Laccases in

Higher Plants

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Abstract

The optimum method of screening for laccase in higher plant leaves was ascertained, and the method used to screen a number of plants. Four fruits from trees of the genus *Prunus* were also screened for laccase. A novel laccase substrate, 4-hydroxyindole, was used as the assay substrate alongside other traditional laccase substrates. Laccase was identified in most of the species studied. 4-Hydroxyindole appears to be a universal laccase substrate. However, most of the higher plant laccases identified did not oxidise syringaldazine, a common substrate for fungal laccases. Yield of laccase was found to be significantly increased by treatment with cell wall-degrading enzymes, particularly Ultrazyme-100 for leaves, and Rapidase® Press for fruit. This observation may have important implications in the study of lignification. The identities of all the enzymes isolated were verified as laccases by inhibitor tests. CTAB was found to inhibit all the laccases studied. Also, a novel laccase inhibitor, N-hydroxyglycine, was studied, and found to only inhibit a small proportion of the laccases studied. The type of inhibition in fruit laccases was identified as non-competitive.

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Abbreviations Used

CTAB	Cetyl trimethyl ammonium bromide
DOPA	Dihydroxyphenylalanine
DPO	Diphenol oxidase
NOG	N-hydroxyglycine
PVP	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone
SDS	Sodium dodecyl sulphate
SHAM	Salicylhydroxamic acid

Chapter 1

Introduction

1 Introduction

1.1 Background

1.1.1 History of laccase

Laccase (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2; also known as *p*-diphenol oxidase; *p*-DPO; *p*-diphenolase) was first described by Yoshida in 1883. It was so named because it came from the lacquer-forming sap of the Japanese lacquer tree *Rhus vernicifera*, and although this sap is usually referred to as "latex", it does not originate in laticifers. Yoshida correctly identified the fraction of the sap (or "Urushi") that catalysed the oxidative coupling of catechol derivatives to form the hard black lacquer used as a wood finish. This fraction he termed a diastase (as the term "enzyme" was not in common use at the time), and noted that the presence of oxygen was required for the "drying" of the lacquer.

However, apart from this early classification, and a subsequent purification of laccase from the related trees, *Rhus succedanea* (Bertrand, 1894) and *Melanorrhea usitata* (Keilin and Mann, 1940), there were essentially no further reports of laccases in higher plants for many years. However, laccase has been reported to be widespread in fungi (Mayer and Harel, 1979). Probably the first laccase identified from a fungus was that described in 1897 by Laborde (Mayer and Harel, 1979). Laccase has been found in almost all Basidiomycetes and in a large number of Deuteromycetes and Ascomycetes (Ferrar *et al* 1995), as well as many of the Fungi Imperfecti (Mayer and Harel, 1979). It is only in the last thirty years or so that laccases have been identified in higher plants, and then only in a few (see 1.3 Laccase in Higher Plants). Even study of the *R. vernicifera* laccase was limited until Reinhammar (1970) perfected a method of purification of laccase from the sap of the tree using cation-exchange chromatography, although Kielin and Mann had performed experiments on *R. succedanea* laccase in 1940, and noted the copper content of the enzyme and its carbohydrate content. However, they wrongly stated that the blue colour of the partially purified enzyme was not due to the copper ion.

1.1.2 General Properties of Laccases

Laccase is perhaps the most suitable name for the enzyme under investigation, since the other names listed above all refer to the ability of the enzyme to oxidise *p*-diphenols. However most laccases are able to oxidise a wide range of substrates, including *p*-diphenols, *o*-diphenols, monophenols, triphenols and ascorbic acid (Mayer and Harel, 1979), as well as other aromatic compounds such as *p*-phenylene diamine, syringaldazine and hydroxylated indole derivatives (Cai *et al* 1993b). Therefore the use of these terms is unsatisfactory except for the fact that it shows the relationship of laccase to the closely related *o*-DPO (or catecholase), whilst showing the important distinction. The substrate specificity of catecholases is much narrower than that of laccases; they are only capable of oxidising *o*-diphenols and some monophenols, and this is a general defining characteristic of *o*- and *p*-DPOs. Hence, the term "laccase" will be used throughout this thesis, along with the related term, "catecholase".

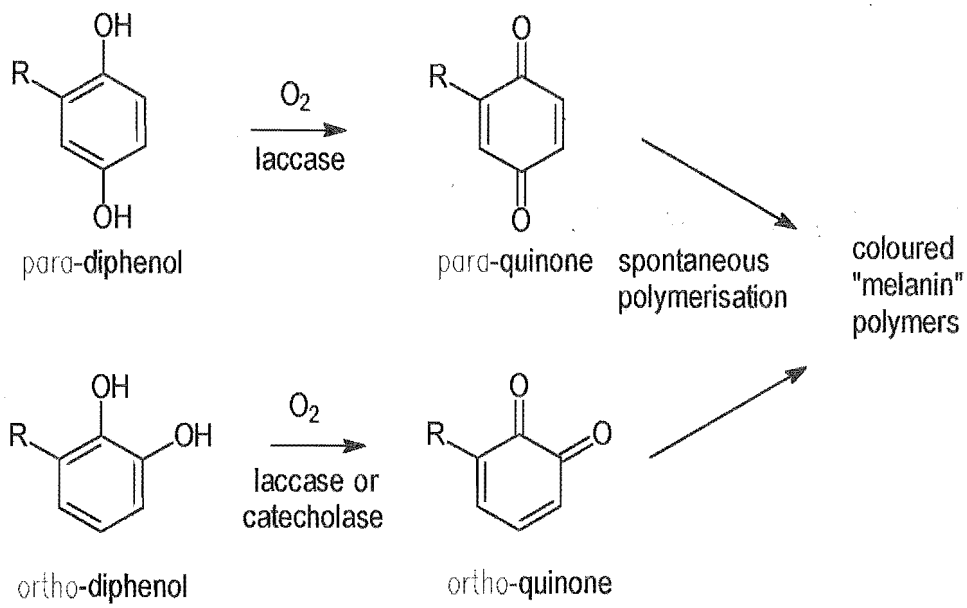


Figure 1.1 Reaction scheme for laccase and catecholase

Laccase is a blue, oxidative, copper-containing enzyme that utilises molecular oxygen as a cosubstrate to perform the oxidation of a variety of substrates (usually a

diphenol) with water as a side product (Ferrar *et al* 1995). The product of the reaction (as well as that of catecholases) is a reactive quinone, which will often spontaneously polymerise to form dark brown or black melanoid pigments, which are characteristic of wounded plant or fungal tissue. The reaction scheme for both enzymes is given in Figure 1.1.

The molecular mass of laccases has been found to vary considerably between organisms, with molecular weights between 36 and 390 kD being reported. However, the main contributor to this variation is probably the carbohydrate moiety, which makes up a large part of the enzyme's mass (10-45%). Typically the primary structure of the enzyme consists of about 500 amino acids (Yaropolov *et al* 1994). However, the amino acid sequence is poorly conserved between species, suggesting that laccases are a "family" of enzymes, i.e. they are not simply descendants of some ancient evolutionary precursor enzyme (Mayer and Harel, 1979). The carbohydrate content of laccases, which tends to be higher in plant laccases than in fungal (Mayer and Harel, 1979), may be involved in solubility or excretion, but there is no evidence for this (Mayer, 1987). There has been a detailed investigation of the carbohydrate moiety in sycamore laccase (Takahashi *et al* 1986), in which the structure of some of the main repeating units of the carbohydrate part of the enzyme were identified, and it was determined that the sugar molecules were joined to the protein itself via the amido group of asparagine residues.

There seems to be some variation in reports about the copper content of laccases, but the most common observation seems to be that there are four copper atoms per molecule of laccase (Yaropolov *et al* 1994) and EPR (Electron Paramagnetic Resonance) studies of both fungal and plant (*R. vernicifera*) laccase have been very useful in this area. Peyratout *et al* (1994) studied the trinuclear type 2/type 3 cluster of the *R. vernicifera* laccase, gleaning information about the active site and inhibitor binding. It seems that the high substrate specificity of laccase may, at least in part, be attributed to the flexibility of this copper-containing active site. Malmström *et al* (1968) also used this method to study fungal laccases. Their results suggested 3 or 4 nitrogen-containing residues (probably histidine) coordinating each copper atom.

1.1.3 Physiological Function of Laccase

In fungi, laccase is usually produced as an extracellular enzyme, and is thought to play a part in lignin decomposition (Yaropolov *et al* 1994), although some fungal laccases have been shown to be capable of oxidising monolignols to form free radicals which are in turn able to combine spontaneously to produce lignin-like structures (Takahama, 1995). The capability of a fungal laccase to perform this reverse reaction is probably largely irrelevant, however, as fungi do not contain lignin, and so it seems that the main role of the enzyme is in decomposition. This ability to produce such an enzyme may be related to the virulence of phytopathogenic fungi (Bar-Nun and Mayer, 1989).

Some fungal laccases are intracellular, but the role of these enzymes is less well understood. Some may play a role in melanin formation (Tanaka *et al* 1992) or, in phytopathogenic fungi, detoxication of plant defence compounds (Mayer and Harel, 1979). A more bizarre role for fungal laccase may be to convert, in the presence of appropriate phenolic accessory substrates, manganese (II) into manganese (III) ions (Archibald and Roy, 1992).

However, the higher plant laccases are the subject of this thesis, and their role is even less well understood. Certainly the function of *Rhus* laccase is to harden and darken the sap of the plant (as is utilised by furniture makers), and this plays a role in plant defence mechanisms and wound healing. It seems likely that the laccase present in the sap of the mango tree (*Magnifera indica*; Robinson *et al* 1993) plays a similar role. Some plant laccases, for example those found in the fruit of some species of *Prunus* (apricots; Dijkstra and Walker, 1991, and peaches; Mayer and Harel, 1968) may be responsible for enzymic browning, a form of plant defence (Ferrar *et al* 1995). Mayer and Harel (1979) also speculated that some plant laccases may be involved in the conversion of endogenous phenols in potential pathogenic fungi to toxic quinones, which would serve as another form of plant defence.

However, the most contestable point concerning the physiological function of plant laccase is the possibility of its involvement in lignification, which seems to parallel

the activity of other oxidative enzymes, in particular the closely related copper-based enzyme catecholase, and also from tyrosinase and peroxidase. To further confuse the issue, one must take into account the fact that there are considerable differences between laccases from fungi and plants, and also from different species. Substrate specificity is one simple way of distinguishing the types of enzymes, and the substrate specificities of *o*- and *p*-DPO have already been discussed above. However, this can often lead to confusion. Syringaldazine was used by Harkin and Obst (1973) to distinguish between peroxidase and laccase in green ash (and other species), but, following evidence that many plant laccases do not oxidise syringaldazine (Joel *et al* 1978; Ferrar *et al* 1995; Walker and McCallion, 1980), doubt has now been cast on these results. To confuse things further, *Rhus* laccase is able to oxidise syringaldazine, whilst sycamore (Sterjiades *et al* 1992) and tobacco (McDougall *et al* 1994) laccases both show a little activity with this substrate. Other substrates give equally confusing results, for example, *p*-cresol, which is oxidised by some laccases but not others (Mayer and Harel, 1979). However, the usefulness of substrate specificity tests for the classification of oxidative enzymes as laccases is greatly increased if a large number of substrates are used, so that a pattern of substrate specificity emerges. Whilst a particular enzyme may not oxidise syringaldazine or *p*-cresol, if it does oxidise *p*-quinol and *p*-phenylene diamine, then chances are the enzyme is a laccase. Another potentially useful substrate, 4-hydroxyindole, has been shown to be oxidised by *Rosmarinus officinalis* and *Pyricularia oryzae* laccase, but not by *Solanum tuberosum* catecholase (Cai *et al* 1993b), but the selectivity of this substrate requires further investigation. At best, substrate specificity studies can only serve as a pointer towards the identity of an oxidative enzyme. Further tests are required, and selective inhibition studies have been shown to be very useful (Walker and McCallion, 1980).

1.2.2 Use of Specific Inhibitors

One of the first specific inhibitors used to distinguish between laccase and catecholase was carbon monoxide (CO), and is still one of the most useful and reliable tests (Cai *et al* 1993a), since CO inhibits catecholase but not laccase.

