Review Article

# Degradation and Synthesis of Lignin and Its Related Compounds by Fungal Ligninolytic Enzymes*[1]

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## Contents



## Preface

Woody biomass is the most abundant organic resource on the earth and 15–36% of this lignocellulosic material in gymnosperms and angiosperms is lignin[1]. Lignin is a phenyl-

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propanoid structural polymer of vascular plants which gives the plants rigidity and binds the fiber cell walls together[2]. Chemical and spectrometric studies of lignin have been performed, and the structure of lignin was determined in the late 1960s[3–6]. Lignin contains various stable carbon-to-carbon and ether linkages between monomeric phenylpropane units. Therefore, efficient utilization of cellulose in the production of ethanol, sugars and protein is hindered by the presence of lignin[7,8]. In addition, its structural complexity has also hindered the production of useful products from lignin.

Little attention has been focused on the use of lignin as a source of other chemicals or the conversion of lignin to more useful products since these can be more easily obtained from petroleum or crude oil[9]. However, the recent energy crisis and the scarcity of crude oil has prompted research activities to develop alternative and renewable feedstocks for polymers or chemicals. Recently, a number of physical and chemical approaches have been suggested for this purpose. However, such processes generally require high temperatures and pressures and are, therefore, costly.

White-rot fungi, most of which belong to the basidiomycetes, are the best lignin degraders among all known microorganisms and secrete extracellular ligninolytic enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase[1,10–12]. These enzymes have been found in culture filtrates of various white-rot basidiomycetes including *Phanerochaete chrysosporium*, Burds.[13], *Coriolus versicolor* (L.: Fr.) Quél.[14], *Phlebia radiata* Fr.[15], *Bjerkandera adusta* (Willd.: Fr.) Karst.[16], *Pleurotus ostreatus* (Jacq.: Fr.) Kummer[17] and *Lentinus edodes* (Berk.) Sing.[18].

LiP catalyzes $H_2O_2$-dependent one-electron oxidation of a variety of non-phenolics to generate aryl cation radicals[13,19]. These radicals undergo subsequent nonenzymatic reactions to yield a variety of final products. MnP oxidizes phenolic substrates to phenoxy radicals in a similar manner to LiP in the presence of $H_2O_2$[20]. Both LiP and MnP are present as a series of isozymes, and contain one mole of iron protoporphyrin IX per mole of enzyme. These are glycoproteins of molecular mass 41,000 and 46,000, respectively[21–26]. Laccase is a copper-containing phenol oxidase, which also catalyzes the oxidation of phenolics and aromatic amines *via* the phenoxy radical formed by one-electron oxidation in a manner similar to peroxidases[27–31].

Biological degradation and transformation of lignin by these enzymes have potential applications to industrial processes as described below:

1. Delignification of wood chips to reduce refining energy in mechanical pulping and consumption of chemicals in kraft cooking[7,8].
2. Delignification of wood, straw and bagasse to increase digestibility by ruminants[32–35].
3. Delignification, bleaching and modification of pulp fibers[36–39].
4. Modifications of different isolated lignins to produce more useful lignins or

chemicals[40,41].

Interestingly, these enzymes have also been found to catalyze the oxidation of halogenated phenolic compounds, polycyclic aromatic hydrocarbons and other compounds which are resistant to microbial attack[11,42]. Therefore, the ligninolytic enzymes may also be useful for the following applications.

1. Treatment of waste bleach liquors to reduce color, toxicity and mutagenicity[43–46].
2. Treatment of soils or waste waters to remove environmental pollutants such as polychlorinated biphenyl (PCB), *p,p'*-dichlorodiphenyl-trichloroethane (DDT) and dioxins[47–51].

Furthermore, the ligninolytic enzymes catalyze both the degradation of aromatic polymer and polymerization of aromatic compounds *via* one-electron oxidation. Therefore, these enzymes may also be useful for development of recycling systems such as transformation of waste polymers to new compounds. The purpose of this study was the development of biotransformation systems of lignin and related compounds using the ligninolytic enzymes of white-rot fungi.

## Chapter I Degradation of Lignin by Ligninolytic Enzymes in Organic Solvents

White-rot fungi are the best lignin degraders among all known microorganisms and secrete extracellular ligninolytic enzymes including LiP, MnP and laccase[1,10–12]. Although many attempts have been made to degrade lignin *in vitro*, ligninolytic enzymes have not been clearly demonstrated to effectively depolymerize lignin in aqueous solution[52]. One of the major difficulties in the biodegradation of lignin with ligninolytic enzymes is in the insolubility of lignin in aqueous solution. Degradation and transformation of lignin and its related compounds by ligninolytic enzymes in organic solvents which can solubilize aromatic compounds are discussed in this chapter.

### I-1 Reaction of lignin peroxidase of *Phanerochaete chrysosporium* in organic solvents

#### I-1.1 Introduction

It has become clear that many enzymes can function in organic solvents as well as in water[53–55]. In organic solvents, proteases and lipases catalyzed reactions which are thermodynamically impossible in water such as peptide syntheses[56] and transesterification[57]. In addition, horseradish peroxidase (HRP) has been found to oxidize phenolics to give the polymerization products[58]. Furthermore, laccase, a ligninolytic enzyme, has been reported to catalyze the oxidation of lignin and substituted phenols in organic solvents[59]. Oxidation of polycyclic aromatic hydrocarbons by LiP in a system containing the lower concentration of organic solvents was also reported[60–62].

In section I-1, the reaction of LiP purified from *P. chrysosporium* in various high

concentration of organic solvents was described and the difference between the reaction in water and in organic solvents was discussed[63].

### I-1.2 Materials and methods

***Production and purification of LiP***

*P. chrysosporium* strain ATCC 34541 was grown statically at 37°C in 200-ml Erlenmeyer flasks with 20 ml of culture medium containing 1% glucose, 0.62% polypeptone, 0.02% yeast extract, 20 mM *o*-phthalate buffer (pH 4.5), 0.005% Tween 80, 100 mM veratryl alcohol, Kirk's salts[64] supplemented with $FeSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_2O$ to a final concentration of 1 mM and 40 polyurethane forms. LiP activity was measured spectrophotometrically as described by Tien and Kirk[22]. One unit of LiP was defined as 1 nmol veratryl alcohol oxidation to veratraldehyde per min. Protein concentration was determined by the method of Bradford[65] using bovine serum albumin as a standard. LiP was purified by the method of Kirk *et al.*[66]. The purified LiP had a pI of 4.1 and a molecular weight of 40,000.

