THE USE OF LIGNIN PEROXIDASES TO DEGRADE LIGNIN IN PLANT CELL WALLS

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by

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ABSTRACT

THE USE OF LIGNIN PEROXIDASES TO DEGRADE LIGNIN IN PLANT CELL WALLS.

The initial objective of this study was to investigate the use of lignin peroxidase (LiP), produced from *Phanerochaete chrysosporium*, to increase the digestibility of barley straw. Initial experiments using purified LiP and subsequent Van Soest chemical analysis of the straw indicated that no increases in digestibility of the straw were achieved. Further investigation into the increases in digestibility achieved by growing white-rot fungi on straws was undertaken. The solid-state fermentation of straw by *P.chrysosporium, Abortiporus biennis* and *Dichomitus squalens* and the analysis of chemical composition over time showed periods of maximum digestibility increases. This enabled the timing of the extraction of enzymes from the fermentations in order to ascertain the presence or absence of lignin peroxidases in these cultures. Protein gel analysis indicated the presence of some lignin peroxidases with a concurrent co-isolation of a large number of other proteins. Due to the complex nature of straw as a substrate and in an attempt gain a clearer picture the direct actions of manganese dependent peroxidase (MnP), a phenol oxidising enzyme produced by *P.chrysosporium*, and LiP on spruce milled wood lignin (SMWL) was investigated. Size exclusion high performance liquid chromatography (SEC-HPLC) analysis of the reaction products showed that both enzymes cause polymerisation and

not depolymerisation of the SMWL, reaction in sodium tartrate buffer (100 mM, pH 5.0), further, it was shown that the balance of products could be affected by changing the rate of reaction. Spectral analysis of the polymerisation products showed differences which could be attributed to the mechanism of action of the enzymes used. Moreover, further data from spectral analyses not only provided conformation that MnP oxidation is via the free phenol groups, but also indicated that LiP oxidation is similar but involves additional mechanisms.

The importance of both MnP and LiP in lignin degradation was underlined by showing a possible synergistic effect on the release of $^{14}CO_2$ from ^{14}C labelled dehydrogenation polymer (DHP) by cultures of *P. chrysosporium.*

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CONTENTS

ABBREVIATIONS AND SYMBOLS

u - micro.

g - acceleration due to gravity (9.81 m s^{-1}).

ADF - Acid Detergent Fibre.

BSA - Bovine Serum Albumin.

GOD - Glucose oxidase.

$HO^{\cdot}$ - Hydroxyl radical.

HPLC - High Performance Liquid Chromatography.

IVOMD - *In vitro* Organic Matter Digestibility.

kDa - Kilodaltons.

LiP - Lignin Peroxidase.

ME - Mercaptoethanol.

MnP - Manganese dependent peroxidase.

NDF - Neutral Detergent Fibre.

PAGE - Polyacrylamide Gel Electrophoresis

POPOP - 1,4-Di-2-(5-phenyloxazolyl)-benzene.

PPO - 2,5-Diphenyloxazole.

SDS - Sodium Dodecyl Sulphate.

SEC - Size Exclusion Chromatography.

SMWL - Spruce Milled Wood Lignin.

UV - Ultraviolet.

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1.1 LIGNOCELLULOSE - THE POLYMER

The trunks of trees are characterised by elongated cells with thickened walls composed of lignocellulose, which gives the cells structural strength and rigidity.

Lignocellulose itself consists of three main components cellulose, hemicellulose and lignin, all of which themselves are polymeric and it is the interaction of these components with one another and their localisation in the cell wall that gives lignocellulose its characteristic properties, Figure 1.1..

1.1.1. CELLULOSE

The largest proportion of the lignocellulose complex is cellulose, shown to be essentially a linear polysaccharide of β 1-4 linked D-glucopyranose units. In the formation of this linkage each unit must be orientated 180° to each other, Figure 1.2.. This orientation allows hydrogen bonding between the CHOH and OH groups and thus prevents any axial movement of the glucose units resulting in a rigid structure, in contrast to the α 1-4 glycosidic linkage in starch molecules, which enables the individual glucose units free rotation. A consequence of this structural rigidity is that the repeating hydroxyl units, on the cellulose chains, are maintained in the same orientation, allowing them to form intermolecular hydrogen bonds, Figure 1.3., interact with one another electrostatically. Such intermolecular bonding leads to the formation of cellulose microfibrils the rigidity of which is typified in the crystalline structure of native cellulose. This crystalline structure does not allow the

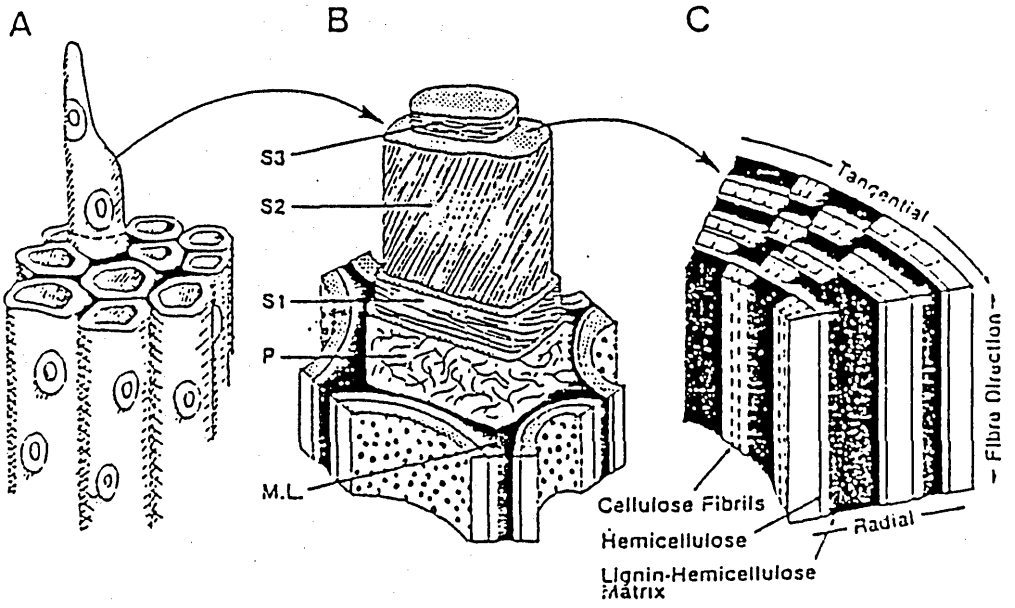

Figure 1.1 Schematic representation of woody tissue.
A: realtionship of individual cells to each other; B: cell wall layers of an individual cell; C: postulated relationship of hemicellulose and lignin in wood fibres, (Goring, 1977).

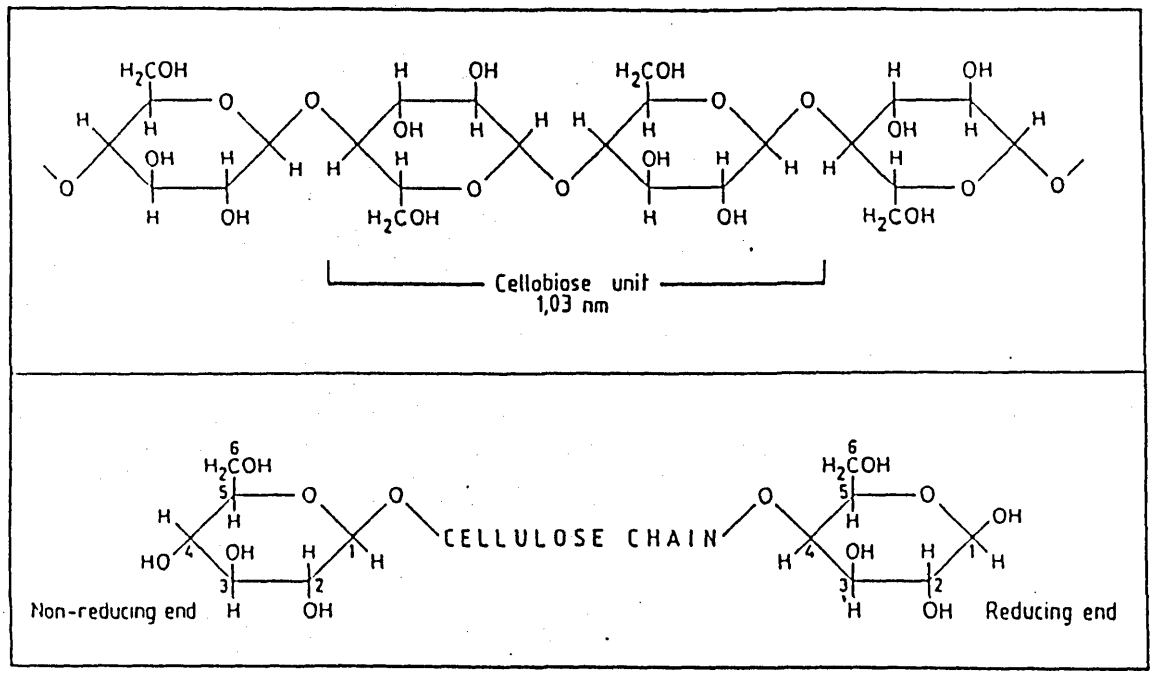

Figure 1.2 **Showing β1-4 linked D-glucopyranose units and the orientation of these units in the cellulose chain** (Fengel and Wegner, 1984).

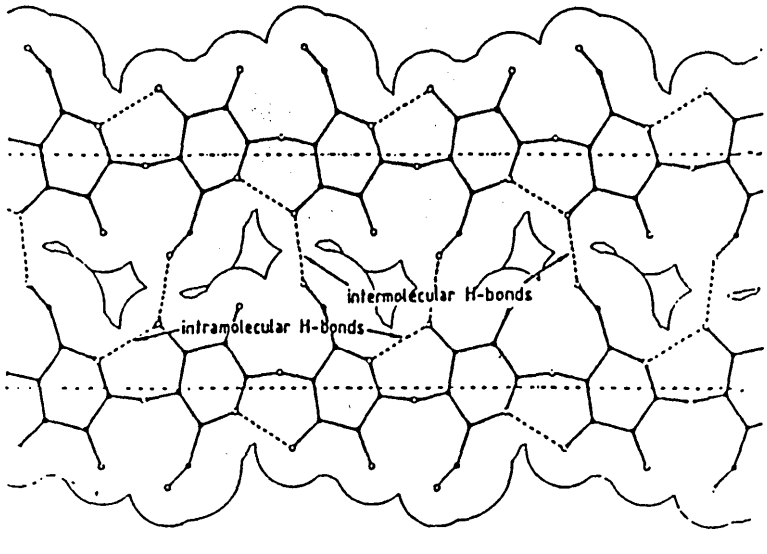

Figure 1.3 **Intermolecular bonding between hydroxl groups in the cellulose microfibrils** (Fengel and Wegner, 1984).

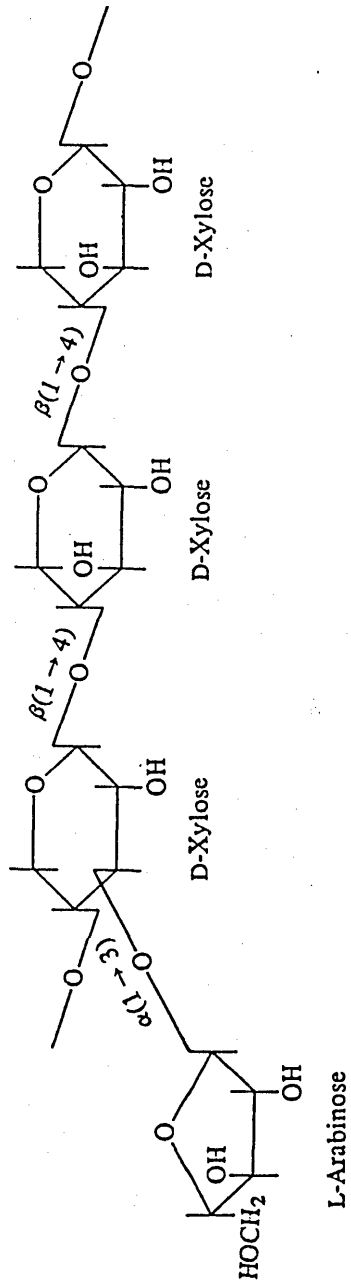

Figure 1.4 Partial structure of an arabinoxylan of angiosperms. (Hall et al, 1982).

penetration of water molecules into the structure and hence, unlike starch, cellulose is water insoluble *in situ*. Despite these close interactions amongst the cellulose chains, these structures do not seem to interact covalently or electrostatically with any other part of the lignocellulose polymer.

1.1.2. HEMICELLULOSE

Hemicelluloses differ from cellulose by consisting of a variety of sugar units, polymerised into shorter chains which are often branched containing sugar acid derivatives, Figure 1.4. They can be found in varying degrees in most plant materials. Hemicelluloses found in dicotyledonous plants are the neutral xyloglucans and the acidic arabinoxylans (M^{c}Niel et al, 1984), whereas, in monocots they tend to consist of acidic arabinoxylans and neutral 1-3α& 1-4β linked glucans (Wada and Ray, 1978) and are frequently found substituted with phenolic acids, such as ferulate and p-coumarate, ester linked to arabinose side chains of the arabinoxylans previously mentioned (Hartley and Buchan, 1979; Mueller-Harvey et al, 1986). It is through similar ester links that hemicellulose, in straws, is associated with lignin (Mueller-Harvey et al, 1986). There is considerable evidence that hemicelluloses have strong bonds with the lignin molecule (Obst, 1982) and one hypothesis suggests that plant secondary cell walls consist of cellulose microfibrils embedded in a ligno-hemicellulosic matrix.

1.1.3. LIGNIN

Lignin is a complex phenylpropanoid polymer which occurs mainly in higher plants where it can form up to 30% of the cell wall

material (Sjostrom, 1981), although this can vary amongst aquatic and herbaceous angiosperms which generally contain less, about 13-20% (Hatakka et al, 1989). Most lower plants such as lichens and algae have no lignin although there has been some evidence of its presence in mosses, although this may only reflect the presence of phenolic compounds and indeed recent work with *Sphagnum magellanium* indicates that they do not contain true lignin (Erickson and Miksche, 1974).

The biosynthesis of the lignin molecule has been subject to a great deal of study over the years (Sarkanen and Ludwig, 1971). Work using alkali-nitrobenzene oxidation of lignin has shown that the lignin molecule is based on the polymerisation of three precusor alcohols, p-coumaryl, coniferyl and sinapyl alcohols (Creighton and Hibbert, 1944). The oxidation of coniferyl alcohol by laccase from *Pasalliota campestris*, used in the form of crude juice extract, yielded a dehydrogenation polymer, DHP (Higuchi, 1958; Freudenburg and Niesh 1968). A similar reaction was shown to occur in the presence of hydrogen peroxide and horseradish peroxidase, the reaction when run to completion gave an insoluble product which was found to have similarities with spruce Bjorkman lignin (Adler, 1977; Bjorkman and Person, 1957). The mechanism itself is complex and is initiated by the dehydrogenation of coniferyl alcohol via a one electron transfer yielding a phenoxy radical. The radicals couple to one another yielding a variety of dilignol products depending on the nature of the radicals involved. However, the dilignol is far from the complex lignin molecule so a further polymerisation must be involved. If the concentration of the monomer is kept low, as is

thought to be so in the lignifying cell (Adler, 1977), then the chances of monomer radicals reacting with another will be decreased and so increases the chances of the monomer radical coupling with the radical of an oxidised dilignol or oligolignol and thus increasing the size of the polymer, a process known as end-wise polymerisation. This form of polymerisation yields several different bond types including those commonly found in the lignin molecule itself. Finally, the involvement of coniferyl alcohol and its related p-coumaryl alcohols in lignin formation has been shown with the use of radiolabelled versions of these alcohols and when fed to a plant they can be shown to be incorporated in the newly formed lignified tissue (Sarkanen and Ludwig, 1971; Gross, 1977).

Because the synthesis of lignin arises from the random coupling of free radicals it does not have a simple repeating structure such as that which characterises cellulose. As a result the structure of lignin cannot be totally defined, however, using oxidative degradation experiments models can be made. One such model for softwood lignin is shown in Figure 1.5. Although this model can not be said to be an exact representation of the lignin molecule it does give some idea as to the complexity of the lignin molecule and the number of different bond types present in the molecule. Analysis of the bond types present in lignin reveals that the most prominent in both hardwood and softwood lignins is β-O-4 ether linkage, accounting for over 50% of the bond types present (Adler, 1977; Glasser, 1980). This high percentage of ether bonds makes lignin extremely stable and can be said to be largely responsible for the characteristic recalcitrance of the lignin molecule.

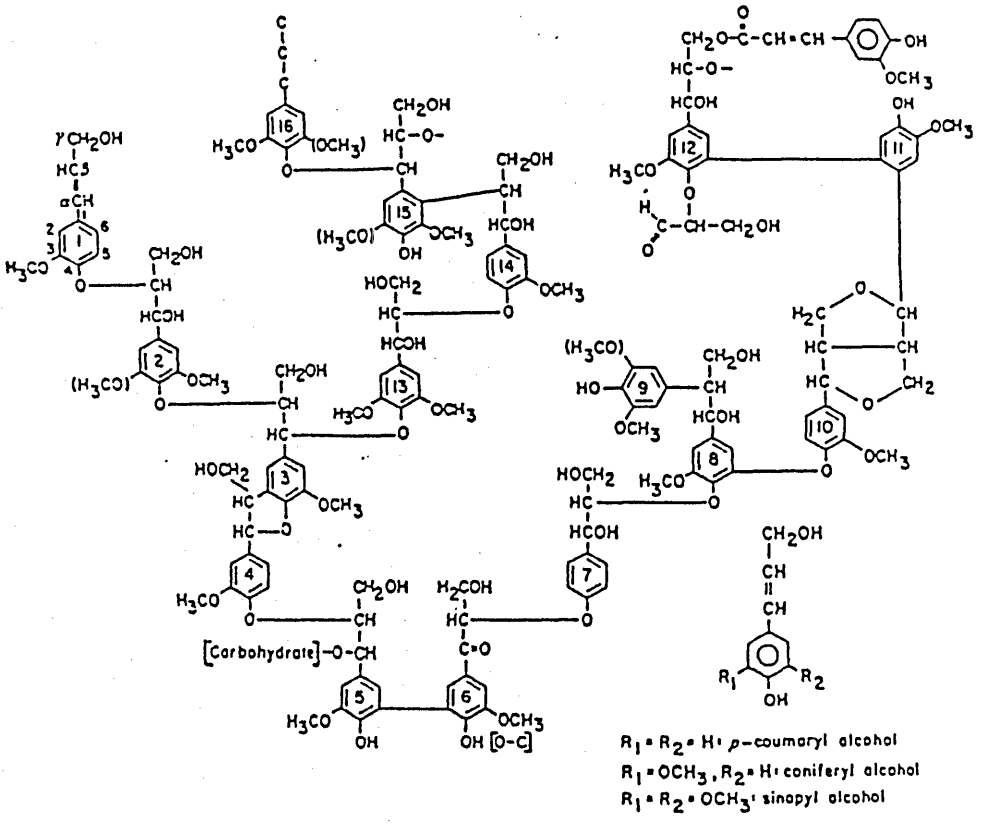

Figure 1.5 Structural model for softwood lignin showing major bond types. (Adler, 1977).

However, it must be remembered that when considering the lignin molecule *in situ* that it forms a matrix with the hemicellulose sugars, with which it is covalently bound (Obst, 1982; Mueller-Harvey et al, 1986). It is this matrix that surrounds the celluose that in effect forms a barrier that retards microbial attack on the cellulose microfibrils.

There are, however, organisms capable of degrading lignin thus enabling them to gain access to the cellulose which becomes a valuable carbon source.

1.2 ORGANISMS CAPABLE OF DEGRADING LIGNOCELLULOSE

Lignocellulose can be degraded by many types of organism including insects,invertebrates, bacteria and fungi. Of these fungi are the most widely studied in relation to their mechanism of lignin degradation.

1.2.1 BACTERIAL DEGRADATION OF LIGNIN

Bacteria known to exhibit lignin decomposing activity include members of the following genera: *Aeromonas; Flavobacterium; Pseudomonas; Bacillus; Streptomyces* and *Nocardia* (Crawford and Crawford, 1980) of these the most studied are the actinomycetes, especially *Streptomyces*. Studies of the degradation by *Streptomyces viridoporus* and *Streptomyces setonii* on Blue spruce (*Picea pungens),* Norway maple (*Acer platanoides)* and Quack grass (*Agropyron repens)*, a soft wood, a hard wood and a grass respectively, have shown that both strains were able to degrade significantly the lignocellulose in all three species (Antai and Crawford, 1981). However, the extent of

degradation of the different plant species was not equal, the grass showed the greatest weight loss of approximately 50% compared to an average of 20% for both the softwood and the hardwood. Similarly, the lignin content of the grass dropped by 40% compared to 33% in the other two woods. Analysis of the chemical transformations occurring during the degradation of isolated spruce lignin, by *Streptomyces viridosporus* and those caused by brown-rot and white-rot fungi (Crawford et al, 1982), showed that changes brought about by *S.viridosporus* most closely resembled those previously reported for white-rotted lignins. Using a variety of techniques, including infra red and ultra violet spectroscopy, it was found that there was a fall in the aromaticity of the lignin to a level similar to that found in white-rotted wood. Consequently, the changes brought about in the lignin molecule were found to be due to intra polymeric ring cleavage reactions, resulting in a degraded lignin that contained branched carbon-carbon chains linked to still intact aromatic rings (Crawford et al, 1982). Although degradation of wood by actinomycetes and higher fungi seem to have similarities, their degradation of grass lignocellulosics appears to differ. Work has shown that actinomycetes in contrast to white-rot basidiomycetes, only degrade grass lignocellulose to a certain level through partial solubilisation, resulting in a lignin rich structure, acid-precipitable polymeric lignin (APPL) (Crawford et al, 1983). On average all APPLs have a molecular weight of about 5 Kd or less and are further characterised by having a hemicellulosic carbohydrate moiety associated with them, thought to account for up 20% of the total material (M^{c}Carthy, 1987). There seems to be some variation in the properties of

APPL produced between bacterial species although this maybe dependant on their method of extraction. Lyophilization yields an APPL with a lower molecular weight than that obtained by acid precipitation (Crawford et al, 1983). The APPL itself is resistant to further decay by the producing organism, however, there is some evidence that other bacteria are able to carry out further limited degradation, although it was found to be recalcitrant to attack by the white-rot basidiomycete *Phanerochaete chrysosporium*. (Pometto and Crawford, 1986).

It has been shown that bacteria other than actinomycetes are capable of degrading APPL produced by *S.viridiosporus* (Seelenfreund et al, 1990). This study showed that *Pseudomonas sp.* and *Enterobacter sp.* attacked the APPL but only the sugar moieties were affected. However, other strains of *Pseudomonas* did attack the aromatic residues of the APPL. The authors suggest that *in situ* these filamentous and non-filamentous bacteria act in consort with one another and since it has been suggested that APPLs resemble humic acids (M^{c}Carthy et al, 1986), they also suggest that these groups of bacteria are involved in the humification of lignocellulosic materials.

Work has continued in the study of actinomycete degradation of lignin with the development of a method to screen genera for their ability to degrade ^{14}C labelled straw (M^{c}Carthy and Broda, 1984) which was found to be a better indicator of lignolytic ability than of their ability to grow on lignin type phenolics as the sole carbon source, which was previously used (Janshekar and Fiechter, 1982).

1.2.2 FUNGAL DEGRADATION OF LIGNIN

Several species of fungi are capable of degrading

lignocellulose to varying extents. Amongst these are Basidiomycetes, Ascomycetes, Phycomycetes, Xylariaceae and mycohrrizal fungi. The most important fungi are to be found in the Basidiomycetes. Wood decay itself is classed in three types: soft-rot; brown-rot and white-rot, the latter has been most studied in respect to its mechanism of enzymic attack on lignin.

The soft-rot fungi, which include ascomycetes and fungi imperfecti, are so characterised because they leave a chain of cavities with pointed ends, in the S_2 layer of the cell wall, arranged in a helical pattern parallel to the orientation of the cellulose microfibrils (Levy and Dickinson, 1980).

Brown-rot fungi leave a characteristic brown colouration in the wood and have been shown to preferentially attack the carbohydrate units of the lignocellulose polymer, whilst only modifying the lignin component, (Kirk, 1975). Studies of lignin left after brown-rot attack have shown extensive demethylation, increased carbonyl content and substantial increase in phenolic hydroxyl groups, thought to arise as a result of the extensive demethylation reactions (Kirk, 1975; Kirk and Adler, 1970). Consequently, it has been suggested that brown-rot degradation of lignin is primarily oxidative, however, unlike white-rot fungi they seem to lack an efficient ring cleavage system which prevents the complete degradation of the lignin molecule (Kirk and Farrell, 1987).

White-rot fungi, not only leave the wood a characteristic white colour, but have also been shown to completely degrade lignin to carbon dioxide with a simultaneous attack on the carbohydrate moieties, within the lignocellulose complex (Otjen and Blanchette, 1982). Despite

this, preferential lignin degradation has been documented for several white-rot fungi (Blanchette, 1984) and for mutants that lack cellulase activity (Eriksson and Goodell, 1974). These selective lignin degraders have been shown to degrade a substantial amount of hemicellulose leaving the cellulose relatively untouched, potentially increasing the digestibility of the material (Blanchette and Reid, 1986).

Much of the work on fungal degradation of lignin has been on white-rot and brown-rot basidiomycetes. However, other groups of fungi have also been identified as having lignin degrading abilities. Several species of mycorrihizal fungi, including *Cenococcum geophilum* and *Rhizopogen luteus,* were capable of degrading both lignocellulose and ring labelled coniferyl alcohol dehydrogenation polymer, DHP (Trojanoski et al, 1984). Whilst these fungi were able to degrade these compounds, the rate of degradation was much slower than that of two white-rot basidiomycetes, *Fomes annosus* and *Sporotricum pulverulentum*, used as a comparison. Similarly, studies on the white-rot Xylariaceae, *Hypoxylon cohearens* and *Xylaria hypoxylon*, have shown that although these fungi decayed sterile wood blocks in a manner visually reminicient of white-rot basidiomycetes, however, the rates observed were considerably slower (Sutherland and Crawford, 1981).

1.3 STRAW AS A SUBSTRATE

It is inevitable that in the large scale production of cereal crops a large amount of straw is also produced, for example in 1983 40% of the total yield from cereal crops in England and Wales was

straw. This is equivalent to 13.44 million tonnes of material, half of which was burnt in the field, a waste of a potential resource. Traditionally straw has been used as an animal feed, and recently this trend has been on the increase, especially in the more densely populated ares of the Earth where there is little land that can be turned over to solely animal foraging. With the populations of these areas unlikely to fall the use of straw as an animal feed will increase.

However, these straws differ from other plant materials used as feedstuffs in that although 70-80% of the dry matter is carbohydrate, it is mainly in the form of less easily accessible structural polysaccharides, as opposed to easily hydrolysable carbohydrates such as starch. Futhermore, because straws are theby-productof a food crop the levels of lignin in the cells tend to be high since harvest of the crop is dependant on the age of the food crop. As mentioned earlier, section 1.1.2., covalent linkages are formed between the lignin molecule and hemicelluloses in the cell wall (Hartley and Buchan, 1979; Mueller-Harvey et al, 1986). This lignin - hemicellulose complex forms a matrix surrounding the cellulose microfibrils. Therefore, the level of lignin is the overriding factor in determining the accessibility of the cell wall polysaccharides to attack by rumen organisms (Brice and Morrison, 1982). Indeed work has shown that examination of cell walls undergoing rumen digestion suggests that due to selective removal of polysaccharide a modified surface layer is formed which due to its high lignin content appears to be recalcitrant to further microbial attack (Chesson and Murison, 1987). It would seem then that if increases in digestibilty of the material are to be achieved then the formation of this modified

surface layer must be prevented. Increases in digestibilty have been achieved by the disruption of the lignincarbohydrate links within the cell wall by chemical pretreatment, section 1.3.2.. Alternatively, oxidation of the lignin molecule macromolecule itself by the pretreatment with fungi, fungal enzymes or other methods may also lead to an increase in carbohydrate accessibility and hence digestibility.

The methods by which the digestibility of lignocellulose is increased can be split into three groups: physical; chemical; and biological treatments.

1.3.1 PHYSICAL TREATMENTS

The physical treatment of straws is mainly achieved by the steam explosion method, in which the straw is subjected to steam at high temperature and pressure (31 bar, 230 °C,) for approximately 1 minute, followed by a fast release of pressure. This is thought to increase the surface area of the material, by disrupting the interaction of the polymers enabling greater enzymatic access, in conjunction with solubilising some of the lignin. However, a large proportion of the hemicellulose can be lost during this process (Katrib et al, 1988). Other methods which avoid this loss of hemicellulose have also been used. Ball milling or grinding not only increases the surface area of the fibres but is also reported to break some of the ether bonds, within the lignin molecule (Scalbert and Monties, 1986) thus enabling greater enzymatic access to the carbohydrate moieties.

1.3.2 CHEMICAL TREATMENTS

Chemical methods for improving the digestibility of straws and other roughages have been suggested as early as 1921, with a method involving the soaking of feed material in vats of sodium hydroxide followed by washing with water. Unfortunately, not only was this process not commercially feasible due to the high chemical cost, but more important was the loss of 20-30% of dry matter through leaching during the washing process. The process was improved upon with the development of a spraying technique (Ferguson, 1942), making the method cheaper and avoiding the loss of dry matter through leaching. Sodium hydroxide is not the only substance to be used in this manner and since then many groups have investigated the feasibility of other chemicals for the upgrading of straws for animal feeds (Morrison, 1988; Klopfenstein et al, 1972; Saarinen et al, 1959; Brice and Morrison, 1982). A study comparing the effect of a range of chemical treatments, including: sodium hydroxide; ammonia; chlorite; and potassium hydroxide, found that all these reagents were capable of effecting an increase in the dry matter *in vitro* digestibility of barley straw (Scalbert and Monties, 1986). Furthermore, it was only those reagents which primarily attacked the core lignin that gave residues with greatly increased degradabilities (Scalbert and Monties, 1986). The mechanism of action of these chemicals is thought to be, as in the case of sodium hydroxide, the solubilisation of part of the core lignin and the swelling of the cellulose chains enabling better enzymatic penetration. The key factor being the partial or total loss of the lignin (Chandra and Jackson,

1971). Despite this a recent study has shown that N-methylmorphine-N-oxide, an organic solvent, was capable of increasing the saccharification of straw (Brice and Morrison, 1982). What is more important is that levels of digestibility comparable to those obtained with alkali were realised, despite the fact that this compound does not remove lignin. It is thought to cause disorganisation of the ultrastructure of the cell wall and so facilitate enzyme penetration.

There has, however, been some doubt as to the importance of the increases *in vitro* digestibilities obtained in these studies (Ferguson, 1942; Ford et al, 1987). In a study, with sheep, chlorite treated straw, although highly degradable *in vitro* and *in saccho* in the ovine rumen, was very poorly degraded when used in a feeding trial (Morrison, 1988). This poor digestibility was attributed to inhibition of fungal growth in the rumen, and hence full digestion of the feed was not possible. This is in contrast with earlier work which reported that chlorite pulps were successfully used in a feeding trial although, in this case the treated feed only accounted for 50% of the food provided (Saarinen et al, 1959). This mixture may have prevented the inhibition of the fungi in the rumen and hence the trial appeared successful.

In one feeding trial it was found that barley straw that had been treated with 5% sodium hydroxide for 1 hour increased the *in vitro* digestibility of the feed (Han and Smith, 1978). However, further treatment of the straw with *Cellulomonas sp.* and *Alcaligenes faecalis* decreased the feed efficiency and digestibility even though the protein content of the feed was increased, this probably being due to the loss of cellulose content during the growth of the organisms. In a recent

study on the combined effects of chemical and biological treatment of bagasse, it was found that chemical treatment in conjunction with autoclaving gave large increases in digestibility of the material (Al-Ani and Smith, 1988). Similarly, the effect of post sodium hydroxide fermentation with *Phanerochaete chrysosporium*, a white-rot basidiomycete, was investigated. The results indicated that treatment of the bagasse after sodium hydroxide treatment had a beneficial effect on its overall digestibility, above that already achieved by alkali alone. This increase could possibly be due to the added loss of lignin brought about by *P.chrysosporium* which the fungi above were not able to achieve.

