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Faculty of Chemistry and Chemical Engineering

Sclerotinia sclerotiorum laccase: biochemical characterization and applications

- PhD thesis -

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Table of Contents of the entire thesis

Keywords: laccase, *Sclerotinia sclerotiorum*, enzyme regulation, protein purification, copper-containing protein, adduct, copper complexes, radicals, prooxidant

Aims of the thesis

The present thesis has five main objectives which were stated in a preliminary form before the work was started and suffered several adjustments during the work proceeded. Each of these objectives contains several main steps which were foreseen in the research thesis project or was established during the ongoing procedures.

1. *Determination of optimum conditions considering maximum laccase activity in liquid culture of Sclerotinia sclerotiorum*
    - Evaluation of several types of liquid mediums upon laccase secretion;
    - Assessing the influence of numerous important parameters/conditions upon laccase production by the fungus such as: pH, N and C sources, time, several inducers;
    - Evaluation of some important physiologic relevant factors upon laccase regulation in order to bring some insights of its physiologic role;
2. *Isolation, purification and characterization of Sclerotinia sclerotiorum laccase*
    - Establishment of suitable protocols for laccase isolation and purification using chromatographic and electrophoretic facilities;
    - Determination of specific activity and biochemical properties (K_M, k_{cat}, optimum pH and temperature, thermostability, substrate selectivity) of the purified enzyme;
    - Spectral characterization of the purified enzyme (UV-vis, CD, EPR, MS);
3. *Elucidation of the mechanism of the reactions catalyzed by the pure enzyme*
    - Characterization of possible reaction intermediates and their kinetics;
    - Study of enzyme – substrate interaction;
4. *Applications of the Sclerotinia sclerotiorum laccase*
    - Establishment of protocols suitable for evaluations of prooxidant and antioxidant activities of polyphenols and some natural extracts;
5. *Theoretical and experimental studies of model compounds for laccases*
    - Evaluation of reactivity towards some ligands and laccase substrates of some copper complexes;
    - Theoretical studies of laccase copper centers concerning their reactivity and spectral behaviour.

General Introduction

Proteins having one or more copper ions as cofactors play very important roles in cellular metabolism of all living organisms. They are involved in photosynthesis, oxidative phosphorylation, homeostasis of metal ions and catabolism of many nutrients. The main reactions involving copper proteins are electron transfer, this due to copper ability to exist in two oxidation states Cu^{+} and Cu^{2+}. Copper centers in proteins have such a coordination sphere provided by the polypeptidic structure so that the transition from one oxidation state to another to be thermodynamically feasible.

The simplest copper dependent proteins are azurins and plastocyanines, they are usually involved in electron transfer reactions. Other more complex proteins, with copper ions in the active sites, such as galactose oxidase, nitrite reductase, ceruloplasmin, ascorbate reductase, bilirubin oxidase and last but not least laccase, are involved electron transfer reactions from reduced substrates to electron deficient molecules.

Lacase (p-diphenols: dioxigen oxidoreductase) is an oxidoreductase (EC 1.10.3.2) with four copper ions in two active sites, which catalyzes the oxidation of reduced substrates usually phenols or aromatic amines, coupled with the reduction of molecular oxygen to water. Lacase is one of the oldest enzymes ever studied, it was described for the first time by Yoshida in 1883 and categorized by Bertrand in 1895 as a copper containing oxidase. However, only in recent decades, when it was discovered that laccases are part of the enzymatic arsenal involved in wood degradation by white rot fungi, study of these enzymes has greatly increased. A more recent intrest in this enzyme is its envolvment in the virulence of some phytopathogenic fungi, as is the case of the present thesis.

Currently, this enzyme is the central subject of many worldwide research groups, due to scientific curiosity and its high potential in numerous applications in biotechnology and bioanalytical chemistry.