***LiP activity assay in organic solvents***

The LiP activities in water-miscible organic solutions were determined spectrophotometrically using a reaction mixture containing 14 mM 3, 3′-dimethoxybenzidine, 0.25 mM $H_2O_2$, 70% of organic solvent and the enzyme solution in a final volume of 1 ml. Activities in water were also determined using 3, 3′-dimethoxybenzidine dihydrochloride as substrate. In the case of the reaction in water-immiscible organic solvents, LiP in 20 mM succinate buffer (pH 4.5) was first dried in 10 ml glass test tube under air flow at room temperature. Immediately after that, 0.7 ml of 20 mM substrates dissolved in different organic solvents were added and the reaction was started by addition of 0.3 ml of the organic solvents saturated with $H_2O_2$. The reactions were monitored by absorbance of the reaction products at 444 nm. One unit of LiP activity in organic solvents was defined as the amount of enzyme which increases the absorbance of 1.0 per 1 minute. Protein was determined by the method of Bradford[65].

***Analysis by electron spin resonance (ESR)***

ESR spectra were recorded with a JEOL JES-RE2X ESR Spectrometer at room temperature, using an aqueous flat cell. Further details are given in the figure legend.

### I-1.3 Results and discussion

As summarized in Table 1, LiP exhibited activity of 3, 3′-dimethoxybenzidine oxidation in 70% aqueous solutions of water-miscible organic solvents including ethylene glycol, diethylene glycol, methylcellosolve and acetone. The activity of LiP in ethylene glycol and methylcellosolve media were 7.6 and 1.6 times higher than that observed in water. The activity in acetone and diethylene glycol media were found to be slightly lower than that in water. However, reactions in *N*, *N*-dimethylformamide (DMF) and water-immiscible organic solvents resulted in loss of activity. These findings show that some glycols are

Table 1. Abctivities of LiP in organic solvents.

| Solvents | Relative activity (%) |
|---|---|
| Water | 100 |
| 70% Acetone | 90 |
| 70% Methanol | 2 |
| 70% 2-Propanol | 10 |
| 70% Dioxane | 13 |
| 70% DMF | 0 |
| 70% Pyridine | 3 |
| 70% Ethylene glycol | 761 |
| 70% Methylcellosolve | 165 |
| 70% 1, 2-Dimethoxyethane | 35 |
| 70% Diethylene glycol | 93 |
| 70% DEGME | 68 |
| 70% DEGDE | 38 |
| Chroroform | 0 |
| Ethylacetate | 0 |
| Benzene | 0 |
| Toluene | 0 |

LiP activities were assayed using 3, 3′-dimethoxybenzidine and 3, 3′-dimethoxybenzidine dihydrochloride as substrates in organic solvents and in aqueous solution, respectively. One unit of enzyme produces an arsorbance of 1.0 for 1 minute. Specific activity of LiP was 124 U/mg in water. DMF: *N*, *N*-Dimethyl-formamide; DEGME: Diethylene glycol monomethyl ether; DEGDE: Diethylene glycol diethyl ether.

favorable solvents for the oxidation of 3, 3′-dimethoxybenzidine by LiP. Activity of oxidation of veratryl alcohol, most widely used substrate of LiP, was found to be negligibly low in ethylene glycol medium.

Effect of organic solvent concentration on oxidation of 3, 3′-dimethoxybenzidine by LiP was also investigated to determine the optimal reaction condition. The specific activity of LiP in the reaction mixture containing 0, 50, 60, 70, 80 and 90% of ethylene glycol was 124, 238, 420, 944, 418 and 38 U/mg, respectively. Thus, an enhancement in catalytic activity was observed in the concentrations of 50–80% ethylene glycol. These results show that 3, 3′-dimethoxybenzidine is oxidized efficiently in the reaction mixture containing 70% ethylene glycol.

Significant differences between water and 70% ethylene glycol media were observed in the oxidation of 3, 3′-dimethoxybenzidine. In the oxidation of 3, 3′-dimethoxybenzidine by LiP in water, two peaks appeared initially at 372 and 700 nm. After reaching the maximum, their absorption decreased progressively by being replaced by a peak at 444 nm (Fig. 1A). On the contrary, in the ethylene glycol medium, the absorption maximum