1.3.3 FUNGAL TREATMENTS

The use of fungal treated lignocellulose as a cattle feed is not a new concept. In southern Chile "Palo Podrido" has been used for many decades as an animal feed. It is obtained from white-rotted logs of *Drymis winteri, Eucryphia cordifolia, Laurelia philippiyna* and *Northofagus dombeyi*. The fungi, *Ganoderma appalantum* and *Armillariella sp.*, responsible for rot of these woods were identified, along with quantification of the lignin contents and digestibilities before and after decay (Zadrazil et al, 1982). Remarkably, the *in vitro* digestibility of the starting wood ranged from 0 to 3.1% which reached a high of 77% post fermentation in conjunction with a reduction of the lignin level to 1%. Obviously, from these results the potential of fungi to increase the digestibilities of poor feeds is high. However, not all wood degrading fungi have been found suitable for this purpose, brown-rot fungi have been shown to actually decrease the digestibility of the straw (Zadrazil,

1984).

The choice of fungus to treat straw in an attempt to increase its *in vitro* digestibility is of the upmost importance (Zadrazil et al, 1982; Zadrazil, 1980). Most commonly used are basidiomycetes, however, these do not all have the same mode of action on the material (Zadrazil et al, 1982; Zadrazil and Brunnert, 1982). From work on the solid-state fermentation of straws by these fungi it is clear that they can be split into four groups (Zadrazil, 1984):

1) Those that decompose the substrate without lignin degradation, resulting in a digestibility less than the starting material. Examples of which are the brown-rot fungi such as *Agrocebe agerita* and *Flammulina velutipes.*
2) Those that increase the *in vitro* digestibility by degrading the lignin but only partially decomposing the other components. These include such fungi as: *Dichomitus squalens, Abortiporus biennis* and *Pleurotus sajor caju.*
3) Fungi that decompose all the components but have negative effect on the *in vitro* digestibility.
4) Fungi that decompose all the components rapidly, but have no significant effect on digestibility, such as *Phanerochaete chrysosporium.*

It would seem therefore that fungi capable of decreasing the lignin content, whilst maintaining a low level of organic matter loss, in the form of cellulose and hemicellulose, would be the most desirable for

use in a solid state fermentation system designed to increase the rumen digestibility of straw. However, it has been shown that the increases in digestibility achieved by these fungi are subject to influence by external factors such as gaseous metabolites, temperature, nitrogen source, water content and light (Zadrazil and Brunnert, 1981; Zadrazil and Brunnert, 1980; Kamra and Zadrazil, 1986; Levonen-Munoz and Bone, 1985). Work with *Pleurotus spp.* has shown that an increased temperature from 22°C to 30°C had an adverse effect on lignin degradation in conjunction with an increase in organic matter loss, resulting in loss in digestibility of the material (Zadrazil and Brunnert, 1981). Similarly, addition of an external nitrogen source, as ammonium nitrate, resulted in a final material that had an *in vitro* digestibility lower than that of the starting material, whilst in the absence of external nitrogen the digestibility increased (Zadrazil and Brunnert, 1980). It is not only the composition of gaseous phase but also its flow through the system that is important in maintaining efficient solid state fermentation (Levoen -Munoz and Bone, 1985; Kamra and Zadrazil, 1985). It has been shown that the excessive passage of air or oxygen through the substrate results in increased organic matter loss during fermentation of wheat straw (Kamra and Zadrazil, 1986). Furthermore, the same authors have also shown that lignin degradation is increased in an atmosphere of oxygen as opposed to air resulting in a net increase in digestibility of the substrate. Therefore it is clear that the successful solid state fermentation of straw requires the consideration and balance of a number of factors.

Although, effective treatments have been achieved on a

laboratory scale problems have been encountered when attempts have been made to scale up the process (Zadrazil, pers. com.; Levonen-Munoz and Bone, 1985). Increases in *in vitro* digestibilities have found to be more than halved on scale up i.e. +25% on lab scale +9% larger scale (Zadrazil and Brunnert, 1981). Reasons for this drop in efficiency may be due to the increased difficulties in maintaining optimum conditions, air flow, temperature etc., on a larger scale (Zadrazil, pers. com).

Due to the difficulties encountered in maintaining optimum conditions for fungal growth, whilst still maintaining low organic matter loss and high lignin depletion, it would seem that the use of lignin degrading enzymes obtained from these fungi may offer another alternative. The advantages being the possibility of direct attack on the lignin without any loss of organic matter through growth of the fungus and shorter time span of treatment, fungal treatments can last up to 60 days, with enzymatic treatments potentially being effective within hours.

1.4 MECHANISM OF LIGNIN DEGRADATION

Originally diffusible activated forms of oxygen, hydroxyl radicals ($HO^{\cdot}$), were thought to be responsible for lignin degradation by white-rot fungi (Hall, 1980; Kirk and Nakatsubo, 1983). The involvement of hydrogen peroxide in the lignolytic system of the white-rot basidiomycete *Phanerochaete chrysosporium* was implicated (Fourney et al, 1982), later confirmed with the discovery that the addition of catalase inhibited lignin degradation by this fungus (Faison and Kirk 1983; Kutuski and Gold, 1982). It was also suggested that $HO^{\cdot}$ radicals were generated from hydrogen peroxide by metal ions. Furthermore, $HO^{\cdot}$

radicals generated from Fentons reagent (1M H_2O_2 / 10 mM $FeSO_4$) were shown to oxidise lignin (Gold et al, 1983) and that oxidation of lignin was inhibited by $HO^{\cdot}$ scavengers (Bes et al, 1983). Further evidence in support of $HO^{\cdot}$ involvement was provided when it was shown that a $HO^{\cdot}$ generating system (glucose/glucose oxidase/Fe^{2+}) was able to degrade a lignin model dimer to yield products similar to those produced by *P.chrysosporium*.

Since these hypothesises were made the discovery, purification and characterisation of extracellular enzymes from *P.chrysosporium* (Tien and Kirk, 1983; Tien and Kirk, 1984; Glenn et al, 1983; Harvey et al, 1985a; Kuila et al, 1985; Renganathan and Gold, 1986) has led to the proposal that lignin degradation in white-rot fungi is an enzymatic process and that $HO^{\cdot}$ radicals are not soley involved in an important reaction in lignin degradation by these fungi (Kirk et al, 1985).

1.5 LIGNIN DEGRADING ENZYMES

1.5.1 LIGNIN PEROXIDASE

The simultaneous isolation of an extracellular enzyme from lignolytic cultures of *P.chrysosporium* by two groups in 1983 (Tien and Kirk, 1983; Glenn et al, 1983), opened a new area in the elucidation of the mechanism of lignin breakdown by white-rot fungi. The enzyme was found to be capable of oxidising methoxylated lignin and lignin model compounds (Tien and Kirk, 1983; Glenn et al, 1983). Though originally thought to be a unique hydrogen peroxide requiring oxygenase; due to

the incorporation of molecular oxygen in model compound degradation products (Hammel et al, 1985), it was later established that the enzyme was in fact a classic peroxidase ,a glycoprotein with a molecular weight of 42 kDa but with an unusually low pH optimum of pH 3.0, generally referred to as lignin peroxidase, LiP (Harvey et al, 1985a; Kuila et al, 1985; Renganathan and Gold, 1986). Since its isolation the enzyme has been shown to contain an iron III protoporphyrin IX prosthetic group and to exist in at least 10 isoenzymic forms, with most studies being carried out on the isoenzyme H8, although all the isoenzymes seem not to differ in their functionality towards lignin (Farrell et al, 1989). The introduction of a spectrophotometric assay based on the oxidation of veratryl alcohol, a secondary metabolite of the fungus, to veratraldehyde (Tien and Kirk, 1984) has enabled the screening of other white-rot fungi for LiP enzymes; similar peroxidases have been isolated from several other lignolytic white-rot fungi (Biswas-Hawkes et al, 1987; Dodson et al, 1987; Jonsson et al,1987; Hatakka et al 1987).

The mechanism of action of LiP on its substrates, phenolic and non-phenolic electron rich aromatic rings, has been established as a one electron oxidation process (Harvey et al, 1985; Kuila et al, 1985). During the redox cycle of LiP the native enzyme is oxidised by hydrogen peroxide to compound I, an oxo-iron (IV) porphyrin radical cation (Harvey et al, 1989) . One electron reduction, achieved by removing an electron from the substrate, produces compound II, an oxo-iron (IV) porphyrin complex, further one electron reduction of compound II yields native enzyme, although in the presence of excessive hydrogen peroxide compound II can be further oxidised to compound III, a less reactive

intermediate, although if veratryl alcohol is also present it has the capacity to convert compound III back to the native enzyme, maintaining the redox cycle (Harvey et al, 1987; Cai and Tien, 1989; Wariishi and Gold, 1989). It has been suggested that veratryl alcohol can not only maintain the redox cycle of the enzyme, but the radical cation produced from it is also capable of acting as an oxidation intermediate between the enzyme and less accessible parts of the lignocellulose polymer and compounds not directly oxidisable by lignin peroxidase itself (Harvey et al, 1986). It is the ability to form radical cations in lignin model compounds which in turn result in the breaking of the C_{α}-C_{β} bond, an essential reaction in lignin degradation, that has added weight to the claim that lignin peroxidase is important in lignin breakdown. The involvement of these enzymes has been further implicated with the observation that the addition of exogenous LiP to lignolytic cultures of *P.chrysosporium* increases the rate of $^{14}CO_2$ release from ^{14}C labelled lignin (Haemmerli et al, 1986; Huynh et al, 1986; Liesola et al, 1988). However, *in vitro* the enzyme has been shown to polymerise lignin (Haemmerli et al, 1986), this is thought to arise from its ability to polymerise phenolic compounds, a reaction which unfortunately results in the inactivation of the enzyme (Harvey and Palmer, 1990).

The enzyme is also capable catalysing a range of other reactions including: aromatic ring opening, demethoxylation, hydroxylation, decarboxylation and phenol coupling reactions (Harvey et al, 1985).

Initially, only small quantities of the enzymes were available, however, with the development of techniques that enable the growth of

the fungus in submerged culture larger quantities have now been made available (Leisola and Fiechter, 1985; Linko et al, 1986; Asther et al, 1987; Kirkpatrick and Palmer, 1987). Consequently, both the amino acid sequence of the enzyme (Tien and Tu, 1987) and its crystalline structure have been ascertained (Kaspar et al, 1989). Furthermore, studies on these enzymes have now been extended from the purely biochemical to the field of molecular biology (Raeder et al, 1989).

1.5.2 MANGANESE DEPENDENT PEROXIDASE.

Along with lignin peroxidases another group of extracellular peroxidases are also expressed by cultures of *P.chrysosporium*, and are generally referred to as manganese dependent peroxidases (MnP) (Paszcynski et al, 1985). Like lignin peroxidase these enzymes show classic peroxidase spectral properties and have been shown to contain an iron IX protoporphyrin. Like lignin peroxidases they are produced by secondary metabolic cultures of *P.chrysosporium*, achieved by allowing the cultures to reach either nitrogen or carbon deficiency, however they are secreted slightly before lignin peroxidase (Gold and Glenn, 1988). They have been shown to also be glycoproteins but with an average molecular weight of 46 kDa (Gold and Glenn, 1988). Their mechanism of action involves the oxidisation of Mn^{2+} ions to Mn^{3+} ions, however, the stability of the Mn^{3+} ions depends the presence of an appropriate chelating agent such as an α hydroxy acid (Paszczynski et al, 1988). The chelated Mn^{3+} ions act as non specific oxidant for a variety of organic compounds, Table 1.5.1. It is the ability of these ions to oxidise phenolic compounds that is thought to be the most important role of

these enzymes in lignin degradation.

Table 1.5.1 Assay substrates for Manganese peroxidase (MnP), adapted from Paszczynski et al, 1988.

Substrate	Wavelength(nm)	E($M^{-1}cm^{-1}$)
TMPD[a]	610	11,600
Vanillylacetone	336	18,300
Syringic acid	260	8,050
Guaiacol	465	12,100
Syringaldazine	525	65,000
Coniferyl alcohol	263	13,400

[a] N,N,N,N-Tetramethyl-1,4-phenylenediamine(-2HCL)

As mentioned earlier LiP is capable of oxidising phenolic compounds but with a subsequent inactivation of the enzyme, it is thought then that the role of the MnP is to oxidise the phenolic lignin and reaction products, without inactivation, whilst the LiP is able to oxidise the non-phenolic components of the molecule. This synergistic action of these enzymes is thought to be the basis of lignin degradation by white-rot fungi. An alternative role for these enzymes has been suggested through their ability to produce hydrogen peroxide from the oxidation of either glutathione, NADPH or dithiothreitol (Glenn and Gold, 1985). However, the suggestion that these enzymes are responsible for hydrogen peroxide production for LiP is unlikely to be the case.

1.5.3 OTHER ENZYMES INVOLVED.

The origin of the hydrogen peroxide required by both LiP and MnP has not yet been established, however, several potential sources

have been identified. Both glucose-1-oxidase (gox) and glucose-2-oxidase have been implicated (Kelly and Reddy, 1986; Erickson et al, 1986). However, both these enzymes are intracellular and although gox$^-$ mutants lost their ability to degrade ^{14}C labelled lignin (Kelly et al, 1986) it has still not be established that these enzymes are responsible for exogenous hydrogen peroxidase production. A more likely candidate is exogenous glyoxal oxidase which is capable of producing peroxide from -hydroxycarbonyl and dicarbonyl compounds, moreover, it is also thought that fragments from LiP cleavage of lignin can also act as substrates (Kersten and Kirk, 1987; Kersten et al, 1989).

Laccase, a copper containing polyphenol oxidase, has also been implicated in lignin biodegradation, although the enzyme has been shown to cause both depolymerisation and polymerisation of lignin (Green, 1977; Kwai et al, 1988; Bourbonnais and Paice, 1990).

Other enzymes thought to be involved in lignin biodegradation include cellobiose:quinone oxidoreductase (CBQase) which reduces quinones and possibly phenoxy radicals, products of lignin degradtion by LiP and MnP, with the simultaneous oxidation of cellobiose to cellobionolactone. The removal of these being essential to prevent their reoxidation by LiP and MnP which would lead to net lignin polymerisation (Ander et al, 1990).

1.6 AIMS OF THE THESIS

The initial aim of this project was the use of lignin peroxidase from *Phanerochaete chrysosporium* to increase the digestibility of straws for use as animal feeds, as an alternative to the existing chemical and

physical methods, see earlier.

As can be seen from the earlier sections the digestibility of straws is dependant on the interactions of the lignin molecule with the hemicellulose and cellulose molecules in the lignocellulose complex. This is because the cellulose microfibrils are effectively inaccessible to the rumen organisms since they are embedded in a recalcitrant matrix of lignin. It was hoped, therefore, that lignin peroxidase would be able to disrupt this matrix, through degradation or modification of the lignin as well as breaking lignin-hemicellulose bonds, allowing greater enzymatic access in the rumen. Further studies on the interaction of Lignin peroxidase and lignin at a molecular level were also initiated. It was hoped that these studies would lead to a better understanding of the interactions between the enzyme and the lignin molecule and so provide a clearer picture as to how these enzymes could be used to increase the digestibilities of straws.

CHAPTER 2

MATERIALS AND METHODS

2.1 GROWTH AND MAINTENANCE OF ORGANISMS

Cultures of *Phanerochaete chrysosporium* ATCC 24725 were maintained on 4 % Potato Dextrose Agar slopes, 30 °C with 10 day cultures being used as sources of spore inoculum.

Abortiporus biennis (84C PRL) and *Dichomitus squalens* (70A PRL) were maintained on 3% malt agar slopes at 25°C, with 7 day cultures being used as sources of inoculum

2.2 ENZYME PRODUCTION

Due to the need for a plentiful supply of enzyme for this project a comparison of three methods of production was undertaken in order to ascertain which would provide the most plentiful and reliable supply.

This was achieved in three ways :

Method 1 : This was essentially the semi-continuous method of Kirkpatrick and Palmer (Kirkpatrick and Palmer, 1987) except that cultures were carbon limited using 0.2 % Glucose and 5.1 mM Nitrogen.

Method 2 : As for method 1 but no foam was used and a spore suspension of 3.3×10^7 spores / ml was used for inoculation.

Method 3 : As for method 1 but the 45 cubes of foam were replaced with 45 cubes of nylon net, 1 cm^3 (Linko et al, 1987).

Enzyme activty was monitored after induction of the cultures with veratryl alcohol (Kirkpatrick and Palmer, 1987), the flasks being sampled by removing 1 ml aseptically and assayed for lignin peroxidase activity as in section 2.3.1. Data for enzyme production can be seen in Figure 2.1

2.3 MEASUREMENT OF ENZYME ACTIVITY

2.3.1 Lignin peroxidase activity :

A spectrophotometric assay based on the oxidation of veratryl alcohol to veratraldehyde was used (Tien and Kirk, 1983). One unit of enzyme being that able to produce 1 umole of veratraldehyde from 1 umole veratryl alcohol per ml enzyme per minute.

2.3.2 Manganese dependent peroxidase activity :

This was achieved by measuring tetraguaiacol formed at 470 nm from the oxidation of guaiacol in the presence of hydrogen peroxide (5 ul, 10 mM) and manganese II ions (10 ul, 10mM $MnSO_4$) at pH 5.0 (0.1 M Sodium tartrate buffer), using 1ml cuvette, 1cm light path, ambient temperature. One unit of enzyme being that able to produce 1 umole tetraguaiacol per ml enzyme per minute.

2.4 ENZYME HARVESTING

On days 5,6,7,and 8 the contents of the flasks were aseptically poured through three layers of sterile muslin into a flask over ice, taking care not to lose any of the cubes of foam, nylon net or any pellets. Fresh 1:10 diluted media (200 ml) were added to the flasks

which were then returned to the incubator for further incubation and enzyme analysis (Kirkpatrick and Palmer, 1987).

2.5 ENZYME CONCENTRATION

After harvesting the extracellular media was passed through an Amicon hollow fibre concentrator, exclusion limit 10 kDa, achieving a 20 times concentration of the media.

2.6 ACETONE PRECIPITATION

After passing through the Amicon the concentrate was placed in a freezing bath and cold acetone (-10 °C) added to 25 % with stirring. After a few minutes any precipitated polysaccharride was removed with a glass rod. Acetone was then added to 66 % and stirred for 20 min. The precipitated protein being centrifuged at 25,000 g for 20 min at -10 °C. After carefully pouring off the supernatant the pellet was resuspended in cold (4 °C) potassium phosphate buffer (10 mM, pH 7.0). The resultant redissolved protein being dialysed against two changes of 8l deionised water for 18 hrs.

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was carried out on slab gels according to the method of Laemmli (Laemmli, 1970) , using a discontinuous buffer system. Separated proteins being visualised with Coomassie Brilliant Blue R.

2.8 VAN SOEST CHEMICAL ANALYSIS OF STRAW

All analyses described below were carried out by the analytical department of the University of Reading.

Once the samples were freeze dried they were stored at -20°C. Before analysis all the samples were left at room temperature for at least 48 hrs. In all analyses several replicates were used (4-6) and the average used to calculate the result presented as g/Kg organic matter.

2.8.1 Dry Matter (DM)

This was achieved by drying the material to constant weight at 105°C, then left to cool in a desiccator.

2.8.2 Ash and organic matter (OM)

This was determined by igniting the dried samples in a muffle furnace (Electric Carbonite Furnace Ltd. Bomford, Sheffield) at 550°C overnight, the weight loss during ashing was deemed to be the OM fraction.

2.8.3 Neutral and acid detergent fibre (NDF & ADF)

These were carried out using the forage fibre analysis of Van Soest and Wine 1967 and Goering and Van Soest 1970 (Van Soest and Wine, 1967; Goering and Van Soest, 1970). For each 1.0 g of sample was refluxed for 1 hr. with 100ml of the appropriate detergent, appendix 1, then filtered under suction. The residues were washed with hot distilled water followed by acetone, then dried to constant weight at

105°C.

% NDF or ADF = (weight of residue / weight of sample) x 100

2.8.4 Lignin determination

This was achieved by treating the ADF residue with permanganate solution for 1.5 hrs., the solution then being removed by suction and the sample treated for 1 hr. with demineralising solution (see appendix I). Once white, the fibre was sucked dry and washed with boiling distilled water followed by 80% industrial methylated spirit and then acetone. The resultant fibre then dried to constant weight at 105°C. Lignin content was calculated as follows:

$$\% \text{ Lignin} = \frac{\text{weight of ADF residue - weight of fibre}}{\text{weight of sample}} \times 100$$

2.8.5 Cellulose content

The residues from the lignin determination were ashed in a muffle furnace at 550°C. The cellulose content being determined by:

$$\% \text{ Cellulose} = \frac{\text{weight of lignin fibre - ash weight}}{\text{sample weight}} \times 100$$

2.8.6 *In vitro* digestibility

The two stage *in vitro* procedure of Tilley and Terry (1963) was used for assessing the digestibilities. Initially, a portion of the sample is finely ground and incubated anaerobically with buffered rumen liquor for 48 hrs. The liquor is then acidified to pH 2.0 with hydrochloric acid killing the bacteria therein which are further digested by the addition

of pepsin for 48 hrs. The insoluble residue is filtered, dried and ignited. The organic matter content of the residue is subtracted from that of the original yielding an estimate of organic matter digested.

This was essentially the method used in this study except:

1. 1 ml of ammonium sulphate (1 N) was added to each tube during the initial incubation to ensure that the growth of the rumen micro-organisms was not limited.
2. Plastic rather than glass tubes were used.
3. After the initial 48 hrs. the liquor was allowed to cool to room temperature to stop fermentation. After addition of 1 ml of 2N sodium carbonate the tubes were centrifuged at 2500 rpm for 7 min. The supernatant was then filtered through a fine nylon cloth, any residue on the cloth being returned to the tube.

A standard of ryegrass hay of known digestibility was included in each run. Runs with standard deviations (+ - 3%) from the usual IVOMD were discarded and the analysis repeated.

Rumen liquor was obtained from the same donor animals (3 rumen fistulated heifers) throughout the study. The animals were fed rye grass hay *ad libitum* and supplemented with 200g/day of commercial concentrate (18% protein). The results presented as a mean of 4-6 repeated determinations of *in vitro* organic matter digestibility (IVOMD).

2.9 SOLID STATE FERMENTATION

For each fungus four 2l Erlenmyer flasks were each inoculated with 4 agar plugs (size 6 cork borer) each containing 75g of air dried

barley straw and 225 ml water previously autoclaved (15 min, 15psi), stoppered with non-absorbent cotton wool. The cultures were incubated at 25°C in the dark with a flask for each fungus being removed for analysis at 30, 45, 60 and 90 days. The contents of each flask were removed by breaking up the material with a plastic rod and then subjected to Van Soest analysis and *in vitro* organic matter digestibility studies, section 2.8. Four identical but uninoculated flasks were incubated and sampled in the same way to provide a basis for comparison.

2.9.1 Extraction of enzymes from inoculated straw.

At 30 days one flask of each fungus was extracted with stirring with 500ml 0.1 M Borate buffer pH 8.4 containing 1 M KCl and 0.1% Tween 80 for 2 hrs. After this they were filtered through 3 layers of muslin and the filtrate spun for 10 min at 27,000 g, 4°C. After discarding the greyish pellet the brown supernatants were placed in an Amicon hollow fibre concentrator with a 10 Kdalton cut off. After being reduced to a volume of approximately 100ml the concentrates were placed in dialysis tubing and dialysed overnight against 20l deionised water.

Post dialysis the samples were subjected to acetone precipitation at 66% acetone, section 2.6, after resuspending the pellet in Potassium phosphate buffer 10mM pH 7 the samples were dialysed overnight against 20 L deionised water. On removal from dialysis the samples were spun for 10 min at 48,000 g and the dark brown pellet discarded. The supernatant was freeze dried and the resulting powder

resuspended in 5ml of deionised water and the samples assayed for veratryl alcohol oxidase (Lignin peroxidase) and manganese dependant peroxidase (guaiacol oxidation) then subjected to SDS Polyacrylamide Gel Electrophoresis. Extracts obtained from cultures grown for 45 days were treated in the same manner as above.

2.10 PREPARATION OF MILLED WOOD LIGNIN

Preparation of the wood :

Spruce sawdust was ground in Moulinex grinder, and sieved through a 500 um sieve.

Ball-milling :

50 g of ground wood was placed in each ball pot and milled for 14 days on a rotary ball-mill.

Extraction :

The milled wood was dispersed in 250 ml of dioxane-water solution (100 ml dioxane / 4 ml water) and shaken for 48 hrs. Dioxane was removed by centrifugation (40,000 g for 10 min) and the wood re-extracted twice more in fresh dioxane solution.

Purification :

The combined extracts were evaporated to low volume on a rotary evaporator (maximum temperature 55 °C). The final solvent removed by freeze drying. The residue was dissolved in acetic acid containing 10 % water, 20 ml solvent for each gram of lignin. The solution was then added dropwise to a large volume of water with constant stirring (250 ml water / 20 ml lignin solution). The creamy white precipitate then being filtered on a Buchner funnel and dried under a hot air flow for 2

hrs. The lignin was then easily scraped off the paper, and dissolved in a solution of dichloroethane-ethanol (2:1), 20 ml solvent / g lignin. This solution was added dropwise to ether (distilled over sodium), 250 ml / g lignin. The solution then centrifuged (10 min at 40,000 g) and the ether discarded. The lignin was then dispersed immediately in fresh ether and centrifuged as before; the ether discarded and the lignin dried in a vacuum desiccator.

2.11 ACETYLATION OF MILLED WOOD LIGNIN

100 mg of lignin was dissolved in 1 ml of pyridine, and 2 ml of acetic anhydride added. After 48 hrs this solution was added to 25 ml of distilled water in a round-bottomed flask, the contents stirred for 1 hr, and the acetic acid and water removed by vacuum distillation. The residue (containing a little pyridine) was dissolved in 2 ml of dioxane containing 0.1 ml water, and this solution then dripped into 20 ml of vigorously stirred water. The precipitated lignin was recovered by centrifugation (10 min, 40,000 g), washed by reprecipitation into water and dried.

2.12 LIGNIN CONCENTRATION CURVE

In order to study the relationship between the concentration of lignin and its absorption maximum at 280 nm 10 mg of spruce milled wood lignin, SMWL, were dissolved in 1 ml 1N NaOH and then diluted to 0.1 mg / ml with 0.2 M glycine / NaOH buffer, pH 10. This solution was then used to obtain absorption maxima at 280 nm for various

concentrations of lignin. All reading were taken using a Perkin Elmer 555 spectrophotometer, 1cm light path.

For acetylated lignin the same procedure was used.

2.13 IONISATION DIFFERENCE SPECTRA

Aliquots of each sample were diluted to equal concentration with either 0.1M sodium phosphate, pH 6 or 0.1M sodium borate, pH 12 and scanned with the pH 12 aliquot in the sample cuvette and the pH 6 aliquot in the reference position. These resulting spectra are ionisation difference spectra.

2.14 REDUCED IONISATION DIFFERENCE SPECTRA

For these spectra the samples are incubated with 25 mM sodium borohydride at pH 11-12 overnight at room temperature. After incubation the samples were diluted and scanned as above, section 2.15. Free phenol content being calculated using Goldschmidt,s equation (Goldschmidt, 1954):

% Free phenols = Absorptivity ($l.g^{-1}cm^{-1}$) at 300 nm x 0.414

2.15 COLUMN CHROMATOGRAPHY OF LIGNIN

2.15.1 LH 20

Sephadex LH 20 (Pharmacia Ltd.) was presoaked overnight in 1,4-dioxane and then packed under gravity in a 1cm x 50 cm column, the final bed height being 36.5 cm. A flow rate of 0.6 ml/min of 1,4-dioxane was used to load and run the samples the column being calibrated with polystyrene standards, 0.02 mg/ml.

2.15.2 Biogel SX-1

Biogel SX-1 (Bio-Rad Ltd.) was preswelled overnight with 1,4-dioxane and packed under gravity in a 1 cm x 50 cm column with the resultant bed height being 34.5 cm. Samples were loaded at a flow rate of 0.48 ml/min and elueted with 1,4-dioxane. The column was calibrated with polystyrene standards, 0.02 mg/ml.

2.15.3 Zorbax PSM 60S

This column (DuPont) was used in association with a Varian 5000 liquid chromatagraph with tetrahydrofuran as eluent. Samples were loaded through a 10 ul closed loop and eluted at a flow rate of 0.5 ml/min. The eluent being continuously monitored on a Varian 2050 variable detector at 280 nm and any peaks integrated on a spectrophysics SP4290 integrator. The column being calibrated with polystyrene standards, 0.02 mg/ml (DuPont).

2.15.4 Sephadex G-75

Packing material (Sephadex G-75, Pharmacia Ltd.) was allowed to swell overnight in glycine/NaOH buffer (0.2 M, pH 10.0) and then was poured into a 2.6 x 40 cm column (Pharmacia K26/40) and was allowed to pack under gravity. Samples were loaded and eluted at a flow rate of 1 ml/min (Pharmacia P1 pump), eluent glycine/NaOH buffer (0.2 M, pH 10.0). The eluent continuously monitored at 280 nm using a LKB Uvicord II detector, 1 cm light path. Exclusion limit was determined using Blue dextran, 2,000,000 Da. Partial calibration

achieved using polyethylene glycol 6,000 and polyethylene glycol 8,000 (6 kDa and 8 kDa respectively).

2.15.5 TSK G3000PW

This column (Anachem Ltd.) was used in association with a Varian 5000 liquid chromatagraph with glycine/NaOH buffer (0.2M, pH 10.0) as eluent. Samples were loaded through a 100 ul closed loop and eluted at a flow rate of 1.2 ml/min. The eluent being continuously monitored on a Varian 2050 variable detector at 280 nm and any peaks integrated on a spectrophysics SP4290 integrator. Exclusion limit determined using Blue dextran (2×10^6 Da) = 3.2 min. Partial calibration achieved using polyethylene glycol 6,000 and polyethylene glycol 8,000 (6 kDa and 8 kDa respectively) = 6.8 min and 6.6 min respectively.

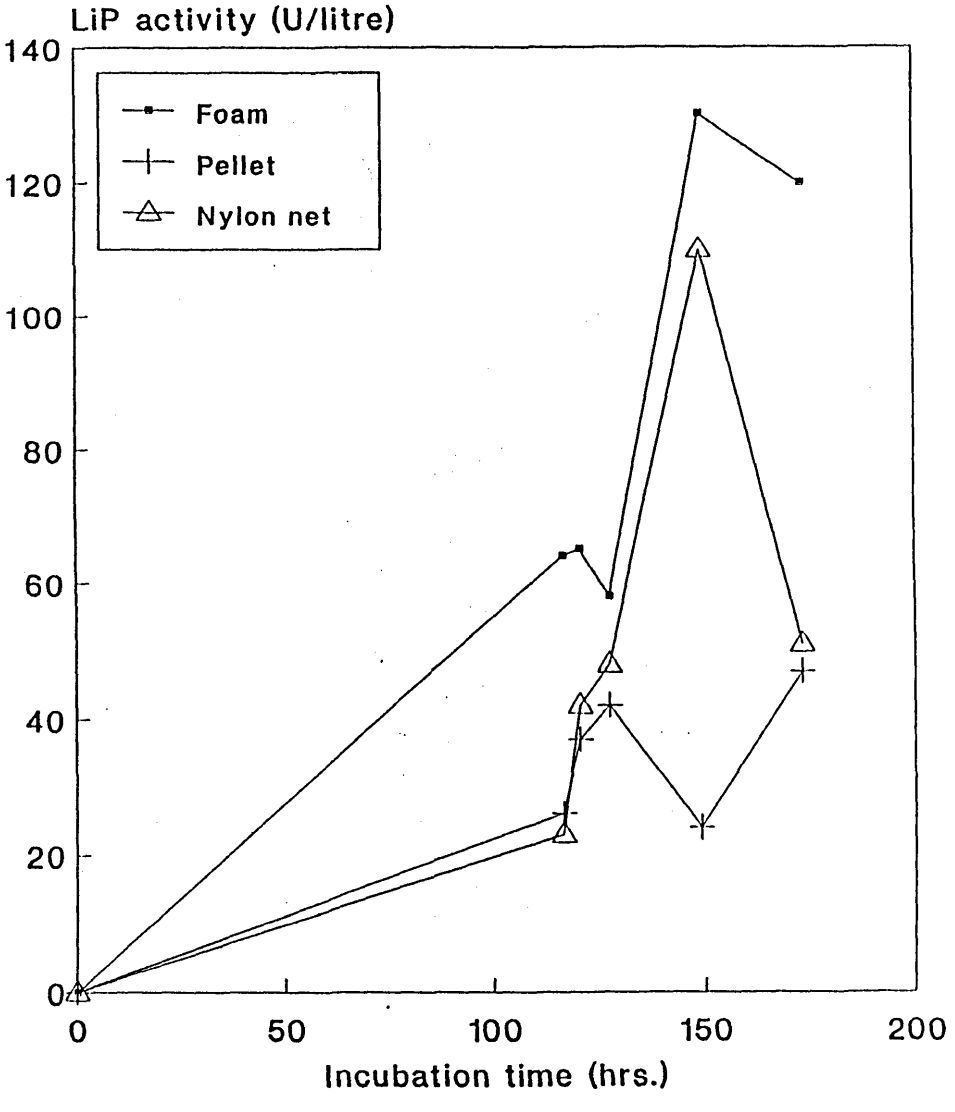

Figure 2.1 Effect of submerged culture technique on the amount of LiP produced by *P.chrysosporium.*

CHAPTER 3
A STUDY ON THE INFLUENCE OF LIGNIN PEROXIDASE ON BARLEY STRAW

3.1 TREATMENT OF BARLEY STRAW WITH LIGNIN PEROXIDASE OR SODIUM HYDROXIDE

3.1.1 INTRODUCTION

The improvement of the digestibility of straw has largely been confined to physical and chemical treatments, Chapter 1. Despite the effectiveness of these methods their large scale use could be hazardous and provide an undesirable source of pollutants. As discussed earlier, Chapter 1, biological methods involving the use of fungi or enzymes could provide a more environmentally acceptable option, for the upgrading of straw, given that an effective and economic technique could be found. In this experiment, run in collaboration with K.Kazhaal at Reading University, lignin peroxidase (LiP) from the white-rot fungus *Phanerochaete chrysosporium* was used in an attempt to assess its potential ability to increase the digestibility of barley straw. Included in the same experiment was a treatment with sodium hydroxide to validate the analytical techniques used.

