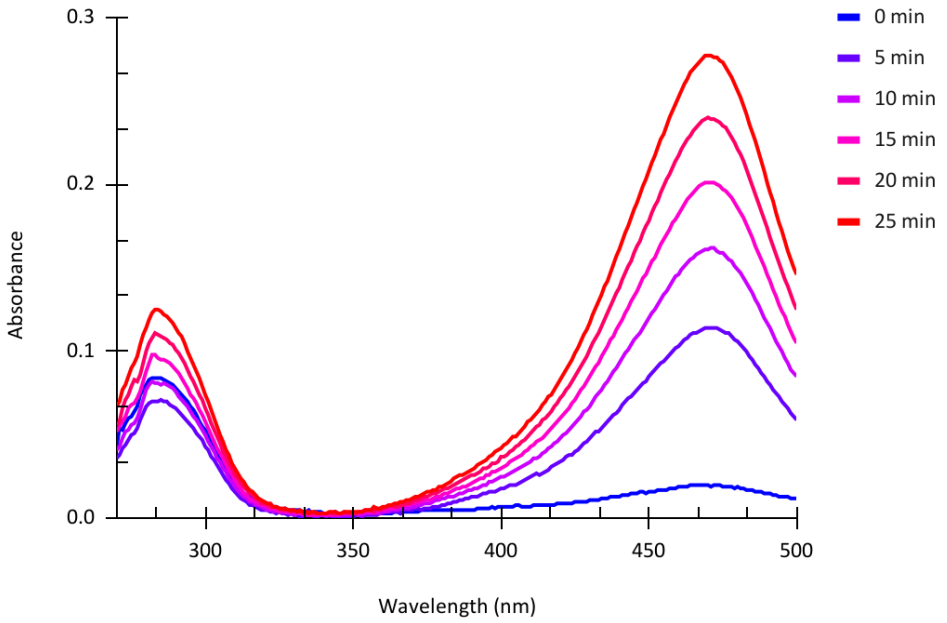

Figure 17. SVLAC spectral assay with DMP was carried out using 2 mM DMP and 10 nM SVLAC. SVLAC spectral assay of DMP conversion to CL did not detect side products. The increase of CL absorbance at 469 nm wavelength and the changes of HCL and DMP absorbance at 280 nm wavelength are in agreement with the three-step model of DMP conversion to CL.

