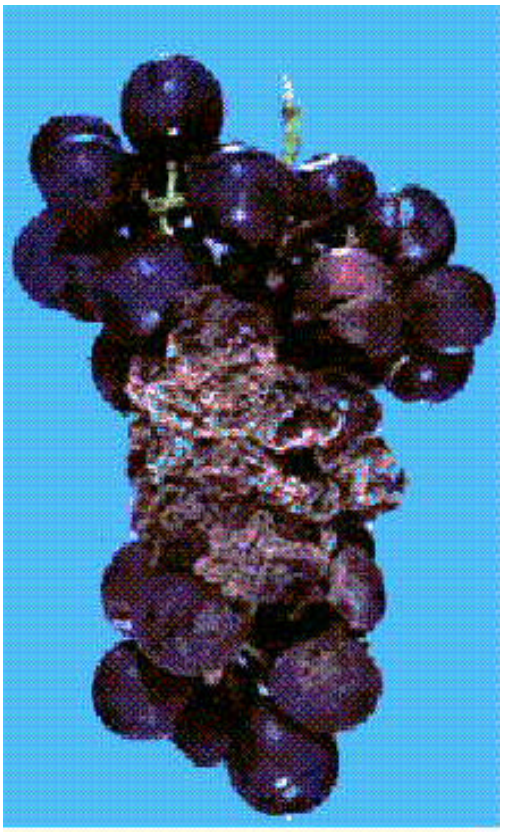
Studies for the Prevention of Red Wine Browning – development of a molecular control for the Copper Enzyme laccase



Final Report to Grape and Wine Research and Development Corporation

Project Number: UF 96/1

Principle Investigator: Dr Lisa Martin

Research Organisation: Flinders University, Adelaide

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Executive Summary

Background

The fungal infection of *Botrytis cinerea* on wine producing grapes can result in both favourable and deleterious effects on fruit quality. In its *ignoble rot* form infection of red wine grapes has a detrimental effect, almost always associated with a loss of quality and often resulting in browning of the wine, rendering it undrinkable! The oenologically dangerous by product of *Botrytis* infection is the copper containing metalloenzyme laccase; a member of the Blue Copper Oxidases (BCO). Although other oxidases are also found in *Botrytis* affected wines, laccase is exemplified by its resistance to the classical anti-enzymic agents, such as, SO_2 and is remarkably stable to heat treatment.

Relevance and Application to the Wine Industry

The wine industry is seasonally devastated by *Botrytis cinerea* infection. Unusually wet, overcast and humid conditions during growth season can result in a total 'down grading' of red wine production in the affected areas with losses of hundreds of thousands of dollars to local and export markets. As reported at the "Managing Bunch Rot" conference in Mildura in 2000, an A$8 million loss was reported in the Mudgee region alone, as fruit was so infected by *Botrytis cinerea* that it was more economical to leave it on the vine, than to harvest the crop. Unseasonal humidity accompanied by an extended spell of hot weather during the 2001 vintage, provided ideal condition for infection and it looks as though yields could once again be affected as found during the past 3 years. Regardless of the outcome in 2001, the problem lies dormant and must be addressed if the industry is to maintain its current growth and its international impact.

Although huge effort has been rallied during times of severe *Botrytis* infection, mostly through extensive vineyard spraying programs, at best, the infection can only be managed. Once in the must, the options for treatment of juice or must are limited. The only effective treatment for *Botrytis* infected samples in the winery is pasteurisation or heat treatment of the must at 80º. The result of treatments of this kind can have a devastating effect on wine quality; premium quality wines is lose their aroma and flavour structures and are rendered, in effect, 'cask grade' after treatment.

Summary of Key Outcomes

Our study was able to achieve the design, preparation and characterisation of synthetic "model complexes" for the triangular copper centre of laccase. Treatment of these model complexes with small molecules, such as, boron trifluoride and hydroxide, enabled us to show that copper-atoms similar to those in laccase, would bind these molecules and this caused the properties of the coppers to be modified.

The use of a commercially available laccase from *Rhus vernicifera* was explored as a protein model for *Botrytis cinerea* laccase. Purification protocols for laccase were developed as existing methods proved unsuccessful and confirmation of pure laccase was obtained using SDS Page-gel electrophoresis. In addition, this technique confirmed the molecular weight of laccase from *Rhus vernicifera* is similar to that published for *Botrytis cinerea* laccase. The laccase activity was checked using the Novo test which indicated that the protein was active.

We employed electrochemical techniques to determine the manner in which *Rhus vernicifera* laccase participated in oxidative reactions. This technology has an advantage that only nano-molar amounts of sample are required for measurments and also the experiments has a pico-amp sensitivity. To date, researchers have detected only a single copper, type 1 (T1), which is surprising as there are 3 types of copper in laccase (T1, T2 & T3). Although we have previously demonstrated this technique as an effective detector for redox chemistry of proteins with low-molecular weights, such as, ferredoxins & adrenodoxins, it was found to be unsuitable for larger enzymes, such as, laccase. Thus, we modified our strategy to include the immobilisation of the laccase enzyme onto a gold surface, thereby enabling for the first time, three oxidation waves to be observed. This development is significant as it is now possible to treat samples of laccase immobilised in this manner with chemicals and observe the responses. If a particular molecule binds to one of the copper atoms and prevents one of the oxidation reactions from occurring we would observe this in the electrochemical response. If the chemical does not react, we would expect the response to remain unchanged. We applied this new method to explore and define the redox the function of the laccase with small molecules in order to develop a molecular treatment for laccase infected wines. Time and resources limited this study to the investigation of only one potential inhibitor for the oxidative reaction of laccase, that of sodium azide. Initially, the electrochemical experiment is performed without azide and the three oxidative processes attributed to laccase are seen. Then, azide is added to the immobilised laccase enzyme and the electrochemical experiment repeated. Little change was observed in the first or second oxidation processes, however, the third process has been dampened and becomes quite different to the original wave. This shows that the copper responsible for wave 3 is the site in which azide binds at the 'active site' of laccase.

We anticipate that in the near future it will be possible to probe laccase with a wider range of chemicals in a manner similar to that described here.

These experiments have used laccase from *Rhus vernicifera* and established a method to directly examine the protein electrochemical response *in vitro*. In addition, we have shown that a small molecule will bind at the active site of laccase, thereby preventing the oxidative process of one copper from occurring. This binding demonstrates the principle of a molecular treatment for laccase infected wines. Also these results can be used to develop a Biosensor system to detect laccase in the laboratory. Future developments are also foreseen using laccase isolated from *Botrytis cinerea* infected wine samples, thus addressing the original long-term objective of this study.

Project Details

Project Title:	Studies for the Prevention of Red Wine Browning – development for a molecules control for the copper enzyme laccase
Field of Research:	GWRDC Program 3: Wine grape and wine: quality and specification
Research Organisation:	Flinders University
Name of Administrative Contact:	Grants Officer, Office of Research, Flinders University
Project Supervisor:	Dr L Martin, Senior Lecturer in Chemistry Flinders University GPO Box 2100, Adelaide, 5001, S.A.
Research Staff:	Project A: D Elliot & J Joannou Project B: S Brinkmann & D Wood
AWRI Staff:	Dr's P Williams, E Waters, Y Hayasaka
GWRDC Funding:	$32,800
Date commenced:	July 1998 (Pilot project commenced January 1997)
Date completed:	December 2000

Background

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Project Objectives

Explicit in the aims of this research project is the development of a 'molecular control mechanism' capable of 'turning off' laccase that results in the browning of red wines. This project aims to furnish information on laccase activity and reactivity towards various substrates and we anticipate progress in this research will be of an advantage to the Wine Industry in the short term. Specifically, we aim to,

1. Use biomimetic model complexes for the triangular copper containing active site of laccase to determine the reactivity towards small molecules. Thus, be able to develop a molecular treatment for red wines affected by *Botrytis cinerea* by deactivation of the enzyme, laccase, believed to be responsible for browning of red wine.

2. Use commercially available plant laccase from *Rhus vernicifera* to model laccase from the fungus *Botrytis cinerea*. Reactivity studies designed to reproduce the red wine

browning reaction will provide an understanding of how the metalloenzyme Laccase damages red wine quality.

Introduction

The Blue Copper Oxidases (BCO) catalyse the oxidation of a variety of substrates (ascorbate, polyphenols, aromatic poly-amines etc.) reducing molecular oxygen by multi-electron transfer to water. They are widespread in nature, occurring both in fungi (laccase), plants (laccase and ascorbate oxidase) and in animals (ceruloplasmin). The biological function for the plant and fungal enzymes is consistent with an oxygen control mechanism eg. laccase oxidases phenols in the presence of oxygen to radicals which spontaneously polymerise to a protective structure, similar to blood clotting in animals.

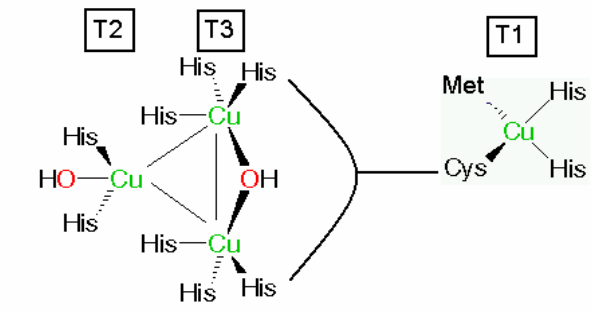


Figure 1. Thethree types of copper atoms at the active site of laccase.