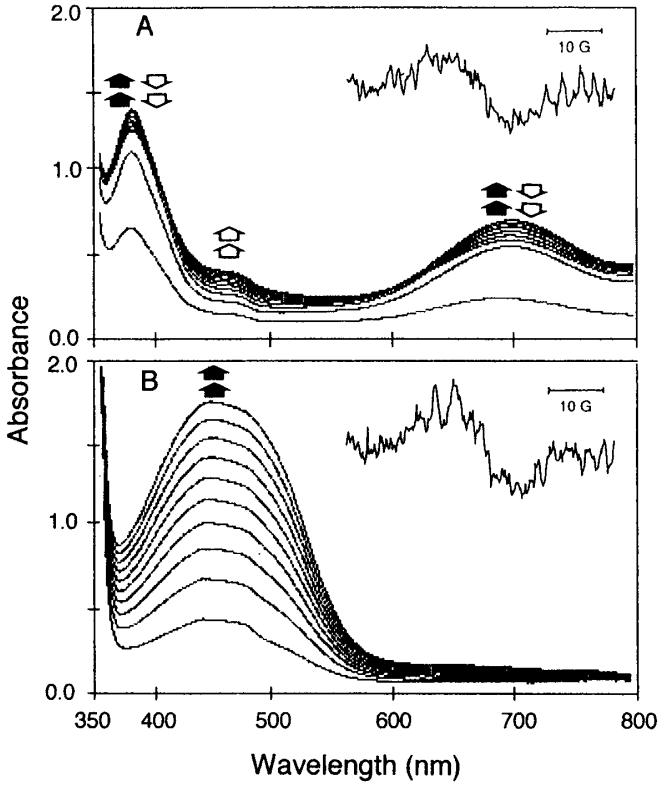


Fig. 1. Absorption and ESR spectra during the oxidation of 3, 3′-dimethoxybenzidine by LiP. Absorption spectra: (A) reaction mixture containing 14 mM 3, 3′-dimethoxybenzidine dihydrochloride, 5 mM $H_2O_2$, and 0.60 $\mu$g/ml LiP in 1 ml of water. (B) reaction mixture containing 14 mM 3, 3′-dimethoxybenzidine, 5 mM $H_2O_2$, and 0.15 $\mu$g/ml LiP in 0.1 ml of 70% aqueous ethylene glycol medium. Reactions were initiated by addition of $H_2O_2$ at room temperature, and spectra were recorded every 30 seconds. ESR spectra (inset): (A) reaction mixture containing 14 mM 3, 3′-dimethoxybenzidine dihydrochloride, 5 mM $H_2O_2$, and 0.38 $\mu$g/ml LiP in 0.1ml of water. (B) reaction mixture containing 14 mM 3, 3′-dimethoxybenzidine, 5 mM $H_2O_2$, and 0.38 $\mu$g/ml LiP in 0.1 ml of 70% aqueous ethylene glycol medium. Instrumental conditions were: center field, 3390 G; scan range, 40 G; field modulation width, 0.2 G; receiver gain, 10×103; sweep time, 8 min.; time constant, 1 sec.; and microwave power, 20 mW.

appeared at 444 nm initially increased to a maximum with increasing time of the reaction (Fig. 1B). The spectral change observed in water was well coincident with that observed in the oxidation of 3, 3′-dimethoxybenzidine at pH 3.7 and 6.0[67] and 3, 5, 3′, 5′-tetramethylbenzidine at pH 5.0[68] by HRP. The product which gave an absorption maxima at 372 and 700 nm was envisaged to be an intermediate charge-transfer complex (III) of the diamine (I, the substrate) and the diimine (IV) as shown in Fig. 2. ESR analysis demonstrates the formation of free radicals both in water and ethylene glycol media as shown in Fig. 1A and Fig. 1B, respectively. This radical (II) could be formed by a one-electron oxidation of 3, 3′-dimethoxybenzidine, which exists in equilibrium with the charge-

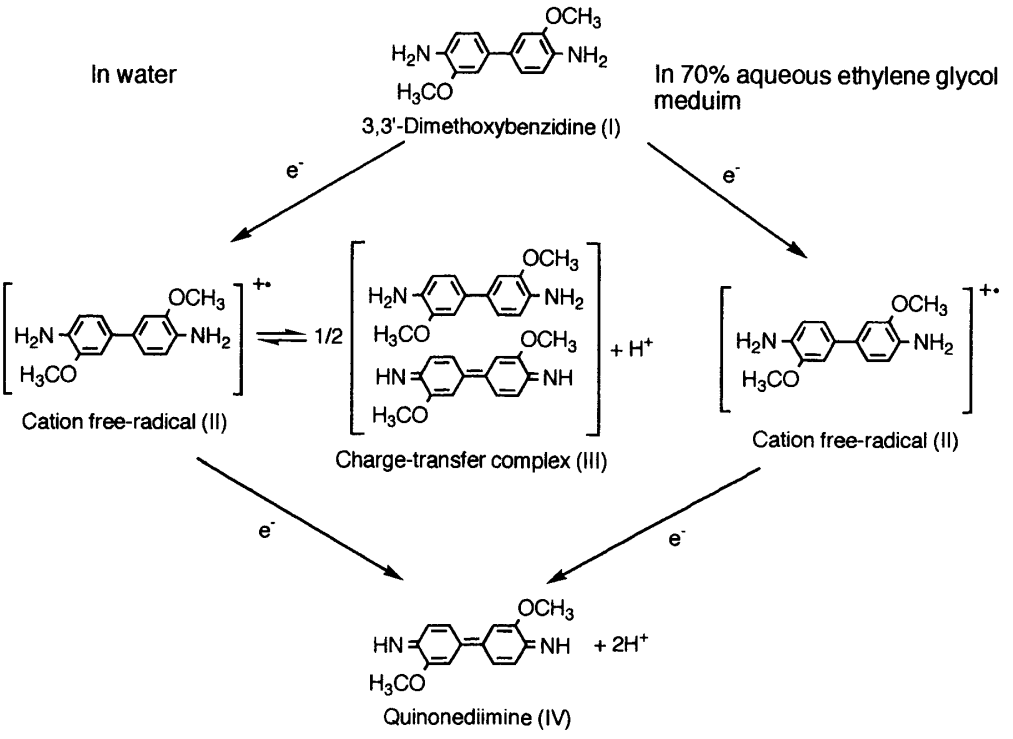


Fig. 2. Proposed oxidation mechanism of 3, 3′-dimethoxybenzidine by LiP in water and 70% aqueous ethylene glycol media.

transfer complex (III) in water. The product which gave the absorption maximum at 444 nm was proposed to be the stable final product (IV) of the reaction which was formed by two successive one-electron oxidations of the substrate. In the ethylene glycol medium, the diimine was speculated to be formed directly from the substrate by two successive one-electron oxidations without formation of the charge-transfer complex, or *via* the cation free-radical which exists in more rapid equilibrium with the charge-transfer complex than in water. The fact that ESR signals were observed in the 3, 3′-dimethoxybenzidine oxidation by LiP also indicated that the first one-electron oxidation by LiP proceeded more rapidly than the following non-enzymatic one-electron oxidation. Further investigation is needed to determine the oxidation rate of each step.