3.1.2 The effect of LiP, veratryl alcohol and hydrogen peroxide on the chemical composition and digestibility of barley straw.

To study the effect of lignin peroxidase on straw a number of treatments were set up as detailed in Table 3.1.1. For each treatment 10g of milled (1.0 mm) non sterile barley straw was placed in a 250 ml erlenmyer flask and 100 ml sodium acetate buffer (200 mM, pH 3.5) added. Lignin peroxidase, veratryl alcohol and hydrogen peroxide were then added to the flasks in varying quantities as detailed in Table 3.1.1. The enzyme was produced according to method 1 in Chapter 2 section 2.2 and its activity measured as in Chapter 2 section 2.3.1.

The flasks were shaken slowly (50 rpm) on a Gallenkamp rotary shaker at 25 °C for either : 1, 24, 48 or 140 hrs. To provide a chemical comparison for the enzyme treatments, two seperate flasks were also treated with two levels of sodium hydroxide for the same time periods, Table 3.1.1. At the end of each time interval the contents of the flask were freeze dried, after which Van Soest chemical analyses of the straw cell wall contents and its *in vitro* organic matter digestibility, IVOMD, were carried out using the methodology outlined in Chapter 2 section 2.8.

Table 3.1.1 Experimental design to investigate the influence of LiP, veratryl alcohol and hydrogen peroxide on barley straw.

Lignin peroxidase treatments

Enzyme Level	Enzyme U/10g straw	Acetate buffer (ml)	Hydrogen peroxide (mM)	Veratryl alcohol (mM)
Control	0.0	100	0.0	0.0
	0.0	100	0.15	0.0
	0.0	100	0.0	2.0
	0.0	100	0.15	2.0
Low	0.1	100	0.0	0.0
	0.1	100	0.15	0.0
	0.1	100	0.0	2.0
	0.1	100	0.15	2.0
Medium	1.0	100	0.0	0.0
	1.0	100	1.5	0.0
	1.0	100	0.0	2.0
	1.0	100	1.5	2.0
High	10.0	100	0.0	0.0
	10.0	100	15.0	0.0
	10.0	100	0.0	2.0
	10.0	100	15.0	2.0

Sodium hydroxide treatments

Level	Amount NaOH per 10g straw (g)	Volume water per 10g straw (ml)
Low	0.25	100
High	0.50	100

For the purposes of statistical analysis the data from the 64 enzyme treatments were considered as being a non-replicated 4 x 4 x 4 factorial layout. They were analysed using analysis of variance (Genstat 5, release 1.2, Lawes agricultural trust, Rothampstead Experimental Station, 1987) at Reading University. However since the effects of the NaOH treatments were obvious these were not statistically analysed. Statistical analysis revealed significant interaction between enzyme concentration and time for *in vitro* organic matter digestibility, neutral detergent fibre, acid detergent fibre and lignin contents, $P<0.001$, and cellulose, $P<0.01$. The main effects and means of the enzyme concentration and time on these factors are shown in Table 3.1.2. It would seem that the general trend in the experiment was for there to be a decrease in digestibility of the material. However, results from these analyses for the lignin peroxidase treatments showed that there was no statistically significant effect of the presence or absence of veratryl alcohol or hydrogen peroxide in these treatments, data not shown. This is suprising considering the known roles of both these in the catalytic cycle of lignin peroxidase, Chapter 1. It was hoped that the addition of veratryl alcohol and hydrogen peroxide to some of the treatments would have a positive effect on the results obtained. This was based on the suggested role of veratryl alcohol radical cation as a charge transfer mediator between the enzyme and the lignin (Harvey et al, 1986). Thus it is surprising that it did not have any detectable effect on the efficacy of the enzyme in this study.

The effects of two levels of sodium hydroxide on the

digestibility and chemical composition of the straw can be seen in Table 3.1.3. As can be seen both levels of alkali gave increased digestibilities over all the time periods, the main changes being obtained in a reduction in the levels of lignin and hemicellulose whilst the cellulose content was not being affected. These results from the sodium hydroxide treatments are consistent with previous data since it is known that this compound can cause the partial solubilisation of lignin and breaking the lignin hemicellulose bonds within the lignocellulose complex enhancing the saccharification of cellulose (Chesson, 1981; Chesson and Orskov, 1984).

It would seem then from these initial studies that the addition of lignin peroxidase to straw does not give rise to significant increases in IVOMD as would have been hoped considering the enzymes's suggested role in lignin degradation, Chapter 1. It is possible that the enzyme itself may have become inactivated on addition to the straw due to the presence of phenolic containing compounds in this substrate (Mueller-Harvey et al, 1986). The phenolics would then be oxidised preferentially by the enzyme resulting in its inactivation (Harvey and Palmer, 1990). The second part of this chapter contains a study to investigate the possibility of this occurring under the experimental conditions employed.

Table 3.1.2 Effects of treatments, on cell wall chemical composition (Van Soest analysis) and *in vitro* organic matter digestibility (OMD) (g/Kg OM).

Variate	Time (hrs)	Control	Enzyme level Low	Medium	High	Mean	SED
	1	312	311	327	314	316	[1]4.2
OMD	24	285	288	317	219	295	[2]8.4
	48	287	294	339	320	310	
	140	285	262	256	262	266	
Mean		292	289	310	297	297	
	1	786	803	783	793	791	[1]5.6
NDF	24	780	780	778	789	782	[2]11.3
	48	808	802	737	755	775	
	140	783	797	809	805	799	
Mean		789	796	777	786	787	
	1	543	544	539	534	540	[1]5.2
ADF	24	544	545	519	529	534	[2]10.4
	48	574	573	486	501	534	
	140	542	575	575	575	567	
Mean		551	559	530	535	544	
	1	243	259	244	259	251	[1]5.5
Hemice	24	237	253	259	260	252	[2]11.1
llulose	48	234	229	251	254	242	
	140	241	222	234	230	232	
Mean		238	241	247	251	244	
	1	120	118	112	112	116	[1]3.0
Lignin	24	111	112	104	109	109	[2]6.1
	48	125	116	84	91	104	
	140	107	123	119	107	114	
Mean		116	117	105	105	111	
	1	383	387	385	387	386	[1]4.5
Cellu-	24	393	400	380	387	390	[2]9.0
lose	48	392	402	363	364	380	
	140	397	408	422	422	412	
Mean		391	399	387	390	392	

[1] For comparing main effects means of time or enzyme.

[2] For comparing interaction between enzyme and time.

Table 3.1.3 The effect of NaOH on the composition and digestibility of straw.

Time Hours	NDF	ADF	Hemice-lulose	Lignin	Cellu-lulose	Ash	OMD
Low NaOH (25g/Kg straw)							
1	758	517	242	110	390	57	428
24	716	525	191	109	405	75	513
48	695	494	201	92	389	71	495
140	731	552	179	126	420	70	564
High NaOH (50g/Kg straw)							
1	653	464	189	94	305	68	545
24	649	462	145	87	365	103	661
48	585	457	128	72	371	96	645
140	584	467	117	91	374	120	668
Untreated							
	824	576	248	129	408	36	304

3.2 THE CATALYTIC CAPACITY OF LIGNIN PEROXIDASE IN THE PRESENCE OF STRAW OR STRAW EXTRACTS.

In the light of the results from the previous experiment, section 3.1 Table 3.1, it was decided to investigate the fate of the enzyme when added to straw. In particular an attempt was made to ascertain whether the enzyme maintained its catalytic viability on addition to the straw. To achieve this two experiments were carried out.

3.2.1 The fate of the enzyme

By monitoring enzymic activity on addition to straw and the use of protein gel electrophoresis the fate of the enzyme on addition to straw can be established. The experimental design shown in Table 3.2.1 was carried out.

Table 3.2.1 Design of experiment to determine the fate of lignin peroxidase on incubation with barley straw.

Treatment	Amount straw (g)	Enzyme vol. (ml)	Hydrogen peroxide (ml)	Veratryl alcohol (ml)	Water vol. (ml)	Total (ml)
A	0.0	0.1	0.0	0.0	4.9	5.0
B	0.5	0.0	0.0	0.0	5.0	5.0
C	0.5	0.1	0.0	0.0	4.9	5.0
D	0.5	0.1	1.04	0.0	3.86	5.0
E	0.5	0.1	1.04	0.5	3.36	5.0

Concentration of stock solutions used:

Enzyme = 41 U/ml
Hydrogen peroxide = 48 mM
Veratryl alcohol = 20 mM.

All reactions were carried out in open vessels the last addition to each being the lignin peroxidase. A 20 ul subsample of Treatment A, no straw present, was taken at 0 min. and assayed for veratryl alcohol oxidase activity. All the treatments were left for 2 hrs. at room temperature. At the end of this time period 200 ul subsamples of each treatment were taken and centrifuged, the supernatant was then assayed for veratryl alcohol oxidase activity. For electrophoretic analysis it was necessary to separate the straw from the aqueous media in the remainder of each treatment, this was achieved by centrifugation at 48,000 g, 0°C for 10 min. The supernatants were then dialysed against 16 l of deionised water for 18 hrs. After dialysis the samples were recentrifuged as before and any pellet discarded. To prepare the samples for SDS-PAGE the supernatants were freeze-dried and resuspended in 120 ul SDS-ME (2:1). After boiling in a sealed tube for 3 min. the samples were subjected to SDS-PAGE gel electrophoresis as described in Chapter 2 section 2.7. Molecular weight markers were run concurrently to provide an estimate of the apparent molecular weight of the bands obtained.

It is apparent from Table 3.2.2 that after 2 hrs incubation all the treatments in which lignin peroxidase was added to straw no enzymatic activity was detectable, however, the treatment in which only buffer and enzyme were present, Treatment A, showed no loss of enzyme activity. This indicates that any loss in enzyme activity must be attributed to the presence of the straw.

Table 3.2.2 Effect of incubation of lignin peroxidase with barley straw on its enzymatic capacity.

Treatment[1]	Enzyme added (Units)	Enzyme detected after 2 hrs. (Units)
A	4.1	4.1
B	0.0	0.0
C	4.1	0.0
D	4.1	0.0
E	4.1	0.0

[1] See Table 3.2.1

Protein profiles of the extracts, Plate 3.1, are summarised in Table 3.2.3, although for clarity only one extract containing the enzyme, Treatment C, is shown the others, Treatments D and E, being identical, data not shown. It can be seen that the straw extract alone, Treatment B Track 2, straw alone, showed a number of bands over a large molecular weight range. However, the straw plus enzyme extract, Treatment C Track 4, LiP with straw, although very similar clearly shows a band corresponding to the expected 46 kDa molecular weight of lignin peroxidase used, Track 6. This would indicate that although there was no detectable lignin peroxidase activity the enzyme was still present and not completely degraded. However, it can be seen that the intensity of the band is not as high as would be expected from the 4.1 U of enzyme added, track 9 (1.0 U) is considerably more intense. It would seem, therefore, that the "lost" enzyme has either degraded or bound to the straw itself.

Lignin peroxidases are capable of catalysing reactions associated with lignin breakdown, such as C_{α}-C_{β} cleavage of lignin model

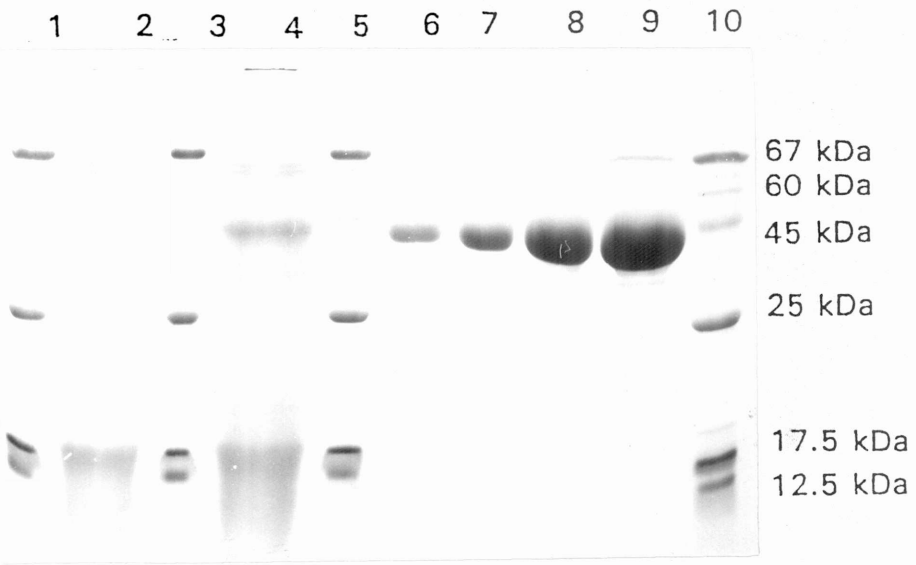

Plate 3.2.1 Fate of lignin peroxidase on addition to barley straw. Tracks 1, 3, 5, 10 standards: Protein standards; Track 2: Aqueous extract of barley straw (Treatment C); Track 4: Aqueous extract of straw to which 4.1 U of LiP was added (Treatment B); Track 6 : 0.125 U LiP; Track 7 : 0.25 U LiP; Track 8 : 0.5 U LiP; Track 9 : 1.00 U LiP.

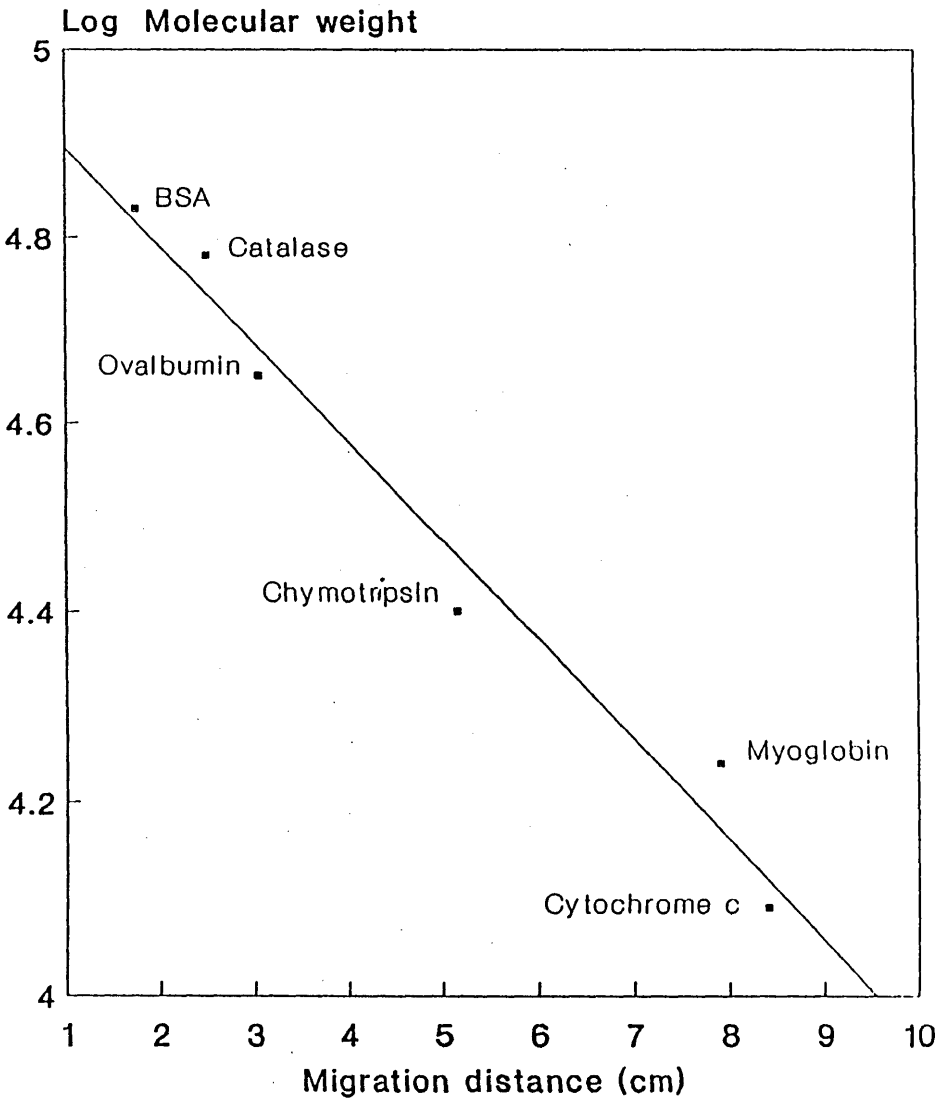

Figure 3.2.1 Log_{10} Molecular weight versus migration distance for the protein standards in Plate 3.2.1. Standards were loaded as a mixture in 50 ul (SDS-ME mixture 2:1) (2.5 ug protein per standard).

Table 3.2.3 Showing molecular weights of main bands in Plate 3.2.1.

Track No.	Sample type	Migration distance (cm)	Estimated Mol.Wt. (daltons)
1	Stds.	N/A	N/A
2	C	N/A	N/A
3	Stds.	N/A	N/A
4	B	3.15	46800*
5	Stds.	N/A	N/A
6	0.125 U LiP	3.15	46800*
7	0.25 U LiP	3.20	46200*
8	0.50 U LiP	3.30	45100*
9	1.00 U LiP	3.33	44800*
10	Stds.	N/A	N/A

*Indicates estimated using data from Fig 2.1. i.e. using equation $y=mx+c$ where y=mol.wt. m=slope (-104) x=migration distance c=y axsis intercept (4.998)
Correlation coefficient= 0.973

compounds, Chapter 1. However, it has also been shown that these enzymes are capable of oxidising phenolic containing compounds such as guaiacol and catechol (Harvey and Palmer, 1990). Moreover, the oxidation of these compounds is one that occurs preferentially to any other reaction that may occur (Harvey and Palmer, 1990). However, during the catalysis of these compounds it has been shown that the enzyme becomes inactivated by causing it to enter the unreactive oxidation state of compound III in its redox cycle, see Figure 3.2.2 below. Since it has been shown that straws contain a large amount of phenolic compounds (Mueller-Harvey, 1986) it is not surprising that the addition of LiP to straw results in the apparent inactivation of the enzyme.

It has been suggested that the presence of veratryl alcohol has the capacity to convert compound III back to the native enzyme, hence maintaining the redox cycle (Wariishi and Gold 1989; Valli et al, 1990). However, this did not seem to be the case in treatment E. This maybe due to the concentration of the veratryl alcohol being too low compared too that of the phenolics present as it has recently been shown for veratryl alcohol to have this effect it has to be present in at least 200 fold molar excess compared to the phenolic compounds present (Harvey and Palmer 1990).

Confirmation that factors within the straw were indeed responsible for the inactivation of the enzyme was obtained by using an aqueous extraction of the straw identical to that of Treatment B, Table 3.2.1. The supernatant from this treatment was added in varying quantities to a lignin peroxidase assay described below :

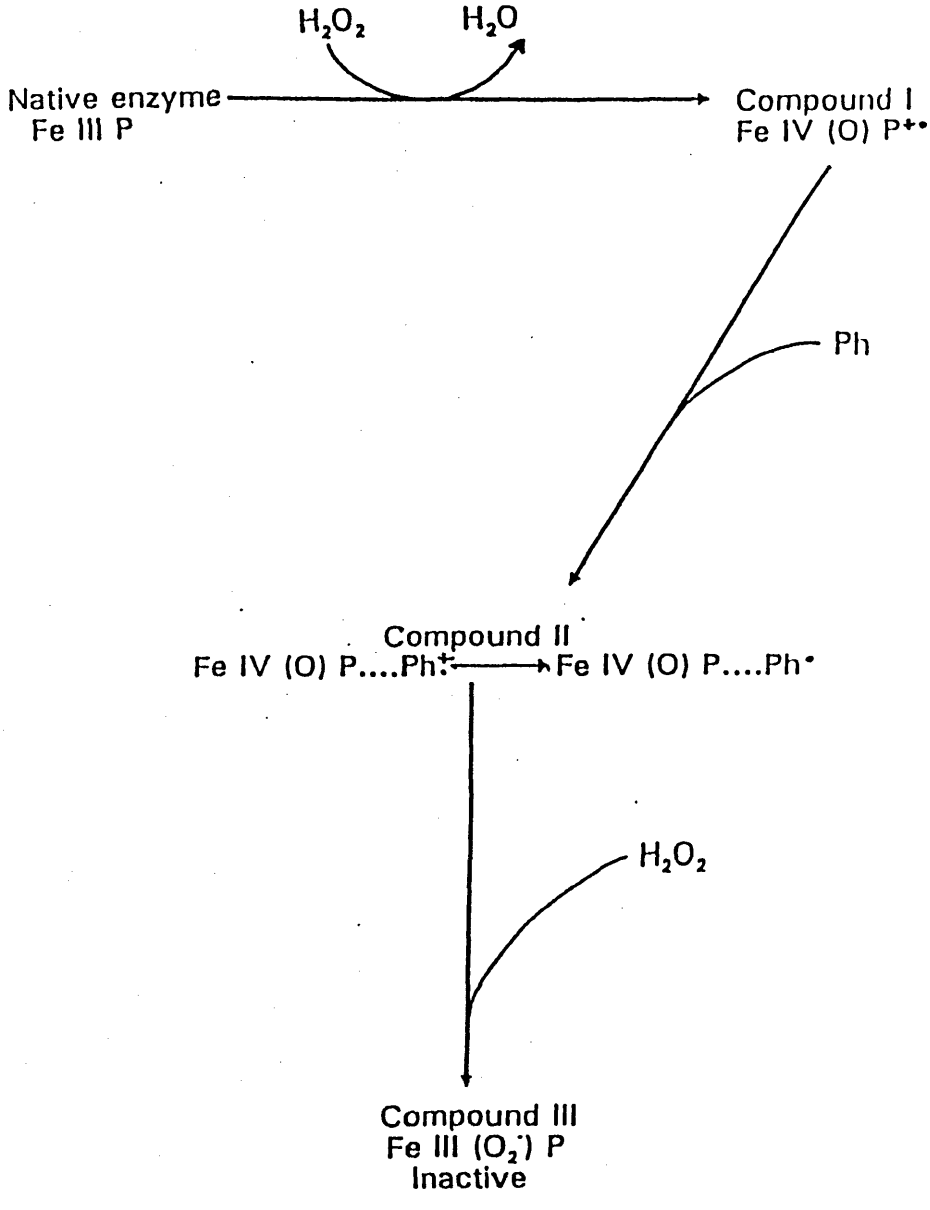

Figure 3.2.2 Catalytic cycle of lignin peroxidase showing inactivation by phenolic compounds. P - porphyrrin; Ph - phenolic compound; Ph• - phenoxy radical.

20 ul Hydrogen peroxide (50 mM)
40 ul Veratryl alcohol (20 mM)
x ul straw extract
4 ul lignin peroxidase (= 7.79U)

The assay being initiated by the addition of the hydrogen peroxide and the appearance of veratraldehyde monitored by increase in absorbance at 310 nm. The results in Figure 3.2.3 shows that the addition of even small quantities of straw extract causes a loss of enzyme activity.

It would seem that the results from both these experiment show conclusively that the addition of lignin peroxidase to straw results in its inactivation. Despite the inactivation of the isolated enzyme, cultures of *P.chrysosporium* are capable of degrading lignin in straw, Chapter 1. It maybe that *in vivo* other enzymes or mechanisms are present which prevent the LiP from coming into contact with the phenols present, or that the phenolics are oxidised by enzymes secreted before LiP. One mechanism for lignin degradation suggests that the LiP does not come into direct contact with lignin but that oxidation is achieved through a redox mediator, in the form of the veratryl alcohol radical cation (Harvey et al, 1986). In fact addition of veratryl alcohol has been shown to enhance the oxidation of spruce milled wood lignin by LiP and hyphae of *P.chrysosporium* (Haemmerli et al, 1986a). Another possibility is oxidation of the phenolics by a second enzyme, namely manganese dependent peroxidase, MnP, which has also been shown to be involved in lignin breakdown (Gold et al, 1984). It is possible that once the MnP has oxidised all the phenolic compounds the LiP can safely oxidise the lignin without inactivation.

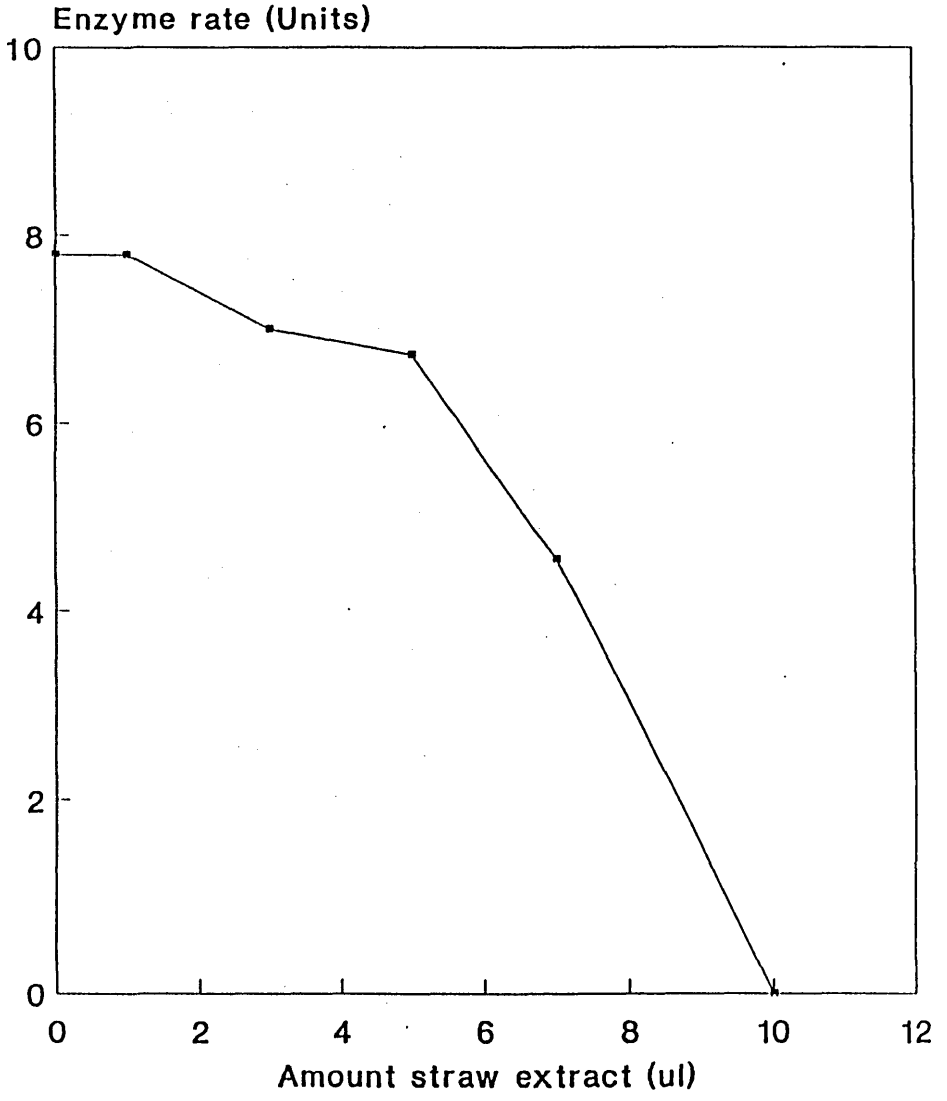

Figure 3.2.3 The effect of straw extract on the catalytic rate of veratryl alcohol oxidation by LiP. Each amount of straw extract was added to a cuvette containing: 1 ml potassium phosphate buffer (20mM, pH 2.75); 40 ul veratryl alcohol (20mM): 4 ul LiP (7.79 U); the reaction initiated with the addition of 20 ul hydrogen peroxide (50 mM), mixed and monitored at 310 nm.

In an attempt to shed some light on these problems the experiment in Chapter 4 was carried out in attempt to ascertain which enzymes and in what quantities are present in solid state fermentation cultures of *P.chrysosporium*.

CHAPTER 4

SOLID STATE FERMENTATION OF STRAW: EFFECT OF FUNGAL TYPE AND FERMENTATION TIME ON CHEMICAL COMPOSITION AND *IN VITRO* DIGESTIBILITY OF BARLEY STRAW. THE EXTRACTION OF ENZYMES FROM SOLID STATE FERMENTATION CULTURES.

4.1 SOLID STATE FERMENTATION - THE EFFECT OF FUNGAL TYPE

Although the effects of fungal type and fermentation time on the *in vitro* digestibility of barley straw have been extensively studied, Chapter 1, it was decided that these experiments were necessary to provide some correlation for the extraction of enzymes from these cultures at periods of maximum increases in digestibility. Since the addition of lignin peroxidases directly to straw resulted in the apparent inactivation of the enzyme, Chapter 3, it was decided to attempt to isolate the enzymes present in solid state fermentations to yield some clue as to which enzymes are most prevalent at periods of maximum digestibility increases and lignin loss. To this end two white-rot fungi, *Abortiporus biennis* and *Dichomitus squalens* were included in the experiment chosen because they have been shown to cause large increases in digestibility (Zadrazil, 1980). They were grown in solid state fermentation along with *P.chrysosporium*, Chapter 2 section 2.9, and the effect of these fungi on the chemical composition of the straw cell wall and the *in vitro* organic matter digestibility monitored, Chapter 2 section 2.8. Subsequent extraction of the solid state fermentation cultures, Chapter 2 section 2.9.1, in order to isolate extracellular enzymes was also undertaken in order to discover whether these fungi in fact produce high levels of lignin peroxidases during solid state fermentation of straws, especially at the time they bring about increased digestibility of the straw.

Analysis of the fermentation profiles of the three fungi reveals distinct differences. Whilst both *A.biennis* and *D.squalens* both utilised the material at a similar rate, *P.chrysosporium* was found to give rise to dry matter losses approximating to twice that of the other two fungi over the same time period, Figure 4.1.1. Closer analysis of the changes in the chemical contents of the straw cell wall over these time periods reveals further differences between these fungi.

There were differences in the degree of hemicellulose degradation for all three fungi. Almost twice as much hemicellulose was lost from the straw incubated with *P.chrysosporium* than with the other two fungi, Figures 4.1.1 and 4.1.2. Furthermore, analysis of the cellulose contents of the straw also reveals a marked difference between *P.chrysosporium* and the other two fungi. It can be deduced from Figures 4.1.1 and 4.1.3 that straw incubated with *P.chrysosporium* showed nearly a 75% loss of cellulose by day 45, compared with a loss of approximately 50% dry matter over the same period. This faster loss of cellulose than dry matter resulted in a reduction of the cellulose content of the straw by a third by *P.chrysosporium*, whilst it would seem that both *D.squalens* and *A.biennis* leave the cellulose content of the straw relatively unchanged, Figure 4.1.3. The significance of this difference in cellulolytic activities becomes clear on analysis of their effect on *in vitro* organic matter digestibility of the straw.

The greatest difference between these fungi is their effect in the *in vitro* organic matter digestibility, IVOMD, of the material. As can be seen in Fig.4.1.4 both *A.biennis* and *D.squalens* have an immediate positive effect on the IVOMD, causing increases of up to

30% by day 30. In contrast, at the same period in time *P.chrysosporium* had caused a drop in IVOMD of approximately 50%. Over the next sampling times both *A.biennis* and *D.squalens* carry on to give increases in IVOMD but not at a similar rate. *P.chrysosporium* also starts to show increases in IVOMD above that on day 30, however, by day 90 it still had not reached the level of the control so that over the whole experiment there was a net loss in IVOMD of the straw. These differences in IVOMD can be related to the cellulolytic activities of these fungi. Both *A.biennis* and *D.squalens* barely alter the cellulose content of the straw, whilst by day 45 *P.chrysosporium* has caused a considerable loss in cellulose content, in the straw, which correlates to the initial fall in IVOMD. However, less cellulose is degraded for the rest of the fermentation period which is reflected in an upward trend in the IVOMD levels.