The X-ray structural analysis of ascorbate oxidase shows that four copper ions are present per sub-unit, arranged as a novel 'triangular' trinuclear cluster with the fourth copper lying remote at some 15 Å distance (see Figure 1). The copper environments are spectroscopically distinguishable. The isolated copper ion is Type 1 (T1, blue Cu), while the triangular cluster comprises a Type 2 (T2, normal Cu) and a Type 3 (T3, coupled binuclear) pair of coppers. The Type 1 and Type 2 sites of laccase *(Rhus vernicifera)* are relatively labile and other workers have shown that the trinuclear cluster is the minimum structural unit capable of oxygen reduction. However, despite these studies no progress has been made in gaining an understanding of the mechanism for the redox process intimate with molecular interaction of the substrate and the triangular metal cluster.

Research Methodology

The project is planned to progress by the following sequence of events. Project A, focuses on the synthesis of model complexes for the copper containing active site of laccase, and Project B uses the commercially available source of laccsse from *Rhus vernicifera*.

Project A: Biomimetic Model Complexes for laccase

During the pilot study we have developed a new ligand and model complexes for the active site of laccase. This successful biomimetic approach will be exploited further to prepare and characterise copper complexes specifically designed to achieve the *triangular* tri-nuclear

copper arrangement (Cu_3) identified for laccase. A feature of this approach is that it includes structural flexibility for the T2 sub-site differentiated ligation observed in structural studies for BCO.

Redox characterisation of these model complexes is an important component of this study as these will provide a database for the types of processes and reactions that trinuclear complexes will undergo. In particular, the reactivity of the complexes towards small molecules (eg. O_2, CO, NO, N_3^-, etc.) are gauged using electrochemistry and spectroelectrochemistry. Those complexes that form stable intermediates will then be characterised. Kinetically unstable transient species are probed using the variable time base technique of cyclic voltammetry.

Project B: Purification & Characterisation of *Rhus vernicifera* laccase

Laccase must operate as a battery, storing electrons gained from sequential oxidation reactions in order to reduce molecular oxygen. Clearly, the redox nature of enzyme function *in vivo* dictates cyclic voltammetry and spectroelectrochemistry as the primary techniques to probe its mechanism and reactivity *in vitro*. For laccase, the ability to delete or substitute metal ions into T1 (Cu→Hg) and T2 (Cu depleted) sites will also provide a further approach whereby the relative contributions of the sites can be assessed relative to the overall enzymic process.

In 1996/97 we initiated a program to develop new strategies by which normally unstable complexes or metalloproteins themselves can be stabilised at the electrode surface. This approach employs the application of co-adsorbing promoter molecules with the protein, in order to encourage the electrochemical response. This research project allows for this approach to be extended and developed further for both the 'model' complexes and the laccase enzyme.

Materials & Methods

Biomimetic Model Complexes for laccase

A general feature of metalloproteins is their extraordinary specificity and selectivity properties and function. Clearly, for laccase both the structural and electronic architecture of the triangular Cu_3 site contribute to the specific coordination and reduction of the substrate, dioxygen. The paucity of simple model complexes containing a *triangular* metal ion environment has provided the impetus for us to examine the synthesis, structure, metal - metal interactions and reactivity towards small molecules.

The X-ray crystal structure of fully oxidised ascorbate oxidase has now been refined to 1.9Å and clearly illustrates the *isosceles triangular* arrangement for three of the copper atoms. Our first generation of model complexes for this Cu_3 active site which incorporates the structural and potentially the reactivity features: the structural disposition for three metal ions in an 'isosceles' triangular array as suggested by numerous examples of binuclear model complexes and which agrees well with the metal - metal distances in the required 3.4 - 4.0 Å range found crystallographically.

The differing coordination environments for the metal ions ie. the T2 and T3 sites, are accommodated by employing a variable heterocycle unit together with two 1,4,7-triazacyclononane (tacn) moieties. This addresses the sub-site differences and will assist in gauging donor atom influences on the electronic structure of the cluster and the implications for substrate binding and ultimately reactivity; an example of this ligand using bisbenzimidazole as the heterocycle, and the facially coordinating tacn ligand, is illustrated in Figure 2.

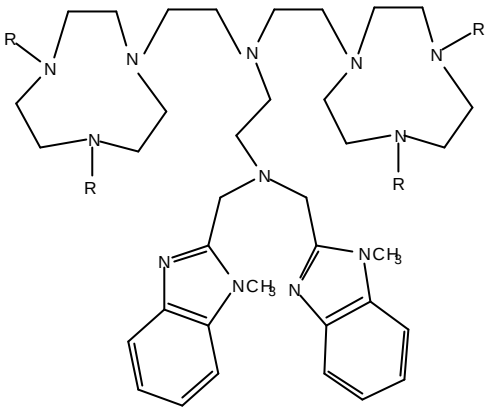


Figure 2: Structural diagrams for the target ligand.

The focus in this project is on the examination of the redox properties associated with a *triangular* cluster arrangement of metal ions is seminal, although physicochemical characterisation in solution and solid states will be employed and are available within the infrastructure at Flinders or through collaboration. In addition to the Cu_3 complexes that are significant, the range of metal ions, such as, Zn^{2+} and Hg^{+} which can be incorporated into the complex will be extended to assess the inherent metal ion contribution to both the structural and electronic properties in a triangular cluster. It was anticipated that the T2 sub-site differentiation built into the model system to provide one copper site containing a more accessible reduction potential than the remaining binuclear copper pair. In addition, comparison with well documented binuclear metal tacn complexes will assist in the dissemination of contributions for the 'T3 and T2' sites for the model complexes.

Model Complexes as Probes for Oxidase activity

The model complexes are good probes for enzymic activity and an important objective of this research project are the reactivity studies. In particular, the reactivity of these model complexes towards small molecules. There is a precedent for this approach, as numerous examples of peroxide, azide, oxo and hydroxy complexes have been prepared with simple mono-, and bi-nuclear coordination complexes; however, only the μ_3-oxo group has been reported with a *triangular* metal arrangement. A specific aim is to determine the significance of the asymmetric binding site with respect to the manner in which coordination of the 'substrate' occurs between the bbz *vs.* tacn moieties (see Figure 2). The binding of substrate molecules to the cluster will involve partially reduced intermediates. Clearly, cyclic voltammetry and spectroelectrochemistry are the ideal techniques to detail the interaction of transient species in solution. We have a BAS 100B electrochemical analyser available for these studies and plan to purchase a Spectroelectrochemical cell suitable for aqueous and non-aqueous studies. This approach should provide valuable insights as to the role of the triangular structural cluster as the catalytic unit.

Direct Electrochemical Characterisation of laccase

Rhus vernicifera laccase is commercially available through Saito & Co, Japan, as an acetone precipitated powder from Japanese lacquer tree (*Rhus vernicifera*) (US$800/kg, minimum order 3 kg). The absorption and epr spectra of laccase has been studied in some detail. In particular, a comparison of laccase with haemocyanin has addressed some aspects of the underlying geometry and electronic structure associated with the T3 centre. In stark contrast, there is a paucity of electrochemical information on the thermodynamic and kinetic basis of laccase function. The dramatic spectral changes (loss of intensity at λ = 600 nm) should return as the electronic relay to the trinuclear cluster occurs. Cyclic Voltammetry and spectroelectrochemistry will effectively probe these redox steps. Laccase shows remarkable stability in non-aqueous solvents that will assist in unravelling the expected proton coupled electron transfer processes.

Electron exchange within the cluster is expected to be fast as will the reduction of bound substrate. Creating an environment where the electron transfer is slowed will assist in our analysis of the enzymic mechanism. Both the T1 and T2 sites in laccase are labile and this offers the prospect of varying the electronic driving force for oxygen reduction. One way in which this lability can be exploited is to produce the T2 depleted (T2D) sample by extraction N,N-Diethyldithiocarbamate. Reconstitution of T2D laccase using copper ions has been successful, although similar attempts using non-copper metal ions have not been reported for the apo-laccase. Electrochemical studies for the T2D sample in the presence of non-copper transition metals ions will illuminate the role played by the T2 centre and perhaps reveal subsite selectivity eluding to the functional role for copper for the enzymes catalytic turnover.

Recent work by Dr Martin in the area of protein electrochemistry detected 'gated' proton transfer coupled to electron transfer process. This process was observed at the 'triangular' Fe_3 moiety of [3Fe4S] ferredoxins. This observation leads us to speculate that the unusual 'triangular' arrangement of metal ions found in Blue Copper Oxidases may facilitate the movement of protons towards the substrate resulting in the reduction of oxygen.