From these findings, the reaction in 70% ethylene glycol medium was found to proceed *via* the one-electron oxidation in similar to that in water. In addition, the difference of the oxidation rate in water and ethylene glycol medium is thought to be deduced from the difference between the intermediate produced by one-electron oxidation of substrate in these solvents. The difference in the oxidation intermediate is explained as follows. 3, 3′-Dimethoxybenzidine has the hydrophobicity, by which solvation of ethylene glycol molecule to 3, 3′-dimethoxybenzidine is more favorable than by of water molecule. In the ethylene glycol medium, the cation free-radical of 3, 3′-dimethoxybenzidine is separated from each

other by solvent molecules surrounding the radical molecule, while in water two 3, 3′-dimethoxybenzidine radicals interacted with each other and a charge-transfer complex is formed. The successive one-electron oxidation of 3, 3′-dimethoxybenzidine radical gives the final product quinonediimine formed in the 70% ethylene glycol medium more rapidly than in water. The mechanism of expression of higher activity in glycols than in water is now under consideration.

## I-2 Effects of organic solvents on the reaction of lignin peroxidase of *Phanerochaete chrysosporium*

### 1-2.1 Introduction

In addition to the observations indicating that many enzymes can function in organic solvents[53–55], the effects of the properties of organic solvents on enzyme function were also investigated to predict optimal solvent and substrate choices for the reactions of enzymes in organic solvents[53,54,69–71]. In previous study, attention is mainly focused on the role of enzyme-associated water in organic solvents and the effects of physical and chemical characteristics of organic solvents such as dielectric constant, miscibility, hydrophile-lipophile balance and propensity for hydrogen bond formation on the catalytic reaction of enzymes including hydrolases and oxidoreductases. The reaction of HRP was also carried out to elucidate the effect of solvent and substrate hydrophobicity on the structure and function in organic solvents[72,73]. However, the effects of organic solvents on the catalytic activity and substrate specificity of LiP are not well understood. Particularly, no systematic evaluation has yet been performed on the effects of the physical and chemical characteristics of organic solvents and substrates including propensity for hydrogen bond formation, hydrophobicity and ionization potential on the reaction of LiP in organic solvents. Elucidation of the effects of these parameters on the reaction will enable optimal choices of substrates and solvents to be made for the reaction of LiP in organic solvents.

In this session, the effects of various physicochemical parameters of organic solvents on the reaction of LiP of *P. chrysosporium* are discussed[74].

### I-2.2 Materials and methods

***Purification of LiP***

LiP was purified from cultures of *P. chrysosporium* strain ATCC 34541 as described in Section I-1.2. The purified LiP had a pI of 4.5 and a molecular weight of 40,000.

***LiP activity assay in organic solvents***

In organic solvents, the activity was measured spectrophotometrically using a reaction mixture containing 14 mM substrate, 0.25 mM $H_2O_2$, desired amount of organic solvent and the enzyme solution in a final volume of 1 ml as described in Section I-1.2. For measurement of substrate specificity in ethylene glycol medium, 0.1 M Na-tartrate buffer (pH 3.0) was added. One unit of LiP activity in an organic solvent was defined as the

amount of enzyme that increased absorbance by 1 per 1 minute.

***Hydrophobicity of substrates***

Hydrophobicity of the substrates was estimated as the partition coefficient of substrate between water and *n*-octanol[75]. *n*-Octanol solutions of substrate and water were placed into test tubes and shaken for 30 min at 28°C. After separation into two phases by centrifugation for 5 min, the absorbance of each phase was measured at 280 nm. The partition coefficient (*K*o/w) was defined by

$$Ko/w = Co/Cw$$

where *C*w and *C*o were the molar concentrations of solute in water and *n*-octanol, respectively.

***Ionization potential of substrates***

Ionization potential of each substrate was measured by the absorption maximum of the charge-transfer complex of the substrate and chloranil[76]. A saturated solution of chloranil in chloroform (2 ml) was mixed with the substrate dissolved in a few drops of chloroform. A colored complex was immediately obtained and its absorption spectrum was recorded on a Shimadzu UV-160A Spectrophotometer. Ionization potential values were calculated from the equation $IP = 1.228\ E_{CT} + 5.038$ (eV), in which the $E_{CT}$ energy corresponds to the absorption maximum of the charge-transfer band of the complex between substrate and chloranil.

***Critical micelle concentration (CMC) of organic solvents***

Critical concentration for micelle formation in organic solvents was measured by a modification of the method of Muto *et al.*[77]. Spectral changes of 7, 7′, 8, 8′-tetracyanoquinodimethane (TCNQ) due to the formation of TCNQ-surfactant micelle complex were measured using Nonidet P-40 as a surfactant. TCNQ (5 mg) was added to 10 ml of mixture containing various concentrations of Nonidet P-40 and organic solvents in L-type tubes. The mixtures were shaken for 72 hrs at 40°C. After shaking, excess TCNQ was filtered off through glass filters. The absorption spectra of the samples were measured. The absorption maxima obtained were plotted against the logarithm of Nonidet P-40 concentration, and the inflection point coincided with the CMC value.

## I-2.3 Results and discussion

***Activity of LiP in organic solvents***

LiP activity in organic solvents was investigated. The best catalytic activity was obtained in a reaction mixture containing 70% ethylene glycol [63]. Therefore, the reaction was conducted in reaction mixtures containing various organic solvents at 70%.

As summarized in Table 2, LiP oxidized 3, 3′-dimethoxybenzidine in reaction mixtures containing 70% water-miscible organic solvents including ethylene glycol, diethylene glycol, methylcellosolve and acetone. The LiP activity in ethylene glycol was 3.7-fold higher than that observed in water. In contrast, the activities in aqueous acetone, methylcellosolve

Table 2. Activity of LiP in vaorious organic solvents, $E_T$ (30) and CMC values.