Content of the thesis

The first chapter describes the most recent research on the overall structural features of laccases as well as on the structures and properties of the active sites, along with the currently proposed mechanisms of reaction. Laccase (*p*-diphenol:dioxygen oxidoreductase), one of the oldest discovered enzymes, contains four copper ions in two active sites and catalyzes a monoelectronic oxidation of substrates such as phenols and their derivatives, or aromatic amines, coupled to a four-electron reduction of dioxygen to water. The catalytic mechanism was studied for decades but is still not completely elucidated, especially in terms of the reduction of dioxygen to water. The key structural features of this enzyme are under research in several groups using techniques such as X-ray diffraction, electron paramagnetic resonance (EPR) spectroscopy, site-directed mutagenesis. The high interest in laccases is explained by the large number of biotechnological applications. Their distribution in nature, the physiologic role, most used methods for purification and biochemical properties and parameters used for their characterization are also described. Numerous applications of laccases such as textile industry, wood processing paper production, pharmaceutical and chemical industries and others are described. Some biological aspects regarding *Sclerotinia sclerotiorum* phytopathogenic fungus and reasons for using this organism as laccase source are presented at the end of the chapter. In the last part of the chapter some copper complexes used as models for laccase active sites are discussed.

The second chapter describes the factors affecting the production of laccase from the phytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary. The carbon/nitrogen ratio appears to be of great importance. Rather than a simple nutrient-rich nitrogen source, yeast extract behaves as a true laccase upregulator, apparently acting via a stress pathway. *Chelidonium majus* extract, a known antifungal agent, acts in a similar manner. The compound(s) in the yeast extract responsible for enhancing laccase synthesis are suggested to be hydrolysable small organic molecules. Both extracts reduce biomass and sclerotia development and enhance laccase production, leading to an increase in laccase activity by one order of magnitude compared to controls. The pH of the medium, a well-known virulence regulator for this fungus, also acts as a true laccase regulator, though via a different mechanism. The effect of pH appeared to be linked to the acidification kinetics of the extracellular medium during fungal development. A number of other known laccase inducers were found to enhance laccase production at most two-fold.

Chapter three contains information regarding the production, purification and characterization of a laccase from the phytophathogenic fungus *Sclerotinia sclerotiorum*. This laccase is identified by mass spectrometry with a sequence coverage of 74.9% (458/577 AA) revealing that the protein is identical or highly homologous to a predicted oxidoreductase from this species (A7EM18 in the Uniprot database); the closest homologous protein previously isolated from a fungus is the Melanocarpus albomyces, with only 35% identity. The UV-vis spectral features of this laccase classify it as a "yellow" one. The EPR spectrum nevertheless demonstrates resemblance to blue laccases – including the type 1 center not detectable in UV-vis spectra. The presence of type 3 coppers was proven by fluorescence spectrum and by 330 nm band in UV-vis. The purified laccase has an apparent molecular mass of 70 kDa and appears as a monomer. The values of K_M and k_{cat} were determined for ABTS, 2,6-dimethoxyphenol, p-phenylenediamine and guaicol and are typical of a laccase. The optimal pH value is around 4 except for ABTS, for which activity is linearly increasing with acidity. The high laccase activity in liquid culture makes Sclerotinia sclerotiorum a useful source of laccase for practical applications.

In chapter four it is provided the first evidence that the yellow laccase isolated from *Sclerotinia sclerotiorum* is obtained from a blue form by covalent, but nevertheless reversible modification with a polyphenolic product. Yellow laccases lack the typical blue type 1 Cu absorption band around 600 nm, but are nevertheless multicopper oxidases with laccase properties. After separating the polyphenols, a typical blue laccase is obtained. With ABTS as model substrate for this blue enzyme, a purple adduct is formed with a spectrum nearly identical to that of the 1:1 adduct of an ABTS radical and Tyr. This modification significantly increases the stability and substrate affinity of the enzyme, not by acting primarily as bound mediator, but by allosteric activation that also alters the type 1 Cu site. Thus, *S. sclerotiorum* yellow laccase is an intrinsically blue multi-copper oxidase that autocatalytically activates itself upon first encounter with a radical-forming aromatic substrate.

The fifth chapter contains numerous results regarding the application of the purified enzyme on antioxidant and prooxidant properties of some phenolics and propolis extracts. A transient species may be detected with UV-vis and EPR spectroscopy during turnover of a laccase with quercetin; this species is assigned as a quercetin-derived radical, based on EPR spectra as well as based on UV-vis similarities with previously reported data on a quercetyl radical obtained via a non-enzymatic route. The formation and decay of this species correlate well with the prooxidant reactivity manifested by flavonoids in the presence of laccase. An assay for the prooxidant reactivity of natural compounds is proposed based on the results reported here; this assay has the advantages

of using a biologically-relevant process (hemoglobin oxidation), and of not needing added oxidizing agents such as peroxide or superoxide. Correlations, or the lack thereof, between the prooxidant parameters and the redox potentials, antioxidant capacities and lipophilicities, are analyzed. New assays for antioxidant activity of natural extracts are also described. It can be noted that the laccase employed in this study does display structural and reactivity-related similarities to a range of other proteins, which includes ceruloplasmin.