Methodologies for Protein Studies at Electrode Surfaces

Application of multi-disciplinary approaches to both research and technology advancements has now become well established. In the area of protein research, electrochemistry and scanning probe microscopy have been introduced and have established “track records” for elucidation of metalloprotein structure, reactivity and function. This technique has been successful in our hands and we are well placed for the purification, immobilisation of redox proteins using direct electrochemistry. Our experience with direct electrostatic attachment of the protein onto the electrode surfaces is employed as is the emerging methods and new strategies for obtaining a direct protein electrochemical response for large enzymes and proteins, such as, laccase. The aim of this strategy is to provide a combination of conditions in which the protein's tertiary structure can be maintained together with the structure of the redox active components under investigation. An advantage of direct electrochemistry is that the interaction at the electrode surface is sensitive to environmental conditions such as, pH or temperature which provides data as to the thermodynamic and kinetic basis for proton transfer and electron transfer reactions.

Electrospray techniques are essential for the characterisation of both biomimetic model complexes and the laccase protein samples and this is available through the AWRI.

Results & Discussion

Project A: Biomimetic Model Complexes for the active site of laccase

The aim during the initial phase of the pilot research project was to achieve the synthesis of a 'symmetric' model complex of the copper containing metalloenzyme laccase. This together with the physicochemical characterisation of several metal complexes has been achieved and the data confirming this new material is presented in this report.

Synthesis of a 'Symmetric' ligand to model the copper active-site of laccase

The synthesis and characterisation of zinc model complexes for this project have been developed using a synthetic route in which a range of sub-site differentiated ligands can be developed. During the earlier stages of this project several major synthetic problems were identified, and these were mostly associated with preparing ligands containing a range of different functional moieties. The synthetic strategy was found to be sound, and progress during the first year indicated that the synthesis of the 'first generation' laccase model complexes, was viable.

The synthetic scheme is outlined in Figure 3 and illustrates the reaction scheme used to prepare the new molecule that has the propensity to form tri-angular trinuclear complexes. This feature is unique to the class of Blue Copper Oxidases (BCO) that includes laccase. Confirmation of the ligand structure was provided by the ^{13}C NMR technique and for the first target molecules, L1, contains 9 resonances. On reduction of L1 the sample gives a simpler spectrum for the L2 ligand that is characterised by only 6 carbon signals. This confirms the highly symmetric nature of the new ligand.

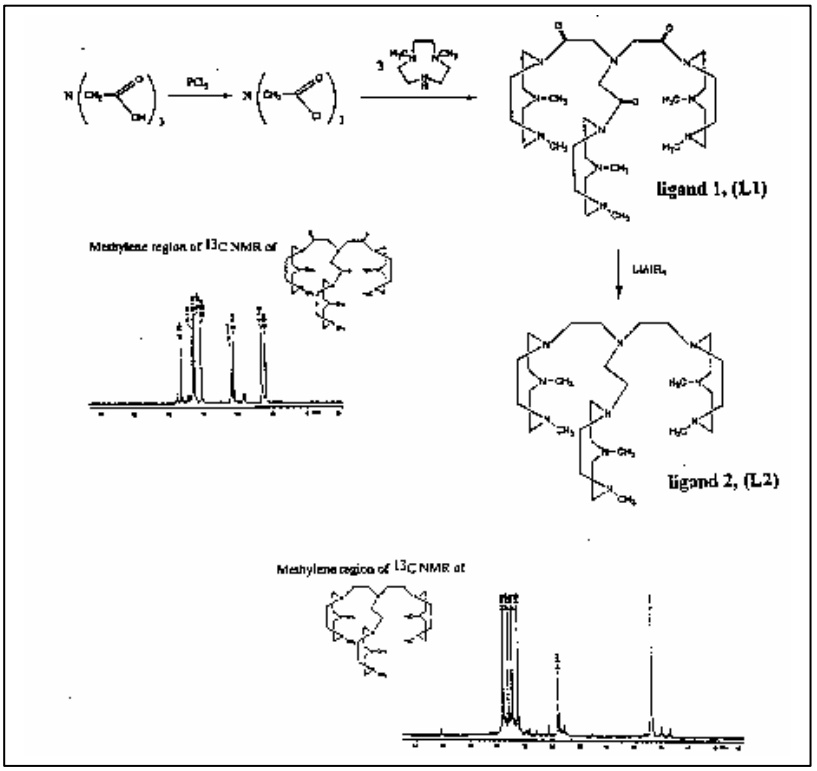


Figure 3: Synthesis & ^{13}C NMR data for Symmetric Ligand (L2)

Characterisation of a Zinc complex of L2

The metal binding properties of our new ligand were determined using NMR as the technique of choice. This technique requires the use of a diamagnetic probe and therefore zinc was selected instead of the paramagnetic copper in order to characterise the complex structure in solution.

Zinc perchlorate was added to a solution of deuterated water containing the ligand L2 and the NMR spectrum recorded. ^{13}C NMR data for the ligand and the complex formed with zinc perchlorate were as expected. All the carbon signals were shifted upfield which is significant and consistent with the binding of the metal to the ligand, L2. Furthermore the binding of the metal is occurring in a symmetric manner as illustrated by the small number of carbon resonances in the spectrum.

Our research was directed specifically to achieving the triangular coordination environment found for the enzyme laccase. Initial studies employed the acetate group to encourage bridge formation between the metal ions. Acetate has been shown to be effective as a bridge due to its propensity to spontaneously self assemble and bridge metal ions forming bi-nuclear complexes. The results obtained for these experiments using NMR together with Electrospray Ionisation Mass Spectrometry (EIMS) at the AWRI. The NMR data indicate that a symmetric coordination environment has been achieved, with only six resonances present in the spectrum, each that can be ascribed to the ligand, L2. In addition, the EIMS data collected at the AWRI identify and confirm that a complex at the mass expected for a tri-angular trinuclear complex is present in the sample. This information is obtained from calculations suggesting that a Parent ion for the simplest complex will occur at m/e 1137 mass units. The spectrum indicates that the ion of highest mass that can be identified in the solution occurs at 1139; only two mass units apart.

The accumulative data presented so far supported our research approach and confirms that we have prepared a new symmetric ligand that offers the tri-angular coordination environment at the active site of the metalloenzyme, laccase. The studies using the zinc metal ion as a NMR probe provides evidence that the ligand, L2 will form a coordination complex and that this complex will react with small molecules. The success of our strategy provided encouragement that the project will succeed as indicated in the original research proposal.

Part II:

The strategy of designing biomimetic model compounds for the trinuclear copper arrangement at the active site for laccase was continued using molecules that were able to spontaneously self-assemble to form triangular copper complexes. Specifically in this approach, we aimed to characterise the interaction of di-oxygen (or other small molecules) with the three copper atoms in order to gain an understanding of the relationship between the structure of the laccase with its reactivity with oxygen and hence its function *in vivo.*

We prepared the compound, $[Cu_3PAD_3(\mu\text{-}OH)](CF_3SO_3)_2$, which illustrates well the strong desire to form an oxygen atom bridge between the three copper atoms. Characterisation using EIMS, NMR, UV-vis-NIR absorption and infrared spectroscopy were used to elucidate the charcteristics of the complex in both solid and solution states. In addition we were able to grow crystals of X-ray quality needed to define the molecular structure. Furthermore, we achieved the X-ray structure determination for this copper complex that showed the presence

of the anticipated triangular arrangement of atoms, about a coordinated oxygen molecule; see Figure 4.

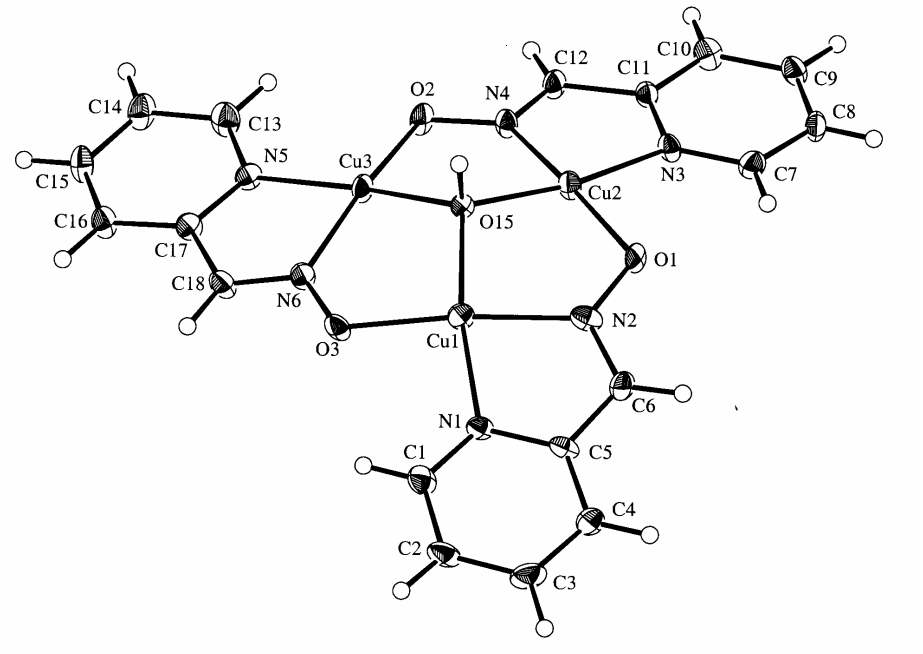


Figure 4: Molecular Structure of the Model Compound, $[Cu_3PAD_3(\mu\text{-}OH)](CF_3SO_3)_2$ for laccase showing the triangular arrangement of copper atoms.