However, this method is rather unwieldy and slightly dangerous, and many other chemical inhibitors have now been identified which are able to differentiate between laccase and other enzymes. Salicylhydroxamic acid (SHAM) and, to a lesser extent, cinnamic acid inhibit catecholase but not laccase (Allan and Walker, 1988). This study is especially relevant here, as the plant laccases used were from *R. vernicifera* and *Picea pungens*. Walker (1968) noted that fungal laccase was inhibited by quaternary ammonium compounds (QACs), such as cetyl trimethyl ammonium bromide (CTAB), while catecholases remained unaffected. The reverse was true with polyvinyl pyrrolidone (PVP). This study was later expanded to include a wide range of laccases and catecholases from both plants and fungi, and the results were the same (Walker and McCallion, 1980). However, only three plant laccases were used in the study, from peach (*Prunus persica*), *R. vernicifera* and *P. pungens*. It would be useful to expand this study to include a greater number of higher plants, to confirm these results. A more recently identified inhibitor is N-hydroxyglycine (Murao *et al* 1992), which has been shown to inhibit fungal laccase (from *Coriolus versicolor*), but not tyrosinase, peroxidase or catecholase. This inhibitor needs to be tested with plant laccases. The same researchers also noted that another laccase inhibitor, kojic acid, showed similar properties of specific inhibition, making it useful for identifying laccases, but it does have the drawback of also inhibiting tyrosinase. 4-Hexylresorcinol is another inhibitor which can distinguish between laccase and tyrosinase, which has been shown to inhibit the latter but not the former (Dawley and Flurkey, 1993). SHAM does the same (Dawley and Flurkey, 1993). Also of use in distinguishing between laccase and catecholase is the anion detergent sodium dodecyl sulphate (SDS), which has been observed to activate catecholases (probably by releasing the enzyme from cell membranes) but no activation has been observed with any laccases yet studied (Walker and McCallion, 1980). Part of this research project will attempt to throw more light on the question of specific inhibitors and activators of laccases in higher plants.

1.3 Laccase from Higher Plants

1.3.1 Occurrence and Classification

When listing sources of higher plant laccases, it is important to know on what basis the classification was made. *Rhus vernicifera* contains a laccase, and the enzyme draws its name from the common name of the plant. Many studies have been carried out on the enzyme, and it remains of a reference point for other laccases. Other members of the family Anacardiacaea (to which the *Rhus* genus belongs) have also been found to contain laccase in the secretory ducts which characterise the Anacardiacaea. Joel *et al* (1978) found laccase in other members of the family; *Magnifera indica* (mango), *Schinus molle, Pistachia palaestina* and *Pleiogynium timoriense*. However, the classification was made solely on the basis of substrate specificity and thus is questionable. However, a more recent study on mango laccase (Robinson *et al* 1993) showed no activation by SDS, whilst there was inhibition by CTAB, which substantiates the claim that this enzyme is a laccase.

Laccase has also been found in other fruits besides mango. An early study reported the presence of laccase in tomato (Mathan *et al* 1965) but this was almost certainly a catecholase, as the substrate used to identify this supposed laccase was catechol (an *o*-diphenol). Interestingly enough, a "tyrosinase" was also reported, based on the ability of plant extracts to oxidise D,L-DOPA. Other reports of laccase in fruit are more reliable. In 1968 Mayer and Harel identified an enzyme from peaches that oxidised *ortho*- and *para*-dihydric phenols and quinol, but not *p*-cresol or *p*-phenylene diamine. Classification of this enzyme as a laccase was based on the fact that usual catecholase inhibitors had no inhibitory effect. CTAB was used, but it was employed as a method of removing pectic substances from fruit extracts and not as an inhibitor. This enzyme is almost certainly a laccase, but more inhibitor studies would be useful to confirm this, as was done by Dijkstra and Walker (1991) in their study on enzymatic browning in apricots. These researchers identified laccase on the basis of substrate specificity (the enzyme oxidised quinol, toluquinol and *p*-phenylene diamine) and inhibitor and activator studies (inhibition by CTAB, no inhibition by PVP, and no activation by SDS). This is strong evidence that the

enzyme is a laccase, and it also lends support to the idea that the enzyme from another species in the same genus (apricots and peaches are both members of the *Prunus* genus) is also a laccase, despite differing substrate specificities.

Another key paper, upon which much of the work in this investigation was based, is Cambie and Bocks (1966), who used a simple extraction and assay method to screen a large number of gymnosperms for laccase, concentrating particularly on the family Podocarpaceae. Many of the plants screened were found to contain a laccase-like enzyme, using the basis of the ability of simple leaf extracts to oxidise the test substrate, 2,6-dimethoxyphenol. No other attempt at further classification was made. Since many of the Podocarpacea studied were found to contain such an enzyme, it may be useful to investigate these species further, using the more stringent criteria detailed above. Those species which did not contain laccase should also be investigated, since the example from different species of *Prunus* shows that substrate specificity (in the case of the podocarps, the ability to oxidise 2,6-dimethoxyphenol) was not necessarily the same from laccases from plants of the same genus.

Other plant sources of laccase are horse chestnut (*Aesculus parviflora*; Wosilait *et al* 1954), *Zinnia elegans* (Liu *et al* 1994), tobacco (McDougall *et al* 1994) and mung bean (*Vigna radiata*; Chabanet *et al* 1994). Only the last of these examples was backed up by inhibitor tests. Finally, a highly promising source of plant laccase has been found in suspension cultures of plant cells, which seem to produce an extracellular laccase which is released into the medium and hence easily recoverable and purifiable. The first and most intensely studied so far was from sycamore (*Acer pseudoplatanus*). Bligny and Douce (1983) reported the isolation of a 97 kD enzyme with 45% carbohydrate content from cell suspension culture media of sycamore, which showed substrate and inhibitor specificities of laccase, in fact quite similar to *Rhus* laccase. Further characterisation of this laccase was carried out by Driouich *et al* (1992), who studied the carbohydrate content and localisation of the enzyme. A second example of laccase from cell suspensions was reported by Cai *et al* (1993a), who used substrate specificity and specific inhibitors to identify the PPO (polyphenol oxidase) activities they had isolated as laccase.

1.3.2 Localisation and Extraction

These two topics are intimately related, since the purification of an enzyme is made easier by knowing where the enzyme is located in the cell. As late as 1979, it was noted that all plant laccases known at the time were cytoplasmic (Mayer and Harel, 1979). However, it has been noted that a cytoplasmic enzyme may be difficult to distinguish from one embedded in the cell wall (Bligny and Douce, 1983). Refer also to the work carried out by Chabanet *et al*, who used rabbit serum directed against laccase to localise laccase in lignifying and lignified cell walls. The localisation of laccase in this part of the plant has important consequences for enzyme extraction procedures. Stephens and Wood (1974) used an endopectate-*trans*-eliminase from *Erwinia carotovora*, to degrade a particular fraction of the cell wall of potato tuber preparations and found increases in enzyme activities correspondingly. Laccase was not one of the enzymes studied, but this experiment showed the potential for using cell wall-degrading enzymes for purification of cell wall-bound enzymes. Laccases from the sap of members of the Anacardiaceae would probably not benefit from this type of extraction, but many other laccase sources may do. Liu *et al* (1994) used a cell wall-degrading enzyme preparation from *Trichoderma reesei* to aid in the extraction of laccase from *Zinnia elegans*, and evidence from other workers that laccase is bound to cell walls supports the use of these enzymes whenever extracting plant laccases. Part of this thesis will investigate this question. Furthermore, Cambie and Bocks (1966) noted that laccases from gymnosperms were attached to the particulate fraction, and Dijkstra and Walker (1991) found the same in apricots, although they suggested that the enzyme might be attached to the cell membrane. However, it is interesting to note that the enzyme from peaches was reported to be a soluble enzyme not attached to the particulate fraction (Mayer and Harel, 1968), but a later paper described the use of a pectate lyase to aid in the purification of laccase from peaches (Harel and Mayer, 1970). They attributed the corresponding increase in laccase activity to a reduction in the amount of interfering pectic substances in the extract. Therefore the effect of cell wall-degrading enzymes in fruit extracts should also be investigated.

However, there is some doubt about the efficacy of this method. McDougall *et al* (1994) found that although the xylem of tobacco contained laccase-like enzymes attached to the cell wall, treatment with "Driselase" (a powerful cell wall-degrading enzyme mixture) did not result in any detectable activity in extracts; which suggests the inability of this method to produce a soluble laccase.

1.4 Comparison between Plant and Fungal Laccase

Clearly, laccases from plants are different from fungal laccases in one important respect; they have a different physiological function, and this has been discussed already (Section 1.1.3). It therefore seems likely that there may be fundamental differences in the physical and biochemical properties of laccases from these two sources. It has been noted already that plant laccases tend to have a higher carbohydrate content than the fungal enzyme, and the substrate specificity of the two groups, while also varying between different fungal laccases and different plant laccases, seems to vary even more. Trends of substrate specificity also emerge. The ineffectiveness of syringaldazine with higher plant laccases has already been noted, yet it is used routinely for the qualitative and quantitative estimation of laccase content in fungi (Leonowicz and Grzywnowicz, 1981) and in solutions such as grape musts and wines where fungal laccases may be present (Grassin and Dubourdieu, 1989), but the response of plant laccases to syringaldazine is, in all laccases yet discovered, apart from *Rhus vernicifera*, slow at best. *p*-Cresol is a similar substrate, but there is less of a clear trend between fungal and plant laccases.

Another key area in which plant and fungal laccases seem to differ is that of pH optimum. Fungal laccases tend to be most active between pH 3-4 (Benfield *et al* 1964; Rehman and Thurston, 1992; Bocks, 1967), while plant laccases tend to be higher than this, around pH 5-7 (Benfield *et al* 1964; Robinson *et al* 1993; Dijkstra and Walker, 1991), although some are lower, such as *Cryptomeria japonica* laccase, which displayed an optimum at pH 4.0 (Cambie and Bocks, 1966). However, as yet there has been no clear indication as to any other major differences between the two groups of laccases, and as such they remain classified as the same enzyme. Patterns

of inhibition, as far as they have been studied, appear identical, but this requires further investigation.

1.5 Aims and Objectives

To summarise, the aims of this research were as follows:

1. To develop a reliable method of screening for laccase activity in higher plants, that will be specific, and also positively identify the enzyme as a laccase and not as some other oxidative enzyme. Substrate specificity and inhibition patterns will need to be used.
2. To identify novel sources of laccase in higher plants.
3. To identify substrates and inhibitors which are specific for all types of laccases, those which are only specific for higher plant laccases, and those which are specific for fungal laccases. Hopefully this data will clarify the issue of the differences between fungal and plant laccases, and also provide useful information on new substrates and inhibitors which may prove useful for the identification and classification of laccases.
4. To test the hypothesis that higher plant laccases are bound to the cell wall and may be released with cell wall-degrading enzymes.

Chapter 2

Evaluation of Screening Methods

2 Evaluation of Screening Methods

2.1 Introduction

2.1.1 Practical Considerations

In order to develop an extraction procedure that could be used for the screening of the leaves of a number of higher plants, it was first necessary to test all methods that might be deemed suitable, with a small number of plants. In past studies, there is variation in the plant organ used and in the extraction procedure. The laccase from *R. vernicifera* is extracted from the sap (Yoshida, 1883; Reinhammar, 1970). However, the presence of laccase in the sap (or latex) of a plant seems to be confined to members of the Anacardiaceae (Joel *et al* 1978). Also, it was desirable to find a quicker and easier method of screening a large number of plants, and the plant organ most suited to this method is the leaves. Cambie and Bocks (1966) used leaves (or needles) in their study of gymnosperms. Therefore leaves were used as the plant organ when selecting a suitable extraction method.

2.1.2 Possible Extraction Procedures

At this stage, it was not considered necessary to obtain purified preparations of laccase; extracts showing laccase activity were all that were required. Therefore complicated purification techniques such as those employed by Reinhammar (1970), involving column chromatography were not necessary. Cambie and Bocks' simple method involved the homogenisation of leaves in distilled water, with subsequent filtering to obtain an extract. However, this method, whilst detecting a number of "laccase-like" enzymes, would not detect enzymes attached to the particulate fraction of extracts. It also might prove more successful if a buffer was used instead of water.

Acetone precipitation is another method that has proved successful for the extraction of plant laccases. Esterbauer *et al* (1977) used this method to obtain a preparation of

laccase from banana, spruce (*Picea abies*) needles and a species of *Rhus*. Robinson *et al* (1993) used acetone precipitaion to extract laccase from mango fruit. Dijkstra and Walker (1991) have also used this method to extract laccase from apricots (*Prunus armenica*). Typically, commercial sources of *Rhus vernicifera* laccases are acetone-dried powders. This method is useful because it removes most other plant compounds such as lipids, chlorophyll and phenolics, which may interfere with the activity of the laccase enzyme.

Another method which may be effective is the use of cell wall-degrading enzymes, which have been used to release similar enzymes (such as peroxidase) from plant cell walls (Stephens and Wood, 1974). More recently, a cell wall-degrading enzyme preparation from *Trichoderma reesei* was used to extract laccase from *Zinnia elegans* tissue (Liu *et al* 1994). Harel and Mayer (1970) also used a pectate lyase to increase the activity of laccase in peach extracts. The localisation of laccase in the cell walls of other plants (in *Vigna radiata* by Chabanet *et al* 1994 and in sycamore by Driouich *et al* 1992) supports the use of this method.

The use of the insoluble polymer polyvinyl polypyrrolidone (PVPP) to remove plant phenolics may also be useful. Liu *et al* (1994) and Harel and Mayer (1970) have both described the use of this polymer in the extraction of laccase from *Zinnia elegans* and peaches (*Prunus persica*) respectively.

The observation that laccase is excreted into the suspension medium of cell suspension cultures of sycamore (Bligny and Douce, 1983) and rosemary (Cai *et al* 1993), while interesting, will not be used here, due to the cost and time involved in the setup of suspension cultures.