The last chapter of the thesis contains the results regarding molecular modelling of laccase active sites and the experiments describing the reactivity of some copper complexes used as models for type 2 copper sites. Laccases contain a blue mononuclear copper center known as 'type-1', and thought to be the primary electron acceptor from organic substrates during the catalytic cycle. A small group of laccases are also known that lack the 600 nm band and hence the blue color ("yellow laccases"). In first section it is reported the use of semiempirical (ZINDO/S-CI) calculations in order to simulate UV-vis spectral parameters for the laccase type 1 copper, attempting to assign geometrical and electronic structure elements that may control the color of this site. The ~600-nm band of the type 1 copper is confirmed to arise mainly from sulfur-to-copper charge transfer, and strong distortions allowing for its displacement by more than 200 nm and/or its dissolution are identified. In the second section some copper porphyrinates are analysed with respect to its reactivity towards some laccase substrate and some other redox active compounds. Copper porphyrinates are generally known to display a less diverse reactivity compared to their iron counterparts. It is examined a water-soluble copper porphyrinate for its ability to engage in reactions involving axial ligation to the copper or possible redox cycling. Although UV–vis spectra indicate an expected lack of reactivity, electron paramagnetic resonance spectra (EPR) reveal an unexpected wealth of changes in electronic structures at the copper, induced by potential ligands such as imidazole or nitrite, but also by seemingly unexpected candidates for ligands, such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and guaiacol, as well as by dithionite. An important function of many copper-containing proteins is activation of O_2 and subsequent substrate oxidation. The Cu (III) oxidation state is generally considered to be less accessible because of the highly positive Cu (III)/Cu (II) redox potentials with typical amino acid ligands. In the last part it is employed density functional (DFT) calculations to explore to what extent copper (III) may be accessed in a biologically-relevant coordination environment around a mononuclear copper center, by breaking the oxygen-oxygen bond in a copper-(hydro) peroxide complex. In agreement with previous findings on copper models with related coordination patterns, the formally high-valent copper complex produced by O-O bond cleavage appears to in fact harbor both oxidizing

equivalents on the ligands. The potential energy surface for such a reaction reveals that with the three-histidine binding motif at the copper, O-O bond cleavage is not impossible, but rather disfavored thermodynamically.

General conclusions

- Optimal conditions under which the *S. sclerotiorum* laccase can be produced were determined. The carbon and nitrogen sources and C/N ratio appear to be of great importance for laccase production in this fungus. Rather than a simple nutrient-rich nitrogen source, yeast extract behaves as a true laccase inducer/upregulator, apparently acting via a stress pathway. *Chelidonium majus* extract, a known antifungal agent, acts in a similar manner. The pH of the medium, a well-known virulence regulator for this fungus, also acts as a true laccase regulator, though via a different mechanism. The effect of pH appears to be linked to the acidification kinetics of the extracellular medium during fungal development. Thus, evidence is shown that this enzyme is involved in stress response pathways, most likely connected to virulence.
- *Sclerotinia sclerotiorum* laccase has been isolated, and its catalytic properties characterized. Notably, although this laccase can be classified as a "yellow laccase" based on the UV-vis spectrum, the "blue" T1 center is nevertheless observable in the EPR spectrum. The extent to which the *S. sclerotiorum* laccase may indeed allow definition of a new type of laccase (neither truly "blue", nor truly "yellow") remains to be explored, especially as for most yellow laccases the EPR spectra have not been reported; should such a class be confirmed, a term such as "mixed blue-yellow" might be appropriate.
- Direct evidence for an example where a blue laccase can be converted to a yellow form *in vitro* by covalent modification at the T1 site, with metabolites produced by the laccase itself was provided. Moreover, this autocatalytic modification significantly improves the structural and catalytic properties of the enzyme. In essence, *S. sclerotiorum* yellow laccase is an intrinsically blue multi-copper oxidase that has activated itself upon first encounter with a polyphenolic substrate. A tyrosine residue was identified near the T1 site, which may be the target of such modifications.
- A transient species may be detected with UV-vis and EPR spectroscopy during turnover of a laccase with quercetin; this species is assigned as a quercetin-derived radical, based on EPR spectra as well as based on similarities with previously reported data. Furthermore, this species correlates well with the prooxidant reactivity manifested by flavonoids in the presence of laccase. An assay for prooxidant reactivity of natural compounds is proposed