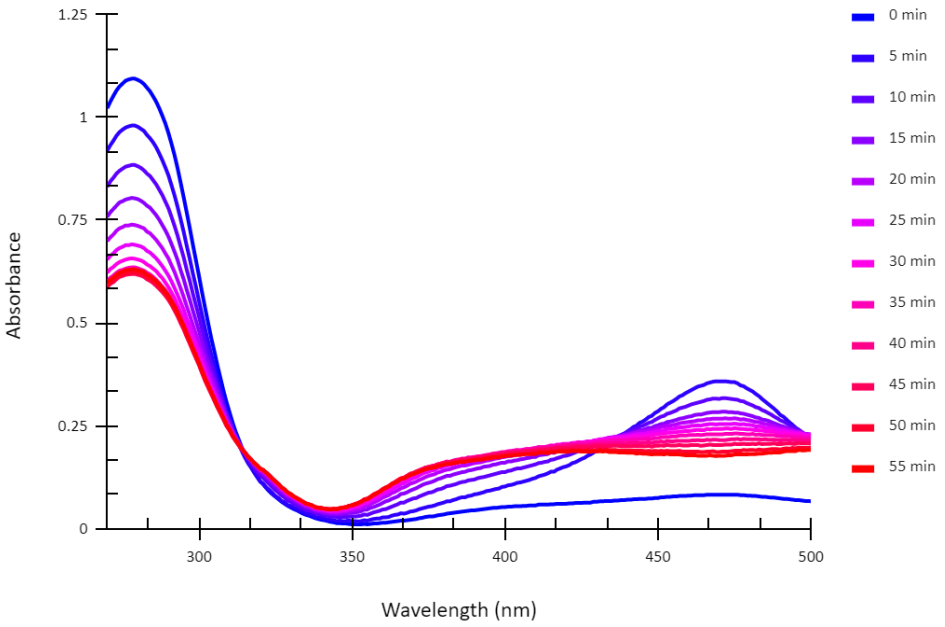

Figure 18. SVLAC spectral assay with HCL was carried out using 60 µM HCL and 5 nM SVLAC. SVLAC spectral assay of HCL conversion to CL detected at least one side product, which absorbed approximately between 350 to 430 nm wavelengths. The decrease of CL absorption at 469 nm deviates from the three-step model of DMP conversion to CL.

3.3 DISCUSSION

DMP is a commonly used model substrate in laccase studies. However, laccase-catalyzed DMP conversion encompasses one non-enzymatic step in addition to two enzymatic steps. It does not pose a problem in screening for DMP oxidase activity, but in order to describe the kinetics of the enzyme, the non-enzymatic step must not be rate-limiting.

Different methods enable to determine the kinetic parameters of different individual steps in SVLAC-catalyzed DMP conversion. In this study three different approaches were used. Firstly, the classical approach, in which case the SVLAC-catalyzed DMP conversion is measured through the formation of CL. This method describes the overall process and covers all three reactions in the pathway. Secondly, the alternative approach proposed by Breslmayr et al. 2018 was used. In that case HCL was used as the substrate instead of DMP and, therefore, this approach describes only the last enzymatic step of the process. Finally, the novel assay proposed in this study was used. This novel assay is based on the measurement of lag time and describes only the first enzymatic step of SVLAC-catalyzed DMP conversion.

The kinetic parameters are summarized in Table 1. It is worth to note that the apparent catalytic rate constant is about one order of magnitude greater when HCL is used as the substrate instead of DMP. In agreement with the findings of Breslmayr et al. 2018, the standard errors are smaller, meaning that the results are more precise.

This approach does not include a non-enzymatic step and, therefore, is suitable for determining the kinetics of laccases. On the down side, the solubility and stability of HCL in water is rather low, which limits the range of substrate concentrations that one can use in the experiments.

The usage of more stable DMP substrate, the description of the first enzymatic step of the conversion of DMP prior to rate-limiting step, independence from the molar attenuation coefficient of the product, lower enzyme concentration usage-enabling k_{cat}^{app} higher value and significantly lower standard errors' rate makes lag time spectrophotometric assay a more reliable method for the evaluation of laccase kinetics.

The description of the second enzymatic step of the conversion of DMP following rate-limiting step, dependence on the molar attenuation coefficient of the product, side product appearance and k_{cat}^{app} lower value makes SVLAC activity assay with HCL substrate a less reliable method for the evaluation of laccase kinetics.

The relatively lower rate of standard errors makes lag time spectrophotometric assay a more reliable method for the evaluation of laccase kinetics. Lower solubility of HCL may be the reason for higher standard errors of SVLAC activity assay with HCL as substrate and reaction of DMP radicals with CL may be the reason for higher standard errors of SVLAC activity assay with DMP as substrate. This was also stated previously by Breslmayr, who used the same method with another enzyme.

Currently there is no easily accessible method available to determine the rate constant of the second, non-enzymatic, step of the overall process. Nevertheless, assumptions can be made based on the comparison of the kinetic parameters of the well-described individual steps and the overall process.

The reciprocals of the catalytic rate constants, transit times, describe the time required for one molecule of substrate to be converted into the product. Transit times are additive, meaning

that the transit time of the overall process is the sum of the transit times of the individual processes.

The rate-limiting step can be determined by finding the step with the lowest transit time that can be calculated for every step by inversely proportional value of k_{cat}^{app} (Figure 19). Rate limiting step is revealed to be a non-enzymatic conversion of DMP radicals to HCL that excludes this assay reliability for laccase kinetics.