2.2 Materials and Methods

In order to select a suitable method of laccase extraction from leaves of a variety of tree species, a number of different methods were tested on the leaves of three different species; *Podocarpus totara*, *Rhus succedanea* and *Rosmarinus officinalis.* These species were chosen because they have previously been found to contain laccase in the leaves, they represent a range of species, and, the case of *P. totara* and *R. officinalis*, the leaves were available in large quantities. Leaves were used to make it easier to screen a larger number of plants than would have been possible had a different part of the plant (e.g. heartwood) been used. The extraction methods studied are listed below.

2.2.1 Buffer Extraction

Leaf pieces (approx. 5mm square, either fresh or freeze-dried) were ground in pH 6.5 0.1M phosphate-citrate buffer using an Ultra-Turrax™ homogeniser (Janke and Kunkel AG) at 4°C. Generally the ratio of leaves to buffer was 1g leaves (fresh weight) to 10mL cold buffer. The resulting liquid was then filtered through Miracloth and centrifuged for 15 min at 4°C and 10 000 rpm. The supernatant was then tested for laccase activity.

2.2.2 Acetone Precipitation

Using the same ratio of leaf to liquid as above, leaves were homogenised in the Ultra-Turrax in a 3:1 mixture of acetone and water respectively, pre-chilled to -18°C. The homogenate was then filtered by suction and the precipitate or "acetone dry powder" washed again with the same cold acetone/water mixture to remove any remaining chlorophyll, lipids, phenolics *etc.*, and the powder left to air dry. However, care was taken not to let the powder dry completely as this can lead to inactivation of some enzymes (Hermann Esterbauer, private communication). The acetone powder was then resuspended in pH 6.5 0.1M phosphate-citrate buffer and stirred for 30 min. The solution was then filtered through Miracloth and centrifuged for 15 minutes at 4°C and 10 000 rpm. The supernatant was then tested for laccase activity.

2.2.3 Grinding in Liquid Nitrogen

In combination with both of the above methods, leaves were frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle, and stirred for various times (see below) in the appropriate liquid, using the same ratios as above. Grinding in liquid nitrogen was seen as an alternative to using the Ultra-Turrax in the above methods.

2.2.4 Use of Cell Wall-Degrading Enzymes

Working on the hypothesis that laccase is a cell wall-bound enzyme, leaf extracts were incubated with various cell wall-degrading enzymes before filtering and centrifugation. After grinding in liquid nitrogen, leaves were placed in buffer solutions containing one of the enzyme preparations, listed below.

(i) Pectinex (from Schweizerische Ferment AG).
(ii) Ultrazyme-100 (from Schweizerische Ferment AG).
(iii) Celluclast® (from Novo Industrias (Copenhagen, Denmark)).
(iv) Cellulase "Onozuka" (from Yakult Honsha Co., Ltd. (Tokyo, Japan)).
(v) Pectinase from *Rhizopus* sp. (from Sigma Chemical Co. (St. Louis, USA)).
(vi) Cellulase from *Trichoderma viride* (from BDH Chemicals Ltd. (Poole, England)).
(vii) Pectinase from *Aspergillus niger* (a 40% solution in glycerol from Sigma).
(viii) "Driselase" from Basidiomycetes (from Sigma).

Driselase is a crude powder containing several cellulolytic enzymes such as laminarase, xylanase and cellulase. Ultrazyme-100 contains a wide range of cellulolytic and pectolytic enzymes. Celluclast contains cellulolytic enzymes such as cellulase and xylanase. Pectinex is a preparation of pectolytic enzymes.

The liquid nitrogen-ground leaves were incubated either at 0°C or at room temperature (22°C) for 2 hr in solutions of these enzymes in pH 6.5 0.1M phosphate-citrate buffer. It was neither practical nor necessary for the concentration of the enzyme preparations to be standardised for the same activity, as at this stage the results were only intended to be qualitative. After the allotted time, the liquid was filtered and centrifuged as before. The ratio of leaf material to liquid in these experiments was 1g liquid nitrogen-ground leaves to 10mL liquid.

Once the most effective enzyme preparation(s) was/were ascertained (i.e. which increased laccase activity), a time course experiment was set up by which *R. officinalis* leaf material was incubated for varying amounts of time in the buffer/enzyme solution and the increase in laccase activity monitored colorimetrically (as below).

2.2.5 Use of SDS

Sodium dodecyl sulphate (SDS) disrupts cell membranes and has been shown to increase catecholase activity from plant extracts (e.g. Ben-Shalom *et al* 1977 and Yamaguchi *et al* 1969), presumably by releasing the latent form of these enzymes from the cell membranes. It was therefore included to test for the presence of *p*-DPOs in the cell membrane and also to serve as a way of discriminating between *o*-DPO and *p*-DPO. It was incubated with liquid nitrogen-ground leaves as a 0.2% solution in pH 6.5 0.1M phosphate-citrate buffer.

2.2.6 Use of PVPP

Polyvinyl Polypyrrolidone (PVPP, or Polyclar AT) is an insoluble polymer that is able to bind phenolic compounds (Loomis and Battaile, 1966). It is sometimes necessary to remove these compounds as they can react with proteins and thus render the enzymes inactive (Loomis, 1974). PVP (the soluble form of PVPP) has been found to selectively inhibit catecholases (Walker, 1968), but insoluble PVP does not have this property. Finally, the removal of natural plant phenolics may allow, by some unknown mechanism, the oxidation of syringaldazine, as it has been reported

that natural plant phenolics from grape inhibit this oxidation (Grassin and Dubourdieu, 1989).

Plant extracts were incubated on ice with different amounts of pre-washed and pre-soaked (in distilled water) PVPP for various times, after which extracts were filtered and centrifuged to remove the PVPP and the supernatant tested for laccase activity.

A second method was also investigated, using a column (200mm x 15mm) containing PVPP. Extracts were run through the column to remove phenolic compounds, the collected fractions being assayed for laccase activity with 4-hydroxyindole.

2.2.7 Enzyme Assay

The extracts obtained by the above methods were assayed for laccase activity in two ways. O_2 uptake was measured using a Clark-type O_2-electrode (Yellow Springs Instrument Co., USA.). The method involved placing a suitably diluted aliquot of the leaf extract into the reaction chamber of the apparatus with 1.5mL pre-aerated 0.1M phosphate-citrate buffer (pH 6.5). The total reaction volume was made up to 2.5mL with aerated distilled water. The reaction chamber was kept at a constant temperature of 30°C. The reaction was initiated by injecting 0.5mL of substrate (10mM toluquinol in most cases) into the reaction chamber from which oxygen is excluded. The solution is stirred magnetically and the partial pressure of O_2 monitored by a potentiometric chart recorder. Initial uptake of O_2 relative to controls using boiled leaf extract (i.e. denatured enzyme) indicated the presence of laccase.

The second method involved using microtitre plates with 300µL wells, and similar proportions of reagents to the O_2-electrode assay. However in this case, the substrates used were 4mM syringaldazine, 10mM guaiacol, 10mM toluquinol, 10mM 2,6-dimethoxyphenol and, when it became available, 10mM 4-hydroxyindole. The progress of the reaction was monitored by observing the colour changes in the solution, and quantified by colorimetric analysis using a Labsystems Multiskan

MCC/340 microtitre plate reader. Colour changes which indicated laccase activity were pink (λ_{max} = 525nm) for syringaldazine, blue (λ_{max} = 615nm) for 4-hydroxyindole, orange for toluquinol and 2,6-dimethoxyphenol and brown for guaiacol and *p*-phenylene diamine. Many laccase (and catecholase) substrates auto-oxidise in contact with atmospheric oxygen, so care had to be taken to ensure that any colour change was due to laccase and not to auto-oxidation.

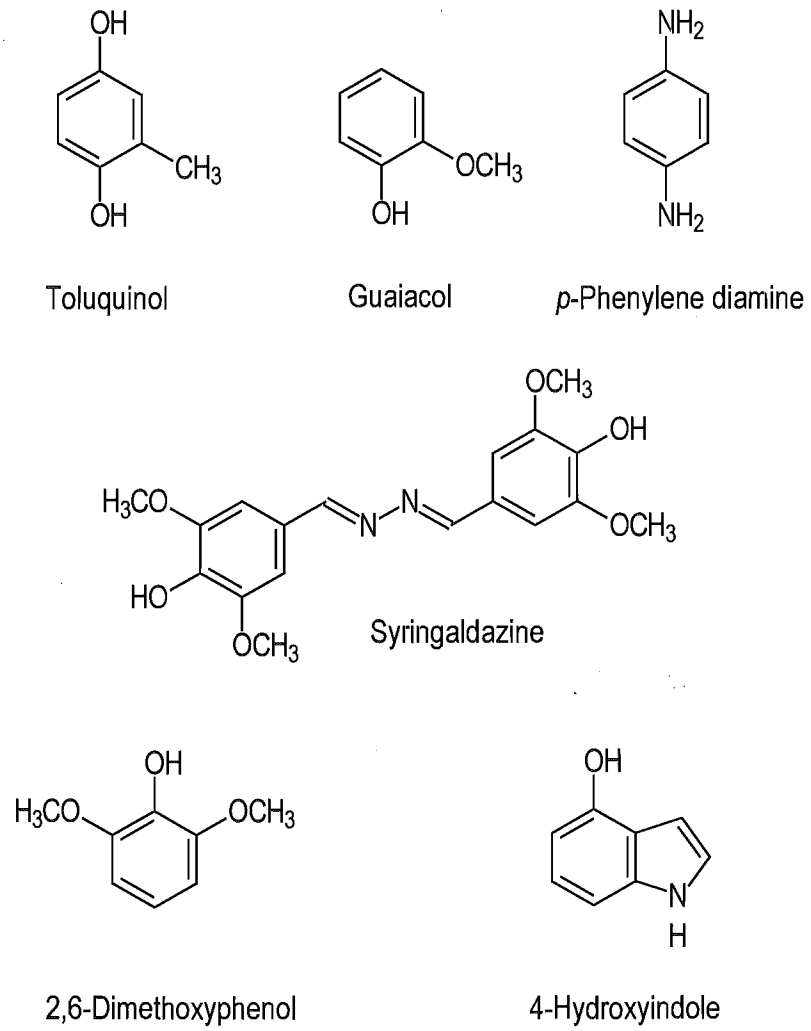


Figure 2.1 Laccase substrates used in this study

Catecholase was also assayed for, particularly when SDS was involved, with D,L-dihydroxy phenylalanine (D,L-DOPA) and 4-methyl catechol, using the same methods.

2.3 Results & Discussion

Most of the results in this section were from extracts of either *P. totara* or *R. officinalis*. The reason for this was that there were only a very small amount of *R. succedanea* leaves available, due to the rarity of the plant in Christchurch. The only available specimen (in the Christchurch Botanical Gardens) was very small and therefore little progress was made in the development of a suitable screening method by the time the very limited supply was exhausted.

2.3.1 Simple Buffer Extraction

There were a number of problems with this method, not the least of which was the very low laccase activity that was obtained. Only toluquinol and 4-hydroxyindole showed any sign of oxidation, and this was only after several hours. Another problem was that the fibrous nature of the leaves meant that they tended to clog the Ultra-Turrax, making the method rather inefficient with respect to time. There was no difference between fresh or freeze-dried leaves. Centrifuged extracts showed more activity than uncentrifuged, as well as being easier to handle, since a clearer extract showed colour changes more effectively.

2.3.2 Acetone Precipitation

This method was very effective for removing natural phenolics, which can interfere with colorimetric assays, particularly when the desired oxidation product is an orange or brown colour. However, this method proved to be fairly poor at extracting laccase. Again the activity was very low, with only toluquinol and 4-hydroxyindole being oxidised after several hours. The acetone fraction was recovered and assayed for laccase activity, which proved comparable to that from the acetone precipitate. It would seem that this method is not effective. The laccase does not seem to be precipitated out from the acetone/water solution in the first stage of the extraction. One reason for this may be the high glycosylation ratio of the protein. Laccases (both plant and fungal) have been shown to be highly glycosylated (Ferrar *et al* 1995) and plant laccases tend to have a higher carbohydrate content than fungal laccases. Sugar groups attached to protein molecules tend to be situated on the outer surface of

the protein in its tertiary structure, and this may affect the solubility of the protein, in turn altering its property of precipitation from a cold 3:1 mixture of acetone and water.

2.3.3 Grinding in Liquid Nitrogen

The use of liquid nitrogen was reasonably labour intensive, but it was far superior to the use of the Ultra-Turrax, and, most importantly, it yielded improved laccase activity relative to results using the Ultra-Turrax. In light of the hypothesis that laccase may be bound to the cell wall, this result is understandable, as the act of grinding may break up the cell wall.

2.3.4 Use of Cell Wall-Degrading Enzymes

This was probably the most significant result from this section. Several of the enzymes used increased the yield of laccase activity with 4-hydroxyindole, and the best of these proved to be Ultrazyme-100, as shown in Table 2.1.

Table 2.1 Effect of cell wall-degrading enzymes on laccase activity with 4-hydroxyindole.