Although, Figure 4.1.5 shows that the lignin content of the straw remained constant for all three fungi, there was a loss of lignin, in the straw, which paralleled the loss of dry matter, Figure 4.1.1.
This study shows that the balance of extracellular enzymes present at the time periods 30 and 45 days, could play a role in the mechanism of action of these fungi in degrading straw during solid state fermentation. This could lend some understanding as to how enzymes could be used to achieve similar levels of IVOMD in straws. Such a study was undertaken the results of which are presented in the second part of this chapter, section 4.2.

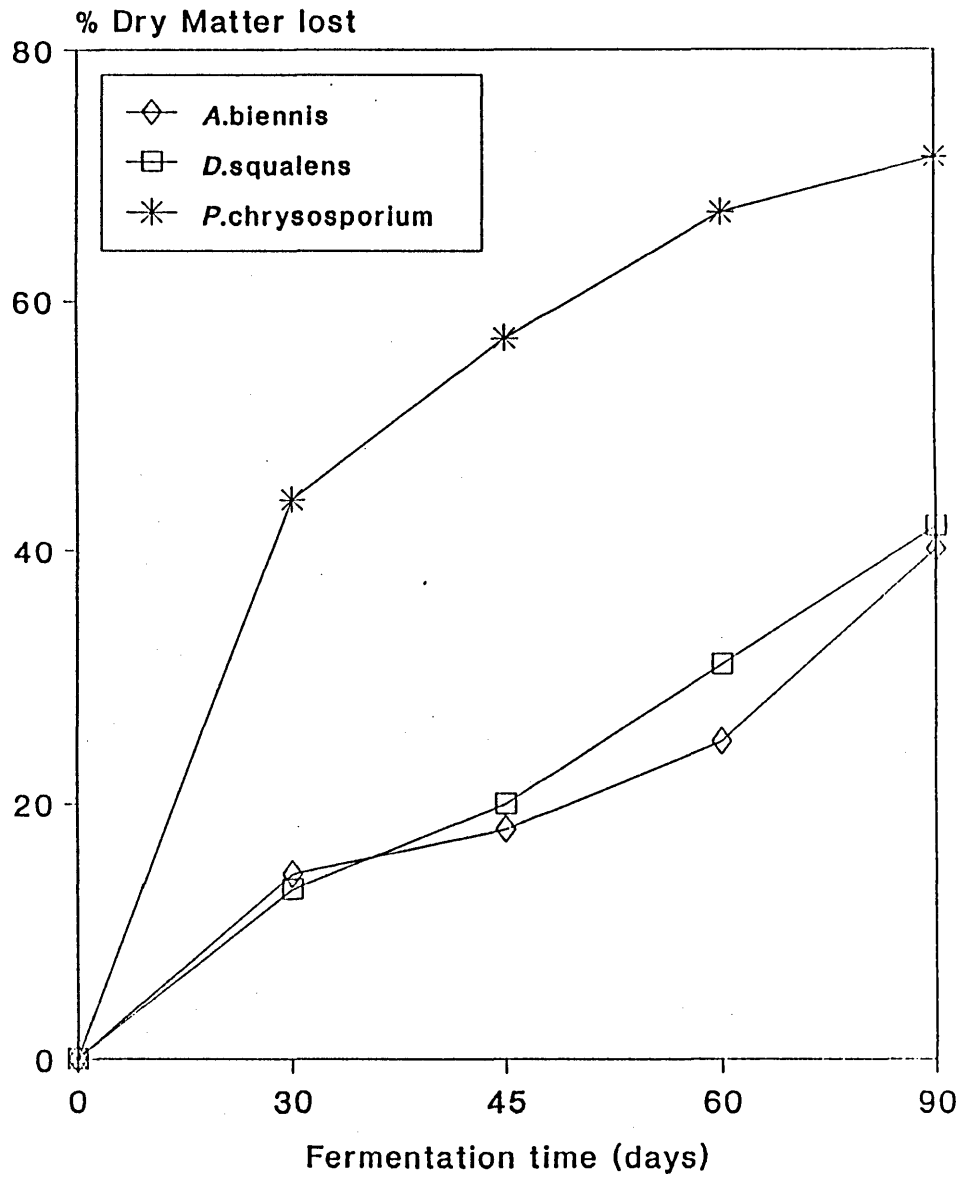

Figure 4.1.1 Change in dry matter of barley straw with time during solid state fermentation with three white-rot fungi (section 4.1).

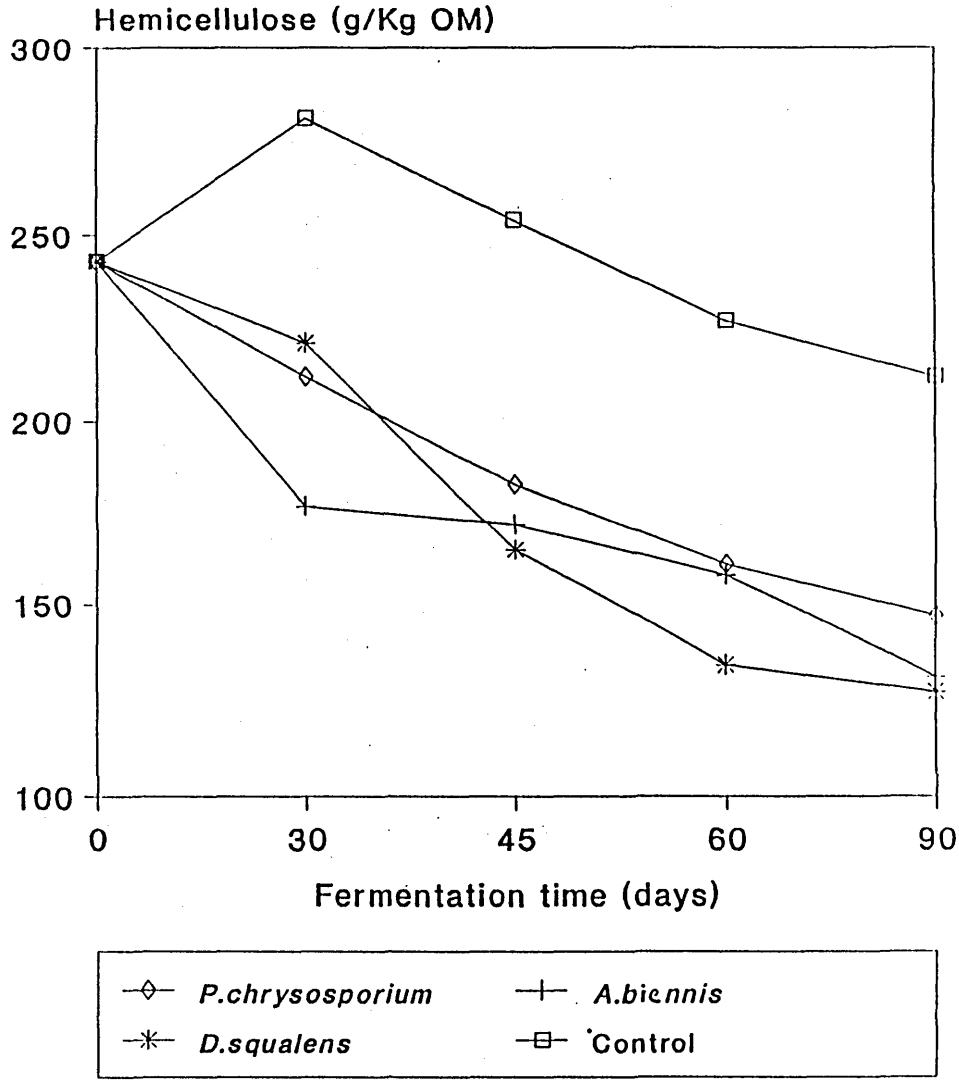

Figure 4.1.2 Change in hemicellulose content of barley straw with time during solid state fermentation with three white-rot fungi (section 4.1).

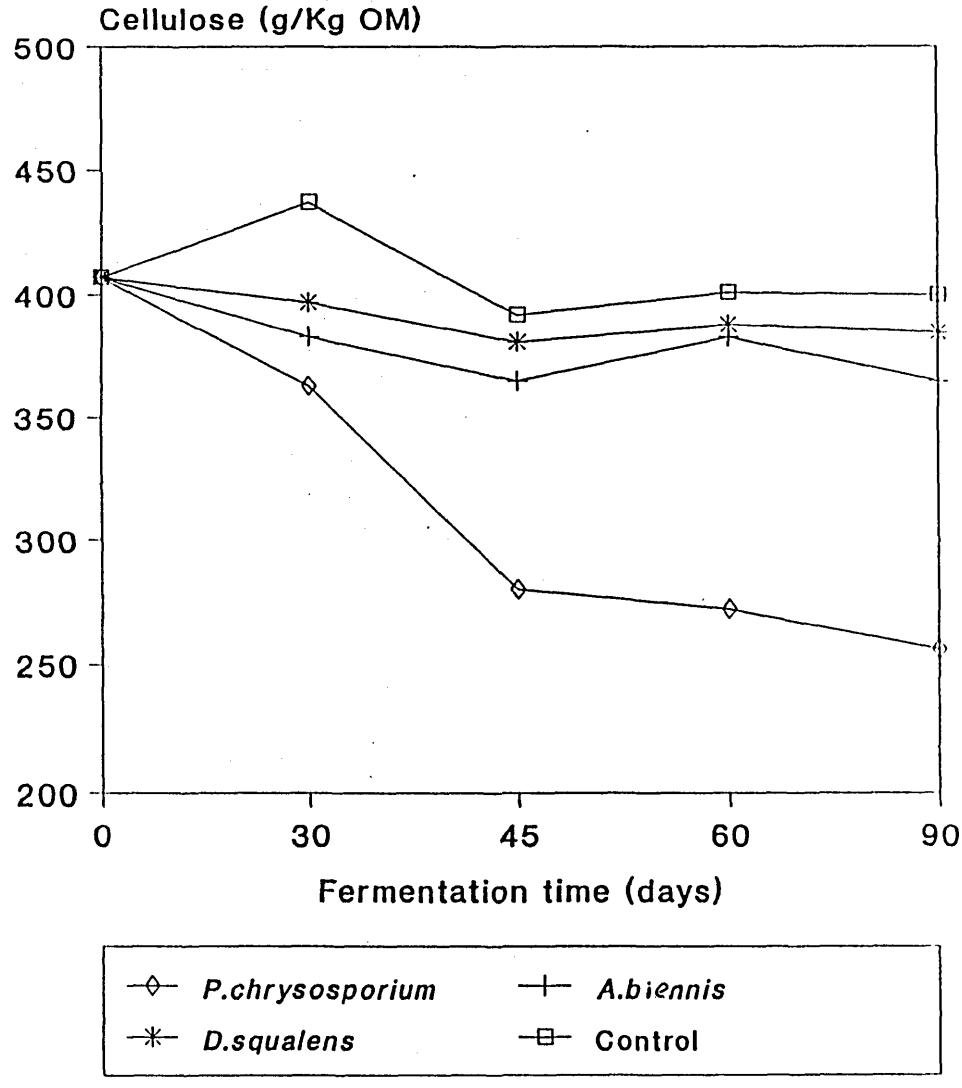

Figure 4.1.3 Change in cellulose content of barley straw with time during solid state fermentation with three white-rot fungi (section 4.1).

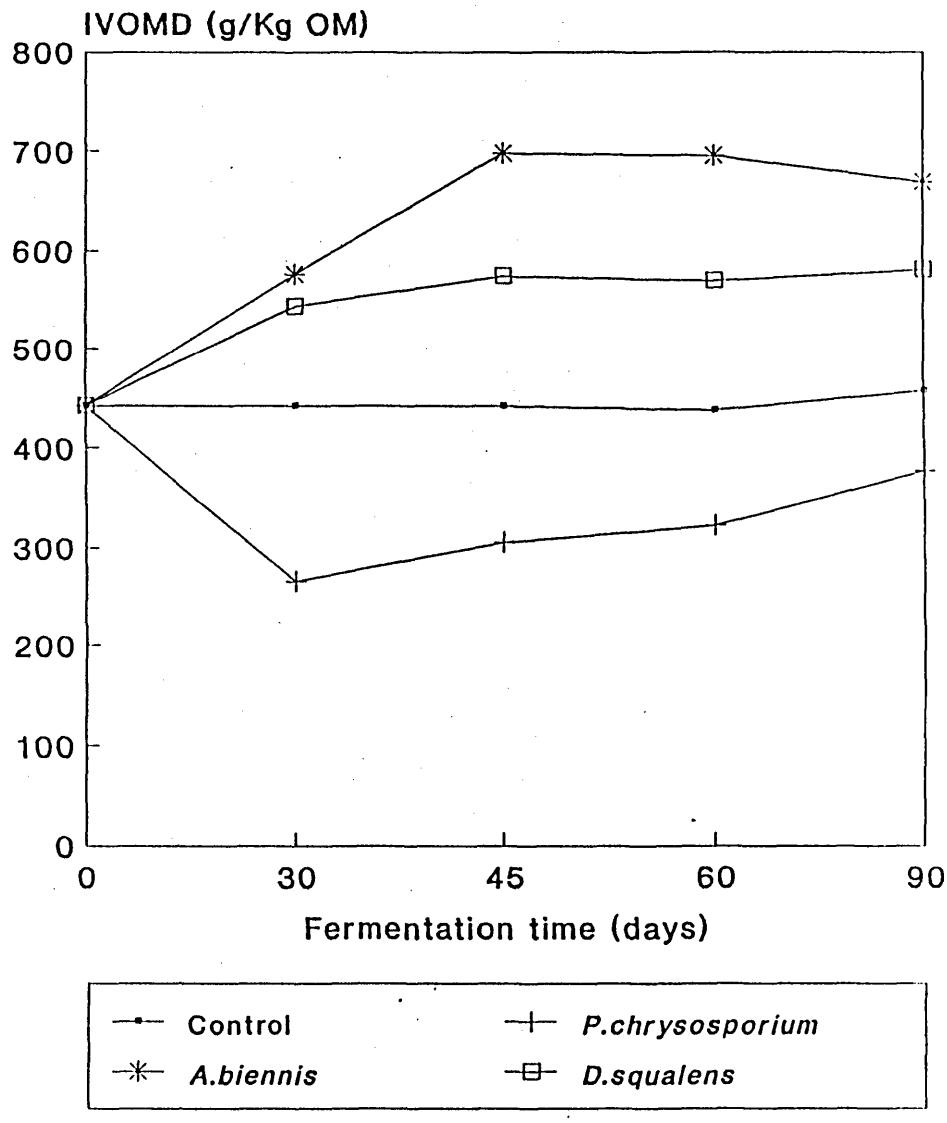

Figure 4.1.4 Change in *in vitro* organic matter digestibility of barley straw with time during solid state fermentation with three white-rot fungi (section 4.1).

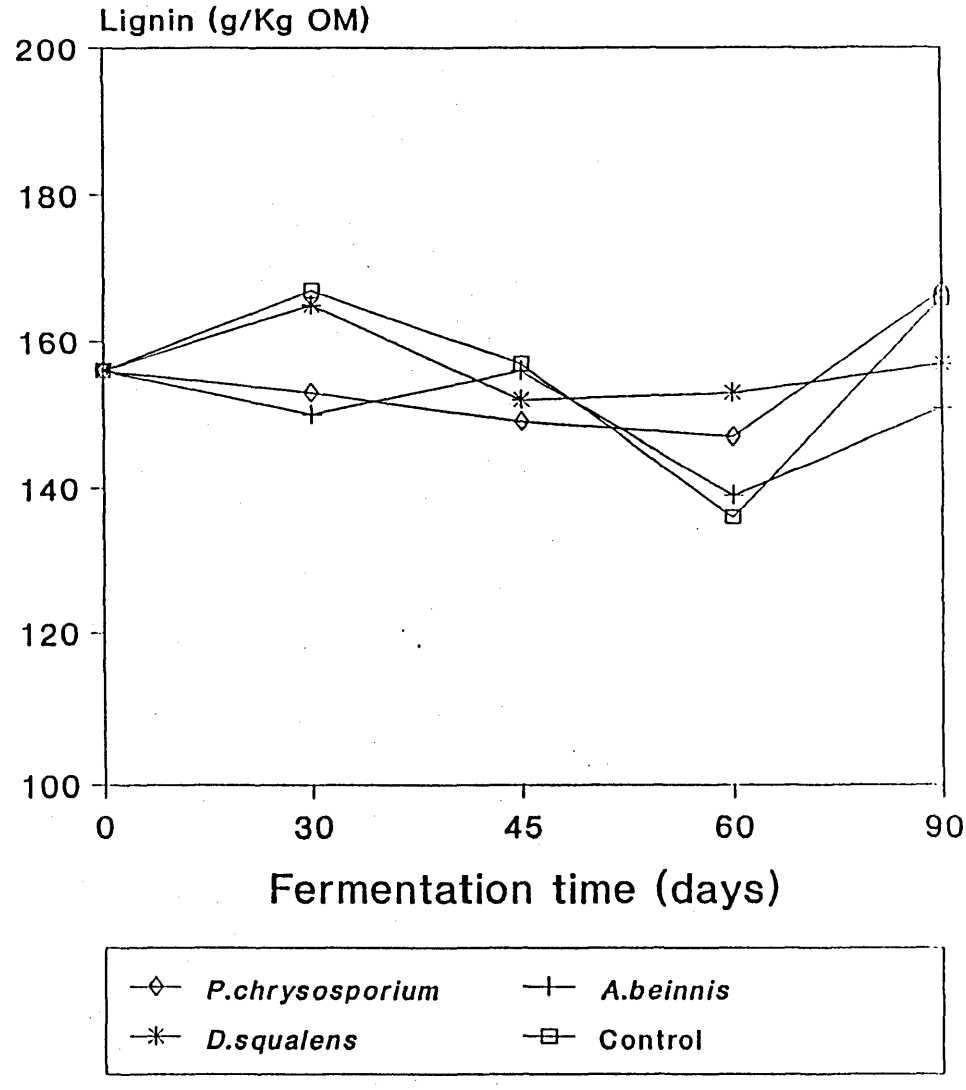

Figure 4.1.5 Change in lignin content of barley straw with time during solid state fermentation with three white-rot fungi (section 4.1).

4.2 EXTRACTION OF FUNGAL ENZYMES FROM SOLID STATE FERMENTATION CULTURES OF *P.chrysosprium* AND *A.biennis.*

From the previous data obtained in section 4.1 it can be seen that increases in organic matter digestibility were apparent for both *D.squalens* and *A.biennis* at 30 and 45 days, section 4.1 Figure 4.1.4, whilst at these times the cultures of *P.chrysosporium* showed a decrease in digestibility. Extraction of the cultures at these times could prove interesting and may give some knowledge as to the role of lignin degrading enzymes at these times.

Barley straw was placed in 2L Erlenmyer (75g + 200ml deionised water) and then autoclaved at 115 psi for 15 min. Each was then inoculated with 4 agar plugs (7 day plates, 4% potato dextrose agar) of either *P.chrysosporium* or *A.biennis* and then incubated at 25 °C. A flask of each culture was harvested at 30 and 45 days after inoculation, Chapter 2 section 2.9.1.

It can be seen in Table 4.2.1 and Table 4.2.2 that from these cultures and under these conditions no detectable LiP activity was found, however, as was clearly shown in Chapter 3 that aqueous straw extract has a detrimental effect on LiP activity, most probably due to phenolics in the straw causing inactivation of the enzyme as discussed earlier, Chapter 3 section 3.2. Interestingly, the only enzymatic activity detected was manganese dependent peroxidase, MnP, although extracts from *A.biennis* did show some manganese independent phenol oxidase activity. This other phenol oxidase activity could be due to the presence of contaminating Mn^{2+} ions in the extract which are eventually lost through the purification steps. However, the protein gel represented in

Plate 4.2.1 does show bands that correspond to the known molecular weight range of lignin peroxidases, 42-47 Kd (Farrell et al, 1989), though it must be noted that these molecular weights are also close to those that correspond to that of the manganese dependant peroxidase (Paszczynski et al, 1986), can only be seen in the extracts from *A.biennis* and not in those from *P.chrysosporium*, Tables 4.2.3 and 4.2.4.

It would seem then that the only detectable enzyme activities are those of phenol oxidase activity, MnP. Although this is not completely surprising in the light of the results in Chapter 3, section 3.2, what is unusual is the absence of any bands in both gels that could possibly correspond to the molecular weight of lignin peroxidase, LiP, in the extracts from *P.chrysosporium*. A possible explanation could be that because of the presence of large number phenolic compounds in the straw any LiP produced is kept very closely associated with the fungal hyphae; the enzyme then oxidising the lignin through a mediator such as veratryl alcohol as suggested in the literature (Harvey et al, 1986). This close association being so tight that the extraction procedures used here are not sufficient to remove it at all or in sufficient quantities to be seen on a gel against the background the bands from the straw extract itself. The suggested role of the MnP or laccase would possibly be to protect the LiP from phenolics by oxidising them first and hence they are probably freely secreted and hence easily extractable and thus detectable as can be seen in this experiment.

It would seem that there is potentially a complex interaction between LiP and MnP in the oxidation of lignin. Further investigation and

understanding of these interactions would seem to be essential before these enzymes could be used for increasing the *in vitro* digestibility of straws.

Table 4.2.1 Progress of units for extraction after 30 days

Step	LiP activity		MnP activity				
			$+Mn^{2+}$		$-Mn^{2+}$		$\%-Mn^{2+}$
	U/ml	Units	U/ml	Units	U/ml	Units	
A.biennis							
Harvest	0.00	0.00	0.82	330.8	0.23	92.6	28.0
Post Amicon	0.00	0.00	2.55	285.1	0.19	22.3	7.8
Post Dialysis	0.00	0.00	1.74	195.3	0.24	2.67	13.7
Post Acetone	0.00	0.00	0.19	9.1	0.00	0.00	0.0
P.chrysosporium							
Harvest	0.00	0.00	0.02	6.2	0.00	0.00	0.0
Post Amicon	0.00	0.00	0.04	4.6	0.00	0.00	0.0
Post Dialysis	0.00	0.00	0.03	4.6	0.00	0.00	0.0
Post Acetone	0.00	0.00	0.19	10.5	0.00	0.00	0.0

For rates marked as -Mn the Manganese sulphate was omitted from the reaction mixture in an attempt to establish background levels of phenol oxidases.

Table 4.2.2 Progress of units for extraction after 45 days

Step	LiP activity		MnP activity				
			$+Mn^{2+}$		$-Mn^{2+}$		$\%-Mn^{2+}$
	U/ml	Units	U/ml	Units	U/ml	Units	
A.biennis							
Harvest	0.00	0.00	0.36	182.0	0.33	165.4	90.8
Post Amicon	0.00	0.00	2.12	254.0	0.18	21.8	8.3
Post Dialysis	0.00	0.00	0.86	129.0	0.18	27.3	21.1
Post Acetone	0.00	0.00	0.10	9.5	0.00	0.00	0.0
P.chrysosporium							
Harvest	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Post Amicon	0.00	0.00	0.04	4.3	0.00	0.00	0.0
Post Dialysis	0.00	0.00	0.05	8.3	0.00	0.00	0.0
Post Acetone	0.00	0.00	0.13	7.8	0.00	0.00	0.0

or rates marked as -Mn the Manganese sulphate was omitted from the reaction mixture in an attempt to establish background levels of phenol oxidases.

Table 4.2.3 Protein profiles of extracts of solid state fermentation by *A.biennis* (Plate 4.2.1)

Track No.	Molecular Wieght (Daltons)	Migration Distance (cm)
30 day extracts		
2	80,000	2.90
	47,500	6.00
	47,000	6.05
	46,500	6.10
	40,500	6.80
	36,000	7.40
	30,000	8.30
	21,000	10.0
45 day extracts		
	74,000	3.80
4	46,000	6.15
	45,000	6.30
	43,000	6.50
	40,000	6.80
	36,000	7.40
	30,000	8.35
	24,000	9.50
	20,500	10.2

N.B. Molecular weights calculated from standard curve in Figure 4.2.1 using the equasion $y=mx+c$ where y=mol.wt m=slope (-7.74) x= migration distance c=y axsis intercept (5.12) Correlation coefficient=0.978

Table 4.2.4 Protein profiles of extracts for solid state fermention by *P.chrysosporium* (Plate 4.2.1)

Track No.	Molecular Weight (Daltons)	Migration distance (cm)
30 day extracts		
6	99,000	2.55
	58,000	4.95
45 day extracts		
8	97,000	2.45
	59,000	4.90

N.B. Molecular wieghts calculated as Table 4.2.3

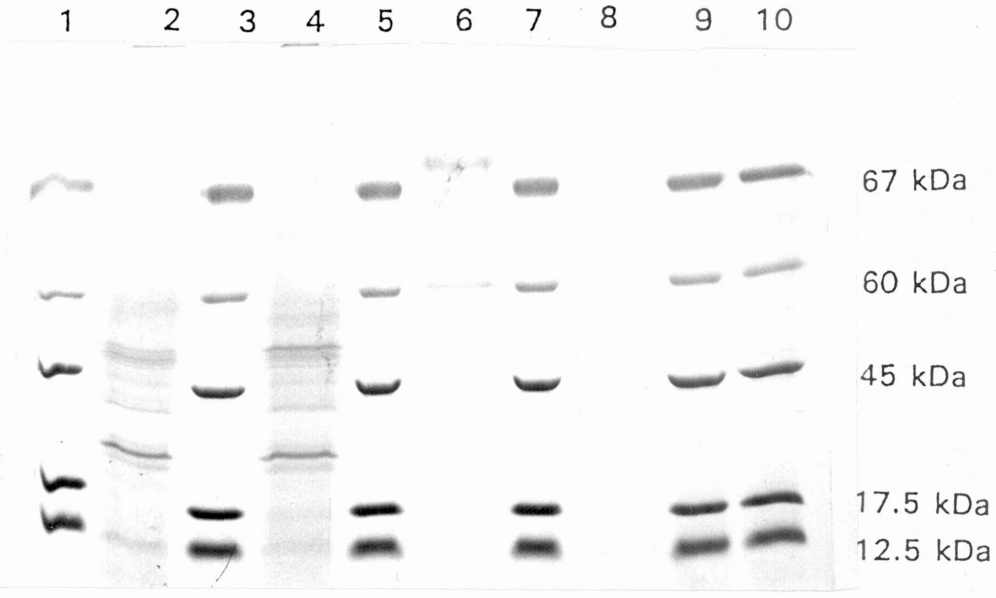

Plate 4.2.1 Extracts from solid state fermentation cultures of *P.chrysosporium* and *A.biennis*.
Tracks 1, 3, 5, 7, 9, 10 standards; Track 2 : Extract of *A.biennis* 30 day cultures; Track 4 : Extract of *A.biennis* 45 day cultures; Track 6 : Extract of *P.chrysosporium* 30 day cultures; Track 8 : Extract of *P.chrysosporium* 45 day cultures.

CHAPTER 5

PREPARATION, CHARACTERISATION AND CHEMICAL MODIFICATION OF SPRUCE MILLED WOOD LIGNIN. REACTIONS OF SPRUCE MILLED WOOD LIGNIN WITH LIGNIN PEROXIDASE AND MANGANESE DEPENDENT PEROXIDASE

5.1 INTRODUCTION

The work described so far has involved the use of straw as a substrate. However, because of the complexity of the results obtained it was decided to investigate the interaction of these lignin degrading enzymes with isolated lignin.

Spruce was selected as a source of milled wood lignin since much is already known about its molecular weight and spectral chracteristics (Goring, 1971; Fengel and Wegner, 1977). This would enable a better understanding of changes taking place in the lignin molecule as a result of enzyme action upon it.

The preparation and characterisation of Spruce milled wood lignin is described together with the influence of acetylation on these properties.

Work with ^{14}C DHP lignins have demonstrated the importance of both lignin peroxidase and manganese dependent peroxidase in lignin biodegradation (Haemmerli et al, 1986a+b; Huynh et al, 1986; Leisola et al, 1988). However, it has also been shown that the *in vitro* reaction of lignin peroxidase and milled wood lignin results in the polymerisation and not the depolymerisation of the lignin (Haemmerli et al, 1987). In this chapter the effect of lignin peroxidase and manganese peroxidase on milled wood lignin will also be studied as with the reaction products of both enzymes.

5.2 CHARACTERISATION OF SPRUCE MILLED WOOD LIGNIN AND ACETYLATED SPRUCE MILLED WOOD LIGNIN

5.2.1 Molecular weight determination of isolated lignin.

Spruce milled wood lignin was isolated as described in Chapter 2, section 2.10 and its molecular weight determined by various methods of size exclusion chromatography, Chapter 2 section 2.15. Since any product from enzymatic reactions with lignin would likely to be chemically modified the effect of acetylation, Chapter 2 section 2.11, on the molecular weight of the polymer was also studied.

It is clear from Table 5.2.1 that the molecular weight estimate of the lignin varied depending on the type of column used.

Table 5.2.1 Molecular weight estimations of the SMWL and acetylated SMWL by various column media.

Column type	Eluent	Estimated Molecular weight (daltons)	
		SMWL	Acetylated SMWL
SEC			
Biogel SX-1	THF*	3400	-
LH 20	THF	<2000	-
Sephdex G-75	Glycine/NaOH**	>8000	>8000
HPLC-SEC			
Zorbax PSM 60S	THF	1200	2800
TSK PW3000	Glycine/NaOH	>6000	>8000

* THF - Tetrathydrofuran
** Glycine/NaOH - 0.2 M Glycine NaOH buffer pH 10.

Similar variations in the estimated molecular weight for spruce milled wood lignin, determined by column chromatography, have been previously reported in the literature (Fengel and Wegner, 1977). However, ultracentrifugation methods have determined the average molecular weight of spruce milled wood lignin to be approximately 11000 daltons (Bjorkman and Person, 1957). As can be seen from Table 5.2.1 the columns using organic solvents as eluents gave a molecular weight estimate considerably lower than this. This apparent low molecular weight of the lignin maybe due to association complexes being formed between the lignin and the column support material, thereby increasing the retention time and decreasing the apparent molecular weight. It is thought that in organic solvents the lignin interacts with the column packing material via the hydroxyl groups on the lignin (Faix et al, 1981). Suggested methods to overcome this problem include acetylation of the lignin, hence reducing the number of hydroxyl groups on the lignin, and the addition of lithium chloride (LiCl) to the eluent (Connors, 1980; Faix et al, 1981). It would seem from the results presented here that acetylation of the lignin did give an increased estimate of the molecular weight of the lignin, over and above that due to the addition of acetyl groups to the lignin. However, in this case the addition of LiCl to the eluent did not seem to have any effect on the molecular weight obtained (data not shown). Nevertheless, the molecular weight estimations obtained using the columns with aqueous eluents, G-75 and TSK G3000PW, both gave much higher molecular weight estimations and closer to 11000 daltons, that reported for SMWL (Bjorkman and

Person, 1957). The choice of G-75 or TSK G3000PW columns for analysis of lignin is clear, since it would seem that any interaction between the lignin and column support is reduced in aqueous eluent. Furthermore, the use of these columns enables the analysis of all the reaction mixture and so avoids any potential loss of breakdown products that might occur should the lignin have to be extracted into organic solvent prior to analysis.

Despite these drawbacks HPLC-SEC provides a fast and sensitive method for comparative evaluations of the macromolecular properties of lignins.