Oxidation and the Browning Reaction

In addition to the techniques already described to characterise the model compound, $[Cu_3PAD_3(\mu\text{-}OH)](CF_3SO_3)_2$ for laccase, we used the electrochemical technique of cyclic voltammetry to examine the behaviour of the model compound under oxidation and reduction conditions. The preliminary data showed that several oxidation processes were possible for this model compound, Figure 5. The redox profile for $[Cu_3PAD_3(\mu\text{-}OH)](CF_3SO_3)_2$ compound was altered after treatment with some small molecules. When boron trifluoride was added to the model compound, the first of these oxidation processes ceased to occur, however, addition of another small molecule, thiocyanate to the model compound did not remove this first oxidation process. Figure 6 shows the redox response for the $[Cu_3PAD_3(\mu\text{-}OH)](CF_3SO_3)_2$ complex after treatment with boron trifluoride. There is a clear change in the redox response for the molecule suggesting that the boron trifluoride has in some way modified the copper metal region of the molecule. Studies are continuing to explore these processes and to define the characteristics of the interaction of a small molecule with the triangular arrangement of copper atoms found in laccase.

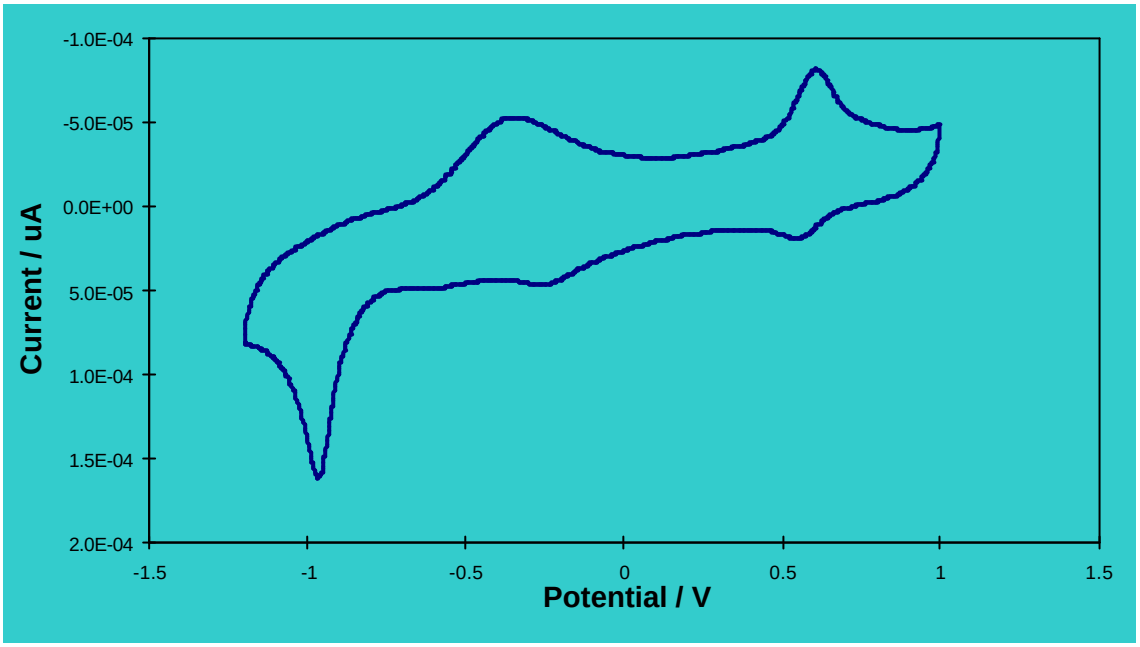


Figure 5: Cyclic voltammogram for the untreated $[Cu_3PAD_3(\mu\text{-}OH)]^{2+}$ showing three clear reduction and two oxidation processes are present.

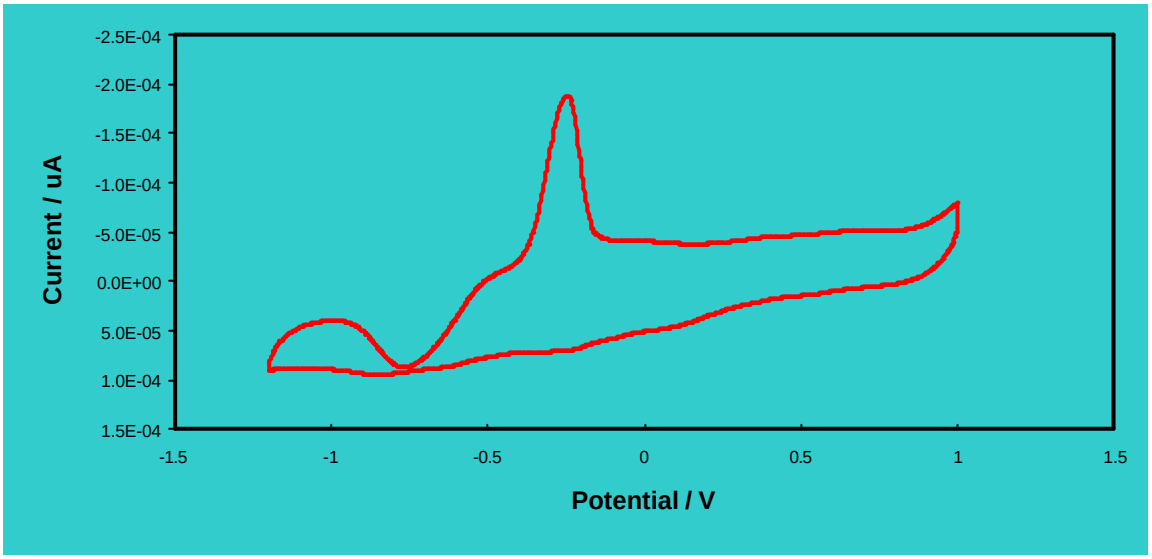


Figure 6: Cyclic voltammogram for the $[Cu_3PAD_3(\mu\text{-}OH)]^{2+}$ complex treated with boron trifluoride and indicating the loss of the three reduction processes. Only one clear oxidation processes is now present.

PROJECT B: *Rhus vernicifera* laccase

In the next stage of this project the use of a readily available source of laccase from the Japanese lacquer tree, *Rhus vernicifera* to explore the molecular interactions that we observed using model complexes with a protein sample for laccase. The focus of the new approach remains on the development of a simple treatment for vignerons to use for *Botrytis cinerea* affected juice or must. Initially we developed a purification protocol for the laccase and

characterised the enzyme using SDS-PAGE and 2D-gel electrophoresis, enzyme activity assays and determined the pH and reactivity profile for the enzyme. As we are one of only a few laboratories in Australia who have developed direct protein electrochemical methods for measuring the reactivity profile of metalloproteins adsorbed onto an electrode surface, this was the technique of choice for these studies.

In particular we monitored the reactivity of laccase using an electrochemical method, cyclic voltammetry. The value of this technique is that it is sensitive to oxidative (redox) reactions and in addition to how they are occurring – both these facets contribute essential data needed to design a molecular control mechanism for laccase. These results indicated that indeed small molecules can bind to the copper atoms and that this is a viable strategy to use for the laccase enzyme directly.

Laccase purification and characterisation

Treatment prior to Purification

Laccase protein from the plant *Rhus vernicifera* was purchased as a crude acetone precipitate and required extensive purification. Initial reconstitution of laccase acetone precipitate powder was achieved by suspension in 0.1 M potassium phosphate buffer, pH 6. Previous studies used 0.01 M potassium phosphate buffer, pH 6. Comparisons were made between the visible appearance of the reconstituted powder in 0.01 M buffer, used in previously described methods, and in 0.1 M buffer. The visual appearance of the filtered solution reconstituted in 0.01 M buffer was browner than that reconstituted in the 0.1 M buffer, which was significantly greener in appearance, suggestive of the presence of more blue coloured proteins. This also might suggest that either the plant pigments present in the acetone powder are less soluble in the more highly concentrated buffer, or the laccase was more soluble in the 0.1 M buffer. Precipitated laccase was later found to be readily soluble in pure water, therefore the earlier suggestion is more likely.

The reconstituted acetone precipitate powder was very turbid and contained large quantities of solid material. This was filtered in several batches through a 0.45 mm filter membrane. The filtrate was then concentrated from 30 mL down to 4.5 mL

Gel Filtration Chromatography

Gel filtration chromatography separates macromolecules according to their size. These media work primarily on an exclusion principal where a protein size that cannot penetrate the bead space of the matrix is eluted in the void volume of the column.

The concentrated laccase solution was applied directly to the top of the gel filtration column matrix. Glycerol was used as a loading medium for the impure laccase solutions and was found to have no effect on the catalytic activity of laccase. The addition of 10% glycerol to the raw laccase solution yielded a more dense liquid which, when applied directly to the top of the gel matrix, settled through to the bottom of the buffer layer on top of the gel matrix with minimum diffusion into the buffer (see Figure 7). This resulted in a clearly visible band of sample following adsorption onto the column and ensured better resolution as the spread of sample was kept to a minimum.