Enzyme Preparation	*Podocarpus totara*		*Rosmarinus officinalis*	
	ΔA_{620}/hr	Increase (%)	ΔA_{620}/hr	Increase (%)
Control	0.034	(100)	0.023	(100)
Pectinex	0.020	NI	0.066	278
Ultrazyme-100	0.141	415	0.172	728
Celluclast®	0.033	NI	0.023	NI
Cellulase "Onozuka"	-	-	0.038	161
Pectinase from *Rhizopus* sp.	-	-	0.026	NI
Cellulase from *Trichoderma viride*	-	-	0.021	NI
Pectinase from *Aspergillus niger*	-	-	0.020	NI
Driselase from *Basidiomycetes*	-	-	0.023	NI

NI = No significant increase.

Ultrazyme-100 contains strong pectolytic enzymes which break down the pectic fraction of the cell wall. Whether the large difference in its effect on laccase activity between this enzyme and other pectinases was due to a difference in the specificity of the enzymes present in each preparation, or whether it was due to the Ultrazyme preparation being more concentrated is unclear, since no data was available about the strength or composition of the Ultrazyme preparation.

Whatever the reason, it is clear that laccase was being released from the cell wall, and that the available activity with respect to 4-hydroxyindole was increased by this treatment. Use of this enzyme should be included in any future screening procedure.

(N.B. Control experiments showed that Ultrazyme-100 was incapable of oxidising 4-hydroxyindole.)

Further study was carried out on Ultrazyme action, and Figure 2.2 shows the effect of increasing Ultrazyme concentration on laccase yield. It seems clear that further increases in concentration would increase laccase yield, but the cost of Ultrazyme was prohibitive, and in the interests of economy, it was decided that a concentration of 0.05g/mL was sufficient for any further experiments.

2.3.4.1 Time Course Study of Ultrazyme Action

The results of the time course study for release of latent laccase from the cell walls of *Rosmarinus officinalis* can be seen in Figure 2.3. The results show an optimum time of incubation of approximately 2 hr, and similar results were obtained with *Podocarpus totara.* Presumably this time is a compromise between the optimum amount of laccase being released from the cell wall, and the minimum amount of laccase being spontaneously degraded at room temperature. It was noted that the laccase preparations obtained in this section tended to lose activity if left at room temperature. Even at 4°C all activity was lost after about two weeks.

Figure 2.2 Effect of Ultrazyme concentration on laccase activity in *Podocarpus totara* extracts

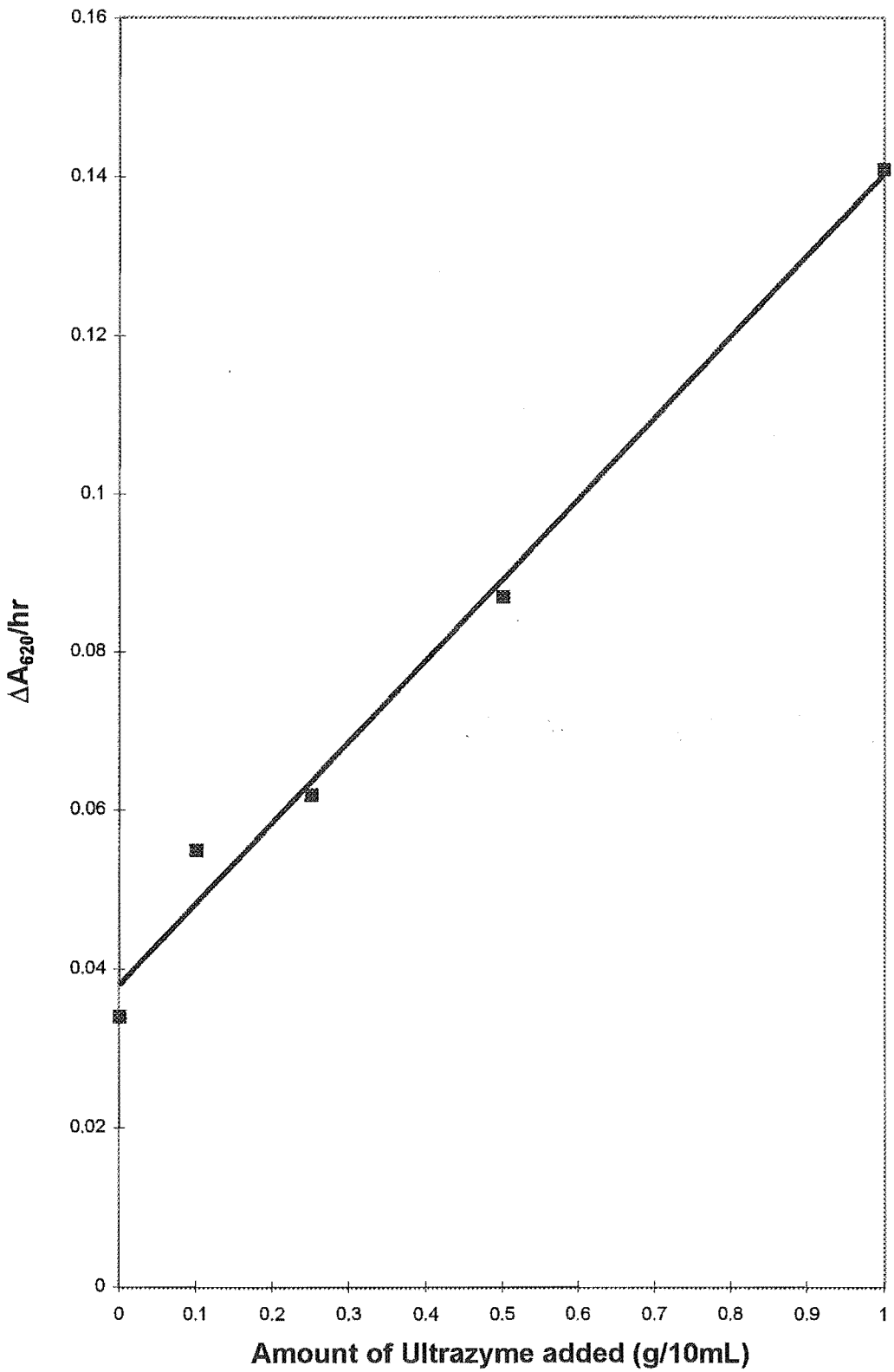
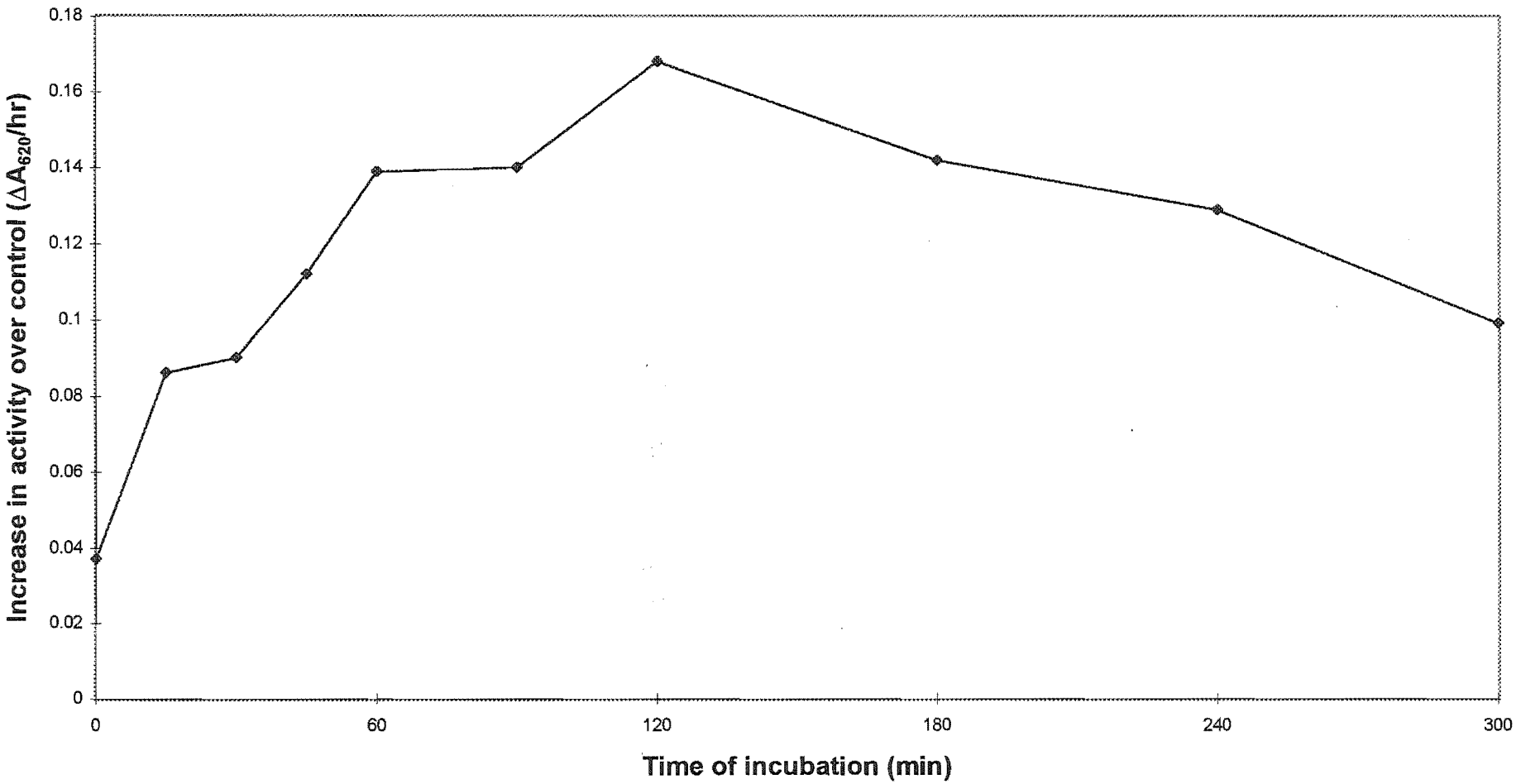


Figure 2.3 Time course of Ultrazyme action on *Rosmarinus officinalis*



2.3.5 Use of SDS

SDS did not activate laccase, and there was no increase in activity over controls. All colorimetric results with 4-hydroxyindole were within experimental error. This is some evidence that the enzyme isolated was not a catecholase, and it shows that any enzyme present was not attached to cell membranes.

2.3.6 Use of PVPP

Addition of PVPP was observed to remove natural phenolics from the plant extract, as could be seen by noting the colour of the extract, particularly after several days at high pH. (High pH promotes the spontaneous oxidation of phenolics; Loomis, 1974.) Column chromatography on plant extracts was the most effective method of phenolic removal, but this method had the disadvantage of diluting the enzyme extract and the process was also time-consuming.

Incubation of extracts with PVPP proved less effective at removing phenolics, but most of the phenolics were removed. The optimum ratio for this treatment was found to be 0.45g of PVPP for every gram of leaves used in the original extract.

In either case, the removal of natural phenolics did not yield higher laccase activities than controls. Therefore this treatment, though perhaps useful in other fields of enzymology, was not of any use here.

It should be noted also that the use of PVPP did not yield lower laccase activities than controls. There was no appreciable difference in activities, as might have been expected had the observed activity been due to the action of a catecholase. This observation lends further support to the theory that the enzyme being studied is a true laccase.

Finally, there was no increase in activity with syringaldazine, as might be predicted if the lack of activity of plant laccases with this substrate was due to inhibition by plant phenolics. Therefore, although plant phenolics may still be responsible for inhibition of fungal laccases (Grassin and Dubourdieu, 1989), the lack of activity of plant

laccases with syringaldazine appears to be a matter of substrate specificity and differences in enzyme properties, which may be a general difference between plant and fungal laccases, although this theory requires further investigation, along with the suitability of 4-hydroxyindole as a test substrate for higher plant laccases.

2.3.7 Evaluation of Test Substrates

As mentioned above, syringaldazine proved to be an unsuitable test substrate for laccase in the three plants assayed, however, it would be useful to investigate the oxidation of syringaldazine in other plants. 4-Hydroxyindole was the most reliable test substrate, and this also needs to be tested on a wide range of plants. Toluquinol was often oxidised, and guaiacol was sometimes oxidised. The other laccase substrates showed no appreciable activity. There was no oxidation of catecholase substrates by any of the plant extracts.

2.4 Conclusion

Each method of extraction can now be evaluated for its suitability in the screening procedure. There were three criteria for inclusion in the final method. Firstly, the method should release laccase into a form which may be assayed effectively (hopefully in a soluble form). Secondly, any method used should be simple and rapid, to facilitate the screening of a number of higher plant leaves. Lastly, it would be desirable for the method to include suitable controls, so that the effect of each treatment could be observed.

The most significant finding from this section is the use of Ultrazyme as a method for increasing the yield of laccase in extracts from higher plants. The apparent release of "latent" laccase from plant cell walls could have significant implications in the study of lignification, and needs further research over a wider range of plants.

Treatment with SDS, whilst not observed to increase laccase activity in any of the plants studied, could be useful as a way of investigating further the release of latent forms of the enzyme from the cell wall. The inclusion of SDS may show if laccase was also released from the cell membrane and also serve as a method of discrimination between laccase and *o*-DPO.