| Solvents | Specific activity (U/mg) | $E_T$ (30)[a] (kcal/mol) | CMC (mM) |
|---|---|---|---|
| Water | 159 | 63.1 | 0.2 |
| 70% Acetone | 37 | 42.2 | —[c] |
| 70% Dioxane | 0 | 36.0 | —[c] |
| 70% DMF | 0 | 43.8 | n.d.[d] |
| 70% Ethylene glycol | 591 | 56.3 | 142.8 |
| 70% Methylcellosolve | 52 | 52.3 | 112.2 |
| 70% 1, 2-Dimethoxyethane | 12 | 38.2 | n.d.[d] |
| 70% THF | 0 | 37.4 | n.d.[d] |
| 70% Diethylene glycol | 118 | 53.8 | 52.8 |
| 70% DEGME | 21 | n.l.[b] | n.d.[d] |
| 70% DEGDE | 5 | 38.6 | 119.1 |
| 70% Pyridine | 3 | 40.2 | n.d.[d] |
| 70% Methanol | 0 | 55.5 | —[c] |
| 70% 2-Propanol | 7 | 48.6 | —[c] |
| Chloroform | 1 | 39.1 | |
| Ethyl acetate | 0 | 38.1 | |
| Benzene | 1 | 34.5 | |
| Toluene | 0 | 33.9 | |

Lip activity was measured as dessbibed in Materials and Methods using 3, 3′-dimethoxybenzidine dihydrochloride and 3, 3′-dimethoxybenzidine as substrates in wateb and in organic solvents, respectively. One unit of enzyme produced an absorbance of 1.0 after 1 minute. Critical micelle concentration (CMC) was measured as described in Materials and Methods. a: Data were taken from reference 79. b: Values notlisted in reference 79. c: These solvents had no obvious CMC value. d: Could not be determined. DMF: *N*, *N*,-Dimethylformamide; THF: Tetrahydrofuran; DGME: Diethylene glycol monomethyl ether, DEGDE: Diethyleneglycol dimethyl ether.

and diethylene glycol media were lower than that in water. However, methanol, tetrahydrofuran (THF), DMF, dioxane and water-immiscible organic solvents resulted in loss of the activity. These findings indicate that some glycols and acetone can be used as solvents for the oxidation of 3, 3′-dimethoxybenzidine by LiP.

The specific activity of LiP in different organic solvent systems was found to correlate well with the Dimroth-Reichardt parameter $E_T$ (30) (Fig. 3) as was reported in the oxidation of pinacyanol chloride by cytochrome *c*[78]. The values of $E_T$ (30) of organic solvents were measured by solvatochromism methods and these values are also listed in Table 2[79]. The solvatochromism method measures a change in the molecular structure of the dye which is representative of the changes in the three-dimensional structures due to solvent-protein interactions[80]. These values have been used for the determination of the denaturation capacity of solvents in the mathematical model of Khmelnitsky[80]. Therefore, the $E_T$ (30)

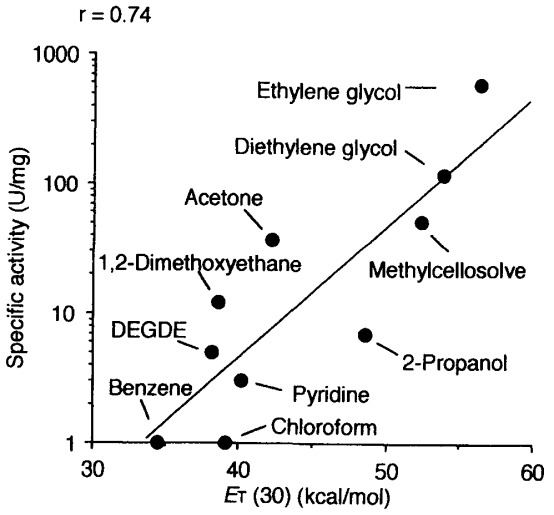


Fig. 3. Relationship between the specific activity of LiP and solvent polarity, $E_T$ (30). The values of $E_T$ (30) were taken from data of reference 79 and are listed in Table 2.

value is thought to be representative of the conformational changes of enzymes in organic solvents. In other words, LiP is thought to retain its activity in organic solvents which do not cause a significant change in its conformation. To investigate the effects of organic solvents on the activity of LiP, further experiments were carried out as described below.

In general, the catalytically active conformation of an enzyme is known to be retained by orienting hydrophobic and hydrophilic amino groups to the inside and the surface of the enzyme, respectively. In aqueous solution, surfactant molecules are also known to exist in the form of micelles. Micelles are fairly monodisperse compact aggregates where the hydrophobic groups of the surfactant molecules are sequestered into the center with the hydrophilic groups facing outwards. The structure of micelles is very likely to resemble those of enzymes, suggesting that organic solvents capable of micelle formation are suitable for the reaction of enzymes in organic solvents. In fact, the abilities of organic solvents to maintain the protein structure have been estimated[81], and the relationships between micelle formation ability of organic solvents and enzyme activity in these solvents have been discussed in several reports[53,82–84]. Therefore, the activity of LiP in organic solvents and the micelle formation abilities of these solvents were investigated to facilitate selection of the best solvent for optimum activity. To examine the micelle formation ability of organic solvents, CMC of the surfactant was measured in various organic solvents. The obtained CMC values of organic solvents are also shown in Table 2. Organic solvents, which have low CMC values, are thought to easily form micelles, whereas micelles are not formed in organic solvents with no CMC value. Ethylene glycol, methylcellosolve, diethylene glycol, and diethylene glycol dimethyl ether (DEGDE) were found to be the micelle-forming solvents, whereas acetone, dioxane, methanol, 2-propanol had no obvious CMC. The

CMC values of THF, diethylene glycol monomethyl ether and 1, 2-dimethoxyethane could not be determined by the method using TCNQ, because Nonidet P-40 reacted with TCNQ.

The activity of LiP was retained in reaction mixtures containing micelle-forming solvents, whereas non-micelle-forming solvents including dioxane, methanol and 2-propanol markedly decreased the activity. Good enzyme activity was also found in reaction mixtures containing acetone. However, acetone has no obvious CMC. The reason for this is not clear at present. Other methods may be available for the measurement of CMC of acetone and organic solvents the value of which could not be determined here.

From these results, the micelle-forming solvents were thought to be suitable for the oxidation of 3, 3′-dimethoxybenzidine by LiP.