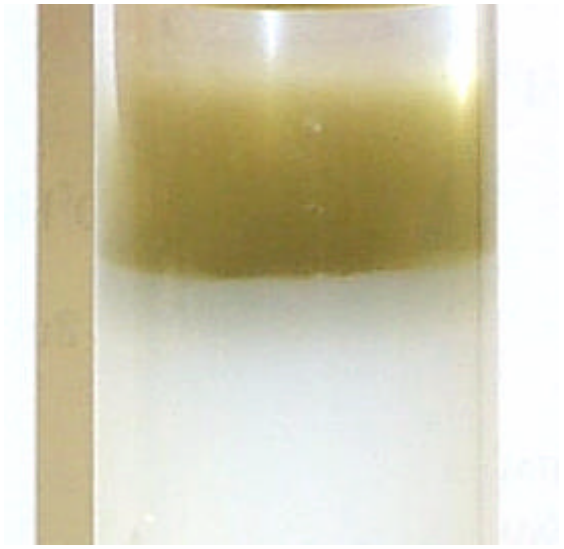


Figure 7: Reconstituted laccase on a Gel filtration column

The filtrate from the 0.1 M solution produced very defined bands, visible as three colours on the gel filtration column. Immediately after the load moved into the gel matrix, three distinct bands were formed. The first band was quite brown and showed no presence of protein on a PAGE gel. Close behind this band was a blue band that showed “laccase activity” (Figure 8). Further behind yet was a blue-green band that had “no laccase activity” and represented the Blue Copper protein, stellacyanin.

Gel filtration chromatography of the 0.01 M buffer solution showed much less resolution between the different coloured bands on the column. This overlap also made it more difficult



Figure 8: Gel column showing the blue laccase band.

to identify the laccase band using UV-Vis spectroscopy. The absorbance peak at 280nm formed by the pigments in solution trailed off through most of the visible spectrum, making detection of the Type 1 copper absorption at 600nm difficult to observe.

The presence of laccase in the eluent was confirmed using a syringaldazine activity assay and also SDS PAGE gel electrophoresis. Every fifth aliquot was subject to gel electrophoresis. Coomassie blue stain was sufficient to detect laccase bands in the PAGE gels, as can be seen in Figure .

The PAGE gels (Figure 9) revealed protein bands at approximately 110kDa, 70kDa and 30kDa. Previous studies reported the 110kDa protein, which shows laccase activity, the 30kDa protein, which was shown to be stellacyanin, however these workers omitted, or did not observe, the 70kDa protein. The elution profile of unpurified reconstituted laccase from a cation exchange column presented in the original purification procedure seems to disprove the presence of another protein. However there were no PAGE experiments run on all the column eluent aliquots in this early procedure, as has been done in this study.

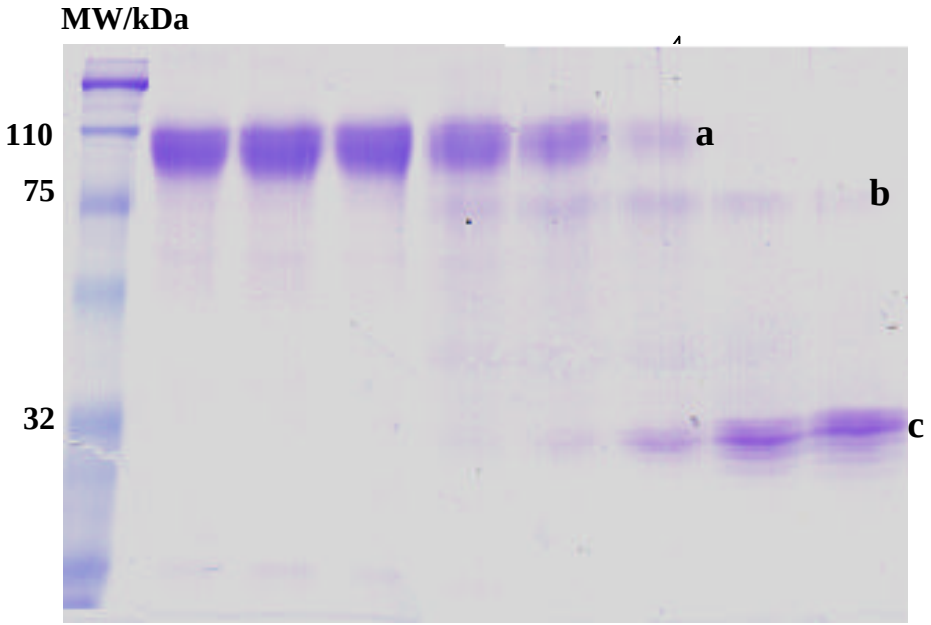


Figure 9. PAGE gel showing elution profile of gel filtration of raw laccase solution. The bands are (a) laccase, (b) unidentified protein, (c) stellacyanin.

Isoelectric Focusing

Isoelectric focusing is an electrophoretic technique that incorporates a pH gradient set up between a cathode and anode, with the cathode at higher pH than the anode. Proteins have amphoteric properties due to the presence of amino acids. At a pH below its isoelectric point a protein will be positively charged, whilst above it will be negatively charged. Therefore, a protein can migrate toward its isoelectric point. Through the use of carrier ampholytes, a pH gradient can be established within a gel matrix by applying an electrical potential. The protein will then migrate toward and 'focus' (concentrate) at its isoelectric point.

Laccase isolated from *Rhus vernicifera* has two reported isoelectric points of 8.55 and 9.28. An isoelectric point of around 8.5 was observed for laccase, as well as another point at around 5.0, which has not been reported for *Rhus vernicifera* laccase. Isoelectric focusing experiments on the laccase sample from the gel filtration purification further confirmed the

presence of a different protein. There were three isoelectric points observed of ~7, 6 and 4 for the protein observed at 70kDa.

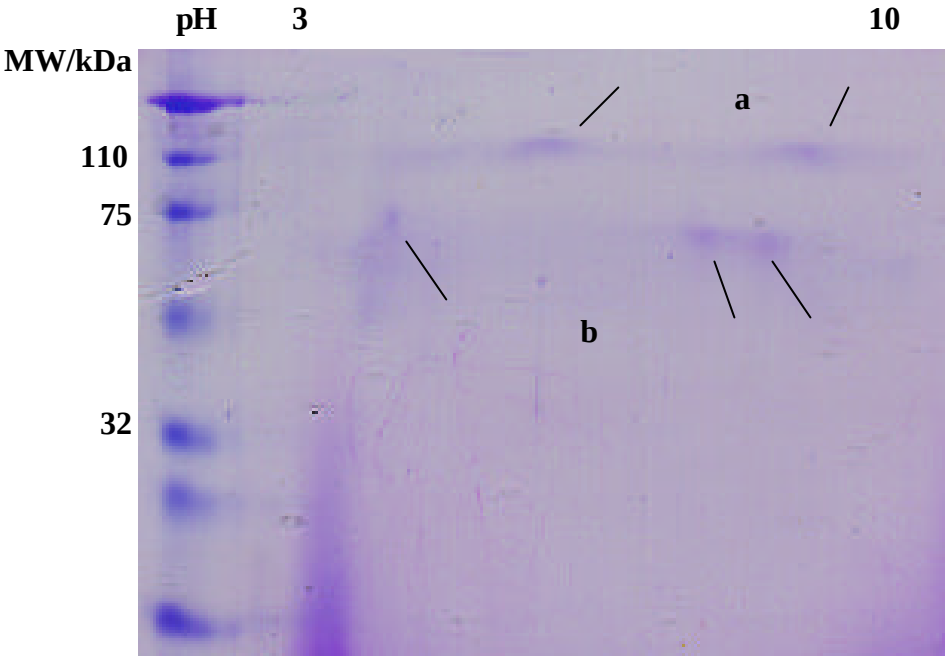


Figure 10: 2-D SDS PAGE gel from isoelectric focusing showing (a) isoelectric points for protein at 110kDa, (b) isoelectric points for protein at ~ 70kDa.

Cation Exchange Chromatography

The results of the isoelectric focusing experiments clearly showed it was necessary to carry out a further purification step. Cation exchange chromatography, run at pH 7, was selected as the most appropriate method for two reasons. Firstly, the isoelectric focusing experiment showed that laccase would be positively charged at pH 7. Cation exchange columns bind positively charged molecules. Secondly, the 110 kDa protein that showed an isoelectric point of 5 would be negatively charged at pH 7 and would not bind to the column. The 70 kDa protein could be avoided simply by combining only aliquots from the gel filtration column that showed presence of laccase on a SDS-PAGE gel.

Aliquots from the gel filtration column that showed laccase activity, but did not show evidence of protein at 70 kDa on SDS-PAGE, were pooled and concentrated to a volume of 3-4 mL, and taken into 0.01 M potassium phosphate buffer, pH 7. This reduced the ionic strength of the solution. This sample was applied to a strong cation exchange column where a blue band formed at the top of the column (Figure 11). A yellowish fraction passed directly through the column and was found to be pigments and tannins. The buffer concentration was then increased to 0.1 M whereby the blue band began to elute from the column. This band had laccase activity and appeared as a single band on the SDS-PAGE gel.