Treatment with PVPP, whilst helpful in removing plant phenolics, was a time-consuming procedure that did not significantly improve laccase yield. Since the use of 4-hydroxyindole gives a blue oxidation product (unlike the oxidation product of any natural phenolic), contamination of extracts by natural phenolics does not seem to pose serious problems. Therefore treatment with PVPP will not be necessary in the screening procedure.

Finally, the best combination of methods seemed to be to use leaves ground to a powder in liquid nitrogen, then incubated with Ultrazyme or SDS in pH 6.5 phosphate-citrate buffer, with subsequent filtering and centrifugation. Acetone

precipitation was not as useful as had been hoped. The final screening method adopted will be detailed in section 2 of the next chapter.

Chapter 3

Laccase in Higher Plants I:

Leaves

3 Laccase in Higher Plants I: Leaves

3.1 Introduction

3.1.1 Screening Procedure

Once the optimum method of screening was determined, the next step was to screen the leaves of a variety of higher plants, mostly gymnosperms, concentrating especially on native New Zealand Podocarps. Plants which had been reported to contain laccase by other workers, and which were readily available, were also studied.

Two hypotheses were tested in the course of the screening procedure. The first was that laccase may be contained in the cell wall, as seemed to be suggested by preliminary results from the previous section. Furthermore, the yield of laccase may be increased by the use of cell wall-degrading enzymes in the course of the extraction.

The second hypothesis tested was that laccases from higher plants do not oxidise syringaldazine, but do oxidise 4-hydroxyindole. Guaiacol and toluquinol were also used as substrates for the sake of completeness, as these are common laccase substrates.

Keeping in mind these two hypotheses, a screening procedure was devised that would test both hypotheses simultaneously.

3.1.2 Selection of Species for Screening

Many Podocarps, whether native to New Zealand or not, have been found to contain a laccase-like enzyme (Cambie and Bocks, 1966), who also found laccase in two other gymnosperms. However, this study did not use cell wall degrading enzymes, and the basis of classification as a laccase was the ability to oxidise 2,6-dimethoxyphenol. Therefore it was worth reinvestigating as many Podocarps as possible, whether or not laccase had previously been identified from these species.

Species investigated by Cambie and Bocks (1966) studied in this investigation included *Podocarpus dacrydioides, P. hallii, P. nivalis, P. totara, Dacrydium cupressinium* and *Cunnunghamia lanceolata.*

Cai *et al.* (1993a) found that *in vitro* cultures of rosemary (*Rosmarinus officinalis*) produced extracellular polyphenol oxidase activities, and that two of these activities exhibited laccase-like properties. This classification was on the basis of substrate specificity and inhibition. Therefore this plant was chosen as one of the species for screening. (N.B. This species had also been part of the previous section.)

Pices species and *Abies* species had previously been found to contain laccase (Allan, 1987) by past researchers in this laboratory, so other representatives of these genera were also screened.

3.2 Materials and Methods

The most effective, efficient and reliable screening method from the previous section was selected. The screening method was also designed to test for the effect of both Ultrazyme-100 and SDS, as well as to gain some data about substrate specificity. The detailed screening procedure, used for all plants, was as follows.

3.2.1 Extraction Method

Three separate Erlenmeyer flasks were set up, each of which contained 20mL of pH 6.5 phosphate-citrate buffer containing either 0.2% SDS, 0.05g/mL Ultrazyme-100, or no treatment (control). 2g of liquid nitrogen-ground leaves or needles were added to each solution and incubated at room temperature for two hours, with occasional stirring. The solutions were then filtered through Miracloth and centrifuged for 15 minutes at 4°C and 10 000 rpm. The supernatant from each treatment was assayed as below.

Each species was screened in duplicate to ensure reproducibility of results, and this duplication was repeated by collecting, if available, a second batch of leaves, harvested at a different time to further ensure the reproducibility.

3.2.2 Enzyme Assay

Each extract was assayed colorimetrically using a microtitre plate assay against four different substrates; 4mM syringaldazine, 10mM toluquinol, 10mM 4-hydroxyindole and 10mM guaiacol. The oxidation of these substrates was observed by monitoring the change in colour as per Section 2.2.7. The oxidation of 4-hydroxyindole was followed quantitatively using a Labsystems Multiskan MCC/340 microtitre plate reader at 620nm. Extracts which showed sufficient laccase activity were also assayed using an O_2-electrode as per Section 2.2.7, with 10mM toluquinol as the substrate.

3.2.3 List of Species Studied

Table 3.1 Sources and collection dates of species screened

Date(s) Collected	Species	Source
Mar 96 & May 96	*Podocarpus totara*	Christchurch Botanical Gardens
Mar 96 & Mar 97	*Cunninghamia lanceolata*	" " "
Mar 96 & Mar 97	*Podocarpus halli*	" " "
Mar 96 & Mar 97	*Dacrydium cupressinium*	" " "
Mar 96 & Mar 97	*Picea stichensis*	" " "
Mar 96 & Mar 97	*Podocarpus nivalis*	" " "
Mar 96 & Mar 97	*Picea pungens*	" " "
Mar 96 & Mar 97	*Abies cephalonica*	" " "
Jan 97	*Prunus domestica*	Private garden in Christchurch
Mar 96	*Rhus typhinia*	Private garden in Christchurch
July 96 & Oct 96	*Picea omorika*	U. of Canterbury Campus
July 96 & Oct 96	*Rosmarinus officinalis*	" " "
Mar 96 & Oct 96	*Podocarpus dacrydioides*	" " "
July 96	*Picea stichensis*	Forestry Power Substation

3.3 Results

3.3.1 Substrate Specificity

Table 3.2 shows the results from the qualitative part of the screening procedure. The results were obtained by visual observation of oxidation by change in colour only.

Table 3.2 Substrate specificity of plant extracts

Plant species	Substrate oxidised?			
	4-hydroxyindole	Syringaldazine	Toluquinol	Guaiacol
Podocarpus totara	+++	-	++	-
Cunnninghamia lanceolata	+++	(+)	+	(+)
Picea omorika	(+)	-	(+)	-
Podocarpus hallii	+	-	+	-
Dacrydium cupressinium	+	-	++	-
Rosmarinus officinalis	+++	-	+++	(+)
Picea stichensis	+++	-	+	-
Podocarpus dacrydiodes	++	-	+++	-
Rhus typhinia	(+)	-	(+)	-
Podocarpus nivalis	++	-	++	-
Prunus domestica	+++	-	++	-
Abies cephalonica	(+)	-	(+)	-
Picea pungens	++	-	++	-

KEY: - no oxidation relative to controls

(+) slight oxidation relative to controls

+, ++, +++ substrate oxidised, number of +'s indicates colour intensity

3.3.2 Quantitative Results/Effect of Treatments

Figure 3.1 shows the quantitative results from all treatments of all plants which showed sufficient laccase activity, as assayed spectrophotometrically, using 4-hydroxyindole as the substrate. *Rhus typhinia* and *Abies cephalonica* showed a slight

trace of laccase activity, and hence were omitted from figure 3.1. However, it should be noted that this small amount of activity was only observed after treatment with Ultrazyme.

Figure 3.2 shows the quantitative results from all treatments of some plants, as assayed with the O_2-electrode, using toluquinol as the substrate. Only those extracts which showed sufficient activity were assayed in this way.

In both figures, results shown are averages over at least four replicates. The amount of variation in activity values was small, and all results are within experimental error.

Figure 3.1 Effect of Ultrazyme and SDS on rate of oxidation of 4-hydroxyindole

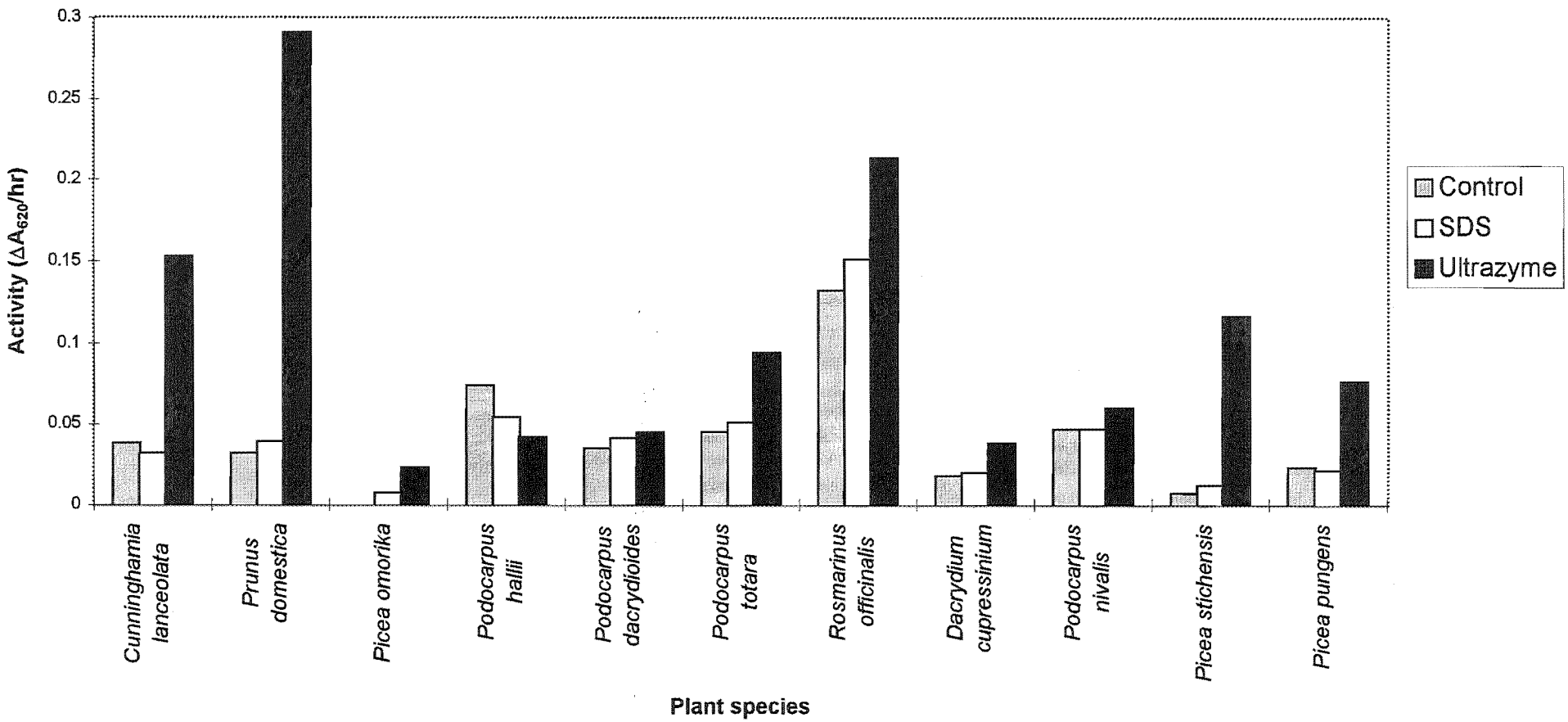
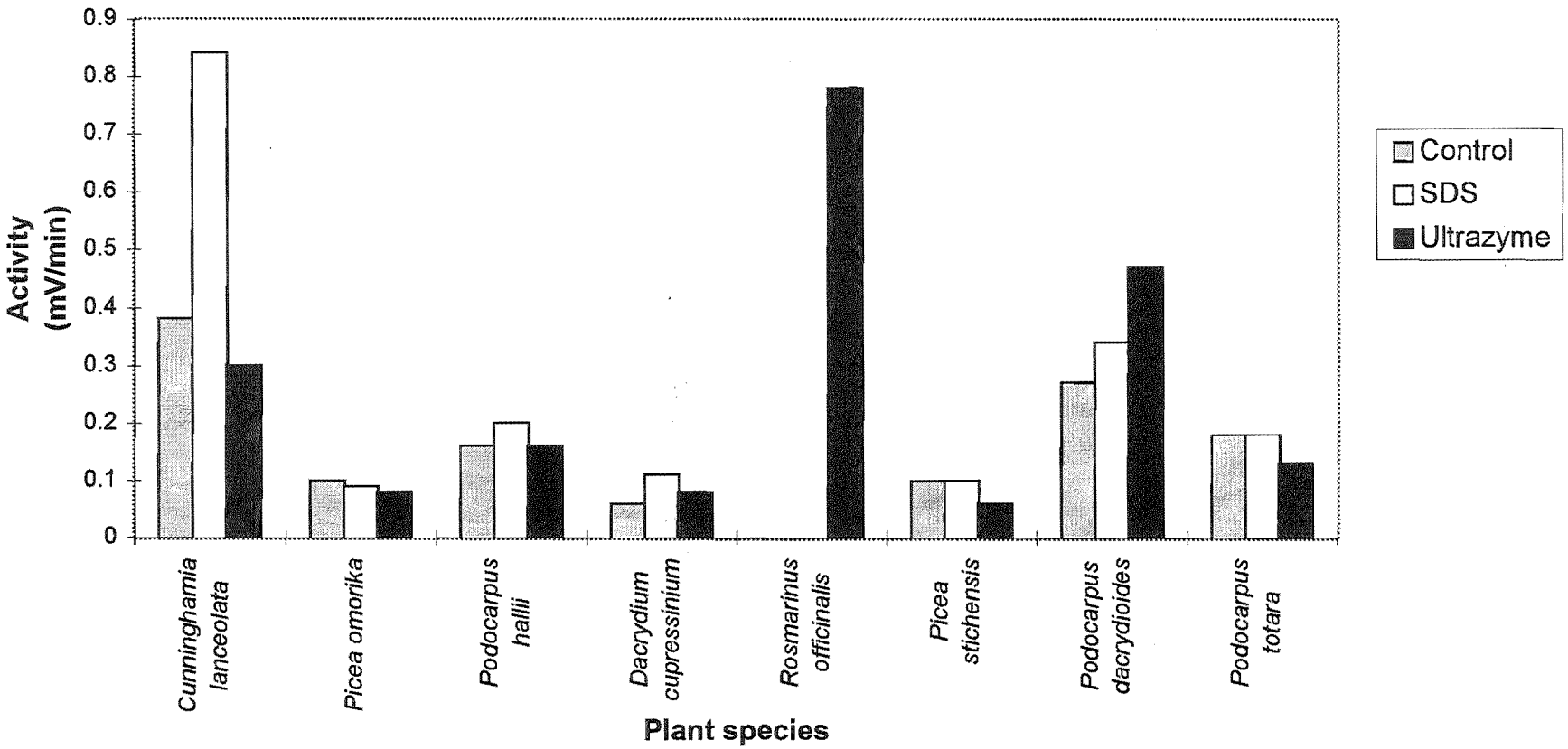