5.2.2 Spectral characteristics

a) Concentration curve

In order to determine the full characterisation of the lignin in any of the different molecular weight fractions isolated in future enzyme work, it was important to have a simple spectrophotometric method for determining the concentration of lignin in these fractions in solution. To this end stock solutions of spruce milled wood lignin and acetylated spruce milled wood lignin were diluted and treated as described in Chapter 2 section 2.12. The relationship between the absorbance at 280 nm and concentration for both types of lignin can be seen in Figures 5.2.1 and 5.2.2. Once plotted and a linear regression performed on the data it can be seen that both spruce milled wood lignin and acetylated spruce milled wood lignin obey Beer's law over this range of concentrations. These graphs can now be used as calibration curves for

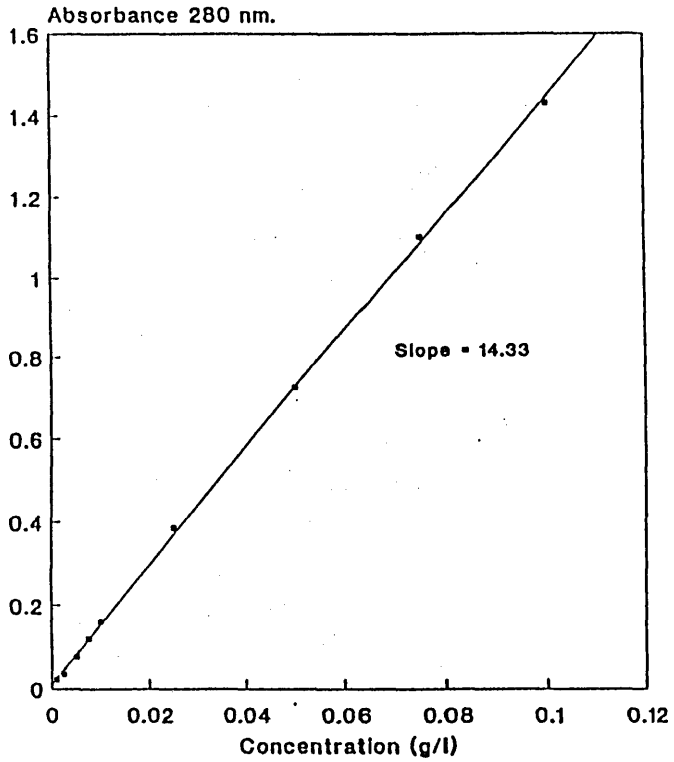

Figure 5.2.1 Graph showing the linear relationship between the concentration of spruce milled wood lignin and its absorbance at 280 nm.

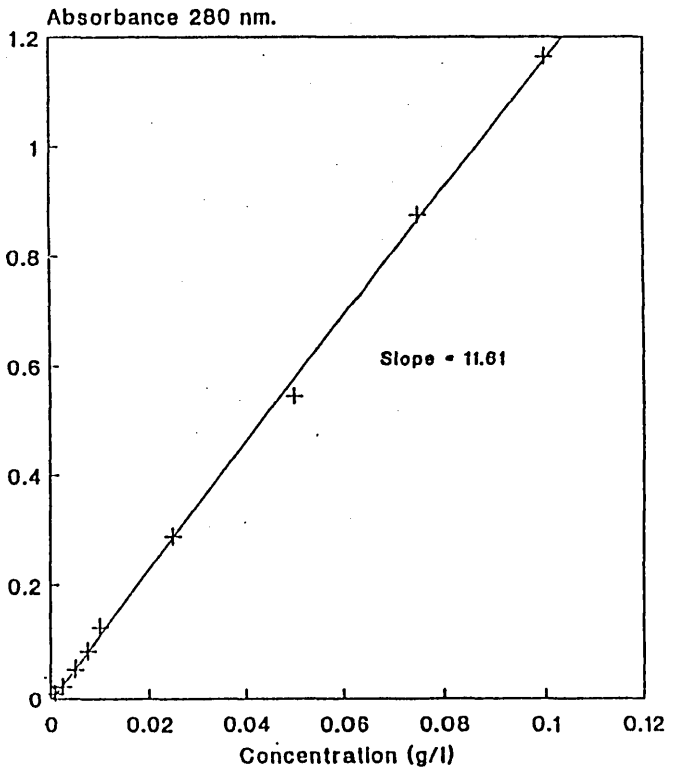

Figure 5.2.2 Graph showing the linear relationship between the concentration of acetylated spruce milled wood lignin and its absorbance at 280 nm.

lignin solutions of unknown concentrations. However, it would seem that acetylation of lignin has reduced the absorbance at 280 nm from 14.33 $l.g^{-1}cm^{-1}$ for native lignin to 11.61 $l.g^{-1}cm^{-1}$ after acetylation, which must be taken into account when calculating lignin concentrations of enzymatically modified lignin fractions.

b) Ionisation difference spectra.

The results of the ionisation difference spectra, IΔS, Chapter 2 section 2.13, on both spruce milled wood lignin and acetylated spruce milled wood lignin can be seen in Figures 5.2.3 and 5.2.4, the results of which are summarised in Table 5.2.2. and Table 5.2.3.

The development by Goldschmidt in 1954 (Goldschmidt, 1954) of a method using the difference spectra of lignin in neutral and alkali solutions to estimate the free phenol content, provided a quick and easy alternative to the lengthy chemical methods previously used. The method is based on the work by Aulin-Erdtman (Aulin-Erdtman, 1949) who showed that the red-shift of the characteristic 280 nm absorption maxima in alkaline solutions is due to the ionisation of phenolic groups in the lignin. Further work with simple phenolic compounds has shown that the maxima of the difference spectra at 250nm and 280 nm are characteristic of phenolate ions of simple substituted aromatic hydroxyl compounds such as eugenol and condendrin (Goldschmidt, 1954). However, if the hydroxyl group is conjugated through the ring with a carbonyl group associated with the C_{α} of the side chain, vanillin, or a conjugated carbon carbon double bond between the C_{α} and C_{β}, coniferyl alcohol, then the characteristic maxima are now at 250 nm and 350 nm (Pew, 1963). Thus when the method

Table 5.2.2 Absorbtivites of the main peaks of reduced and non reduced ionisation spectra for SMWL.

Peak (nm)	Absorptivity ($l.g^{-1}cm^{-1}$)	Apparent % Free Phenol groups
Non reduced.		
350	1.868	-
300	0.805	0.33
Reduced.		
350	0.000	-
300	3.113	1.29

Table 5.2.3 Absorbtivites of the main peaks of reduced and non reduced ionisation spectra for acetylated SMWL.

Peak (nm)	Absorptivity ($l.g^{-1}cm^{-1}$)	Apparent % Free Phenol groups
Non reduced.		
350	0.889	-
300	0.732	0.30
Reduced.		
350	0.000	-
300	0.989	0.409

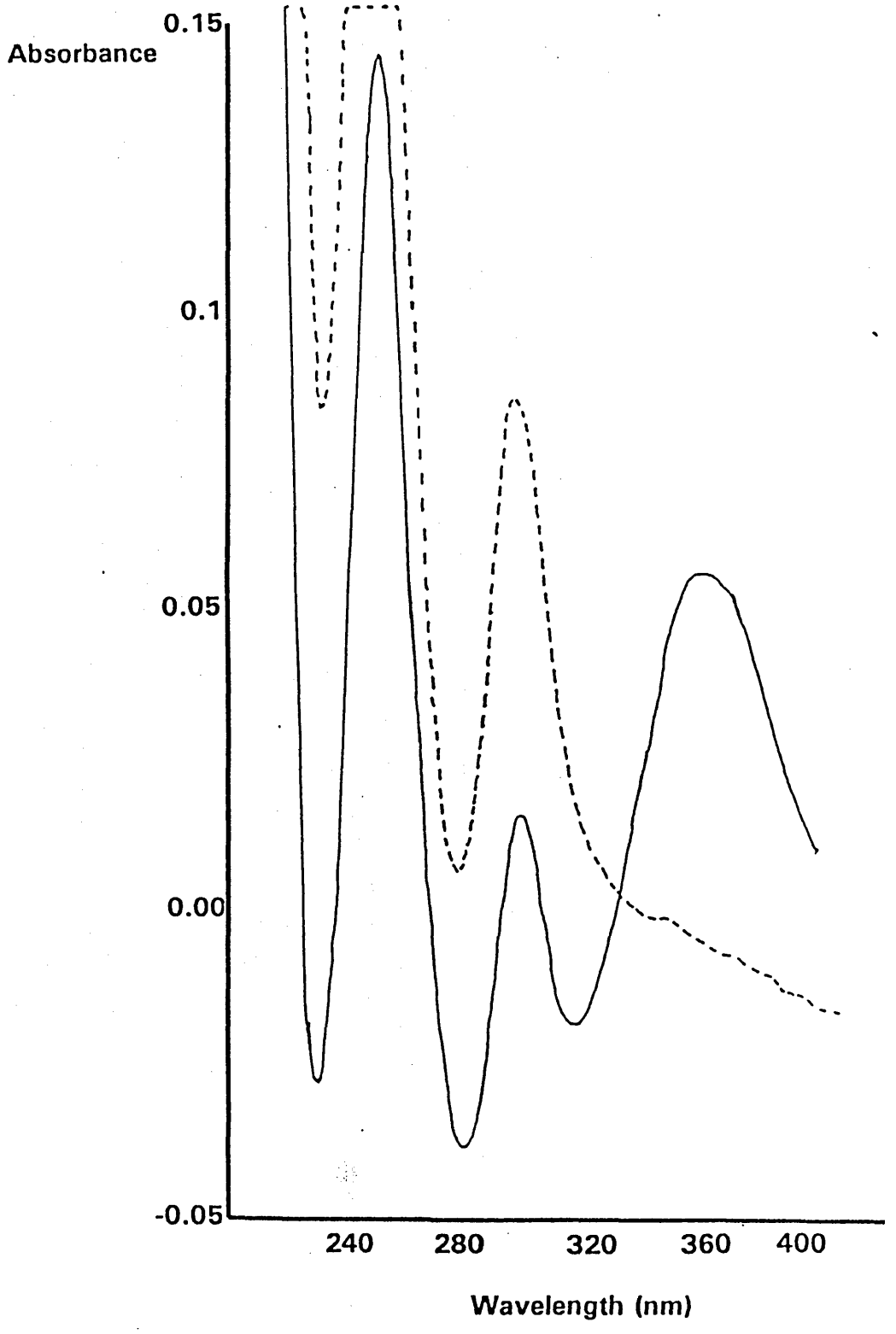

Figure 5.2.3 Ionisation difference spectra for spruce milled wood lignin. A final concentration of 0.027 g l^{-1} was used for each. (——) non reduced spectrum, (----) reduced spectrum.

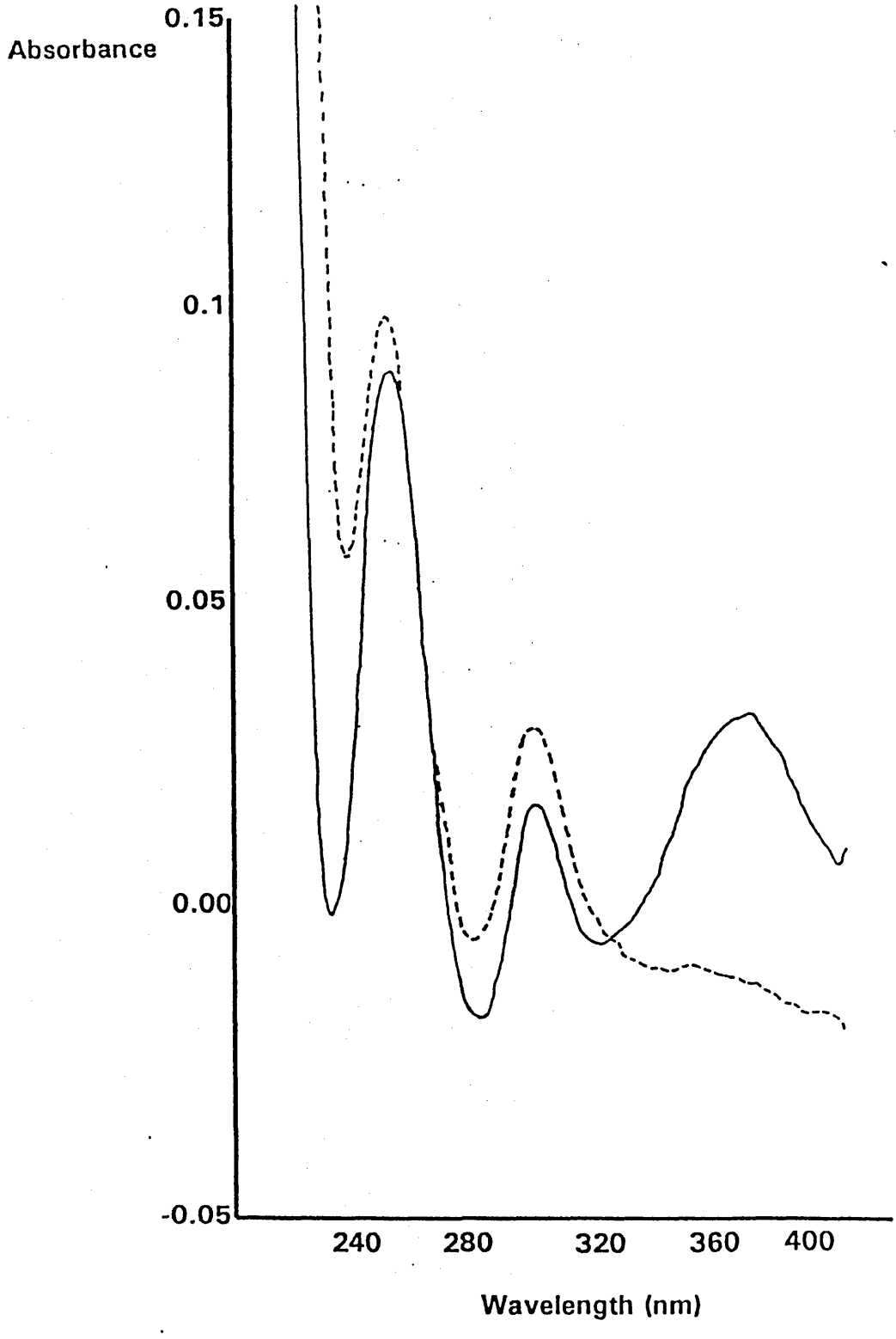

Figure 5.2.4 Ionisation difference spectra for acetylated spruce milled wood lignin. A final concentration of 0.027 g l^{-1} was used for each. (——) non reduced spectrum, (----) reduced spectrum.

is applied to the lignin molecule the spectrum obtained shows peaks from hydroxl groups that are both associated and non associated with the bond types mentioned above. Therefore, direct use of Goldschmidts, equation (Chapter 2 section 2.13), based on the absorption maxima of model compounds not conjugated with carbonyl groups, to estimate the % free phenol content would give an underestimate due to the interference of the phenolic groups that are associated with carbonyl groups. However, chemical reduction of the lignin with borohydride reduces the carbonyl bond (Morton and Adler, 1961), resulting in all the free hydroxyl groups having an absorbance at 300 nm. Therefore, the Goldschmidt equation now applied to the reduced ionisation difference spectra gives a truer estimate of the phenol content of the lignin.

Analysis of the absorptivity data shown in Tables 5.2.2 and 5.2.3, now gives characteristics to the lignin preparations used. Therefore, specific chemical attributes in the lignin molecule can be monitored before and after enzymatic reaction in addition to any change in molecular weight that might be observed.

5.3 OXIDATION OF SPRUCE MILLED WOOD LIGNIN BY LIGNIN PEROXIDASE

5.3.1 Effect of pH on the oxidation of spruce milled wood lignin by lignin peroxidase (LiP).

It has been shown that the optimum pH for lignin degradation by *P.chrysosporium* is pH 4.5, however, in contrast to this it has also been shown that the optimum pH for the degradation of lignin model compounds by lignin peroxidase is pH 2.75. One such compound, veratryl alcohol, is also used as a spectrophotometric assay for lignin peroxidase, LiP (Tien and Kirk, 1983). It has been suggested that the veratryl alcohol radical cation may act as a mediator between LiP and the lignin molecule *in situ* (Harvey et al, 1986). Indeed work has shown that the presence of veratryl alcohol does enhance the oxidation of spruce milled wood lignin by LiP (Haemmerli et al, 1987). Therefore, an experiment to investigate the influence of pH, in addition to the effect of the presence or absence of veratryl alcohol, was perfomed, Table 5.3.1. Despite the fact that, as mentioned earlier, pH 4.5 is thought to be the optimum for lignin degradation, in this experiment pH 5.0 was used since it makes future work involving comparisons with other enzymes easier. Hydrogen peroxide was used to initiate the reactions and further additions (540 nmoles) made every 2 hrs. until the termination of the experiment at 8 hrs. At the end of this time the reaction was stopped with 100 ul 1M NaOH and the samples analysed on a G-75 column as described in Chapter 2, section 2.15.4.

Table 5.3.1 Experimental design for the investigation of the interaction of LiP and SMWL.

Assay	Lignin stock (ul)	V.alcohol (ul)	Enzyme* (Units)	Buffer (ml)	
				A	B
1	100	100	3.0	2.3	
2	100	100	3.0	-	2.3
3	100	0.0	3.0	2.3	-
4	100	0.0	3.0	-	2.3
5	100	100	0.0	2.3	-

*Enzyme = 166 U/ml from carbon limited foam experiment, chapter 2.

Buffer A = 2,2-DMS pH 5.0, 100 mM
Buffer B = 2,2-DMS pH 2.75, 100 mM
Veratryl alcohol = 20 mM

The molecular weight distribution patterns, Figures 5.3.1a+b, show that the oxidation of lignin by LiP resulted in the polymerisation of the lignin with no detectable degradation products apparent. The effect of pH on the extent of polymerisation is also apparent. The degree of polymerisation at pH 5.0 is considerably higher than that obtained at pH 2.75. It was hoped that since pH 2.75 is the pH optimum for veratryl alcohol oxidation that this would, via mediation through the veratryl alcohol radical cation, have an equally apparent effect on the lignin. Indeed the addition of veratryl alcohol did enhance the degree of polymerisation of the lignin at both pHs.

It would seem therefore that despite pH the role of veratryl alcohol in the oxidation of the lignin molecule is an important factor. However, these results must be treated with caution since the solubility of lignin at pH 2.75 is much lower than at pH 5.0 and this may have in

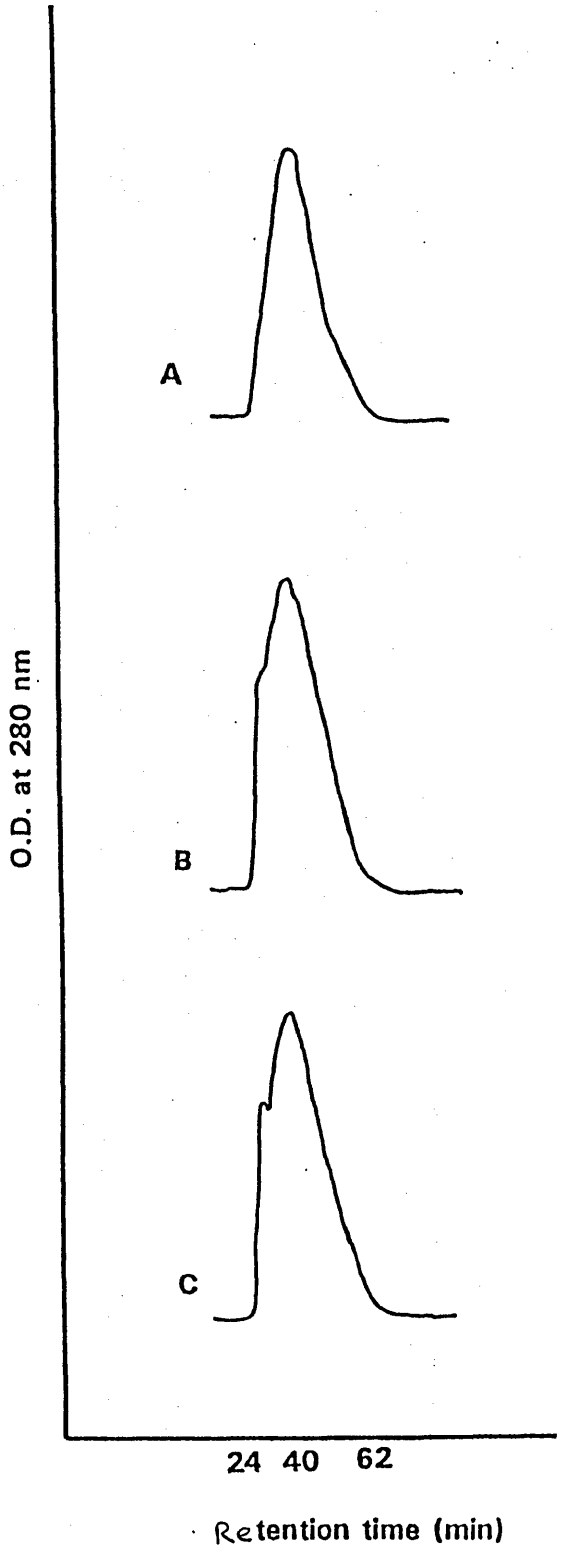

Figure 5.3.1a Elution patterns of SMWL on oxidation by LiP at pH 5.0. Oxidations were carried out as described in section 5.3.1. A: SMWL control; B: SMWL with 2.3 U LiP; C : SMWL with 2.3 U LiP and 0.8mM veratryl alcohol. Samples were analysed on a sephadex G-75 column (Chapter 2, section 2.15.4), flow rate 1ml/min, eluent 0.2 M glycine/NaOH pH 10.0, and monitored at 280 nm continuously. Exclusion limit 20 min (Blue dextran).

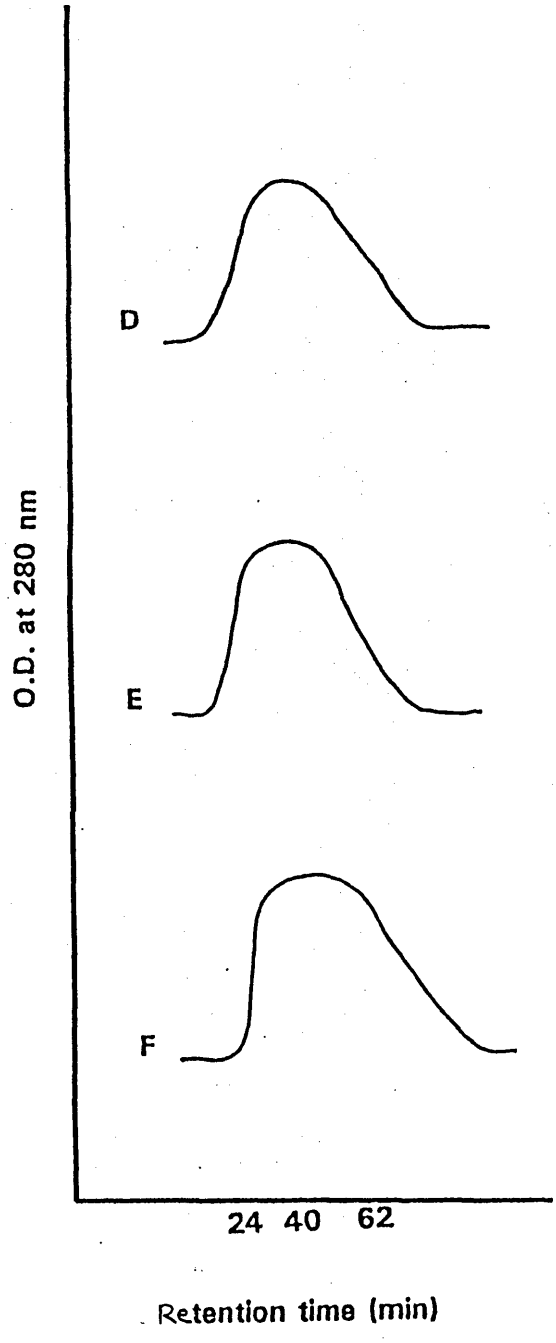

Figure 5.3.1b Elution patterns of SMWL on oxidation by LiP at pH 2.75. Oxidations were carried out as described in section 5.3.1. D: SMWL control; E: SMWL with 2.3 U LiP; F: SMWL with 2.3 U LiP and 0.8mM veratryl alcohol. Samples were analysed on a sephadex G-75 column (Chapter 2, section 2.15.4), flow rate 1ml/min, eluent 0.2 M glycine/NaOH pH 10.0, and monitored at 280 nm continuously. Exclusion limit 20 min (Blue dextran).

fact been responsible for the differences in degrees of polymerisation observed.

5.3.2 Development of an *in situ* hydrogen peroxide generating system using glucose oxidase.

The requirement of hydrogen peroxide in the catalytic cycle of lignin peroxidase is essential, and the source of this hydrogen peroxide *in vivo* is thought to be enzymatic, Chapter 1. In experiment 1, section 5.3.1, the hydrogen peroxide was added to the reaction mixture externally at timed intervals. However, *in vivo* it is more likely that there is a constant supply of hydrogen peroxide to the enzyme. Therefore, in an attempt to mimic *in vivo* conditions a system using glucose oxidase (EC 1.1.3.4) to produce hydrogen peroxide *in situ* during the oxidation of SMWL by LiP was initiated. In order to be able to do this it was necessary to determine the effect of the pH of the reaction mixture on the level of peroxide produced by glucose oxidase. The reactions were performed in a 1 ml oxygen electrode with the glucose oxidase being added last. Oxygen consumption as measured by the oxygen electrode gives an indirect measure of the amounts of hydrogen peroxide produced by using the equation :

$$2\ \text{D-Glucose} + 2O_2 + 2H_2O \longrightarrow 2\ \text{D-Gluconic acid} + 2\ H_2O_2$$

Figure 5.3.2 shows the effect of the concentration of glucose oxidase on the rate of oxygen consumed at pH 5.0 in the presence of lignin. Since for every mole of oxygen consumed one mole

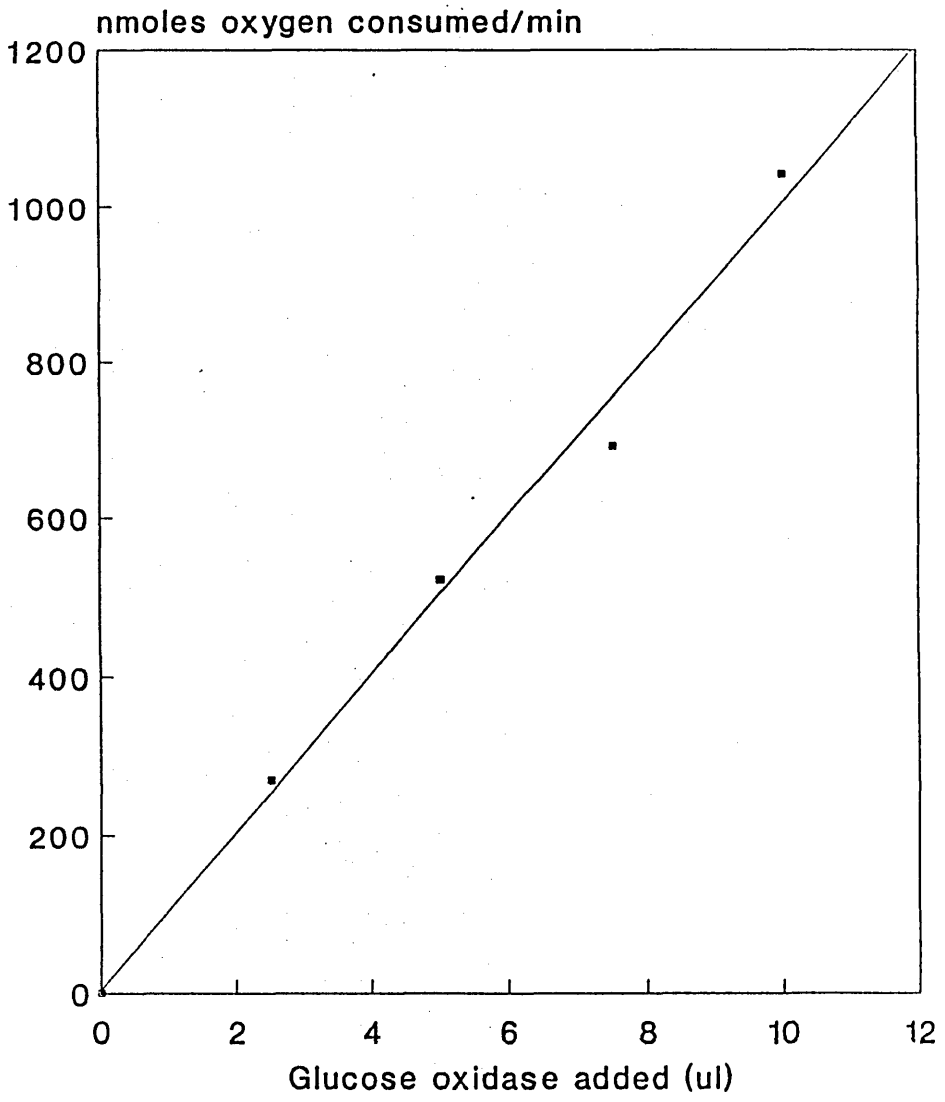

Figure 5.3.2 Showing the relationship between the amount glucose oxidase and the amount of oxygen consumed. Reactions were performed in a 1ml clark oxygen electrode. Each assay contained 800 ul lignin solution (2mg/ml, 0.1M sodium tatrate buffer pH 5.0); 50 ul glucose (0.5M); the reaction was initiated with varying amounts of glucose oxidase (1mg/ml).

of hydrogen peroxide is produced the rate of hydrogen peroxide production can now be calculated.

The development of this system now provides a method of producing hydrogen peroxide *in situ* during the reaction between LiP and SMWL.

5.3.3 Effect of *in situ* hydrogen peroxide generation on the polymerisation of spruce milled wood lignin by lignin peroxidase.

Having established a system for generating hydrogen peroxide, *in situ* with glucose oxidase, it was now necessary to ascertain its effect on the oxidation of spruce milled wood lignin by LiP. Since the results in experiment 1 would seem to indicate that modification of the lignin by the enzyme was enhanced by the presence of veratryl alcohol, the effect of 1,4-dimethoxybenzene on the reaction products was assessed. This compound, like veratryl alcohol, is also shown to form a radical cation on oxidation with lignin peroxidase (Kersten et al, 1985) and has been shown to perform a mediatory role similar to veratryl alcohol (Harvey et al, 1986). However, this radical cation is more stable than that of veratryl alcohol and so any mediatory effect obtained would be increased.

Since SEC-HPLC provides a faster system with greater resolution, analysis of the reaction products was performed on a TSK G3000PW column as described in Chapter 2 section 2.17. In each of the reactions shown in Table 5.2.3, 200 ul aliquots were taken at 30 min, and 60 min and 10 ul of 4 M NaOH added to solubilise the lignin prior to analysis.

Table 5.3.3 Experimental design to determine the effect of a glucose oxidase/peroxide generating system on the interaction between LiP and SMWL.

Assay number	LiP (113 U/ml) (Units)	DMB (20mM) (ul)	Lignin solution* (ul)	Glucose (0.5M) (ul)	GOD (1.0 mg/ml) (ul)
1	00	40	800	50	5
2	2.8	00	800	50	5
3	2.8	40	800	50	5

* Lignin solution = 100 mg in 1 ml 1M NaOH diluted 1:5 with 100mM Sodium tartrate buffer pH 5.0 and the final solution adjusted to pH 5.0

Analysis of the molecular weight distributions from the SEC-HPLC column indicates the polymerisation of spruce milled wood lignin, by LiP, is more complex than that would be suggested by the original analysis in Experiment 1. When 1,4-dimethoxybenzene (DMB) is absent from the reaction, assay 2, two polymerisation products can be seen, one with a retention time (RT) of 5.6 min and one with a RT of 4.9 min, Figure 5.3.3. However, when 1,4-dimethoxybenzene (DMB) is included the balance of these polymerisation products changes, and a further polymerisation product of even higher molecular weight, RT 4.2 min, appears, Figure 5.3.4. For clarity these will from now on be referred to as products 1, 2 and 3 respectively. It would appear that the presence of DMB has enhanced the formation of a polymer of higher molecular weight, product 3, in addition to those observed in the absence of DMB,

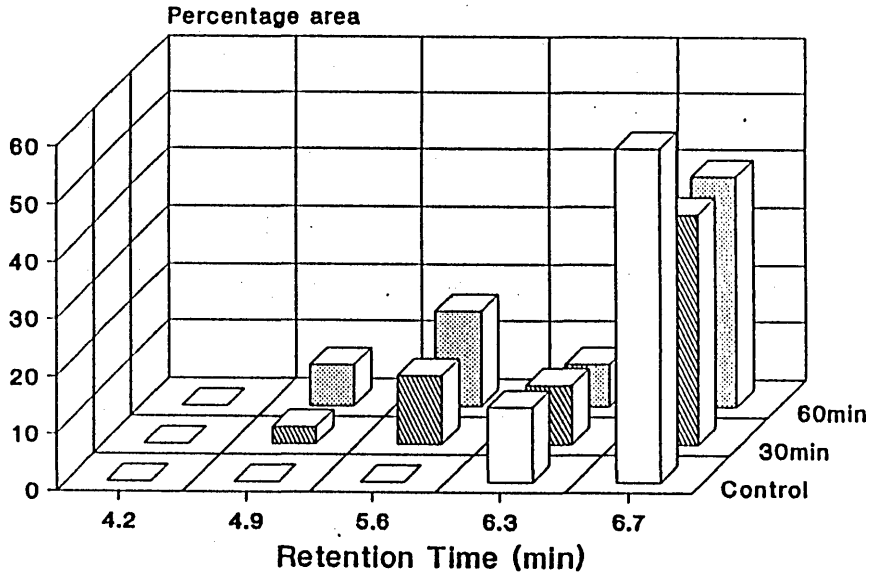

Figure 5.3.3 Schematic view of the elution profile of SMWL on reaction with 2.8 U of LiP in the absence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 25 ul LiP (2.8 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

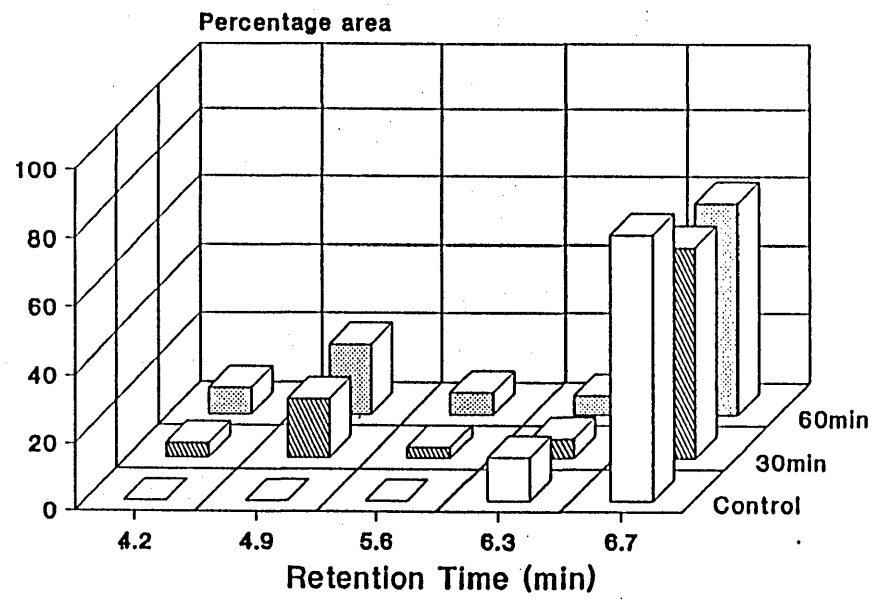

Figure 5.3.4 Schematic view of the elution profile of SMWL on reaction with 2.8 ul LiP in the presence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 25 ul LiP (2.8 U);40 ul DMB (20 mM); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limited.3.2 min (Blue Dextran).

products 2 and 3. It has already been established that the dimethoxybenzene radical cation can perform the same mediatory role as veratryl alcohol in the reaction between LiP and its substrates (Harvey et al, 1986). It is clear then from Figure 5.3.4 that during the oxidation of spruce milled wood lignin by LiP, DMB plays an important role.