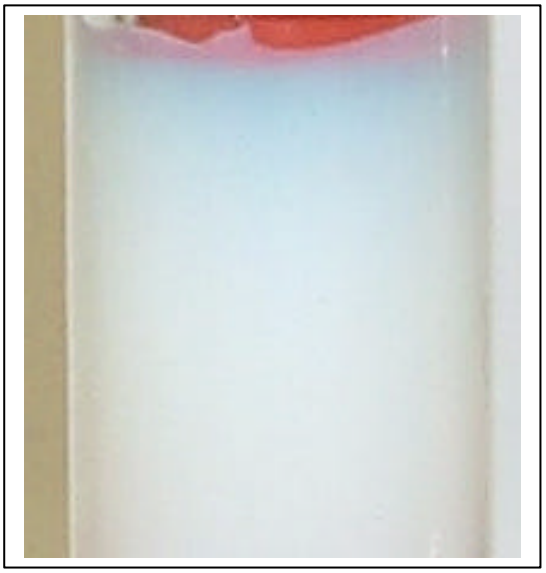


Figure 11: Cation exchange column showing laccase binding as a blue band.

Enzyme Activity Assay

Syringaldazine is used as a direct measurement of laccase activity, denoted by the presence of a pink-purple oxidation product. It is used within the wine industry to measure units of activity of laccase – the NOVO test. Although it must be emphasised by the authors that other chemicals and enzymes can just as readily oxidise syringaldezine. The reaction for the oxidation of syringaldazine is shown in Figure 12.

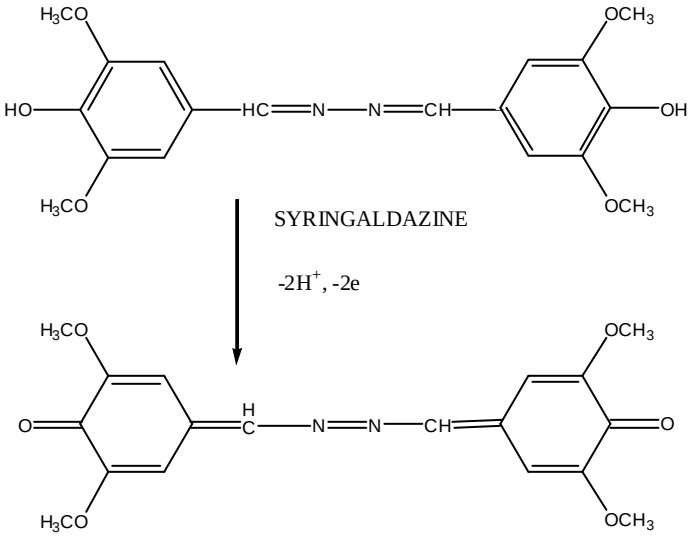


Figure 12: Oxidation reaction of syringaldazine.

In these experiments syringaldazine was used as a means of fast colurmetric detection for the presence of laccase (see Figure 13). This was important in procedures such as concentrating of protein where the condition of the filter membrane could be monitored by assaying both the filtrate and the concentrate for enzyme activity.

Syringaldazine was also used to assess the inhibitory effect of azide on laccase. The oxidation of syringaldazine by untreated laccase was monitored through following the absosrbance of the oxidation product at 530nm as shown in Figure 14.

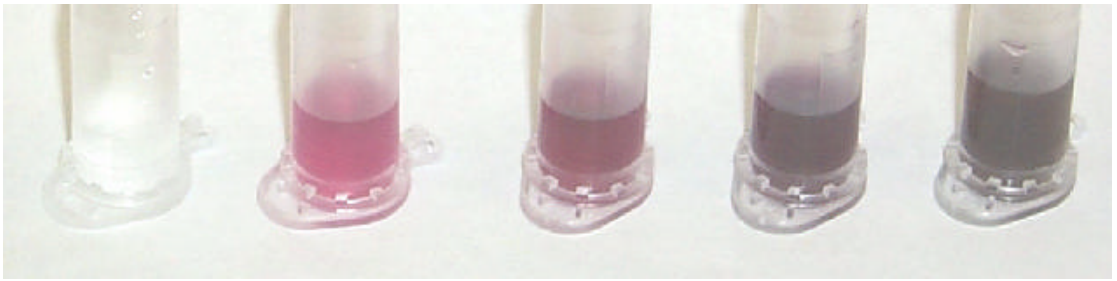


Figure 13: Colour change observed from 0 – 15 min for the oxidation of syringaldazine by laccase.

Upon treatment with excess azide the activity of laccase was observed to drop markedly, although not completely. This was unusual as other workers had reported that inhibition was complete after incubation for 2-10 min.

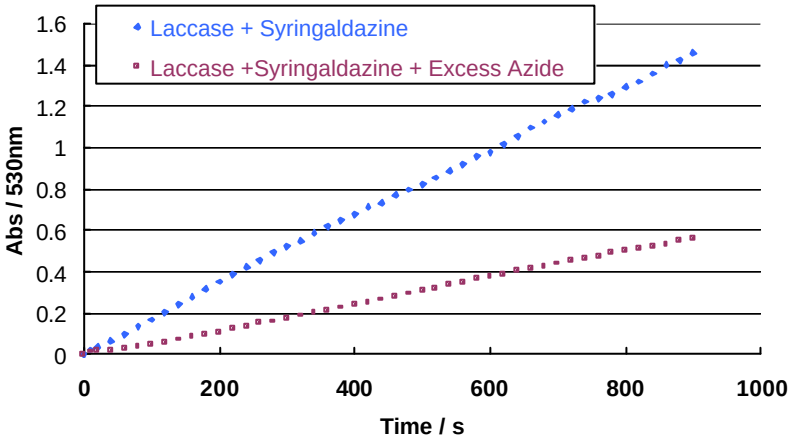


Figure 14: Effect of azide on activity of laccase.

Experiments are designed to probe laccase from *Rhus vernicifera* using our protein electrochemical protocol and finally to find an appropriate gaseous molecule that will bind and thereby prevent the oxidative activity of the laccase enzyme. These results will then be used to develop trials with *Botrytis cinerea* infected wine samples, thus addressing the objective in this study.

Direct Electrochemistry of Laccase

Electrochemical Experiments

Electrochemical techniques are used widely to characterise the redox properties of metal centres. Cyclic voltammetry involves application of a potential through a working electrode that is swept linearly with time, forwards and backwards, between two potential limits. The

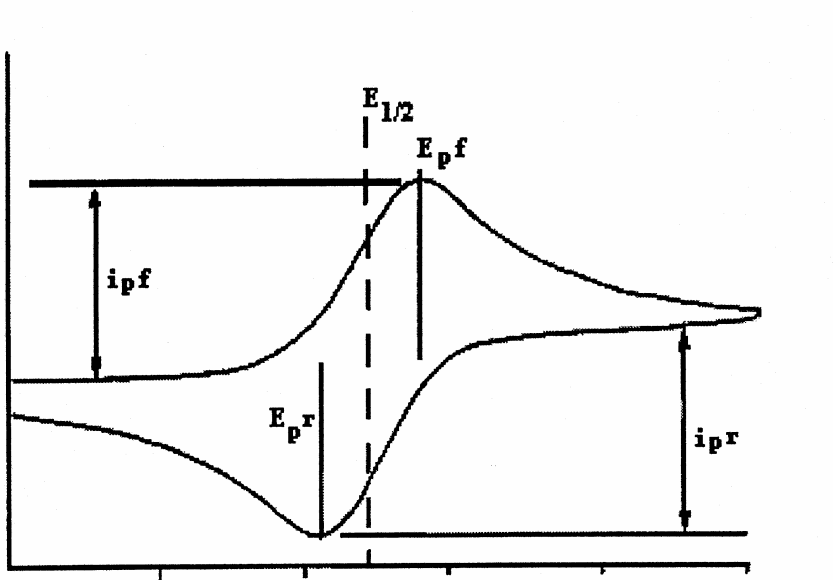


Figure 15: Typical cyclic voltammetric response for a reversible process.

current is monitored as a function of potential, and wave maxima occur as a result of oxidation or reduction of the redox active species. A process is considered reversible if the rate at which the species is scanned does not affect the current maxima. Figure 15 shows a typical cyclic voltammetric response for a reversible process.

A process is also reversible if the difference between the peak potential of the forward curve ($E_p f$) and the peak potential of the reverse curve ($E_p r$) is equal to 59/n mV. A process may be considered quasi reversible if $E_p f - E_p r$ increases as the scan rate increases, and if the peak current for the reverse reaction ($i_p r$) divided by the peak current for the forward reaction ($i_p f$) is approximately 1.0. The process is classed as irreversible if neither of these criteria are met.

Preliminary Electrochemical Experiments

Previous electrochemical studies of laccase have shown that no signal is produced at the glassy carbon, pyrolytic graphite and gold electrodes. This was confirmed in preliminary experiments in this study. A response from laccase may be absent due to strong adsorption of the protein to the electrode resulting in denaturisation and loss of activity. This may be so with the pyrolytic graphite electrode as the charge on the surface is negative because of oxidation of the 'edge-on' surface. At a pH lower than that of the isoelectric point of the protein, the net charge of the protein is positive, allowing it to adhere strongly to the negatively charged electrode surface. It may also be as a result of the relatively bulky size of the protein (110kDa) when compared with smaller proteins such as plastocyanin and ferredoxin (~10-30 kDa) which have been shown to give an electrochemical response. The larger size may mean the protein simply falls off the electrode as the electrostatic interactions are not adequate to hold the protein on the electrode surface.