Figure 3.2 Effect of Ultrazyme and SDS on rate of oxidation of toluquinol



3.4 Discussion

3.4.1 Substrate Specificity

The use of 4-hydroxyindole as a substrate for screening has proved extremely useful. All plant extracts that showed laccase activity oxidised the substrate, and parallel tests with other oxidative enzymes (banana and apple catecholase) plus data from the literature (Cai *et al* 1993b) show that this substrate is specific for laccases. 4-Hydroxyindole has several other advantages as a screening substrate, since it is relatively stable at room temperature and it does not undergo rapid auto-oxidation, compared to other laccase substrates, which allows the detection of very low levels of laccase activity. The detection of activity in extracts from *Picea omorika*, *Rhus typhinia* and *Abies cephalonica* would not have been possible with other laccase substrates. The oxidation of toluquinol relative to controls was questionable as the natural orange colour of the plant extract tended to deepen over long periods of time, making it difficult to distinguish low levels of activity. The second advantage was that the blue colour of the reaction product of 4-hydroxyindole made observation and quantification of oxidation much easier, as there is no interference by natural phenolics in plant extracts. This also eliminated any need to remove phenolics (by the use of PVPP or some other method) from plant extracts.

These results with 4-hydroxyindole build on the initial work by Cai *et al* (1993b), which only investigated its oxidation by one higher plant laccase (*Rosmarinus officinalis*) and one fungal enzyme (*Pyricularia oryzae*). Further tests with 4-hydroxyindole showed that it was also oxidised by *Rhus vernicifera* laccase, and by laccases from *Coriolus versicolor* and *Armillaria mellea*. However, to further validate the specificity and action of this substrate, inhibition tests will be required to positively identify the activity observed as being catalysed by a laccase.

The results of screening with syringaldazine alongside other laccase substrates was also enlightening. It seems that oxidation of this substrate by higher plant laccases was the exception rather than the rule, as only one plant extract showed any activity with the substrate. Syringaldazine is a very stable substrate, with a very slow rate of

auto-oxidation, so it was possible to observe activity over the course of a few days if desired, so it is fairly certain that no extract other than *Cunninghamia lanceolata* displayed any activity with this substrate. Also, the pink colour of the oxidation product is very distinctive and hard to miss. Therefore it would be fair to say that syringaldazine is an ineffective substrate for laccase screening in higher plants, as used by Harkin and Obst (1973).

The same seems to be the case for guaiacol. Only two plants showed any activity with this substrate, and these were very low. As for toluquinol, it remains to be seen whether this substrate is useful, as results in the next section (3.4.2) with toluquinol are inconsistent and confusing. Toluquinol is also less useful than 4-hydroxyindole in that the colour of its oxidation product is orange/brown, which makes it more difficult to distinguish and quantify in a plant extract which may change to a similar colour over time.

The other important result from this section is the detection of laccase in species which had not previously been shown to contain laccase. Cambie and Bocks (1966) detected laccase activity in Podocarps (*P. nivalis*, *P. totara*) and another gymnosperm (*C. lanceolata*) in which laccase has again been detected here. However, the same workers also reported a lack of laccase activity in several species (*P. hallii, P. dacrydioides* and *D. cupressinium*) in which laccase has been detected here. Laccase was also detected in a the leaves of *Prunus domestica*, which had not previously been found to contain laccase by any workers.

3.4.2 Effect of Ultrazyme and SDS

As alluded to in the previous section, the results with 4-hydroxyindole were much more successful then those with toluquinol (as measured with the oxygen electrode). This is a direct result of the properties of 4-hydroxyindole noted in the previous section which allowed accurate and reliable results to be obtained by colorimetric methods. The use of the Multiskan MCC/340 was very useful in this respect as it allowed the simultaneous assay of up to 76 different reaction systems, with duplicates to enhance accuracy. The results shown in Figure 3.1 are therefore

considered reliable and any observed difference due to treatments is likely to be a true effect.

The most notable effect was the increase in laccase activity caused by the Ultrazyme treatment of the majority of plants. Only two (*P. hallii* and *P. dacrydioides*) of the ten species which showed appreciable laccase activity did not show any increase in activity after treatment with Ultrazyme. It is interesting to note that these two species, along with *D. cupressinium* (which gave only low levels of activity),were not found to contain laccase by Cambie and Bocks (1966), using 2,6-dimethoxyphenol assay substrate. These two observations, of the differing substrate specificity, and the differing reaction to treatment with Ultrazyme (which suggests that the enzymes may not be located in the cell wall) may imply that these enzymes may belong to a different class of laccase. However, this hypothesis requires further investigation.

The increases in yield of laccase observed upon treatment with Ultrazyme are strong evidence that these enzymes are located in the cell walls, which may have important implications in the study of lignification. The long digestion time required to release an active form of the enzyme suggests that a "latent" form may be buried inside the cell wall, as opposed to being located on the surface. Any enzyme secreted by a plant cell to participate in lignification could be expected to end up inside the cell wall, and the work of other researchers (Section 1.1.3) has shown that laccases from higher plants are capable of doing this job. The localisation of laccase in the cell wall of most of the plants studied here lends further support to these arguments, since the enzymes are present at the required location, and have shown to be able to perform the oxidation, it seems likely that they might be, at least in part, responsible for lignification. However, there is still no absolute proof either way, and until there is, the only path to be taken is one of hypothesis and circumstantial evidence.

There was no significant observed increase in activity with SDS treatment in any of the plant species studied. This was a form of control which shows that (a) the

enzyme was less likely to be a catecholase (which are usually activated by SDS), and (b) the latent enzyme was not bound to the cell's membranes.

Results with toluquinol (Figure 3.2) are less enlightening. While two results stand out (for *C. lanceolata* and *R. officinalis*), and these results were reproducible, the results do not stand up alongside those obtained through the use of 4-hydroxyindole. This leaves the impression that perhaps a different enzyme may be involved in the oxidation of toluquinol. In general, there was no increase with Ultrazyme except for the surprising result obtained with *R. officinalis* (which was double-checked, as was the *C. lanceolata* result). The increase with SDS for *C. lanceolata* suggests the involvement of a catecholase, but the lack of results from other species means that it is difficult tobe certain. The only conclusion that can realistically be reached from this data is that toluquinol is not likely to be a suitable assay substrate for laccase in higher plants. Certainly in light of other results, the use of 4-hydroxyindole is preferable to toluquinol in screening procedures.

3.5 Conclusion

This section has shown that the hypotheses presented in section 3.1 appear to be correct, at least for the species investigated. Laccases from higher plant leaves can be released by the use of cell wall-degrading enzymes, which suggests that these enzymes are located in the plant cell wall. Also, 4-hydroxyindole has been shown to be a very useful substrate for screening of laccase in higher plants, while syringaldazine has been shown to be unsuitable for this type of investigation. However, the suitability of 4-hydroxyindole as a laccase substrate is yet to be investigated with inhibitor tests.

Furthermore, the identification of laccase in three new species was described, in *P. hallii, P. dacrydioides* and *D. cupressinium.* This characterisation as a laccase also needs to be investigated by the use of inhibition tests, as do all the laccases isolated in the screening procedure.

Chapter 4

Laccase in Higher Plants II:

Fruit

4 Laccase in Higher Plants II: Fruit

4.1 Introduction

4.1.1 Purpose of the Study

The results from the previous section have provided evidence to back up the hypotheses put forward earlier, but this relates only to laccases obtained from higher plant leaves. Section 1.3.1 details the occurrence of laccase in fruits, particularly from the *Prunus* genus, and it would be desirable to extend this study to include fruit. The main focus of this section was to reinvestigate the two hypotheses, these being the unsuitability of syringaldazine as a test substrate for laccase in higher plants, and the hypothesis that laccase is present in the cell wall, and may be released by cell wall-degrading enzymes. Also, there was some effort made to identify novel sources of laccases from fruits, although this was a side issue.

4.1.2 Species Investigated

The species studied in this section were restricted to the genus *Prunus*. Peaches *(Prunus persica)* and apricots (*Prunus armenica*) were identified as containing laccase by Mayer and Harel (1968) and Dijkstra and Walker (1991) respectively. The peach study will be interesting to repeat, as the yield was reported to be increased by the use of pectolytic enzymes (Harel and Mayer, 1970), indicating the usefulness of cell wall-degrading enzymes may also apply to fruit extracts.

There is little data in the literature about the substrate specificity of fruit laccases, particularly relating to the ability to oxidise syringaldazine (although mango laccase is reported to be unable to oxidise the substrate; Joel *et al* 1978). Therefore data obtained in this section on substrate specificity hopefully will answer some questions about where fruit laccases fit; i.e. if they are more closely related to plant or fungal laccases, or somewhere in between.

Also investigated in the hope of identifying novel laccase sources were other representatives of the *Prunus* genus, *P. domestica* (plums) and *P. persica* var. *nectarina* (nectarines).

4.2 Materials and Methods

Extraction of laccase from *Prunus* species was performed by two methods as below. Each method was performed in duplicate to ensure reproducibility. The methods of Mayer and Harel (1968) and Dijkstra and Walker (1991) were used as a guide (both used acetone precipitation) but, since at this stage purification was not necessary, considerable simplification was possible.

4.2.1 Acetone Precipitation

100g fresh stonefruit (including skin but not stone) tissue was homogenised in 100mL pre-chilled (-18°C) acetone for 20 seconds in a Waring blendor. The liquid was divided into four parts, one was used as a control, and one of three treatments was added to the others. To one was added 10mL of 0.02% SDS, to another 10mL of 0.05g/mL Ultrazyme-100 and to a third was added 10mL of a 5:1 dilution of Rapidase® Press (from Gist-brocades, France), a pectolytic enzyme designed especially for the clarification of fruit juices. To the control was added 10ml of distilled water. These preparations were incubated at room temperature for two hr and subsequently filtered through Miracloth and centrifuged for 15 min at 4°C and 10 000 rpm. The supernatant was tested for laccase activity.

4.2.2 Buffer Extraction

The method here was the same as above, except that 100mL of pH 6.5 0.1M phosphate-citrate buffer replaced the cold acetone.

4.2.3 Species Studied

Four species of *Prunus* were studied; *P. persica* (peaches, var. "Spring Crest"), *P. armenica* (apricots) and *P. persica* var. *nectarina* (nectarines) were obtained from local supermarkets, and *P. domestica* (plums) were obtained from a local garden.

4.2.4 Enzyme Assay

The method for laccase assay was the same as that described for leaf extracts detailed in section 3.2.2, except that the O_2-electrode was not used in this case.

4.3 Results

4.3.1 Acetone Precipitaion

This method yielded clear, colourless extracts (except when Rapidase was used, the brown colour of which was carried over to extracts) which showed considerable laccase activity in those species which had previously been identified as containing laccase (i.e. peaches (Mayer and Harel, 1968) and apricots (Dijkstra and Walker, 1991)). However, treatment with SDS and cell wall-degrading enzymes had no effect when incorporated into this method, and tended to decrease the yield of laccase relative to controls. Therefore this method, while useful as a routine laccase extraction procedure in fruits, was not amenable to modification to include cell wall-degrading enzymes.

However, these extracts were useful in the investigation of substrate specificity. Again, 4-hydroxyindole was the most reactive substrate in both species in which laccase was extracted, whilst syringaldazine showed a little activity, but only with apricot laccase. Peach laccase disd not oxidise syringaldazine. Toluquinol appeared to be oxidised by both extracts, as did guaiacol, but to a lesser extent.

Extracts from the other two species showed much less laccase activity. Nectarine extracts showed only very slight laccase activity, and then only with 4-hydroxyindole. Plum extracts showed slightly more activity, and again this was detectable only with 4-hydroxyindole.