***Absorption spectra of LiP in organic solvents***

LiP contains one iron protoporphyrin IX per enzyme as a prosthetic group[85]. Organic solvents are thought to affect not only the conformation of LiP, but also the active site, heme. Therefore, to investigate the effects of organic solvents on the active site structure, absorption spectra of LiP in various organic solvents were measured. The spectra data of LiP in various solutions are shown in Table 3. LiP showed strong absorption at 407 nm with weak maxima at 500 and 630 nm in 20 mM succinate buffer (pH 4.5). Similar spectra were obtained in 70% aqueous ethylene glycol, diethylene glycol and DEGDE medium, indicating that the heme of LiP was little affected by the addition of these organic solvents. However, the intensity of the Soret band was reduced in the presence of 70% dioxane and THF, indicating that these solvents affect the structure of the active site.

Table 3. Spectral characteristics of LiP in water-miscible organic solvents.

| Solvents | Absorption maxima (nm) | | [$\varepsilon$ ($mM^{-1}cm^{-1}$)] |
|---|---|---|---|
| Succinate buffer (pH 3.5) | 408 [186] | 500 [21] | 634 [14] |
| 70% Acetone | 408 [169] | 492 [24] | 648 [Obscure] |
| 70% Dioxane | 407 [ 71] | 570 [Broad] | |
| 70% DMF | 403 [151] | 568 [ 9] | 608 [ 9] |
| 70% Ethylene glycol | 409 [183] | 500 [24] | 636 [ 4] |
| 70% Methylcellosolve | 401 [162] | 472 [Sh 25.0] | 592 [19] |
| 70% 1, 2-Dimethoxyethane | 400 [149] | 580 [20] | |
| 70% THF | 401 [ 44] | 470 [19] | |
| 70% Diethylene glycol | 409 [180] | 502 [24] | 640 [11] |
| 70% DEGME | 420 [112] | 550 [17] | |
| 70% DEGDE | 409 [155] | 500 [19] | 644 [11] |
| 70% Pyridine | 408 [156] | 530 [27] | 558 [27] |
| 70% Methanol | 399 [262] | 488 [Sh 46] | |
| 70% 2-Propanol | 400 [152] | 472 [Sh 26] | 588 [20] |

Sh : Sholder peak ; DMF : *N*, *N*-Dimethylformamide ; THF : Tetrahydrofuran ; DEGME : Diudhylene glycol monomethyl ether ; DEGDE : Diethylene glycol dimethyl ether.

Acetone seemed to have little effect on heme. The other solvents were found to have strong effects on the heme group in LiP, probably due to the rapid complexation of the heme iron with the solvent molecule as a ligand or alteration of the active site structure by penetration of the solvent into the active site of LiP. In addition, the absorption maxima of LiP in ethylene glycol and diethylene glycol gradually changed after a few minutes ; new peaks in the spectra in ethylene glycol and diethylene glycol appeared at 410, 538 and 578 nm and 412, 542 and 576 nm, respectively. These results suggested that penetration of the solvent into the active site occurred gradually even in these solvents.

***Substrate specificity of LiP in ethylene glycol***

Various compounds which have been used as the substrates of laccase and peroxidases were screened as substrates in the reaction in 70% aqueous ethylene glycol medium. As shown in Table 4, all aromatic compounds tested were oxidized by LiP in the presence or absence of ethylene glycol, except for *p*-aminophenol and hydroquinone. Aromatic amines including 3, 3′-dimethoxybenzidine, *o*- and *p*-phenylenediamines and *o*-aminophenol were more easily oxidized in ethylene glycol medium than phenolic compounds. Furthermore, EG/AQ, which indicates the ratio of the increase of the reaction estimated by comparing the activity in the solvents with those in water, of the *o*-substituted phenylenediamine was higher than that of *p*- and *m*-substituted derivatives. These findings show that *o*-substituted aromatic compounds are favorable for the oxidation in ethylene glycol medium, and this suggested that the reactivity of *o*- and *p*-substituted aromatic amines was more efficiently enhanced by electron resonance than that of the *m*-substitutive derivative[86]. Apparent differences in the substrate specificities were found between water and ethylene glycol media ; enzyme activities on 3, 3′-dimethoxybenzidine and *o*-aminophenol were higher in ethylene glycol medium than in water. However, addition of other substrates resulted in loss of activity in 70% aqueous ethylene glycol medium. LiP failed to catalyze the oxidation of veratryl alcohol, which is a non-phenolic compound with high redox potential, in the ethylene glycol medium. This was thought to be caused by the decrease in the redox potential of LiP in organic solvents.

***Effect of ethylene glycol concentration on oxidation of the substrates***

The effects of ethylene glycol concentration on the oxidation the various phenolics and aromatic amines were investigated to optimize the reaction conditions. Interestingly, at concentrations of ethylene glycol between 40–70%, an increase of LiP activity in 3, 3′-dimethoxybenzidine oxidation was observed (Fig. 4A). Similar increases were observed with *o*-aminophenol, catechol and *p*- phenylenediamine as substrates. However, in reactions using guaiacol, 2, 6-dimethoxyphenol, veratryl alcohol, and *m*-anisidine as substrates, an increase in the ethylene glycol concentration resulted in a marked decrease in the activity (Fig. 4B). Similar results were also obtained with *m*-phenylenediamine, aniline, *o*-, *p*-, and *m*-anisidines, *m*-aminophenol, phenol, resorcinol, hydroquinone

Table 4. Substrate specificities of LiP in aqueous and ethylene glycol solution.