5.3.4 Effect of changing the concentration of lignin peroxidase on the oxidation products from spruce milled wood lignin and the influence of 1,4-dimethoxybenzene on these.

Since in Experiment 3 the addition of 1,4-dimethoxybenzene to the reaction had a marked effect it was decided to see if changing the enzyme concentration could produce a similar effect and whether the DMB would still have an effect at these higher enzyme concentrations. Table 5.3.4 shows the range of enzyme concentrations used, although only one control is shown in the table, assay 1, a control was run for each enzyme concentration as they were not run simultaneously. Two reactions were carried out for each enzyme concentration, one in the presence of DMB and one in its absence, 200 ul samples being taken at 30 and 60 min and 10 ul of 4 M NaOH added. The solubilised lignin was filtered through a 0.22 um filter prior to analysis on a TSK G3000PW column, Chapter 2 section 2.15.5.

Table 5.3.4 Experimental design to determine the influence of enzyme levels on the reaction products of the oxidation of spruce milled wood lignin by lignin peroxidase in the presence and absence of 1,4-dimethoxybenzene.

Assay number	LiP (113 U/ml) (Units)	DMB (20mM) (ul)	Lignin solution* (ul)	Glucose (0.5M) (ul)	GOD (1.0 mg/ml) (ul)
1	0.0	40	800	50	5
2	5.6	40	800	50	5
3	5.6	00	800	50	5
4	8.5	40	800	50	5
5	8.5	00	800	50	5
6	11.1	40	800	50	5
7	11.1	00	800	50	5

* Lignin solution = 100 mg in 1 ml 1M NaOH diluted 1:5 with 100mM Sodium tartrate buffer pH 5.0 and the final solution adjusted to pH 5.0.

The effect of the enzyme concentration on the polymerisation products can be split into two groups, one in which DMB was not present in the reaction and one where it was. However, in both groups the only products detected were the three polymerisation products found previously, section 5.3.3., with retention times (RT) of 5.6, 4.9 and 4.2 min, products 1, and 3 respectively. For clarity the elution profiles are not shown, however, the changes in the lignin is represented by the percentage areas of the peaks, obtained from the integrator, and are shown in Figure 5.3.5 - Figure 5.3.10 for both groups.

The variation in the balance of the products in the group in which DMB was not included is summarised in Figure 5.3.11. It is clear from this graph that increasing the amount of enzyme present in the reaction has the effect of increasing the level of the largest polymer,

product 3, produced as a result of the reaction. This is similar to the effect that the addition of DMB had on the level of this product when added to lower levels of LiP, section 5.3.3. This increase in the amount of product 3, RT 4.2 min, would initially seem to be at the expense of the amount of the smallest polymer formed, product 1, RT 5.6 min. However, the level of this product seems to level off after an initial decrease, Figure 5.3.11. Changing the balance of these two polymers, products 1 and 3, would not seem to have any discernable effect on the levels of the intermediate sized polymer, product 2, RT 4.9 min., Figure 5.3.11.

The effect of adding DMB to the reaction on the balance of polymeric products is shown in Figure 5.3.12. The difference in the amount of the largest polymer, product 3 RT 4.2 min, produced is striking. Here it can be seen that as the level of LiP increases, the level of this polymer also increases and is present at higher levels than in the absence of DMB. Furthermore, in conjunction with the higher levels of this polymer, product 3, there is a reduction in the amount of the smallest polymer produced, product 1 RT 5.6 min. The level of enzyme even in the presence of DMB does not seem to have any major effect on the level of the intermediate sized polymer produced, product 2 RT 4.9 min.

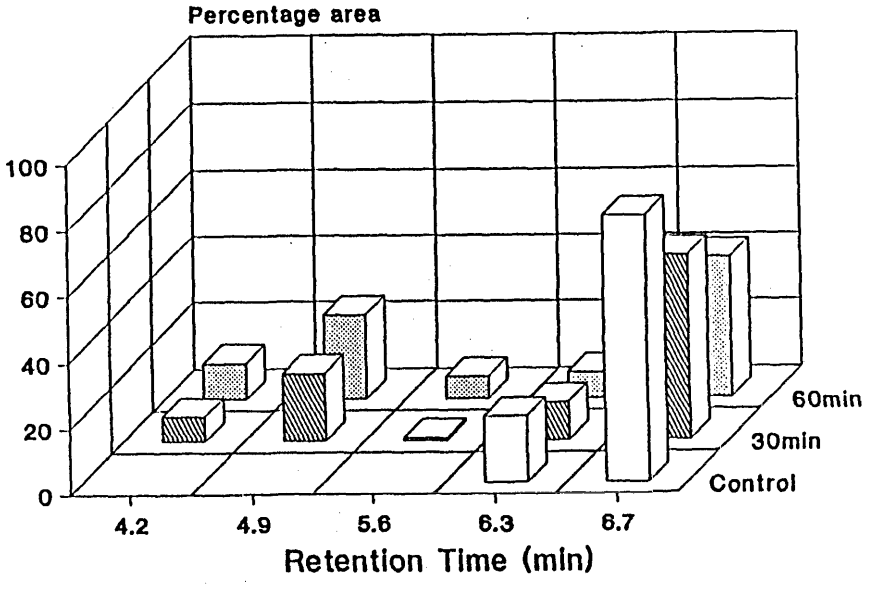

Figure 5.3.5 Schematic view of the elution profile of SMWL on reaction with 5.6 U of LiP in the absence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 50 ul LiP (5.6 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

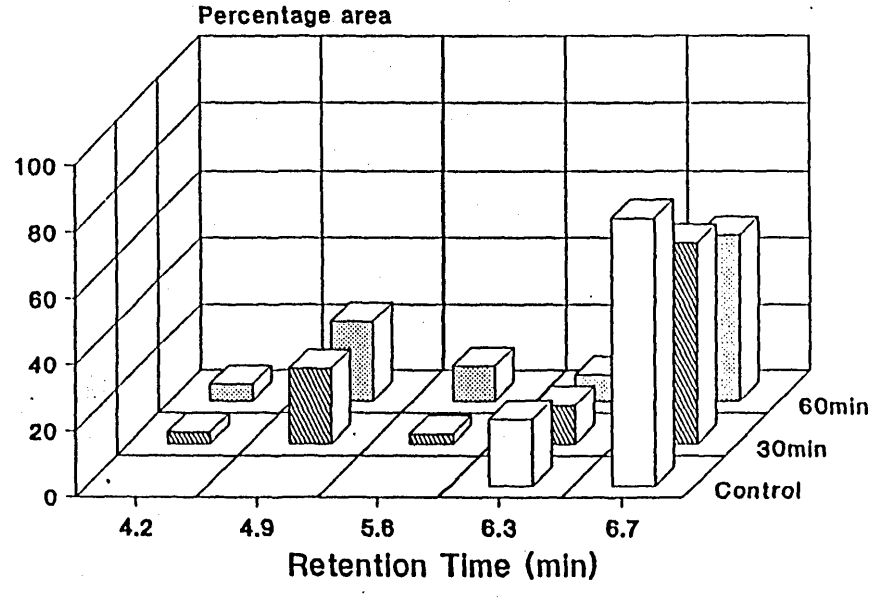

Figure 5.3.6 Schematic view of the elution profile of SMWL on reaction with 5.6 ul LiP in the presence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 50 ul LiP (5.6 U);40 ul DMB (20 mM); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

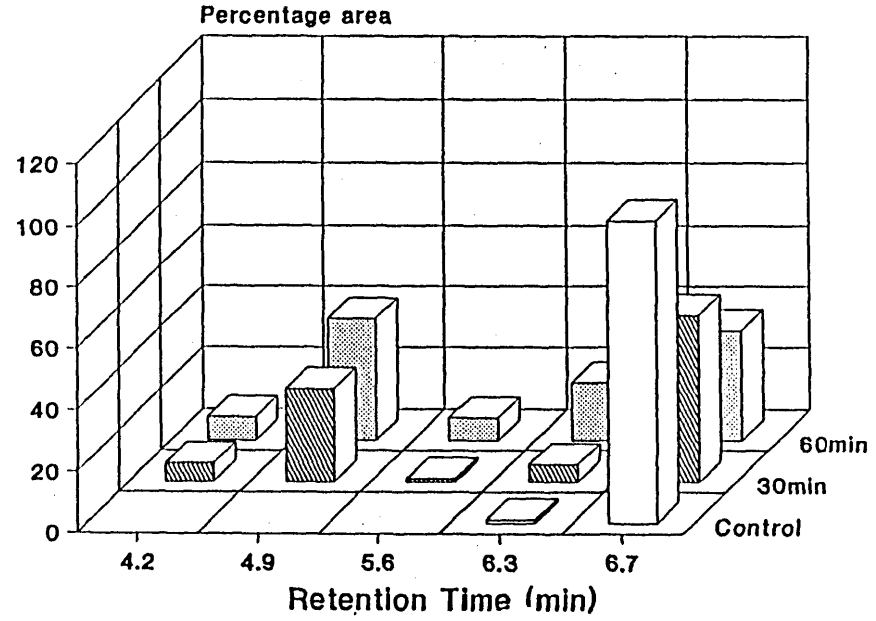

Figure 5.3.7 Schematic view of the elution profile of SMWL on reaction with 8.5 U of LiP in the absence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 75 ul LiP (8.5 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

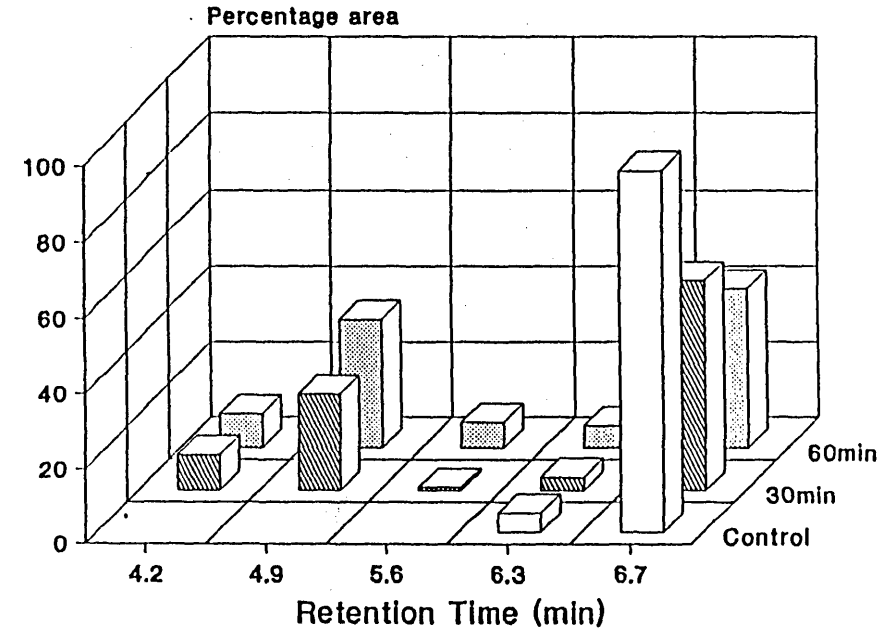

Figure 5.3.8 Schematic view of the elution profile of SMWL on reaction with 8.5 ul LiP in the presence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 75 ul LiP (8.5 U);40 ul DMB (20 mM); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

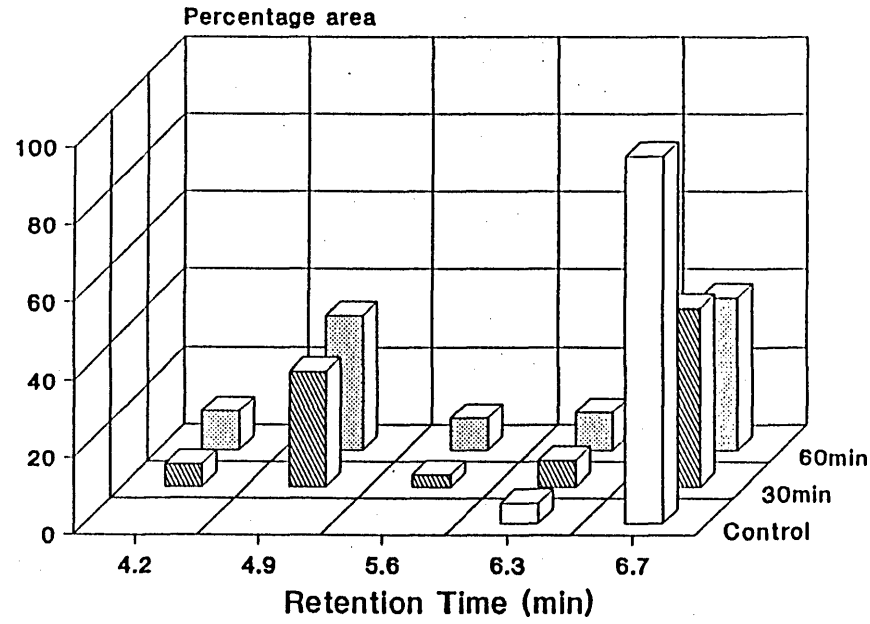

Figure 5.3.9 Schematic view of the elution profile of SMWL on reaction with 11.1 U of LiP in the absence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 100 ul LiP (11.1 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

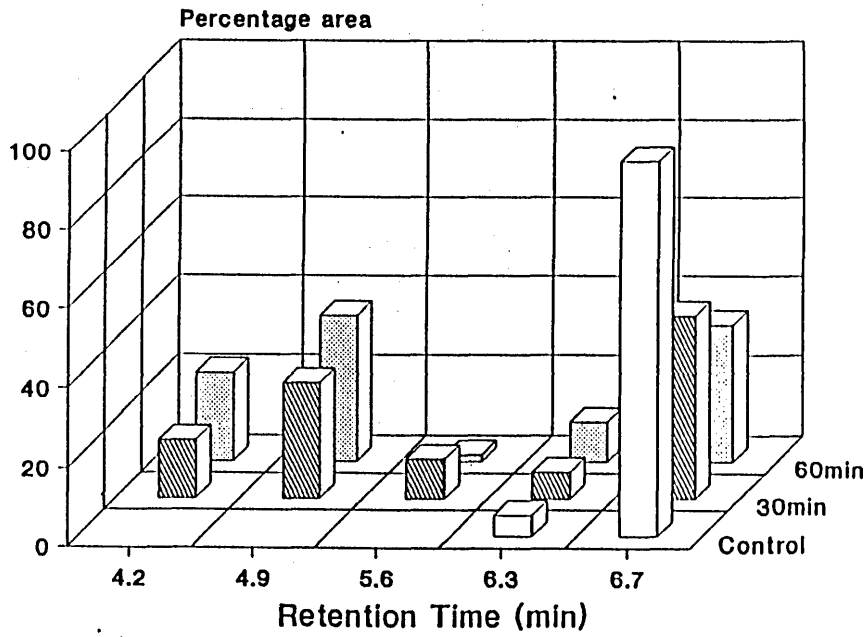

Figure 5.3.10 Schematic view of the elution profile of SMWL on reaction with 11.1 ul LiP in the presence of DMB. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 100 ul LiP (11.1 U);40 ul DMB (20 mM); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

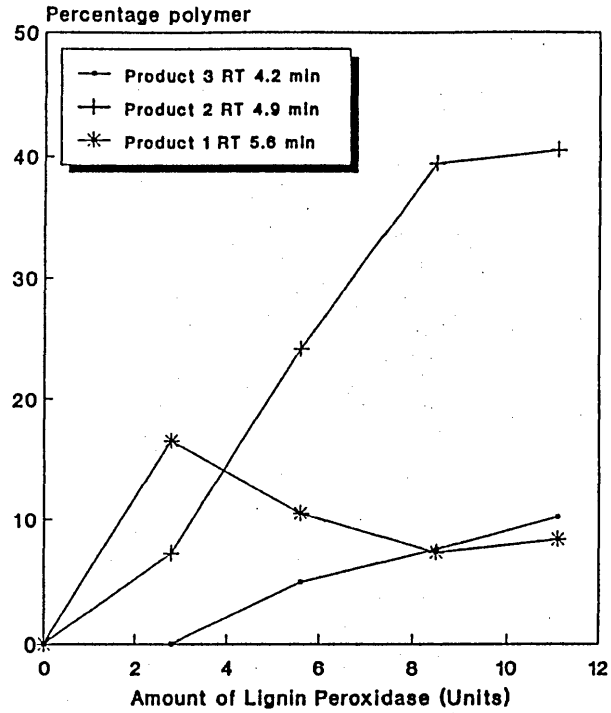

Figure 5.3.11 **Summary of the effect of increasing enzyme concentration on the polymerisation of spruce milled wood lignin by lignin peroxidase.** Only values for 60 min incubation shown.

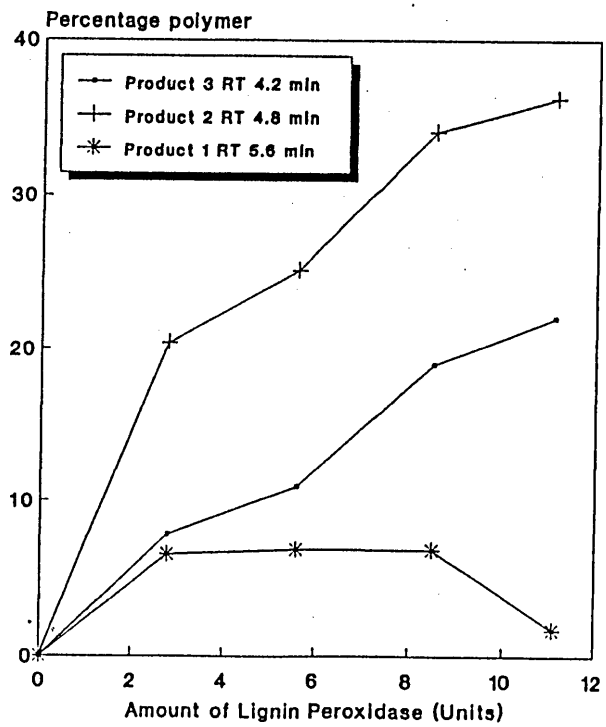

Figure 5.3.12 **Summary of the effect of increasing enzyme concentration on the polymerisation of spruce milled wood lignin by lignin peroxidase in the presence of DMB.** Only values for 60 min incubation shown.

5.4 REACTIONS INVOLVING MANGANESE DEPENDENT PEROXIDASE AND SPRUCE MILLED WOOD LIGNIN.

5.4.1 Analysis of the products from the oxidation of spruce milled wood lignin by manganese dependent peroxidase and the effect of the concentration of manganese ions on these.

As outlined earlier, Chapter 1, the enzyme manganese dependent peroxidase, MnP, is also thought to play a role in lignin biodegradation. This enzyme oxidises its substrates by oxidising Mn^{2+} ions to Mn^{3+} ions which are the effective oxidative agent (Glen and Gold, 1985; Paszczynski et al, 1986). In fact the depolymerisation of lignosulphonates by MnP and chemically generated Mn^{3+} ions has already been shown (Huynh et al, 1986). The importance of this enzyme is further underlined with its isolation from the solid state fermentation cultures in Chapter 4. It would be of interest therefore to study oxidation of spruce milled wood lignin by MnP. The effect of changing the concentration of the Mn^{2+} ions in the reaction and hence the level of the ultimate oxidant Mn^{3+} ions on the oxidation of lignin would also be of interest.

To achieve these aims the experiment described in Table 5.4.1 was performed. Reactions were initiated with the addition of glucose oxidase and run and analysed as for the lignin peroxidase experiments in section 5.3.4.

Table 5.4.1 Experimental design for the investigation of the oxidation of spruce milled wood lignin by manganese dependent peroxidase and the effect of the concentration of Mn^{2+} ions on these.

Assay number	MnP (78 U/ml) (units)	Lignin solution* (ul)	Glucose (0.5M) (ul)	GOD (1.0 mg/ml) (ul)	[$MnSO_4$] in assay (mM)
1	0.0	800	50	5	0.000
2	1.6	800	50	5	0.005
3	1.6	800	50	5	0.05
4	1.6	800	50	5	0.5
5	1.6	800	50	5	5

* Lignin solution = 100 mg in 1 ml 1M NaOH diluted 1:5 with 100mM Sodium tartrate buffer pH 5.0 and the final solution adjusted to pH 5.0.

5.4.2 Discussion of the oxidation of spruce milled wood lignin by LiP and MnP.

Analysis of the molecular weight distributions indicates that as in the case of lignin peroxidase, LiP, oxidation of lignin, section 5.3, only polymerisation products were detected with no apparent depolymerisation products being found. Most striking is the effect that the concentration of Mn^{2+} ions has on the distribution of polymerisation products. This effect can be seen in Figures 5.4.1 - 5.4.4. and is clearly summarised in Figure 5.4.5, the individual oxidations. Again as in the case for the oxidation of spruce milled wood lignin by LiP, a variety of oxidation products are seen, that is a lower molecular weight polymer, RT 5.56 min and an intermediate molecular weight polymer, RT 4.66 min. These are directly comparable to the retention times of the polymers obtained from the LiP oxidations, section 5.3, and will be referred to also as products 1 and 2 respectively. However, it must be

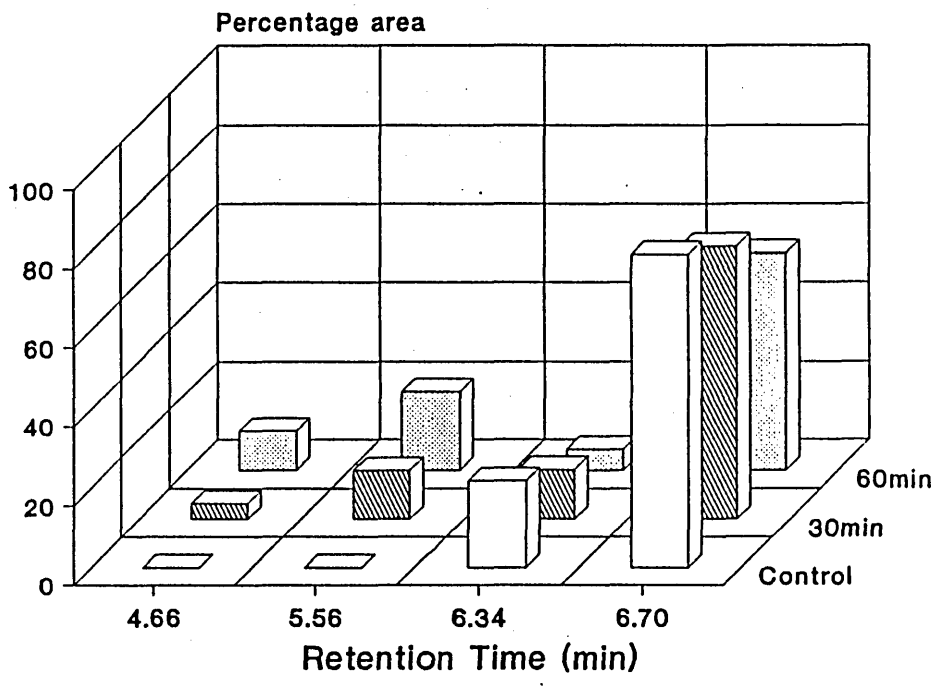

Figure 5.4.1 Schematic view of the elution profile of SMWL on reaction with MnP in the presence of 0.005 mM Mn^{2+}. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 20 ul MnP (1.6 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

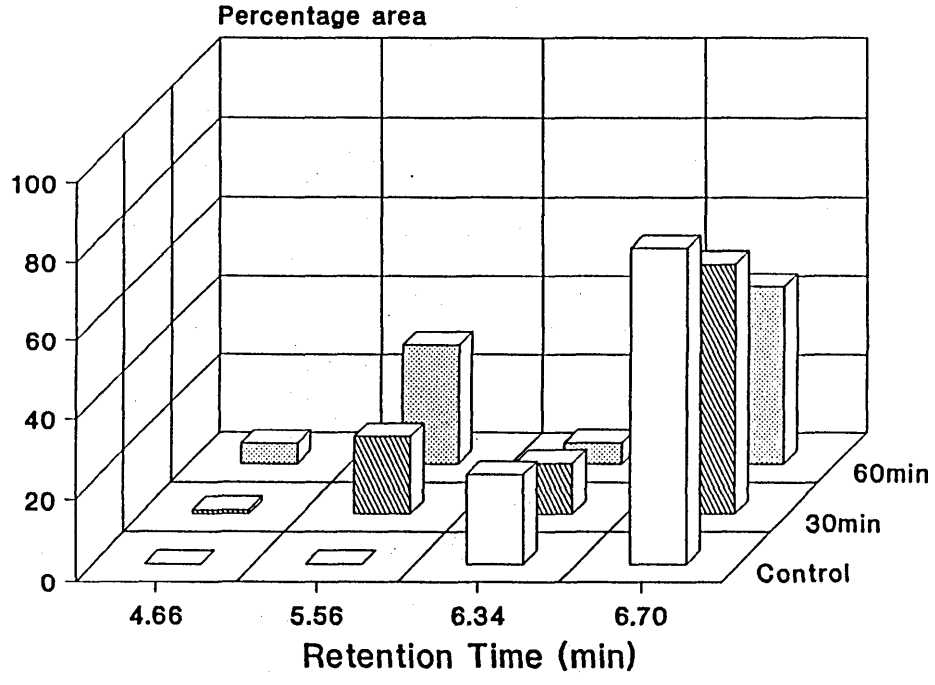

Figure 5.4.2 Schematic view of the elution profile of SMWL on reaction with MnP in the presence of 0.05 mM Mn^{2+}. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 20 ul MnP (1.6 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

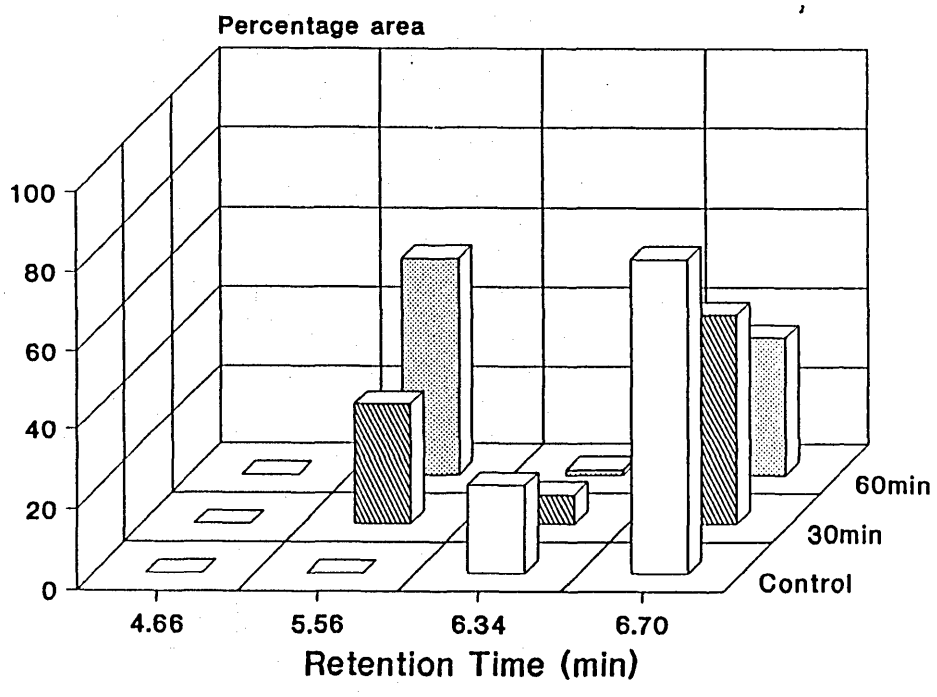

Figure 5.4.3 Schematic view of the elution profile of SMWL on reaction with MnP in the presence of 0.5 mM Mn^{2+}. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 20 ul MnP (1.6 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

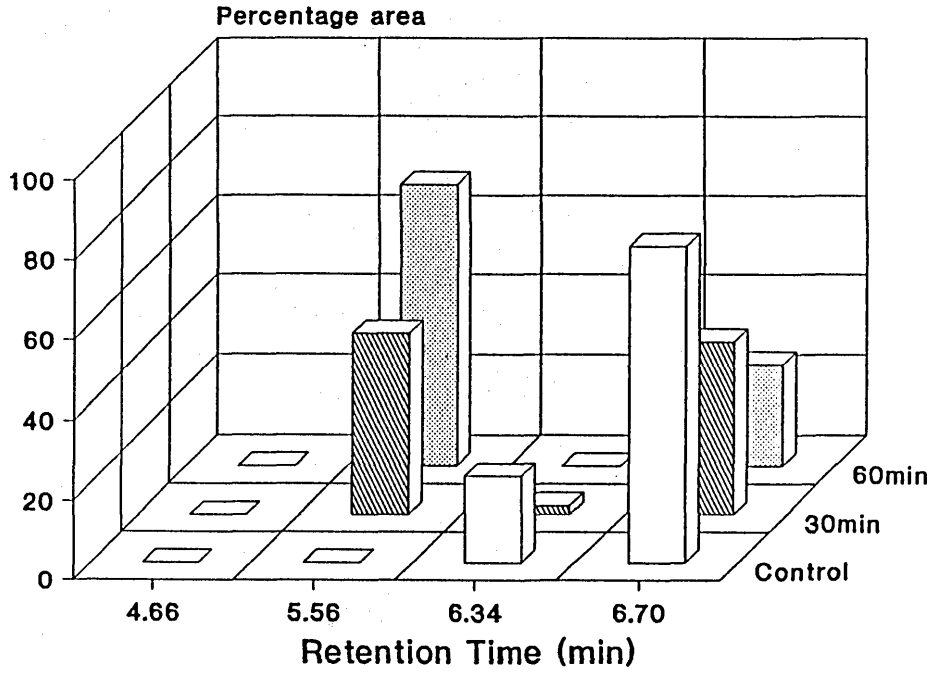

Figure 5.4.4 Schematic view of the elution profile of SMWL on reaction with MnP in the presence of 5 mM Mn^{2+}. Assay contained 800ul lignin solution (2mg/ml, 0.1M sodium tartrate buffer pH 5.0); 50 ul glucose (0.5M); 20 ul MnP (1.6 U); the reaction initiated with the addition of 5 ul GOD (1mg/ml). 200 ul samples were taken at 30 and 60 min with 10 ul NaOH (4M) being added prior to filtering (0.22 um) and analysis on a TSK G300PW column (100 ul injection loop). Eluent being glycine/NaOH buffer (0.2M, pH 10) and monitored at 280 nm. Exclusion limit 3.2 min (Blue Dextran).