There was also no electrochemical response observed in experiments that made use of the promoters poly-L-lysine and polymyxin B sulphate. These promoters have been shown to co-adsorb proteins such as ferredoxin and plastacyanin at the electrode surface, and have been used to successfully facilitate direct electron transfer in these redox enzymes.

The lack of response from laccase at the electrode surface prompted investigation into alternate methods by which to optimise interaction between electrode and enzyme. The use of self-assembled monolayers to immobilise large enzymes covalently to an electrode surface had resulted in successful direct electrochemistry of the enzyme. The following results are presented using 3-mercaptopropionic acid (MPA) as the immobilising agent.

Direct electrochemistry of laccase immobilised on the SAMs

All electrochemical experiments were carried out under anaerobic conditions. Direct current cyclic voltammograms of the laccase immobilised onto the SAM of a MPA-modified gold electrode in 50mM potassium phosphate buffer at pH 7 showed well-defined electrochemistry in the scan range 25 – 500 mVs^{-1}. Electrochemical response varied slightly between electrodes, however, in most experiments, there were at least two oxidation waves present at all pH values, and usually a broad single reduction wave. In some experiments three oxidation waves were observed and an additional reduction wave associated with this third wave also observed. Occasionally there was only one wave present in the initial scan of a freshly prepared electrode. However, when the scan rate was reduced to 10 mVs^{-1}, a cyclic voltammogram recorded, then the scan rate increased, the second oxidation wave, and sometimes a third oxidation wave, became visible. There was still usually only a broad reduction wave associated with the first two oxidation waves.

A response of more than one wave in direct current cyclic voltammograms of laccase has not been reported previously. The electrochemistry of laccase already reported shows only weak responses (usually in the 2-8 μA range) at a gold electrode and a pyrolytic graphite electrode compared to those described in this study.

pH dependence studies of the direct electrochemistry of laccase

The electrodes showing three oxidation waves in the cyclic voltammograms were then used in pH dependence studies. The direct current cyclic voltammograms of the immobilised laccase under different pH conditions was recorded. pH conditions were adjusted by removing the buffer from the cell and replacing it with that of an appropriate pH.

The first oxidation wave and its associated reduction wave were present in all scans under all conditions. Usually, at pH 7, the second oxidation peak was quite small and its associated reduction wave was undefinable, as it appeared to merge with the first reduction wave. The third oxidation wave and reduction wave was reasonably well defined, when present.

Scans run at pH 4 also showed well-defined electrochemistry in the scan range 25 – 500 mVs^{-1}. The initial scan at pH 4, run at a scan rate of 100mVs^{-1}, did not immediately show any change in peak current or potential. After 2-3 cycles the intensity of the second oxidation wave increased. The two merged peaks within with the broad reduction wave associated with the first two oxidation waves also became more defined.

Scans run at pH 9 are shown in Figure 16. These showed a well-defined third oxidation peak, similar to that at pH 4, but there is an overall shift toward a higher potential associated with this peak and its complimentary reverse wave. The second oxidation wave shows considerable flattening and also a shift to higher potential. The peak current associated with the first wave has also been reduced in intensity.

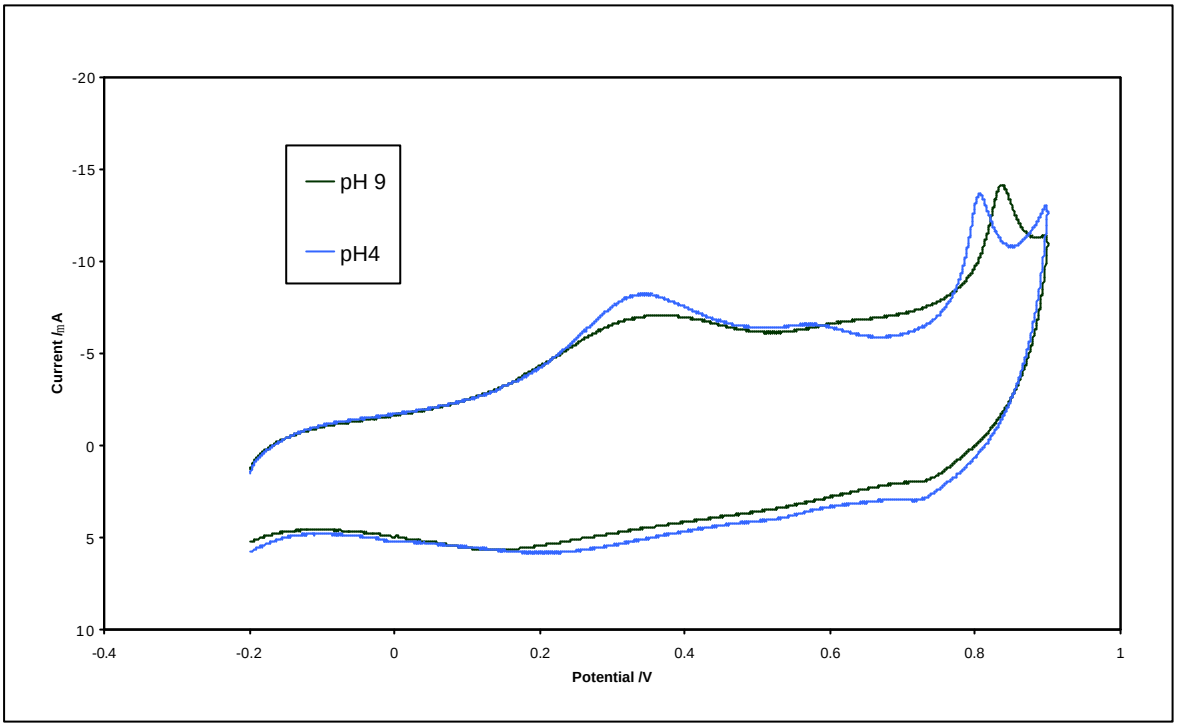


Figure 16: Effect of pH on electrochemical response of laccase immobilised on an MPA-modified gold electrode.

When the buffer was altered back to pH 7 or pH 4 cyclic voltammograms similar to those described above were once again observed. This process of altering pH could be repeated several times before the enzyme began to lose activity. In a one-off experiment, the electrode was left in buffer at pH 9 and room temperature over night. An initial scan gave no response, however, after repeating the scanning process and adjusting to pH 4, two anodic and one broad cathodic peaks were observed.

These observations at varying pH values may possibly be attributed to an alteration of the coordination environment about the copper atoms at the active site. Laccase has been shown to increase its catalytic activity at lower pH values and has a catalytic optimum around pH 4. It has been shown that the hydroxy ligand on the T2 copper must be absent for catalytic turnover to occur. If there is a low proton concentration it may be expected that loss of the hydroxyl in the form of water is much slower. Conversely, if the proton concentration is high, the ease of which electron transfer can occur increases. Given these requirements, a decrease in peak current for the T2 site may be expected due to a slower electron transfer rate. However, the disassociation of a ligand to a redox active species may also result in an alteration in the potential of that species. Since there is both a change in peak current for one peak and a change in potential for the other peak it is difficult to assign the second or third oxidation waves to either the T2 or T3 copper centres using the results from the pH studies.

Effect of inhibitors of laccase on the direct electrochemistry

The electrodes displaying three oxidation peaks were also used in inhibition studies. The direct current cyclic voltammograms of the immobilised laccase with the addition of excess azide were recorded at pH 4, as this gave the best resolution of the three peaks. Azide was added directly to the cell and incubated for 10 minutes at room temperature. The cyclic

voltammograms showed a reduction in intensity of the anodic peak currents for all three peaks and also reduction in intensity of the third cathodic peak. When the azide-containing solution was replaced with fresh buffer, after several cycles at 100mVs-1, the original response was restored.

The significant reduction in peak current observed for the third oxidation wave suggests evidence of an interaction between the azide anion and this copper site. EPR spectroscopy studies of the interaction of azide with the laccase trinuclear copper centre have shown a reduction in the signal associated with the T2 copper(II). In these studies it was suggested there is an equilibrium between the three copper(II) atoms in the trinuclear centre. These observations from the EPR studies, along with the significant perturbation of the third oxidation wave, lead to the tentative suggestion that the third wave can be assigned to the T2 copper site in laccase. The shift in potential with changing pH conditions may be attributed to the presence or absence of a hydroxy ligand, which further supports this assignment in these preliminary experiments.