| Substrates | λ max (nm) | Specific activity (U/mg) | | EG/AQ (%) | IP (eV) | Log ($C_O/C_W$) |
|---|---|---|---|---|---|---|
| | | AQ | EG | | | |
| 3, 3′-Dimethoxybenzidine[a)] | 444 | 182 | 979 | 538 | 6.72 | 1.58 |
| *o*-Phenylenediamine | 440 | 656 | 291 | 44 | 6.95 | 0.00 |
| *p*-Phenylenediamine | 459 | 153 | 142 | 93 | 6.89 | −0.30 |
| *m*-Phenylenediamine | 400 | 17 | 1 | 6 | 7.61 | −0.40 |
| Aniline | 705 | 72 | 5 | 7 | 7.32 | 0.77 |
| *o*-Anisidine | 500 | 34 | 10 | 29 | 7.11 | 0.78 |
| *p*-Anisidine | 550 | 122 | 6 | 5 | 7.02 | 0.45 |
| *m*-Anisidine | 400 | 23 | 0 | 0 | 7.25 | 1.44 |
| *o*-Aminophenol | 440 | 166 | 245 | 148 | 7.45 | 0.47 |
| *p*-Aminophenol | 440 | 0 | 0 | 0 | —[b)] | 0.07 |
| *m*-Aminophenol | 440 | 59 | 1 | 2 | 7.27 | 0.24 |
| Phenol | 399 | 171 | 4 | 2 | 7.77 | 1.65 |
| Catechol | 398 | 23 | 8 | 35 | 7.52 | 0.91 |
| Hydroquinone | —[b)] | 0 | 0 | 0 | 7.23 | 0.36 |
| Resorcinol | 320 | 5 | 0 | 0 | 7.55 | 0.85 |
| Pyrogallol | 300 | 29 | 5 | 17 | 7.50 | 0.12 |
| 1, 2, 4-Benzenetriol | 471 | 0 | 0 | 0 | —[b)] | 0.35 |
| Guaiacol | 465 | 169 | 2 | 1 | 7.51 | 1.01 |
| HQME | 371 | 21 | 0 | 0 | 7.22 | 1.50 |
| 3-Methoxiphenol | 320 | 9 | 0 | 0 | 7.68 | 1.41 |
| 2, 6-Dimethoxyphenol | 468 | 607 | 2 | 0.3 | 7.56 | 0.24 |
| Vanillyl alcohol | 310 | 2 | 0 | 0 | 7.38 | 0.22 |
| Veratryl alcohol | 310 | 128 | 0 | 0 | 7.35 | 0.51 |

The LiP activity was determined using reaction mixtures containing 14 mM substrate, 100 mM sodium tartrate (pH 3.0), the enzyme solution, 0.25 mM $H_2O_2$, and 70% organic solvents. IP and hydrophobicity (Log ($C_O/C_W$)) of the substrates were determined as described in Materials and Methods. The λ max of the reaction products was determined by measurement of absorption spectrum of the mixture reacted for a few minutes. a: The activity was measured usinc 3, 3′-dimethoxybenzidine dihydrochloride and 3, 3′-dimethoxybenzidine as the substrates in water and70% ethylene glycol solution, respectively. b: Not determined. HQME: Hydroquinone monomethyl ether; IP: Ionization potential.

monomethyl ether, 3-methoxyphenol, and vanillyl alcohol as the substrates (data not shown). In general, organic solvents are believed to act to decrease the catalytic activities of enzymes. However, these findings indicate that the activity of LiP in organic solvents depends on not only the nature of solvent but also on that of the substrate.

Similar results were obtained in the reaction of HRP in organic solvents. Ryu and Dordick reported enhancement of the catalytic activity of HRP using *p*-cresol as a substrate in reaction mixtures containing 60–80% of dioxane[87]. They also discussed the effects of

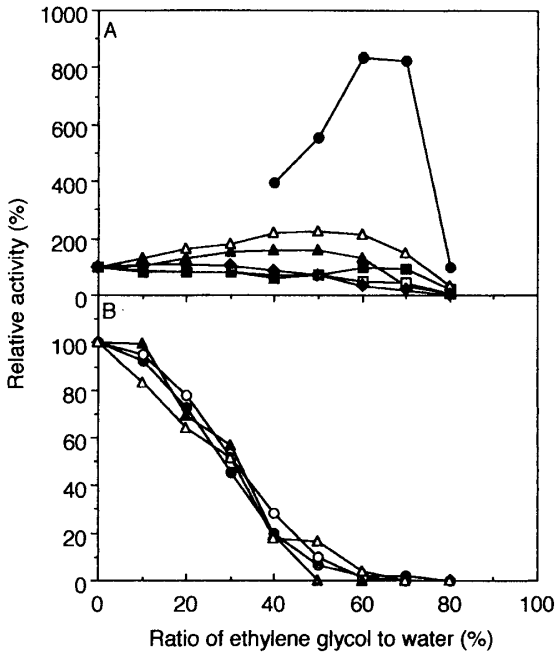


Fig. 4. Effect of ethylene glycol concentration on the oxidation rate of various substrates. The activity of LiP was measured using reaction mixtures containing 14 mM substrates, 100 mM sodium tartrate (pH 3.0), 0.25 mM $H_2O_2$, the enzyme solution, and organic solvent. A: Oxidation rate of 3, 3′-dimethoxybenzidine (—●—), 3, 3′-dimethoxybenzidine dihydrochloride (—○—), *o*-phenylenediamine (—□—), *p*-phenylenediamine (—■—), catechol (—▲—), *o*-aminophenol (—△—), and pyrogallol (—◆—) increase with increasing concentration of ethylene glycol. B: Oxidation rates of veratryl alcohol (—▲—), guaiacol (—●—), 2, 6-dimethoxyphenol (—○—), and m-anisidine (—△—) by LiP decrease with increasing concentration of ethylene glycol.

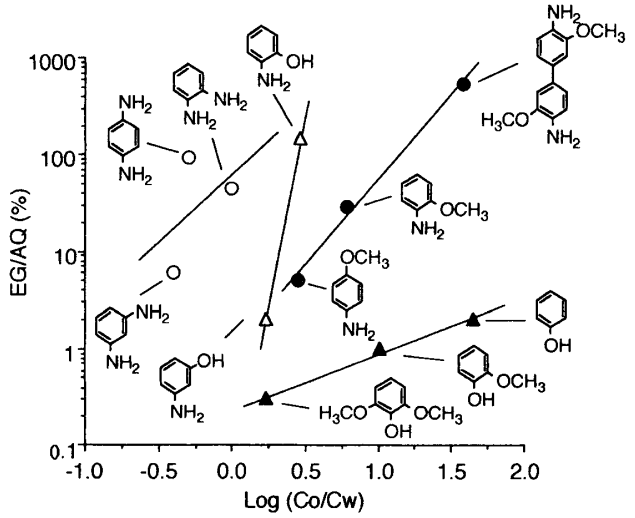


Fig. 5. Hydrophobicity of substrates and LiP activity in 70% aqueous ethylene glycol solution. Correlation coefficients for anisidines, phenols, aminophenols and phenylenediamines were 0.99, 0.99, 1.00 and 0.23, espectively. Hydrophobicity of the substrates was estimated as the partition coefficient of substrate between water and *n*-octanol as described in Materials and Methods.

hydrophobicity of substrate on the catalytic turnover of HRP in water-miscible organic solvents including dioxane and methanol. Analyses of the reaction using the physicochemical properties of substrates can be used to predict optimal solvent and substrate choices for the reaction of LiP in organic solvents.