4.3.2 Buffer Extraction

This gave extracts that tended to be more intensely coloured and, if not treated with pectolytic enzymes, cloudier than acetone precipitated extracts. However, activity levels were comparable, and differences in treatments were seen, as described in the table overleaf. Figures given for increase in activity with Rapidase are approximate averages over all replicates.

	Effect of Treatment (relative to control)		
Plant Species	SDS	Rapidase	Ultrazyme
Peach	Decrease	130% Increase	No effect
Apricot	Decrease	300% Increase	Slight increase
Plum	No effect	Slight increase	Slight increase

Table 4.1 Effect of enzyme treatments and SDS on fruit extracts

Nectarine extracts did not show sufficient activity to make any accurate statements about the effect of treatments, but it should be noted that the only activity detected in nectarine extracts was in those that had been treated with Ultrazyme or Rapidase.

4.4 Discussion

4.4.1 Extraction Procedure

Buffer extraction was the most useful procedure in terms of the results obtained from it, but this was only because attempts to include different types of treatment such as Ultrazyme in the acetone precipitation procedure were not successful. The most useful procedure in terms of routine extraction of purified laccase from fruits is undoubtedly acetone precipitation, as it has the advantage of removing many impurities from the extract. In conjunction with further purification procedures, this method would be desirable for use in any further studies.

4.4.2 Effect of Cell Wall-Degrading Enzymes

The results from this section closely parallel the results from Section 3 except for one important difference; that increases in laccase yield were observed after treatment with Rapidase® Press and no increase was observed after treatment with Ultrazyme. The significance of this is unclear, as both enzyme preparations are thought to be reasonably similar, but the exact composition of each is unknown. However, both preparations contain pectolytic enzymes, and the fact that Rapidase is specifically designed to be effective in fruit extracts (particularly juices) may be the reason that this enzyme preparation was more effective at increasing the yield of laccase from fruit extracts. The observation that a different that a different cell wall-degrading enzyme preparation was effective may be indicative of a difference in cell wall structure between leaves and fruit, or there may be a difference in the exact localisation of laccase in the cell wall of leaves and fruits.

4.4.3 Substrate Specificity

The laccases extracted from fruits of the genus *Prunus* showed similar substrate specificity to the laccases extracted from leaves. Again it seems that oxidation of syringaldazine is the exception and not the rule, as only one out of four extracts showed any activity, and this activity was low. Since Joel *et al* (1978) also found that mango laccase did not oxidise syringaldazine, it seems likely that this substrate is not suitable for the assay of laccases in fruits or leaves.

Again 4-hydroxyindole proved to be the most reliable substrate for the assay of laccase in fruit extracts. Toluquinol was less useful, although it was still oxidised to some extent by most extracts. However, guaiacol proved to be a more effective substrate for fruit laccases than for leaf laccases, although its activity was still lower than the activity with both 4-hydroxyindole and toluquinol. This observation places fruit laccases closer than leaf laccases to the fungal enzyme, although only in this respect, and, in general, fruit laccases seem to be much more closely related to leaf laccases than fungal laccases, at least in terms of substrate specificity, and also probably in terms of their localisation and function.

4.5 Conclusion

It seems that generalisations about higher plant laccases can be widened to include laccases from fruits as well as from leaves, although there are a few minor differences between the two groups of enzymes. The use of 4-hydroxyindole has been shown to be useful in the assay of laccases from fruits as well as from leaves and fungi. The substrate has now been shown to be oxidised by extracts from every known laccase source that has been investigated in the present study. All that now remains is to confirm that the activity is due to the action of a true laccase by the use of specific inhibitor studies.

Chapter 5

Inhibitor Studies

5 Inhibitor Studies

5.1 Introduction

5.1.1 Aims of the Inhibitor Studies

This section sought to perform three functions. Firstly, the oxidative enzymes tentatively identified as laccases in the previous two sections required further characterisation and confirmation of their identity as laccases by using known laccase inhibitors and inhibitors of similar oxidative enzymes (such as catecholase) to establish inhibition patterns that would confirm that the enzymes identified in sections 3 and 4 were laccases.

Secondly, the use of these inhibitors would provide further information on the action of laccase inhibitors on higher plant laccases, which are poorly studied. The effect of cetyl trimethyl ammonium bromide (CTAB), in particular, has only been tested on a few higher plant laccases (peach, spruce and *Rhus vernicifera*), by Walker and McCallion (1980). Other reports have shown conflicting results. Sterjiades *et al* (1992) found that CTAB did not inhibit sycamore laccase, whilst Chabanet *et al* (1994) found that CTAB effectively inhibited mung bean laccase. Dijkstra and Walker (1991) also found that CTAB inhibited apricot laccase. Further information on the selective inhibition of CTAB on higher plant laccases was required. Also, the identification of a novel laccase inhibitor, N-hydroxyglycine, (Murao *et al* 1992) is in need of further study. It has been shown to inhibit fungal laccase from *Coriolus versicolor*, but not any other oxidative enzyme. Studies on this potential selective inhibitor need to be widened to (a) include more fungal laccases, and (b) include higher plant laccases, which have not been studied at all with this inhibitor.

This was the aim of this final section, to investigate the action of a novel inhibitor (N-hydroxyglycine), on higher plant and fungal laccases, and to determine whether or not this inhibitor might prove a useful diagnostic tool in the identification and classification of laccases. This data, along with data from other experiments in this

section, may provide further information about the differences and similarities between fungal and plant laccases.

5.1.2 Other Inhibitors Studied

Apart from CTAB and N-hydroxyglycine, known catecholase inhibitors were also studied, to provide further evidence that the enzymes are laccases. Salicylhydroxamic acid (SHAM) and *trans*-cinnamic acid have been identified as specific catecholase inhibitors (Allan and Walker, 1988), and were used here for this purpose.

5.2 Materials and Methods

Inhibition of laccases identified earlier in this investigation was studied in detail where possible. The low activity of many of the extracts, particularly the leaf extracts, prevented the identification of inhibition type by kinetic studies such as the Lineweaver-Burk plot. However, it was still possible in these cases to establish whether or not inhibition by a particular compound was occurring.

5.2.1 Inhibitors studied

Cetyl trimethyl ammonium bromide (CTAB) was supplied as "Cetavlon" (Cetrimide B.P.) by I.C.I. Ltd., Great Britain. N-hydroxyglycine was kindly synthesised by Glen Foulds of the Department of Chemistry, Canterbury University, according to the method of Jahngen and Rossomando (1982). *Trans*-cinnamic Acid and Salicylhydroxamic acid were also studied.

5.2.2 Inhibition Assay

Kinetic measurements of enzyme inhibition were carried out by microtitre plate analysis as in Section 2.2.7. 4-Hydroxyindole was used as the substrate, at concentrations of 0, 0.67, 1.33, 2, 2.67 and 3.33mM, and with varying concentrations of inhibitor. 50μL of suitably diluted enzyme was present, with the final volume of 300μL made up with pH 6.5 0.1M phosphate-citrate buffer. Initial rates of oxidation obtained as rates of change in absorbance at 620nm were analysed using the enzyme kinetics program ENZPACK™.

In some cases, where colorimetric analysis was not possible (such as the case of CTAB inhibition, which caused micelles to form in the enzyme extract, resulting in cloudy solutions, making accurate colorimetric analysis difficult), and where enzyme activity was sufficient, some quantification of enzyme inhibition was possible using the O_2-electrode. In these cases suitably diluted enzyme was added to the reaction cell, with and without various concentrations of inhibitor, using either 4-hydroxyindole or toluquinol as the substrate. Initial rates of O_2 uptake were used as a

measure of enzyme activity. See Section 2.2.7 for details of the use of the O_2-electrode.

In all other cases, the only indication of enzyme inhibition was a simple observation of the lack of colour formation with the chosen substrate (usually 4-hydroxyindole, since the blue colour of its oxidation product was easy to identify and distinguish from the colour of the enzyme extract). In these cases it was only possible to obtain a simple "yes" or "no" answer.

5.3 Results

5.3.1 General Inhibition Patterns

The formation of micelles in extracts containing CTAB prevented the accurate colorimetric analysis of inhibition, therefore no kinetic plots of inhibition were available. The figures given in the table below were obtained by using the O_2-electrode with 4-hydroxyindole as the substrate. The concentration of CTAB in these cases was 0.66mM except for in the case of *Podocarpus totara*, where 2mM CTAB was used. Other data was estimated by visual observation of colour change.

The percentages for inhibition by N-hydroxyglycine were obtained at a concentration of 0.1M with the Multiskan MCC/340 using 4-hydroxyindole as the substrate.

Table 5.1 Inhibition of plant laccases by various inhibitors

Key: - no inhibition

+,++,+++ inhibition, number of +'s indicates extent of inhibition

Plant Species	CTAB (% inhibition)	SHAM	Cinnamate	N-hydroxyglycine (% inhibition)
Podocarpus totara	55	-	-	-
Cunninghamia lanceolata	43	-	-	-
Rosmarinus officinalis	67	-	-	5
Picea stichensis	44	-	-	5
Podocarpus hallii	100	-	-	-
Podocarpus dacrydioides	++	-	-	-
Dacrydium cupressinium	+++	-	-	-
Podocarpus nivalis	++	-	-	-
Picea pungens	++	-	-	-
Picea omorika	++	-	-	-
Prunus armenica	+++	-	-	69
Prunus domestica	+++	-	-	40
Prunus persica	+++	-	-	74

For comparison, other oxidative enzymes including fungal laccases and plant catecholases, were also assayed with the same inhibitors. 4-Hydroxyindole was used as the substrate with laccases, while 4-methyl catechol was used with catecholase.

Table 5.2 Inhibition of other oxidative enzymes by various inhibitors

See Table 5.1 for Key

Enzyme and Source	CTAB	SHAM	Cinnamate	N-hydroxyglycine
Rhus vernicifera laccase	+++	-	-	-
Armillaria mellea laccase	+++	-	-	-
Pyricularia oryzae laccase	+++	-	-	+++
Coriolus versicolor laccase*				+++
Banana catecholase	-	+++	++	-
Apple catecholase	-	+++	++	-
Mushroom catecholase	-	++	++	-

*From Murao *et al* 1992.

5.3.2 Kinetic Measurements

It was possible to achieve accurate measurements of rates by colorimetric methods only in two cases, those of N-hydroxyglycine with peach and with apricot laccase, from which Lineweaver-Burk Plots could be drawn (Figures 5.1 and 5.2). The results from these graphs show clearly that N-hydroxyglycine with these two sources of laccase gave non-competitive inhibition.