based on these results, which has the advantages of using a biologically-relevant process (hemoglobin oxidation), and of not needing added oxidizing agents such as peroxide or superoxide. Correlations, or the lack thereof, between the parameters obtained from this assay and redox potentials, antioxidant capacities and lipophilicities, are discussed. It was also noted that the laccase employed in this study does display structural and reactivity-related similarities to a range of other proteins, which includes the serum ceruloplasmin, and also displays reactivity similarities with heme-containing peroxidases. In addition, a new more informative and effective scale of antioxidant capacity is obtained by applying PCA on DPPH (2, 2-diphenyl-1-picrylhydrazyl) bleaching kinetic profiles. In order to obtain comparable antioxidant activities, a non-dimensional parameter was generated which is termed the *quercetin factor* (QF), which defines the ratio between quercetin equivalent in mg/L of the assayed propolis sample and the corresponding propolis concentration in mg/L. Further application of this methodology to other botanical extracts will confirm this new method for assessing antioxidant activity.

- The coordinative chemistry of copper porphyrinates may be distinctly more complex than previously described, and that EPR but not UV-vis spectroscopy is the method of choice for investigating this new chemistry.
- Using computational methods, torsion and elongation-type deformations have been identified, which allow a "blue" tri-coordinated type 1 copper center to apparently lose its characteristic 600-nm band responsible for its blue color both by shifting it by more than 200 nm, and, in some cases, by decreasing the extinction coefficients. However, DFT calculations suggest that such distortions might also be detectable with EPR spectroscopy.
- Unlike in related iron or manganese complexes, high-valent states appear not to be achievable via peroxo chemistry in copper complexes – even though O-O bond cleavage per se appears to entail reasonably low energy barriers; this may be interpreted to be due to a difference in redox potentials, which makes the peroxide-derived hydroxo and oxo ligands easier to oxidize than Cu (II).

List of publications on thesis topic

1. Moţ A.C., Damian G., Sarbu C., Silaghi-Dumitrescu R., *Redox reactivity in propolis: direct detection of free radicals in basic medium and interaction with hemoglobin*, Redox Report 14 (2009) 267-274; (IF: 1.732, SRI: 0.478)
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3. Lupan A., Matyas C., Moţ A.C., Silaghi-Dumitrescu R., *Can geometrical distortions make a laccase change color from blue to yellow?,* Studia Universitatis Babes-Bolyai Chemia, 56 (2011) 231-238. (IF: 0.129)
4. Moţ A.C., Pârvu M., Damian G., Irimie F.D., Darula Z., Medzihradszky K.F., Brem B., Silaghi-Dumitrescu R., *A "yellow" laccase with "blue" spectroscopic features, from Sclerotinia sclerotiorum*, Process Biochemistry 47 (2012) 968–975; (IF: 2.627, SRI: 2.267)
5. Moţ A.C., Syrbu S.A., Makarov S.V., Damian D., Silaghi-Dumitrescu R., *Axial ligation in water-soluble copper porphyrinates: contrasts between EPR and UV–vis*, Inorganic Chemistry Communications 18 (2012) 1-3; (IF: 1.972, SRI: 0.905)
6. Imre A., Moţ A.C., Silaghi-Dumitrescu R., *Exploring the possibility of high-valent copper in models of copper proteins with a three-histidine copper-binding motif*, Central European Journal of Chemistry 10 (2012) 1527-1533; (IF: 1.073, SRI: 0.656)
7. Moţ A.C., Silaghi-Dumitrescu R., *Laccases: complex architectures for one-electron oxidations*, Biochemistry (Moscow), accepted; (IF: 1.058, SRI: 0.422)