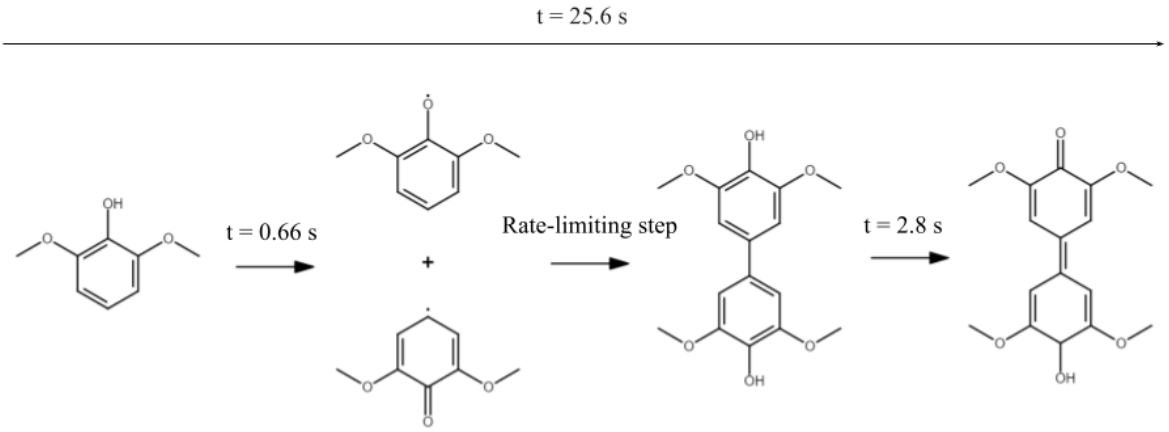

Figure 19 Rate-limiting step of SVLAC-catalyzed DMP conversion to CL: the whole conversion of DMP to CL takes about 25.6 s. It starts with the first enzymatic step with the lowest transit time of 0.66 s. Then it is followed by a rate-limiting step, the non-enzymatic conversion of DMP radicals to HCL. Finally, the second enzymatic step takes place with moderate transit time of 2.8 s (Marvin JS, Google Slides).

Overall, it can be concluded that SVLAC-catalyzed DMP conversion to CL starts with the first enzymatic step with the highest k_{cat}^{app} and the most reliable standard error of the corresponding SVLAC lag time assay with DMP substrate. It is followed by a rate-limiting non-enzymatic step. Finally, the second enzymatic step takes place with intermediate k_{cat}^{app} and relatively reliable standard error of the corresponding enzymatic SVLAC activity assay with DMP substrate. Furthermore, there is a need to evaluate laccase kinetics in different oxygen saturation conditions.

SUMMARY

The kinetics of industrially important laccases has been commonly evaluated by the activity assays of widely used laccase model substrate DMP conversion. DMP is oxidized in three steps with the formation of intermediate radicals that affect assay accuracy. This thesis comprehensively analyzed DMP oxidation and developed a more reliable lag time spectrophotometric assay for laccase kinetics.

The kinetics of every step of DMP conversion to CL has been analyzed with distinct assays. The analysis of k_{cat}^{app} values of different steps of SVLAC-catalyzed DMP conversion revealed that the rate-limiting step is the non-enzymatic conversion of DMP radicals to HCL. This means that the classical DMP assay is unable to describe laccase kinetics. This is in contrast to SVLAC activity assay for HCL, and the developed lag time spectrophotometric assay that are applicable for laccase kinetics and show significantly higher experimental precision. The developed lag time spectrophotometric assay is more reliable for the evaluation of laccase kinetics than HCL-based assay, because it is not affected by side reactions.

DMP conversion to CL still remains challenging to be properly evaluated due to the presence of mass spectroscopically recognised yet spectrophotometrically undistinguishable side products in DMP conversion to CL and spectrophotometrically distinguished side absorbance of unknown origin.

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