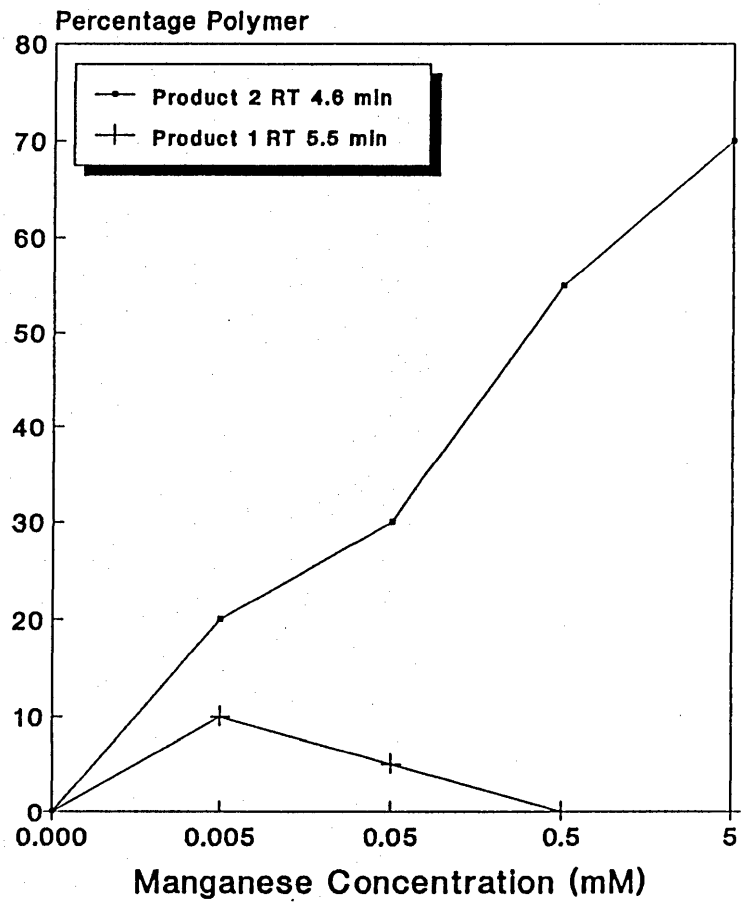

Figure 5.4.5 Summary of the effect of increasing the concentration of Mn^{2+} ions on the polymerisation of SMWL by MnP. Only values for 60 min incubation shown.

noted that there is a total absence of the highest molecular weight polymer, product 3 RT 4.2 min, produced when lignin is oxidised by LiP, section 5.3. Futhermore, the balance of these polymers is dependant on the concentration of Mn^{2+} ions in the reaction mixture, Figure 5.4.5. This can be directly related to the rate of reaction, since levels of Mn^{2+} ions have been shown to be limiting in the oxidation of guaiacol by MnP, Figure 5.4.6. It can be seen in Figure 5.4.1 that with increasing Mn^{2+} ions concentration, and hence reaction rate, the balance of products shifts until only product 1 is produced. For example in the presence of 5mM Mn^{2+} ions over 70 % of the original lignin is polymerised by MnP to product 1, product 2 is absent, Figure 5.4.5. However, in comparison increasing the LiP concentration, had the opposite effect, at 11.1 Units, with no DMB present only 8% of the small polymer, product 1, is produced, Figure 5.3.9.

It is clear from the results obtained earlier in this chapter that the oxidation of lignin by LiP and MnP differ in the nature of the final products. However, the results presented in this chapter may give some indication as to the mechanisms involved. A known characteristic of spruce milled wood lignin is that it contains free phenolic groups, section 5.2. The ability of MnP to oxidise phenolic substrates is well known, (Paszczynski et al, 1988), furthermore, as can be seen from the catayltic cycle of MnP, Figure 5.3.7, this enzyme oxidises Mn^{2+} ions to Mn^{3+} ions, (Paszczynski et al, 1985) which in chelated form, are the effective oxidative agent involved in its enzymic reactions, (Glenn and Gold, 1985; Paszczynski et al, 1986). The process of oxidation of phenolic compounds involves the formation of the phenoxy radical on removal of

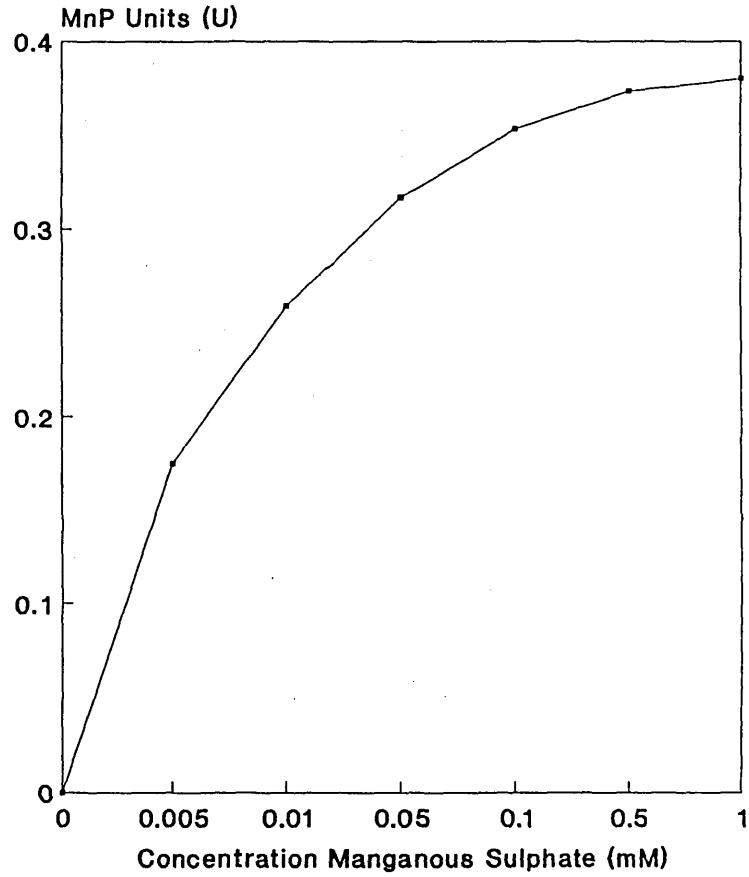

Figure 5.4.6 Effect of the concentration of Mn^{2+} ions on the oxidation of phenolic compounds. Each assay consisted of 1 ml sodium tartrate buffer (100 mM, pH 5.0); 20 ul guaiacol (40 mM, 5% acetonitrile); 5 ul MnP (0.4 U) and varing concentrations of manganous sulphate. Each assay was carried out in 1ml cuvettes and initiated with 5 ul hydrogen peroxide (10 mM), the appearance of tetraguaiacol continually monitored monitored at 470 nm.

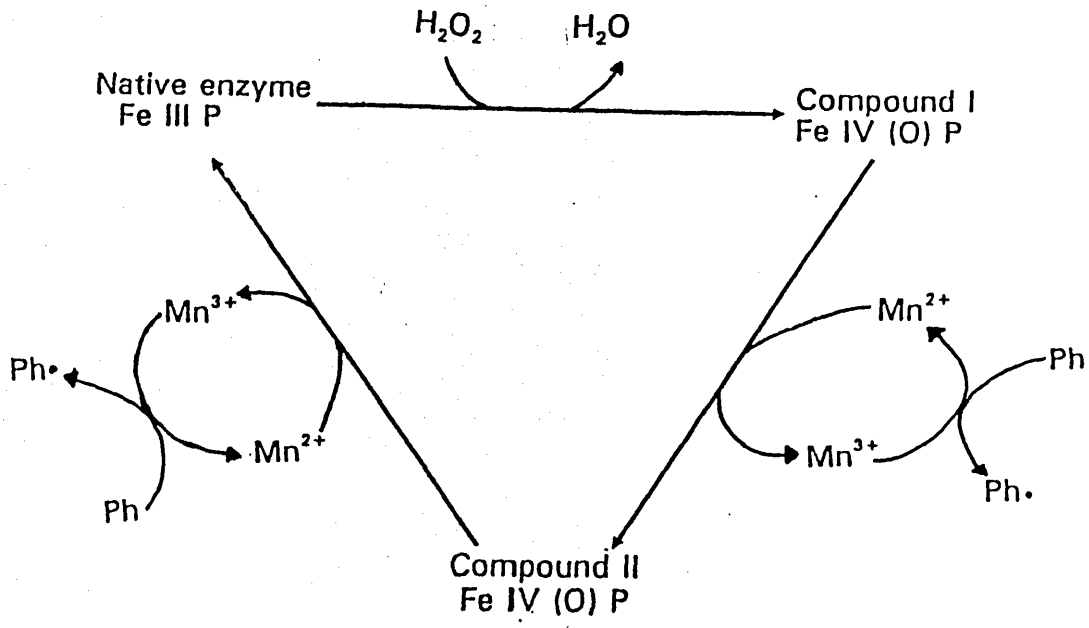

Figure 5.4.7 **Catalytic cycle of Manganese dependent peroxidase showing oxidation of phenolic compounds.** P - porphyrin; Ph - phenolic compound; Ph• - phenoxy radical.

an electron by the Mn^{3+} ions. These radicals couple to one another yielding a variety of products. It is suggested that through this mechanism MnP oxidises lignin leading to polymer formation. Furthermore, from the results in section 5.4.1 it is apparent that the concentration of the Mn^{2+} ions has a marked effect on the size of polymer produced. Clearly the level of these ions would have a direct effect on the rate of phenoxy radical production. It is proposed that at high concentrations of Mn^{2+} ions, due to high levels of phenoxy radicals produced, there is an increased chance of radical species reacting with one another to form product 1. However, at the lower concentrations of Mn^{2+} ions, the corresponding levels of phenoxy radicals are also low, radicals react with one another forming product 1, however, ther is now an increased chance of a phenoxy radical, generated in the original lignin, now reacting with product 1, forming a larger polymer, as seen in the formation of product 2. This effect is similar to one seen in the formation of dehydrogenation polymer, DHP, from coniferyl alcohol, a process known to involve phenoxy radical coupling, (Freudenberg and Niesh, 1968). In this case if the rate of reaction is kept low then the size of the final DHP is large however if the rate of reaction is high then the final DHP is of lower molecular weight, (Freudenberg and Niesh, 1968).

The polymerisation of spruce milled wood lignin by LiP is complex, and although no clear explanation seems apparent there are several possibilities. Again the initial polymerisation of the lignin is through oxidation of the phenolic moieties on the lignin. However, work has shown that lignin peroxidases are capable of oxidising phenolic

substrates and that the oxidation of these substrates is one that occurs preferentially to any other, however, the oxidation of these phenolic containing compounds results in enzyme inactivation, (Harvey and Palmer, 1990). The results shown in Figure 5.3.3, from an experiment in which 2.8 U of LiP and no DMB was included, the main product was product 1 and only a little of product 2. Increasing the enzyme concentration results in the appearance of an additional product, the highest molecular weight polymer, product 3, along with those already present, Figure, 5.3.4. Previous work has shown that LiP is capable of generating phenolic moieties from non phenolic lignin model compounds through three ways : Demethoxylation; C_β ether cleavage and C_α-C_β cleavage in β-O-4 linked models (Schoemaker et al, 1985; Miki et al, 1986; Tien and Kirk, 1984). It is proposed that LiP generates phenolic groups in the initial oxidation products, 1 and 2, and that these are then further oxidised by LiP to form phenoxy radicals, which then polymerise, as decribed previously, leading to the formation of product 3. However, as mentioned earlier the oxidation of phenolic compounds by LiP results in enzyme inactivation, therefore, at the lowest level of enzyme, 2.8 Units Figure 5.3.3, the preferential oxidation of phenolic compounds (Harvey and Palmer, 1990) results in this amount of enzyme being inactivated before generation of phenolic groups in the products can take place. However, increasing the level of enzyme would have the effect of increasing the catalytic capacity in the reaction, and hence the generation of additional phenols in the products can proceed through the reactions catalysed by LiP. The balance of products is consequently changed with a fall in the level of product 1 and an appearance of

product 3, Figure 5.3.11.

The result of adding DMB to the reactions had a similar effect to increasing the LiP concentration, Figure 5.3.12. The addition of DMB to all levels of enzyme caused the balance of polymers to shift to favour the production of product 3, with a concomittant fall in the level of product 1. It is probable that this too may be as a result of increased catalytic capacity of the enzyme due to the presence of DMB. However, there are two possibilities as to how DMB may maintain the catalytic capacity of LiP. The mechanism of inactivation of LiP during the oxidation of phenolic compounds is shown in Figure 5.4.8. Here it can be seen that when compound I reacts with a phenolic substrate a phenoxy radical is formed at the active site of the enzyme. The resulting enzyme-radical complex does not have the oxidative capacity to react with the lignin molecule and further reaction with hydrogen peroxide results in the formation of the inactive compound III and the release of a phenoxy radical. Work with veratryl alcohol (Harvey et al, 1987) has suggested that during the catalytic cycle of LiP compound I reacts with veratryl alcohol to form a radical cation, this remains bound to the active site of the enzyme forming a compound II radical cation complex. If this complex were now to react with a further veratryl alcohol molecule then the enzyme would be returned to the ground state with the simultaneous release of two veratryl alcohol radical cations. However, if the compound II-radical cation complex were to react with a phenolic compound then it would return the enzyme to the ground state with the simultaneous release of a phenoxy radical and a radical cation, Figure 5.4.9. this would have the effect of increasing the catalytic capacity of the enzyme

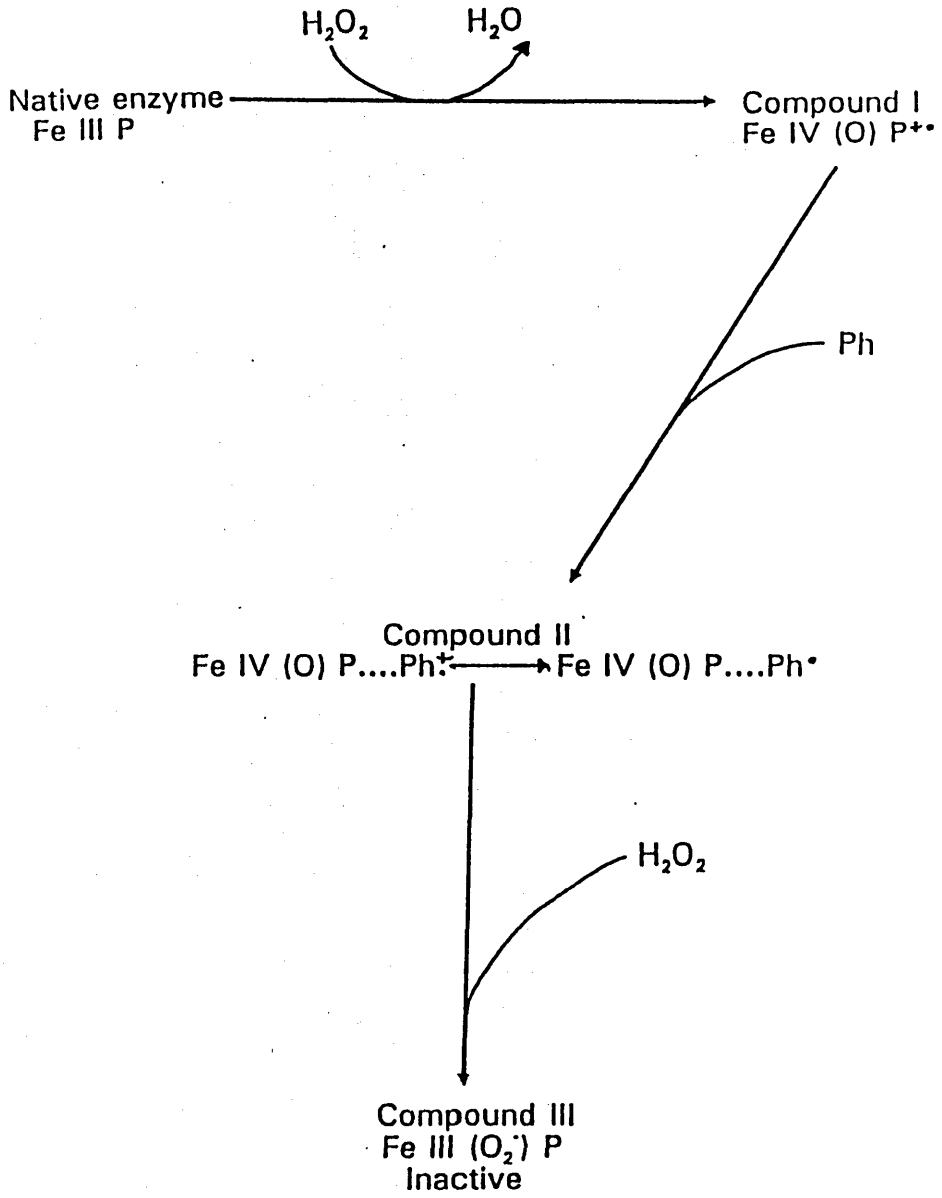

Figure 5.4.8 Catalytic cycle of lignin peroxidase showing inactivation by phenolic compounds. P - porphyrin; Ph - phenolic compound; Ph• - phenoxy radical.

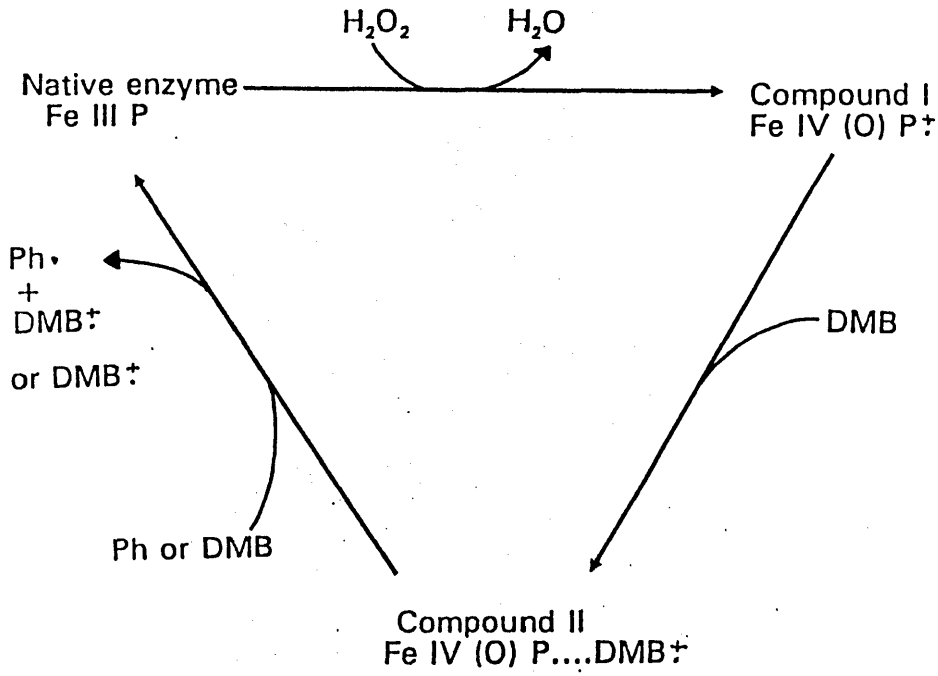

Figure 5.4.9 Possible mechanism of enzyme cycling by DMB during the oxidation of phenolic compounds by LiP. P - porphyrin; Ph - phenolic compound; Ph$\cdot$ - phenoxy radical.

in the system since it would remain active for longer. Because LiP is known to form radical cations from DMB (Hammel et al, 1985) it is likely that this process would also occur in the case of DMB.

An alternative possibility suggests a different route as to how the catalytic capacity of the enzyme is maintained (Valli et al, 1990). This work was based on the oxidation of monomethoxylatedcompounds by LiP, however, since the oxidation of these compounds also results in the formation of compound III, the same principle can be used in this case as with phenolic compounds. It is suggested by these workers that on the formation of compound III there is a further association with hydrogen peroxide to form compound III*, Figure 5.4.10. This is able to react with a dimethoxylated substrate, in this thesis DMB, resulting in the release of a superoxide anion and the return of the enzyme to the ground state. In this way it is proposed that the enzyme is kept catalytically active during the oxidation of phenolic compounds.

One possible role of the manganese dependant peroxidase in lignin biodegradation is the oxidation of phenolics preventing the inactivation of the lignin peroxidase, allowing the depolymerisation reactions catalised by LiP to predominate. However, preliminary experiments with enzyme mixtures yielded no evidence of depolymerisation and the only products observed were those polymers already mentioned, data not shown. Further work on the balances and reaction sequences of these enzymes is therefore necessary.

Isolation and analysis of the polymers generated by both these enzymes could prove interesting and may provide some information as to the mechanism invoved in the oxidation of lignin by

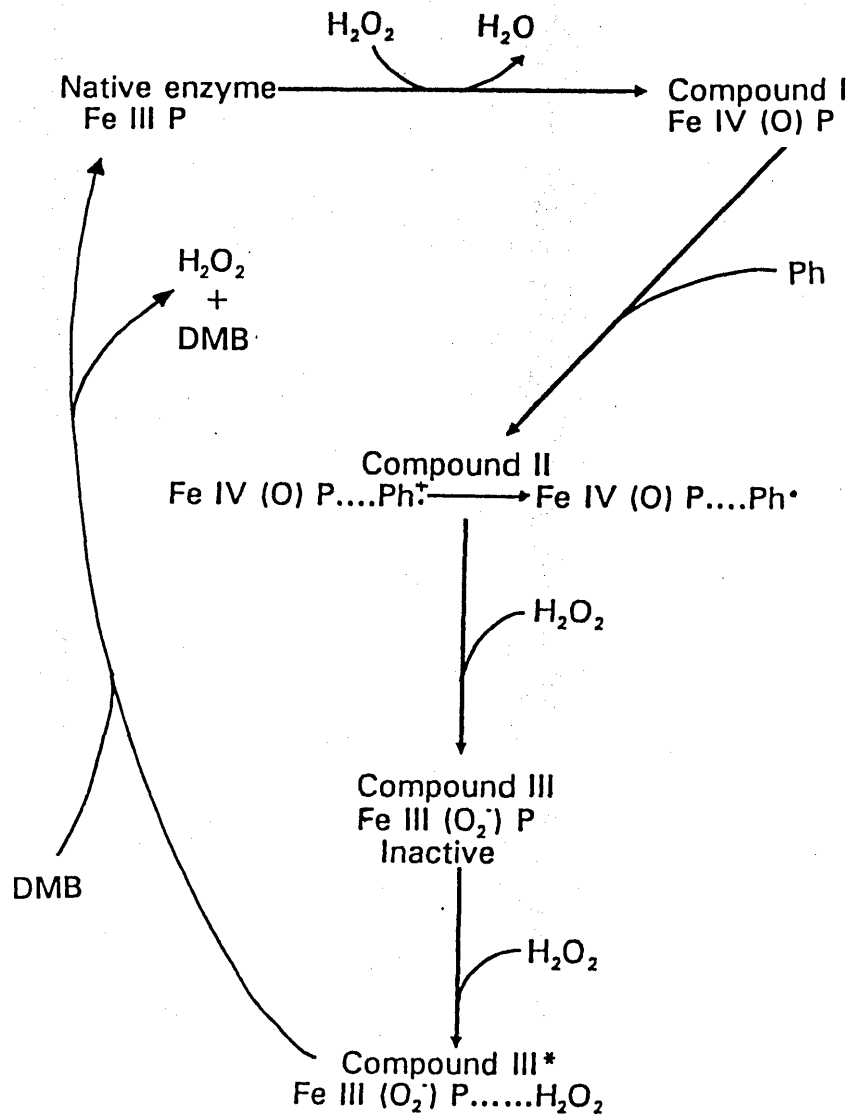

Figure 5.4.10 **Possible mechanism of enzyme cycling by DMB during the oxidation of phenolic compounds by LiP, showing conversion of compound III* to ground state.** P - porphyrin; Ph - phenolic compound; Ph• - phenoxy radical.

these two enzymes.

5.5 ISOLATION AND SPECTRAL ANALYSIS OF POLYMERS FROM THE OXIDATION OF LIGNIN BY LIGNIN PEROXIDASE AND MANGANESE DEPENDENT PEROXIDASE.

Isolation of the lowest molecular weight polymer, RT 5.56, from the oxidation of SMWL by MnP can easily be accomplished since the oxidation products from the reaction can be limited to this one product, section 5.3. However, it is clear from ealier work that preparative isolation on the G-75 column of the polymerisation products from lignin is not sensitive enough to allow the separate isolation of the two higher molecular weight polymers, RT 4.2, RT 4.9, from LiP oxidation of the lignin, since there is always a mixture of the two, section 5.2, as well as some smaller polymer always present. However, by producing the maximum quantity of higher molecular weight polymers, 11.1 Units LiP and DMB, and only using the earliest fractions obtained from the G-75 column it should be possible to isolate a mixture of higher molecular weight polymers, the purity of which can be checked by chromatography on SEC-HPLC.

To achieve these ends two separate assays were initiated. The first was a repeat of assay 5, Table 5.4.1, which was allowed to run for 1 hr and then stopped with the addition of 40 ul of 4M NaOH to solubilise the lignin. The whole assay was then placed on a sephadex G-75 column, Chapter 2 section 2.17. The second was a repeat of assay 6, Table 5.3.4, which was treated as for the first assay above. Only the initial fractions of the first peaks to elute from the column were collected and their purity checked using SEC-HPLC on the TSK G3000PW column,

Chapter 2 section 2.17. As can be seen from Figure 5.5.1 the purity of the smaller polymer, RT 5.23, is fairly high judging by the tightness of the peak. However, the polymer obtained from the LiP oxidation, second assay, shows a broad peak indicating a mixture of polymers were obtained. The retention time obtained for the peak, RT 4.25 min, would indicate that this consists mainly of the higher molecular weight polymer.

Since the purities of the polymers had been established further spectral analyses of these was undertaken. The concentration of the polymers were calculated using the extinction coefficient obtained for acetylated lignin, section 5.2, Figure 5.2.2, since it was clear that chemical modification of the lignin may reduce its absorbance at 280 nm. With this established solutions of equal concentrations, 0.027 mg/ml, were prepared in 0.1M sodium borate pH 12.0, of each polymer and of acteylated and non acetylated lignin and their spectra drawn on a Perkin Elmer 555 spectrophotometer. When these spectra are compared, Figure 5.5.2, it can be seen that the acetylated lignin does not, as expected, have any major differences with that of the unacetylated lignin. However, when the polymers are compared, Figures 5.5.3 and 5.5.4 it can clearly be seen that the spectral properties of these polymers are very different to that of the original lignin. Most striking is the increased absorbance at 320 nm, which disappears on reduction with sodium borohydride, and has been associated with the presence of carbonyl groups (Pew, 1963). This can be seen in both polymers, indicating an increased level of this bond type in both polymers, but more evident in the polymer produced by LiP, Figure 5.5.3. It has been shown that selective introduction of an α carbonyl

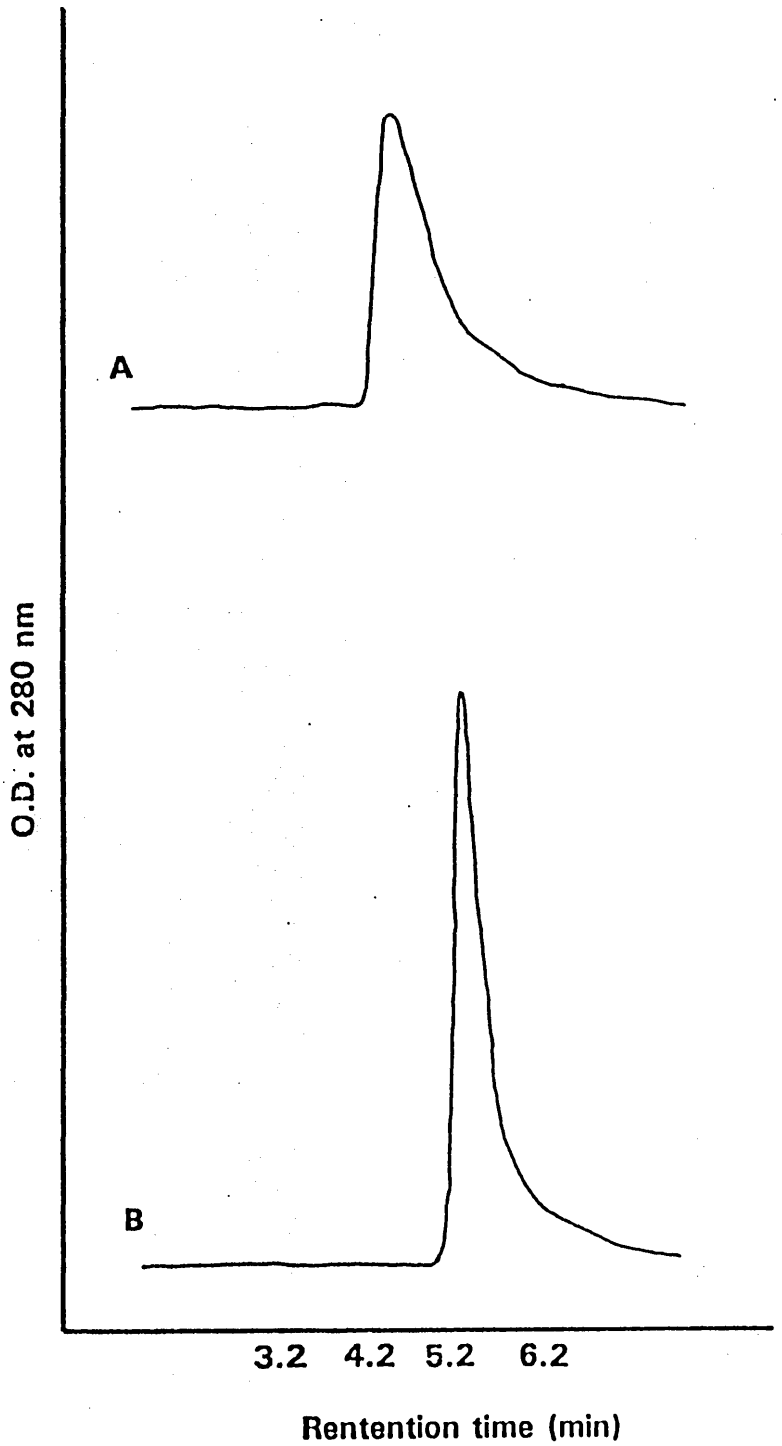

Figure 5.5.1 Elution profiles of the polymers from the oxidation of SMWL by LiP and MnP. A: Large polymer (RT 4.2 min) from the oxidation of SMWL by LiP (section 5.5); B: Small polymer (RT 5.2 min) from the oxidation of SMWL by MnP (section 5.5). Both polymers were analysed on a TSK G3000PW column (Chapter 2, section 2.15.1) except that a 50 ul injection loop was used. Exclusion limit 3.2 min (Blue dextran).

groups enhances the depolymerisation of spruce lignin by cultures of *P.chrysosporium* (Fenn and Kirk, 1984)0. It is possible therefore that the increased carbonyl content of the polymers seen here may *in situ* be a precursory event in the degradation of lignin.

Analysis of the free phenol contents of these polymers showed that they had dropped. This was achieved using the ionisation difference spectra, Chapter 2 sections 2.13 and 2.14, with each polymer having a reduced and non reduced spectra drawn, Figures 5.5.5 and 5.5.6. Percentage free hydroxyl contents calculated from these spectra, Table 5.5.2, show that when compared to those obtained from spruce milled wood lignin, section 5.2, they are as expected much lower, both polymers. Therefore, it can be said that from analysis of these polymers that the polymerisation of lignin by both MnP and LiP does occur via the oxidation of the free hydroxyl groups in the lignin, as suggested earlier. Although this is not suprising for MnP this is the first time it has been shown to be so for LiP.

Table 5.5.2 Percentage free phenol groups for Spruce milled wood lignin, the large polymer (RT 4.2 min) and the smaller polymer (RT 5.2 min).

Lignin Type	% Free phenol gps.*
Spruce milled wood lignin	1.29
Large polymer RT 4.2 min	0.53
Smaller polymer RT 5.2 min	0.38

* Calculated, Chapter 2 section 2.15, from the absorptivities at 300 nm from the reduced ionisation difference spectra

Figure 5.5.2 Straight spectra of non acetylated and acetylated spruce milled wood lignin.
(——) non acetylated, (----) acetylated, 0.027 mg/ml (0.1 M sodium borate, pH 12.0) lignin used for each spectrum.