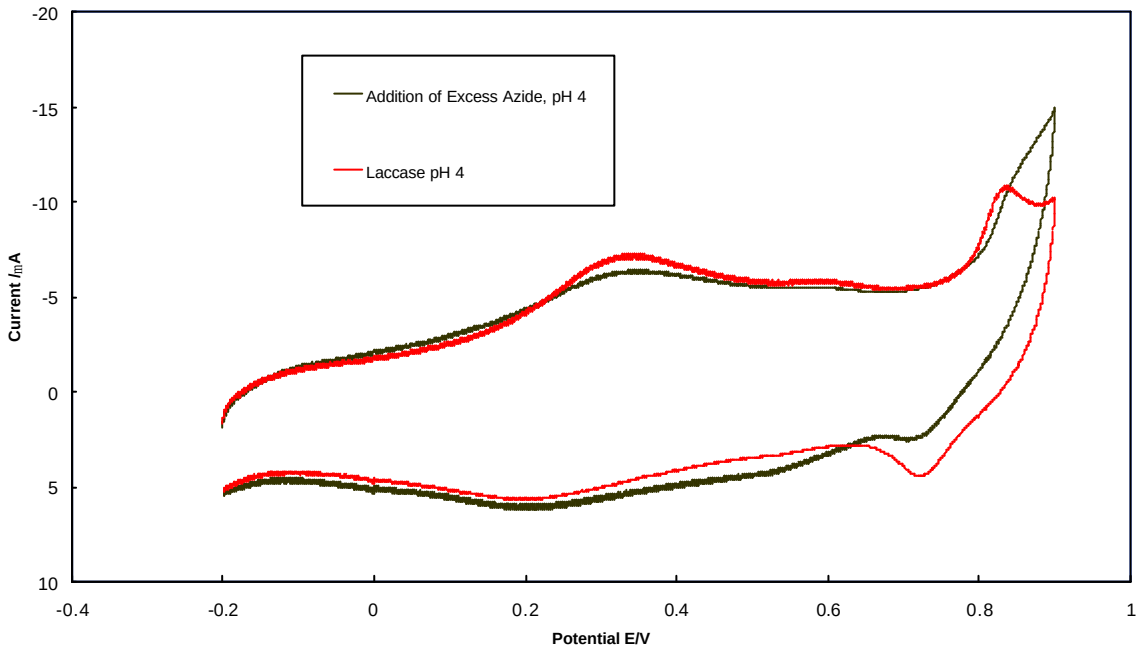


Figure 17: Effect of azide on electrochemical response of laccase immobilised at an MPA-modified gold electrode.

Conclusions

In conclusion, the first ever multi-electron response from the direct electrochemistry of laccase has been achieved. The use of an MPA-modified flat gold electrode to immobilise laccase onto the electrode was found to significantly enhance enzyme response to direct current cyclic voltammetry. This simple method does not produce alterations in the protein's functional properties, and appears to be flexible enough to allow the protein to settle or adjust, resulting in multi-electron transfer. There was shown to be a variation in potential of the second and third waves with different pH conditions, supportive of evidence that laccase

activity alters with pH. In addition, there was also shown to be a significant perturbation of the response of laccase that had been treated with the known inhibitor azide.

Modifications to a well-known purification method for *Rhus vernicifera* laccase have also been developed resulting in the reduction of the number purification steps. This procedure modification also enabled the discovery of a previously unreported protein.

Future Directions

The removal of the T1 and the T2 copper sites in laccase has been used in many reactivity and spectroscopic studies of Blue Copper Oxidases. These investigations of the voltammetric response of Type 1-depleted and Type 2-depleted laccase are continuing funded by University research awards and will provide further insight into the mechanistic aspects of these copper sites.

One of the aims of the larger project that this study supports is to find an inhibitor for laccase. Similar investigations to the azide binding experiments need to be performed using molecules that can be classed as food additives. These could potentially include nitric oxide or nitrous oxide. We anticipate that this research work has provided an important contribution to the knowledge of how laccase oxidises phenols and thus have moved toward the development of a treatment for laccase infected wines.

Summary of Key Outcomes

Our pilot study was able to design, prepare and characterise synthetic "model complexes" for the unique triangular copper centre of laccase. Treatment of these model complexes with small gaseous molecules, such as BF_3, enabled us to show that copper-atoms such as those in laccase, would bind small molecules and this caused the properties of the coppers to be modified.

In our more recent research work, the use of a commercially available laccase from *Rhus vernicifera* was explored as a protein model for *Botrytis cinerea* laccase. Purification protocols for laccase were developed as existing methods proved unsuccessful and confirmation of pure laccase was obtained using SDS Page-gel electrophoresis. In addition, this technique confirmed the molecular weight of laccase from *Rhus vernicifera* is similar to that published and is thus a good protein system in which to compared to *Botrytis cinerea* laccase. The laccase activity was checked using the Novo test which has severe limitations as it is oxidised by a wide range of enzymic and non-enzymic chemicals likely to be found in wines. However, until a more suitable method can be found, we used this test to indicate that the protein was active.

We employed electrochemical techniques to determine the manner in which *Rhus vernicifera* laccase participated in oxidative reactions. This had an advantage that only nano-molar amounts of sample are required and also has a pico-amp sensitivity. To date, researchers have detected only a single copper type, which is surprising as there are 3 types of copper in laccase (T1, T2 & T3), as shown in Figure 1. Although we had demonstrated this method as an effective detector for redox chemistry with low-molecular weight proteins, such as, adrenodoxins, it was unsuitable for larger enzymes like laccase. Thus, we modified our strategy to include the immobilisation of the laccase enzyme onto a gold surface, thereby

enabling for the first time, three oxidation waves to be observed. This development is significant as it is now possible to treat samples of laccase immobilised in this manner with chemicals and observe the responses. If a particular chemical binds to one of the copper atoms and prevents one of the oxidation reactions from occurring we would observe this in the electrochemical response. If the chemical does not react, we would expect the response to remain unchanged. We applied this new method to explore and define the redox the function of the laccase with small molecules. Time and resources limited this study to investigation of only one potential inhibitor for the oxidative reaction of laccase, that of sodium azide. Initially, the experiment is performed without azide and the same three oxidative processes are seen, see Figure 16. Then, azide is added to the immobilised laccase enzyme and the electrochemical experiment repeated. Little change was observed in the first or second oxidation processes, however, the third process (top RHS) has been dampened and is now quite different, showing that this copper site is the site of azide binding to the 'active site' of laccase.

We anticipate that in the near future it will be possible to probe laccase with a wider range of chemicals in a manner similar to that described here.

These experiments have used laccase from *Rhus vernicifera* and established a method to directly examine the protein electrochemical response *in vitro*. In addition, we have shown that a small molecule will bind at the active site of laccase, thereby preventing the oxidative process of one copper from occurring. These results can be used to develop a Biosensor system to detect laccase in the laboratory. Future developments are also foreseen using laccase isolated from *Botrytis cinerea* infected wine samples, thus addressing the original objective in this study.

Project outcomes related to original aims & ojectives

This project involves a multi-disciplinary approach that aims to control the laccase enzyme by a combination of biochemical control with chemical treatment. This has direct application to the Wine Industry for wine treatment.

Biomimetic model complexes indicated that small molecules, such as, boron trifluoride and hydroxide can coordinate to the active sites of the Blue Copper Oxidases, such as, laccase.

This project has demonstrated using plant laccase that a molecular control for vigneron's to 'switch off' the enzyme laccase is possible and that this can be achieved through molecular coordination, effectively blocking the 'active site' and preventing phenolic oxidation which causes browning and spoiling in wines.

Plant laccase has reprodiuced the reactivity characteristics of laccase in *Botrytis cinerea* affected wines. At the current time *Botrytis cinberea* laccase is not commercially available, however we envisage that our research strategy could be pursued in the future using the fungal laccase.

Dissemination of Project Outcomes - Publications & Conference Papers:

Publications in Refereed Journals:

1 D. Elliot, C.L. Price, L.L. Martin and M.R. Taylor, N-2-hydroxyethyl-N,N-bis[1-methyl-2-benzimidazolylmethyl]aminechloro copper (II) chloride hydrate, *Acta Cryst*, (1996), **C52**, 1421-1423.

2 D. Elliot, C.L. Price, L.L. Martin and M.R. Taylor, μ-Acetato-μ-aqua-μ-hydroxo-bis[(1,4-dimethyl-1,4,7-triazacyclononanyl-κ^3N)copper(II)] Diperchlorate, *Acta Cryst*, (1998), **C54**, 1259-1261.

3 J. Joannou, L.L. Martin and M.R. Taylor, Crystal structure of 2-thiophenealdoxime, C_5H_5NOS, *Z. Kristallogr. NCS*, (2000), **215**, 1-2.

4 D.J. Elliot and L.L. Martin, Crystal Structure of bis(1,4-dimethyl-1,4,7-triazacyclononanyl)-μ-chloro-μ-hydroxodicopper(II) perchlorate, *Z. Kristallogr. NCS*, (1999), **214**, 38-40.

5 Dusan Losic, Sandra Brinkmann, Justin Gooding, Joe Shapter & Lisandra Martin, Electrochemistry of the Blue Copper oxidase, laccase reveals three waves from three copper sites, *Bioelectrochemistry Communications*, (2001) manuscript in preparation.

Conference Papers:

1 D. Elliot and L. Martin, (1995), Blue Copper Oxidases - Modelling the Triangular Active Site, RACI, 10th National Convention, Adelaide

2 D.J. Elliot and L.L. Martin, (1996), Modelling the Triangular Copper Site of Blue Copper Oxidases, RACI Inorganic Division '96 Conference, Townsville, Queensland.

3 J. Joannou & L.L. Martin, (1998) Studies for the Prevention of Red Wine Browning: development of a molecular control for the copper enzyme laccase, Australian Wine Industry Technical Conference, Sydney, NSW.

Oral Presentations:

1. L.L. Martin, (1998) Studies for the Prevention of Red Wine Browning: development of a molecular control for the copper enzyme laccase, Australian Wine Industry Technical Conference, Sydney, NSW.