Figure 5.1 Inhibition of peach laccase by N-hydroxyglycine

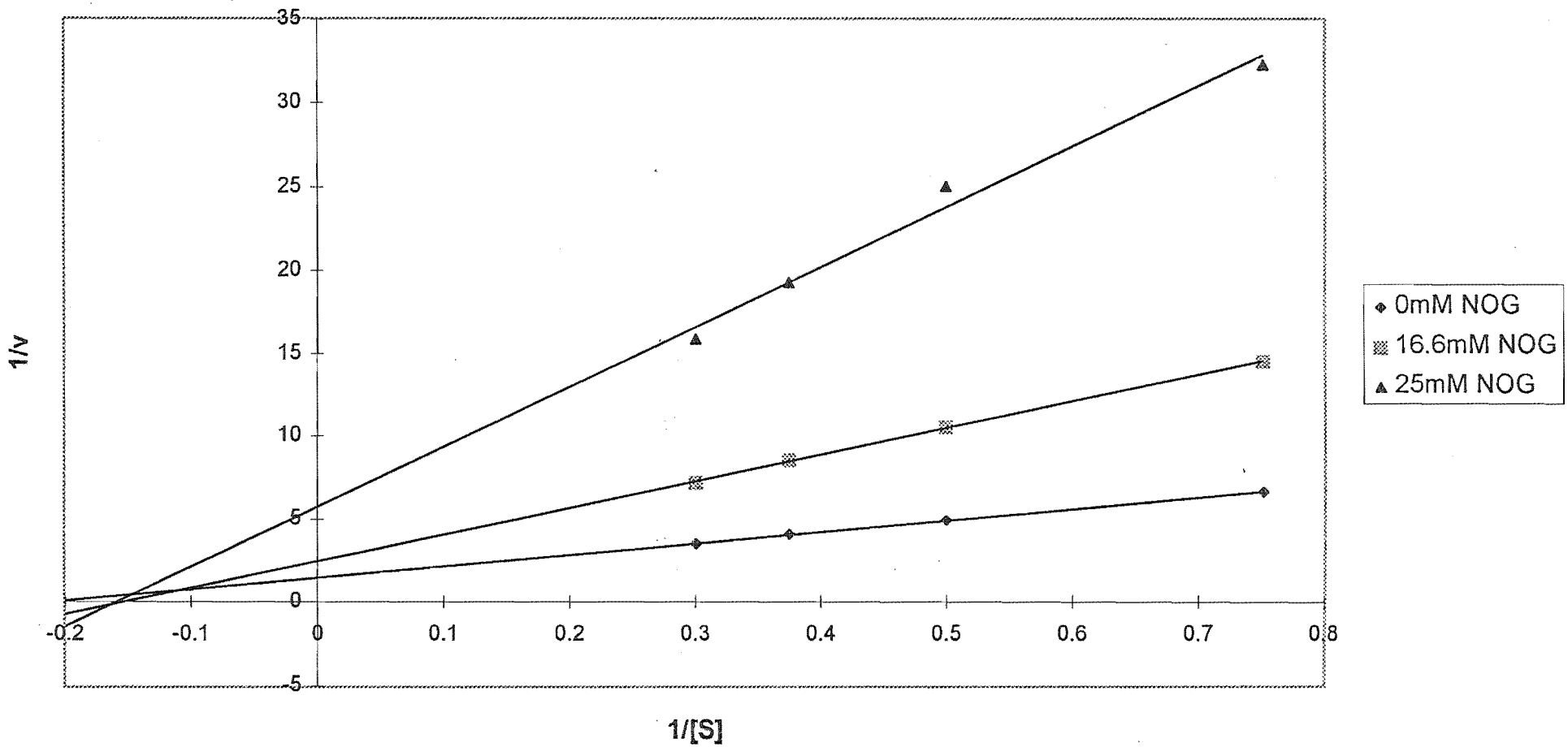
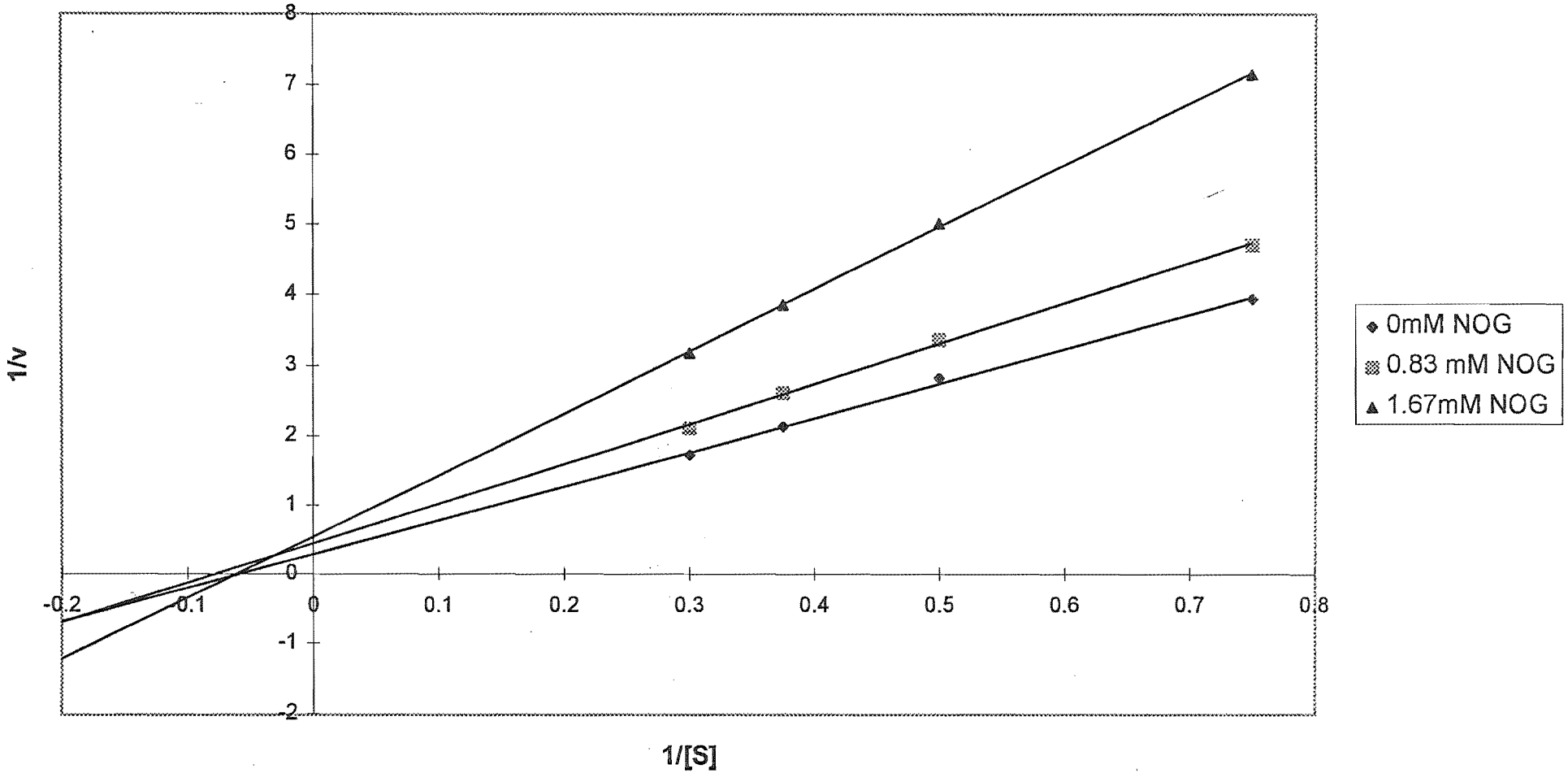


Figure 5.2 Inhibition of apricot laccase by N-hydroxyglycine



5.4 Discussion

5.4.1 CTAB Inhibition

All laccases, both plant and fungal, have been shown to be inhibited by CTAB. This is confirmation that (a) the enzymes tentatively identified as laccases in Sections 3 and 4 were true laccases, and (b) that CTAB is a suitable selective inhibitor for use in the classification of laccases in both plants and fungi. This is also evidence that the oxidation of 4-hydroxyindole was catalysed by a laccase, and not by some other enzyme.

The second point mentioned above is very important in terms of the classification of laccases, as it has been shown that CTAB inhibition appears to be a common property of all the laccases studied. This may prove to be a useful defining characteristic of laccases, despite the differences in substrate specificity and sensitivity to other inhibitors observed both between plant and fungal laccases, and between laccases from different species of plants (or fungi. This could be useful for the identification of oxidative enzymes in future assays, especially in conjunction with other inhibitors of oxidative enzymes, such as SHAM and cinnamate.

5.4.2 N-Hydroxyglycine Inhibition

The above results do not seem to apply to N-hydroxyglycine, as it has shown to be effective only against a small proportion of laccases. Of the plant laccases, only those from the genus *Prunus* were effectively inhibited by the compound (although laccases from *Rosmarinus officinalis* and *Picea stichensis* seemed to be slightly inhibited), even at the high concentration of 0.1M. There was no inhibition of any plant laccases at 10mM. This has also shown to be true for fungal laccases. Murao *et al* (1992) reported that a laccase from *Coriolus versicolor* was inhibited by N-hydroxyglycine, and it was observed that *Pyricularia oryzae* laccase was also inhibited by the compound. However, laccase from *Armillaria mellea* was not inhibited. The inhibitory action of N-hydroxyglycine on fungal laccases requires further study.

However, it seems clear that most plant laccases are not inhibited by N-hydroxyglycine, including laccase from *Rhus vernicifera*, which seems to be less like other higher plant laccases, and more like fungal laccases, on the basis of substrate specificity. This leads to the conclusion that higher plant laccases from fruits are, as a class of enzymes, in some way fundamentally different to laccases from the leaves of higher plants. Whether or not the fruit enzymes share some common structural feature with those fungal laccases that are also sensitive to N-hydroxyglycine inhibition requires further research.

In any case, it has been shown that the mode of inhibition by N-hydroxyglycine on *Prunus* laccases was of the non-competitive type. This was as expected, as N-hydroxyglycine is not a structural analogue of laccase substrates, and so would not be expected to bind at the substrate binding site. However, it could still bind at the active site by binding to the Cu^{2+} cation. However, this mode of inhibitor binding does not seem to be conserved among laccases, and this could explain the observation that many laccases are not inhibited by the compound. The similarity between *Prunus* laccases and some fungal laccases would most likely be an artefact, and not indicate any common ancestry or similar role of the enzyme in the two groups of organisms.

In conclusion, the use of N-hydroxyglycine as a diagnostic tool for laccase identification seems limited. It would not be suitable for the classification of oxidative enzymes as laccases in either fungi or plants, since these results show that a low proportion of laccases, both plant and fungal, are inhibited by the compound.

5.5 Conclusion

This section provides a useful verification of results from Sections 3 and 4. In Section 1.2.2 it was noted that the identification of an enzyme as a laccase relied upon inhibitor tests as well as substrate specificity. The results in this section have provided additional proof that the enzymes extracted from higher plants in Sections 3 and 4 were laccases, while at the same time showing that 4-hydroxyindole is a true laccase substrate, and that CTAB is a universal laccase inhibitor.

Also, this section has answered the questions set forth in Section 5.1 about the novel laccase inhibitor, N-hydroxyglycine, identified by Murao *et al* (1992). While this inhibitor has not been shown to be useful in terms of its ability to act as a specific inhibitor for laccase, it has raised some interesting questions about the properties of laccases in general. While plant and fungal laccases have several differences, there are also differences within each group, and the sensitivity to N-hydroxyglycine is one example. It seems that *Prunus* laccases are different in some way to other higher plant laccases, in their inhibition patterns and their sensitivity to cell wall-degrading enzymes (see Section 4).

Chapter 6

Concluding Remarks

6 Concluding Remarks

6.1 Methods of Screening for Laccase in Higher Plants

The use of 4-hydroxyindole, a novel laccase substrate identified by Cai *et al* (1993b) has proved very useful. Its specificity for laccase seems to be consistent for laccases from all sources, and as such would be very useful as a substrate for use in any future investigations into higher plant laccases, particularly screening a large number of plants. The use of cell wall-degrading enzymes would also be useful, as results from this study have shown that, in some species, laccase cannot be detected without treating extracts with these enzymes before assaying for laccase. Validation of the identity of an enzyme as a laccase can reliably be made by using specific inhibitors, particularly CTAB. Syringaldazine is not an effective substrate for use in the study of higher plant laccases in general.

6.2 Novel Sources of Laccase

A few novel sources of laccase in higher plants have been identified (*Prunus domestica*, both leaves and fruit, *Podocarpus hallii*, *Podocarpus dacrydioides*, and *Dacrydium cupressinium*). However, apart from in the leaves of *Prunus domestica*, which yielded high levels of laccase, especially when treated with Ultrazyme, the yield of laccase from these species was low. This species may prove to be an interesting topic of further study, particularly relating to the difference in the amounts of laccase in the fruits (which gave very low yields) and the leaves.

6.3 Classes of Laccase

The patterns of substrate specificity and inhibitory activity of all the laccases studied here have led to the natural polarisation of laccases into four distinct groups, based on their properties. These groups are listed below, in order of their similarity (i.e. those at each end are the most different from each other).

1. Higher plant laccases from leaves, characterised by the inability to oxidise syringaldazine, and the localisation if the enzyme in the cell wall of the plant. These enzymes are not susceptible to N-hydroxyglycine inhibition.
2. Higher plant laccases from fruit of the *Prunus* genus, characterised by the inability to oxidise syringaldazine, and susceptibility to N-hydroxyglycine inhibition. A slightly different preparation of cell wall-degrading enzymes is required to increase the yield of this class of enzyme than is the case for Group 1.
3. Laccase from *Rhus vernicifera* seems to lie in a class of its own. It is a higher plant laccase, but it shows considerable activity with syringaldazine, and is not located in the cell wall of the plant. It is not susceptible to N-hydroxyglycine inhibition. Laccases from other members of the Anacardiaceae may fit into this group.
4. Fungal laccases, which are characterised by their ability to oxidise syringaldazine. The susceptibility of this group to N-hydroxyglycine is variable. Fungal laccases tend to be extracellular.

There is some crossover between groups, and some laccases do not seem to fit into any group, particularly higher plant laccases which are capable of oxidising syringaldazine, but in general these groups may be helpful when discussing laccases. Also, CTAB inhibition and oxidation of 4-hydroxyindole seem to be common characteristics across all the groups. The following table may be helpful in clarifying the properties of the groups:-

Table 6.1 Inhibition patterns and substrate specificity of various classes of laccase.

	Inhibitors		Activity with substrate?	
Laccase source	CTAB	N-(OH)-glycine	Syringaldazine	4-hydroxyindole
Plant leaves	Inhibited	Not inhibited	Mostly no	Yes
Plant fruits	Inhibited	Inhibited	Mostly no	Yes
Rhus vernicifera	Inhibited	Not inhibited	Yes	Yes
Fungi	Inhibited	Variable	Yes	Yes

6.4 Use of Cell Wall-Degrading Enzymes

The use of Ultrazyme (in the case of leaves) and Rapidase® Press (in the case of fruit) made possible the extraction of adequate levels of laccase from most of the plants studied. This is evidence of the localisation of the enzymes in the cell wall, as hypothesised at the beginning of the thesis. Hopefully the continued use of these type of enzyme preparations will enable the study of higher plant laccases in the future to progress more easily.

However, the most significant aspect of this finding is its implication in the study of lignification. It seems that laccases in higher plants are associated with the cell wall, and this lends support to the hypothesis (reviewed in more detail in Section 1.1.3) that laccase may be involved in lignin formation.

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