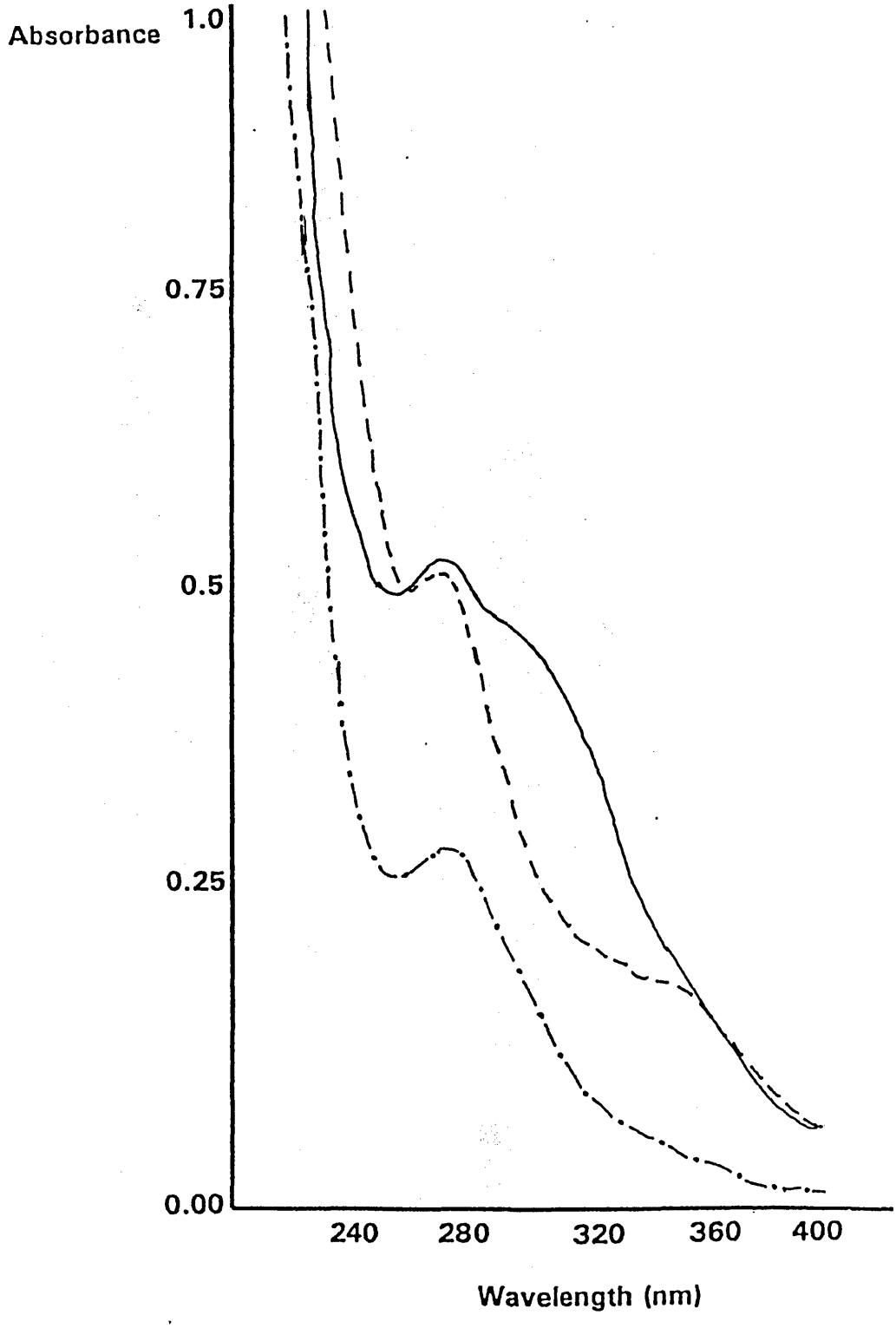

Figure 5.5.3 **Straight spectra of large polymer (RT 4.2 min), comparison with that of SMWL.**
(——) large polymer, (-·-·-) reduced large polymer, (----) SMWL, 0.027 mg/ml (0.1 m sodium borate, pH 12.0) lignin used for each spectrum.

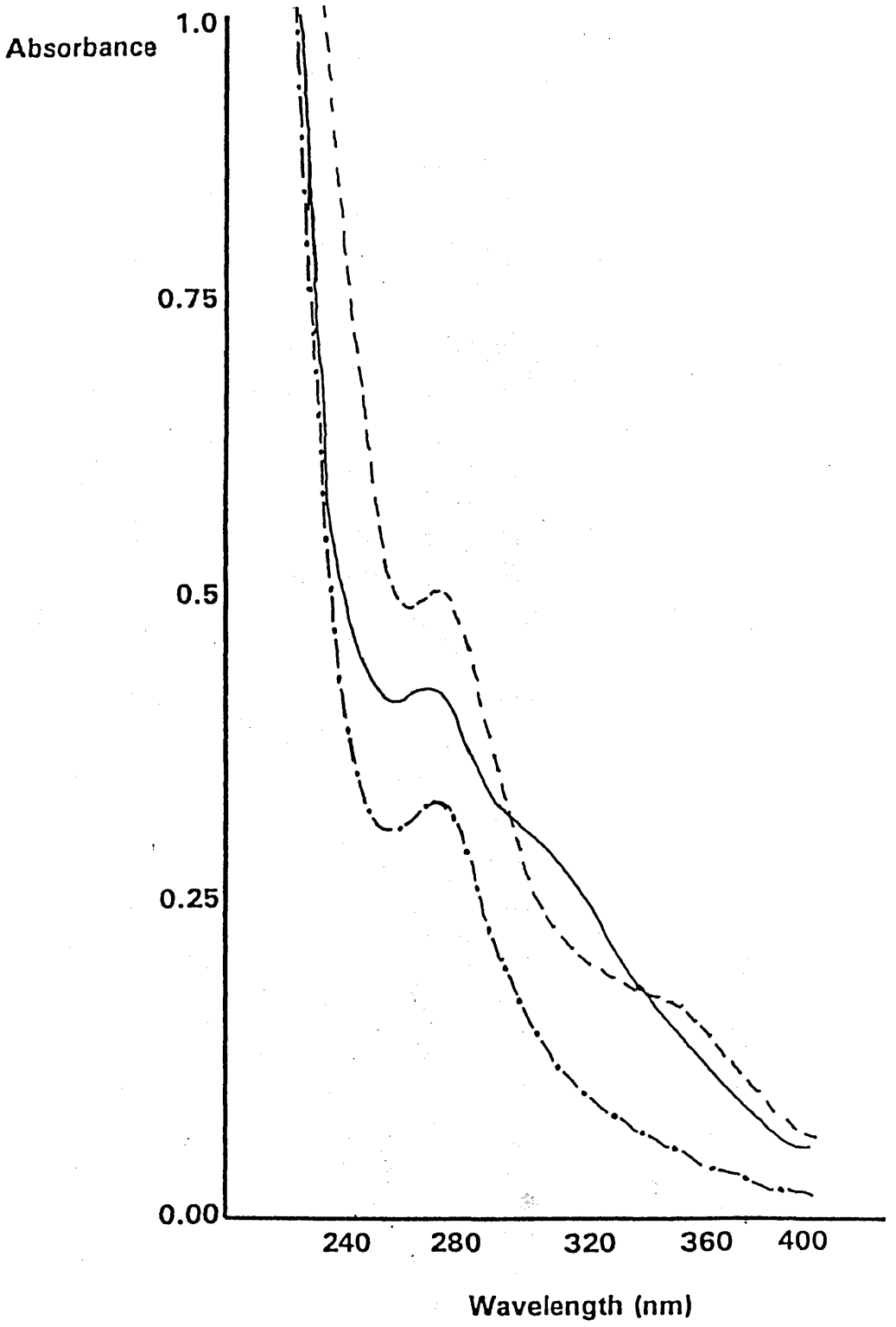

Figure 5.5.4 **Straight spectra of small polymer (RT 5.2 min), comparison with that of SMWL.**
(——) small polymer, (-•-•-) reduced small polymer, (----) SMWL, 0.027 mg/ml (0.1 m sodium borate, pH 12.0) lignin used for each spectrum.

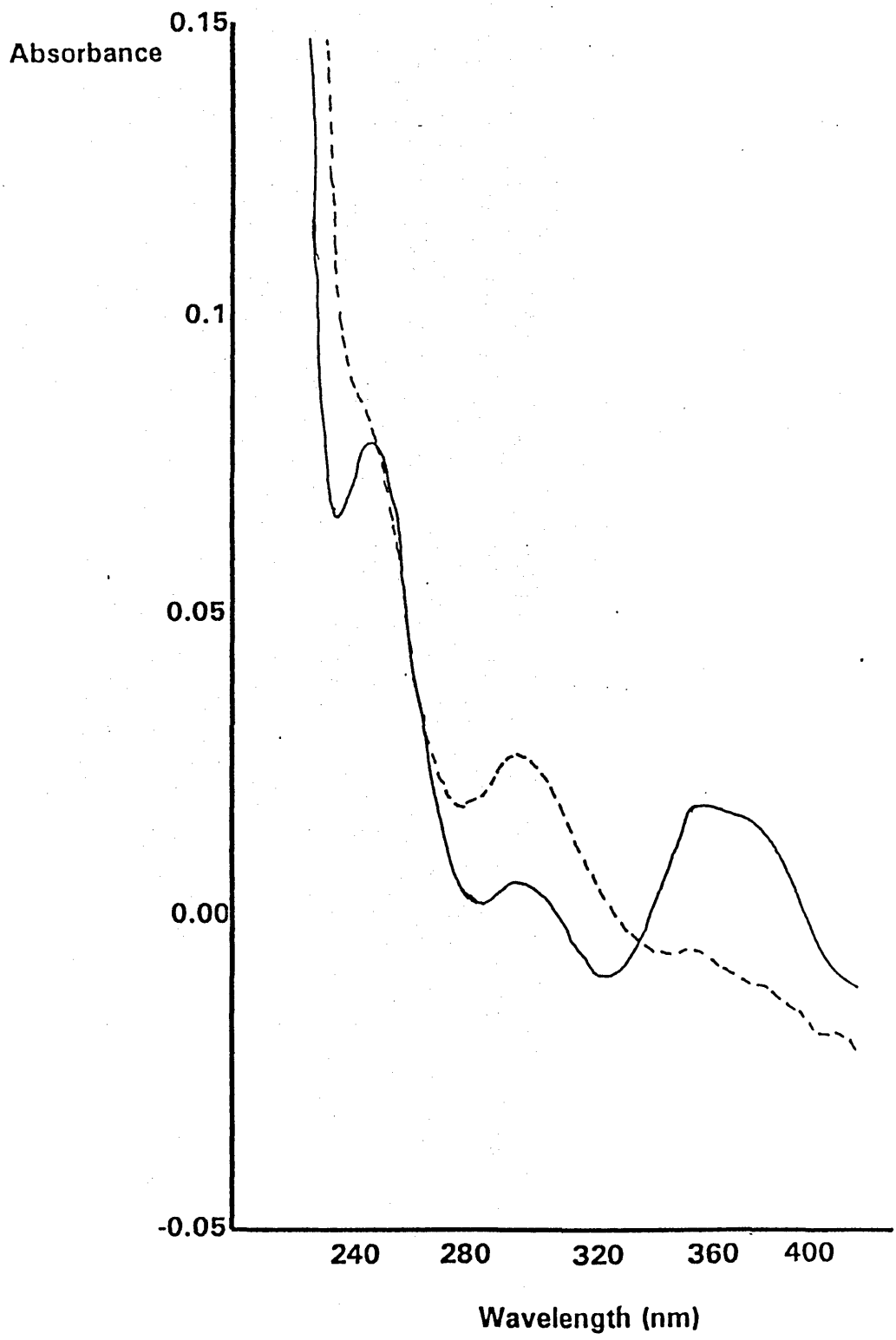

Figure 5.5.5 Ionisation difference spectra for large polymer (RT 4.2 min). A final concentration of 0.027 g l^{-1} was used for each. (——) non reduced spectrum, (----) reduced spectrum.

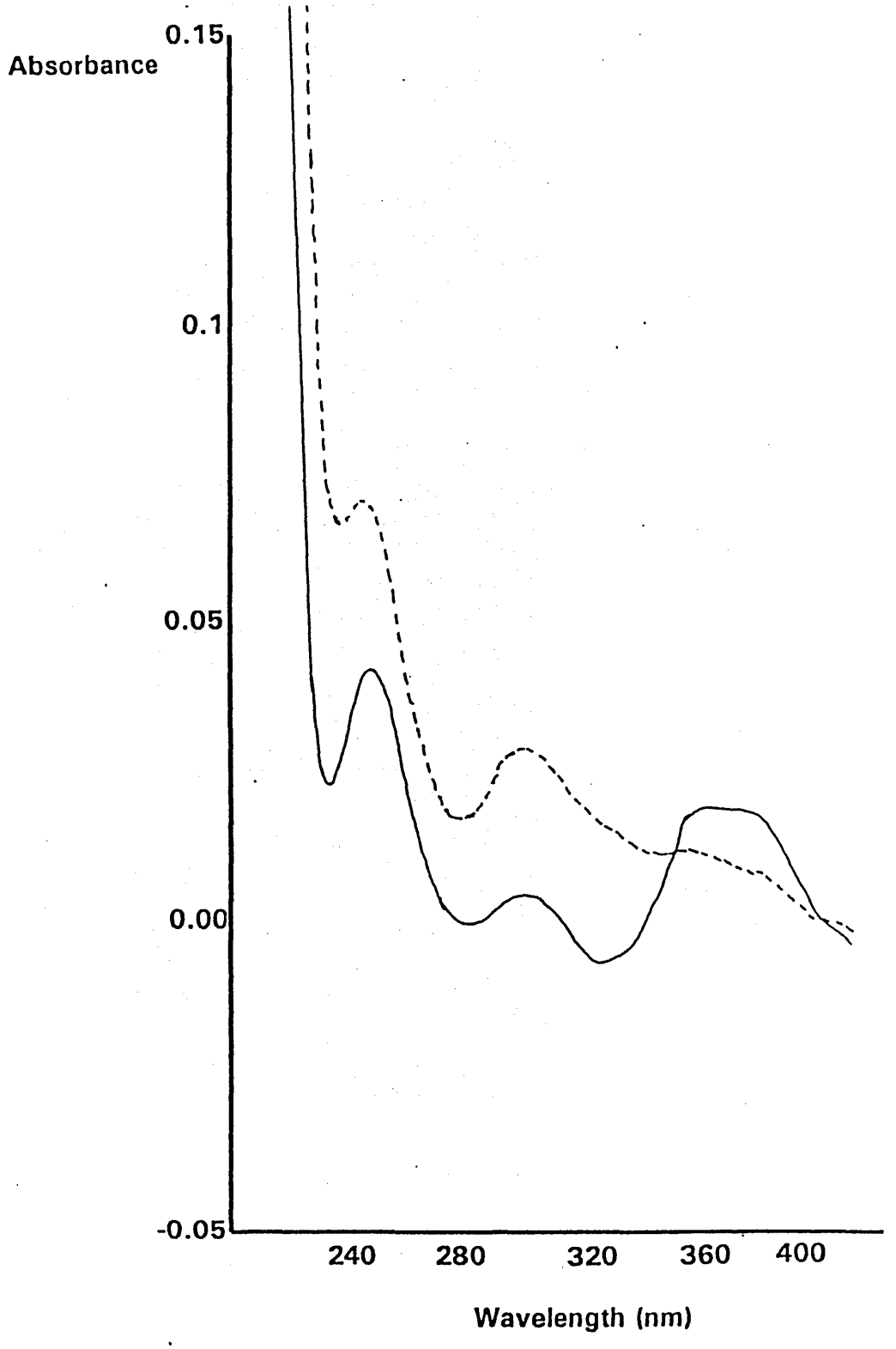

Figure 5.5.6 Ionisation difference spectra for small polymer (RT 5.2 min). A final concentration of 0.027 g l^{-1} was used for each. (——) non reduced spectrum, (----) reduced spectrum.

5.6 EFFECT OF THE ADDITION OF LIGNIN PEROXIDASE AND MANGANESE DEPENDENT PEROXIDASE ON $^{14}CO_2$ RELEASE FROM ^{14}C LABELLED DHP BY LIGNOLYTIC CULTURES OF P.CHRYSOSPORIUM.

In the previous sections it was shown that the oxidation of spruce milled wood lignin in aqueous solution by both MnP and LiP both resulted in further polymerisation of the lignin. However, previous data has shown that these enzymes are important in lignin degradation (Haemmerli et al, 1986; Huynh et al, 1986; Leisola et al, 1988). In order to confirm these observations and to investigate whether the presence of both LiP and MnP in the same reaction would have an additional effect, a set of ^{14}C labelled experiments were initiated. The method used was essentially that of Leisola et al (Leisola et al, 1988) cultures of *P.chrysosporium* were grown and treated as described except that only 2 times concentrated pellets were used. All flasks had 330 bequerels of ^{14}C labelled DHP, 203 Bq mg^{-1}, (from ring labelled coniferyl alcohol, gift from E.Odier.)along with other additions made, see Figure 5.6.1. The flasks were shaken at 30 rpm with air flowing through the flasks at 0.5 ml/min. The exhaust gasses from the flasks being bubbledthrough 10 ml trap/scintillant which was changed every hour. The trap/scintillant was based on that used by Hatakka and Rauva and Tien and Kirk (Hatakka and Rauva, 1983; Tien and Kirk, 1984) and consisted of per litre : 4g PPO; 0.1g POPOP; 2M Ethanolamine; 100ml Ethoxyethanol; 600ml Toluene and 300ml Methanol, (BDH, Scintran grade). As can be seen from Figure 5.6.1 the additon of MnP to these cultures had little effect on the level of $^{14}CO_2$ released from the labelled DHP, whilst the addition of LiP had a marked effect. However, when MnP and LiP were included

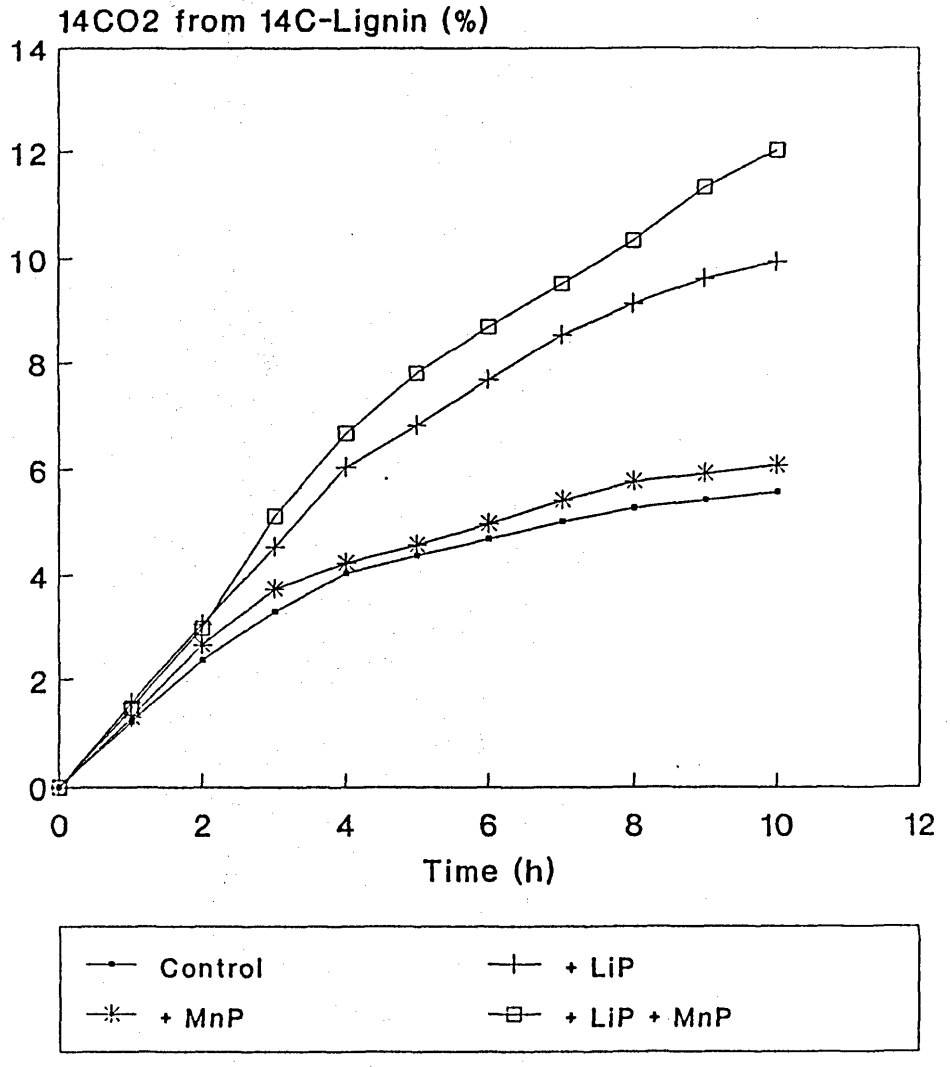

Figure 5.6.1 The effect of LiP, veratryl alcohol and MnP on lignin degradation by *P.chrysosporium*. To active, nitrogen limited, carefully washed mycelial pellets, 250 Bq ^{14}C DHP, 2.5 U of glucose oxidase were added, 75 umol veratryl alcohol. Enzyme additions : LiP (4U); MnP (5U); LiP + MnP (4U + 5U respectively). 0.5 mM $MnSO_4$ was also added to each flask.

in the same flask the increase in $^{14}CO_2$ was even greater than that observed for the cultures in which LiP was added. This would indicate that MnP appears to work synergystically with LiP, indicating an important role for these enzymes in the degradation of lignin.

CHAPTER 6

6.1 GENERAL DISCUSSION

The relationship between lignin, cellulose and hemicellulose in the lignocellulose polymer is an important one, the level and organisation of the lignin being the most important factor in determining the level of digestibility of the material, see Chapter 1. Physical treatments that disrupt the organisational orientation of the polymer afford access to rumen digestive factors to the cellulose core of the lignocellulose polymer, leading to a net increase in the digestibility of the material, Chapter 1. Chemical treatments also cause similar organisational disruption but in addition are thought to break linkages between the hemicellulose and the lignin polymer. The use of selective lignin degrading fungi has also been used, however, some loss of digestible carbohydrate to the fungus also occurs. Furthermore, despite the apparent effectiveness of these methods, in achieving the increased digestibilities in lignocellulosic by products, none appear to be practical on a large scale.

With the discovery and isolation of lignin degrading enzymes from the white-rot basidiomycete *Phanaerochete chrysosporium* the potential for the selective degradation of lignin by these enzymes leading to increases in the digestibility of lignocellulosic wastes became apparent. It would seem, however, that from the results presented in this thesis that the direct addition of lignin peroxidase to barley straw has no observable beneficial effect on the digestibility of the material, Chapter 3. This lack of effect is probably due to the inactivation of the enzyme by substances in the straw, possibly phenolic, Chapter 3. Further analysis of enzyme systems of fungi actively degrading lignin in

barley straw was undertaken, Chapter 4. No lignin peroxidase activity was detected, although, this may have been due to inhibition of the enzyme by phenolic compounds present in the extract. However, another enzyme, MnP, also associated with lignin degradation, was detected in extracts from these cultures, the import of which will be discussed later.

LiP is capable of cleaving C_{α}- C_{β} bonds (Kirk et al, 1986; Renganathan et al, 1985) and aromatic rings (Umezawa and Higuchi, 1986) in a variety of non-phenolic dimeric lignin model compounds by catalysing the formation of corresponding cation radical intermediates (Hammel et al, 1985; Harvey et al,1986). In the lignin molecule this would lead to depolymerisation (Tien and Kirk, 1983; Kern et al, 1989). Indeed the addition of exogenous LiP to lignolytic cultures of *P.chrysosporium* does result in increased $^{14}CO_2$ release, from ^{14}C labelled DHP (Haemmerli et al, 1986b; Huynh et al, 1986; Leisola et al, 1988; Chapter 5, section 5.6). However, the net reaction of LiP *in vitro* is the polymerisation of lignin (Haemmerli et al, 1986a; Chapter 5). From the results in Chapter 5 it would seem that this polymerisation reaction is as a result of phenoxy radical coupling as a consequence of the oxidation of free hydroxyl groups generated on or already present in the lignin. If this coupling could be prevented or reduced then it maybe that the net reaction would be one of depolymerisation. Work with horseradish peroxidase, Hrp, has shown that it is capable of causing the depolymerisation of milled wood lignin in organic solvents but not in water (Dordick et al, 1986). Here the enzyme was modified by reaction with palmatyl chloride so that it would be soluble in organic solvents. On incubation of the enzyme with milled wood lignin depolymerisation was

observed in the organic medium but not in the aqueous medium. It was suggested by these authors that repolymerisation of the free-radical lignin fragments was greatly diminished in organic solvents, compared to aqueous solvents, because the rate constants of solvent quenching of similar free radicals in organic solvents was more than twice as great as that in water (Koras and O'Dricoll, 1975). It is possible that *in vivo* LiP is closely associated with the hyphal wall and surrounded by mycelial slime. This environment would also have a low water content and hence possibly have similar radical quenching properties as that of the organic solvents above. Indeed tight mycelial binding of lignin to fungal cells has been reported during the degradation of lignin by *P.chrysosporium* (Janshekar et al, 1982; Chua et al, 1983). It seemed that the lignin was first bound then degraded. The fungal slime layer may also present a physical barrier to the repolymerisation of the lignin effectively immobilising the free radical fragments. From a preliminary experiment using spruce milled wood lignin, immobilised in agarose, it would appear that LiP and MnP had an interesting effect on the lignin, Plates 6.1.1 - 6.1.3. Most striking is the effect that LiP had on the immobilised lignin, giving it a pink colouration, Plate 6.1.1. What the pink colouration signifies is not known, however, it is clearly different from the darker brown seen in the MnP reaction, Plate 6.1.2, which has been associated with the polymerisation of spruce milled wood lignin (Haemmerli et al, 1986a). Further work on the use of LiP with immobilised lignin or in the presence of high concentrations of carbohydrate is needed.

Other theories as to how repolymerisation from phenoxy

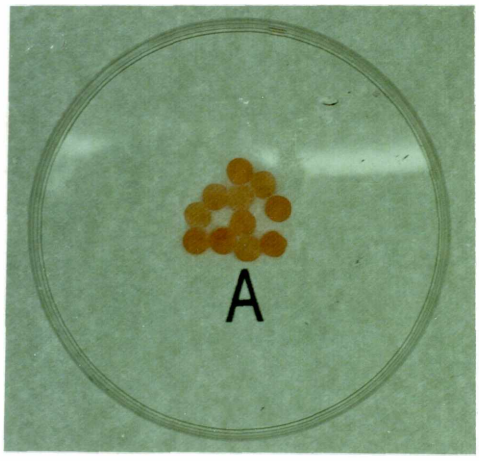

Plate 6.1.1 Immobilised lignin with LiP.

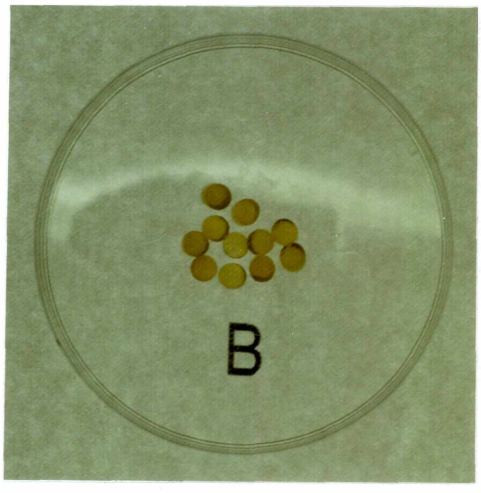

Plate 6.1.2 Immobilised lignin with MnP.

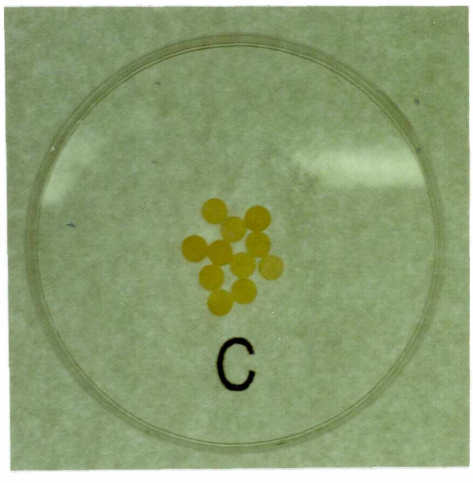

Plate 6.1.3 Immobilised lignin control.

Figure 6.1.1 Effect of LiP and MnP on immobilised spruce milled wood lignin. Each reaction contained : 2mg/ml lignin (0.1 M sodium tartrate buffer, pH 5.0) in 1 % agarose (Sigma Type I); 800 ul sodium tartrate buffer (0.1 M, pH 5.0); 50 ul glucose (0.5 M); 5 ul GOD (1mg/ml). Reaction A also contained : 11.3 U LiP; 20 ul veratryl alcohol (75 mM). Reaction B also contained : 1.6 U MnP; 10 ulmanganese(II)sulphate (100 mM). All reactions were carried out in open stirred vessels for 1 hour.

radical coupling may be prevented are available. It has been suggested that extracellular enzymes other than these lignin peroxidase are normally present *in vivo* and are responsible for preventing this repolymerisation occurring. Recent work with cellobiose: quinone oxidoreductase, CBQase, has shown that this enzyme is capable of reducing the extent of polymerisation of kraft lignin by LiP (Ander et al, 1990). This enzyme reduces quinones and possibly phenoxy radicals with simultaneous oxidation of cellobiose to cellobionolactone (Westermark and Erkisson, 1974). This reduction of phenoxy radicals would reduce the extent of lignin polymerisation and may lead to lignin depolymerisation by these enzymes. This work is however in contrast with earlier work that has shown that CBQase did not prevent the polymerisation of synthetic lignin by LiP (Odier, 1988).

Another theory suggests that lignin peroxidase may not be freely secreted *in vivo* as it is when the fungus is grown *in vitro* (Harvey et al, 1986). Instead it is suggested that the enzyme is bound to the hyphal wall and surrounded by mycelial mucilage. This is thought to prevent contact of the enzyme with phenolic compounds, the enzyme instead oxidising the lignin through the freely diffusible veratryl alcohol radical cation. Subsequent isolation of the manganese dependant peroxidase, another enzyme associated with lignin degradation gave light to further possibilities since this enzyme is capable of oxidising phenolic compounds without inactivation. It is known that when *P.chrysosporium* is grown *in vitro* MnP is produced before the LiP (Leisola et al, 1987). If this situation were to occur *in vivo* it is possible that the role of MnP is to oxidise phenolic compounds present that potentially could cause the

in activation of LiP. Indeed in Chapter 5, section 5. it would seem that there is some synergistic action between LiP and MnP when added to lignolytic cultures of *P.chrysosporium*, demonstrated with the increased levels of $^{14}CO_2$ released by these cultures.

It would seem then that although the importance of these enzymes in lignin degradation is clear, further work on their interactions with one another is necessary. Prevention of the inactivation of LiP by phenolic compounds is clearly important, possibly being achieved by the preincubation of the material with MnP followed by the addition of LiP and a mediator such as DMB and a phenoxy radical reducing enzyme such as CBQase.

However, in the broader aspects of the use of lignin degrading enzyme with forage crops, it must be remembered that the total degradation of the lignin is not necessarily required to achieve increased digestibilities, and that disorganisation of the lignocellulosic matrix may be sufficient.

This may be achieved by the addition of MnP alone to the substrate, the phenol oxidising capacity of this enzyme possibly being sufficient to cause the appropriate amount of disorganisation within the lignocellulosic matrix. However, it is likely that the action of LiP on the lignocellulosic matrix, in the correct conditions, would also prove to be important. A recent study has shown that a crude enzyme preparation from *in vitro* cultures of *P.chrysosporium* is capable of the solubilisation of lignin in straws (Mason et al, 1990). These workers suggest that this lignin solubilisation activity was unlikely to be due to lignin peroxidases, since the experiments were performed in the absence of exogenous

hydrogen peroxide. However, the enzyme preparation used was crude and since the experiments were performed in air the generation of hydrogen peroxide *in situ* cannot be ruled out, and therefore, neither can the action of lignin peroxidases.

It is clear then that much work on the use of lignin peroxidases and enzyme mixtures is needed, if the enzymatic treatment of lignocellubsic wastes for use as feeds is to be successful.

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APPENDIX I

NEUTRAL DETERGENT FIBRE

REAGENTS :	gl^{-1}(water)
Disodium ethylene diaminetetra acetic acid (EDTA)	18.61
Sodium tetraborate	6.81
Sodium lauryl sulphate	30.0
Anhydrous Sodium hydrogen sulphate	4.56
2-Ethoxyethanol	10.0
Sodium sulphite	20.0

ACID DETERGENT FIBRE

REAGENTS :	gl^{-1}(water)
Conc. Sulphuric acid	28 ml
Cetyl trimethyl ammonium bromide (CTAB)	20.0

DEMINERALISING SOLUTION

Oxalic acid	50g
Ethanol	700 ml
12 N HCl	50 ml
Water	250 ml