EG/AQ values for the various substrates plotted versus the hydrophobicity of the substrates used are shown in Fig. 5. Substrates were subdivided into four groups; anisidines, phenylenediamines, phenolics, and aminophenols. A simple direct positive correlation was observed for the reaction of LiP in each group. These results suggested that hydrophobic aromatic compounds are more favorable substrates in 70% ethylene glycol than hydrophilic compounds. Hydrophobic aromatic compounds likely provide an increased binding energy for catalysis over hydrophilic aromatics and the activity increased in less polar media, as was reported in the reaction of HRP by Ryu and Dordick[87].

In addition, ionization potentials of substrates were also reported to be important in the reaction of peroxidase[54,88,89]. The capacity of aromatic compounds to form free radicals is related to their ionization potential. The ionization potential of a molecule is defined as the energy required to completely remove an electron from the neutral particle in its ground state. The removal of one electron from the compound generates a free radical. The ability of LiP to oxidize substrates was also correlated with ionization potential of the substrates. Therefore, in addition to hydrophobicity, ionization potentials of the substrates

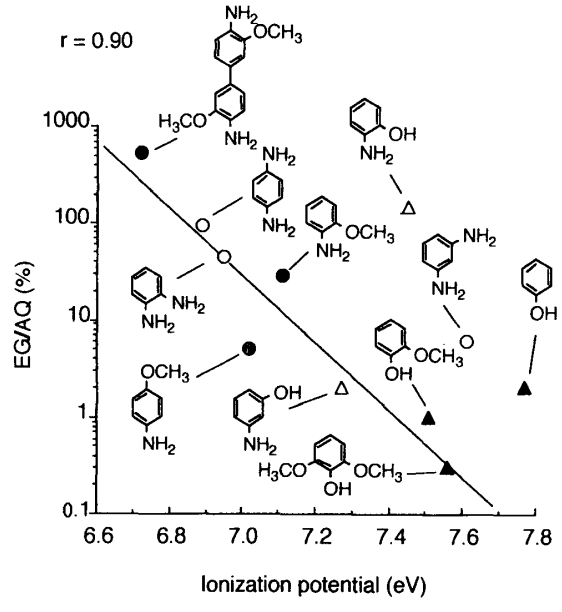


Fig. 6. Ionization potentials of substrates and LiP activity in 70% aqueous ethylene glycol solution. Ionization potentials of the substrates were obtained from the charge transfer absorption spectra of chloranil and the substrates as described in Materials and Methods. EG/AQ: (activity in 70% aqueous ethylene glycol solution/activity of buffer solution) ×100.

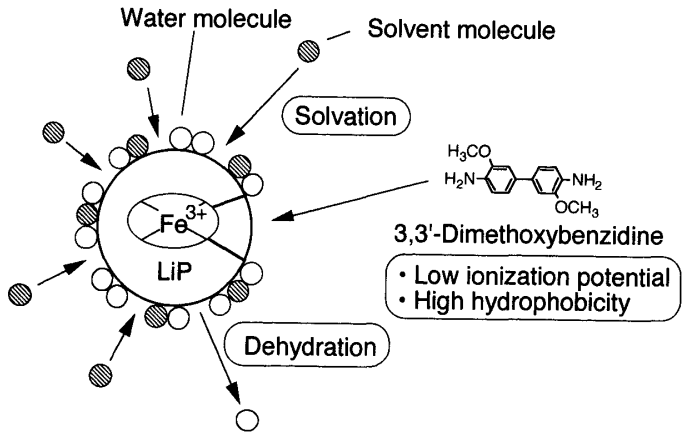


Fig. 7. Reaction of LiP in reaction mixtures containing water-miscible organic solvents.

is thought to be also important for the increase in activity of LiP in reaction mixtures containing water-miscible organic solvents.

Figure 6 shows EG/AQ values for the various substrates plotted *versus* the ionization potential. There was a general trend toward higher activity in 70% aqueous ethylene glycol medium with lower ionization potential.

A possible mechanism of the reaction of LiP in organic solvents is shown in Fig. 7 based on results obtained in this study. In general, enzymes are known to have a hydration shell. In reaction mixtures containing water-miscible organic solvents, distortion of the hydration shell caused by introduction of organic solvent into the enzyme solution upsets the system of interactions supporting the native conformation, which results in the loss of catalytic activity. However, water-miscible organic solvents such as ethylene glycol, diethylene glycol and methylcellosolve, which possess sufficient solvation capabilities are thought to easily solvate the enzyme molecule in place of water molecules. In addition, these solvents are thought not to disturb the environment of the active center. Moreover, 3, 3′-dimethoxybenzidine was found to be the most suitable substrate of all the substrates tested, because it has high hydrophobicity and low ionization potential. Such substrates are thought to provide increased binding energy resulting in an increase in the activity in these water-miscible organic solvents[87].

## I-3 Chemical modification of lignin peroxidase of *Phanerochaete chrysosporium*

### I-3.1 Introduction

Recently, chemical modification of enzymes has been extensively studied as a way to change their physical and chemical properties. Particularly, lipases and proteases modified with polyethylene glycol (PEG) were shown to be soluble and active in organic solvents, and have been used to perform several enzyme processes of practical importance, such as transesterification, syntheses of esters, and syntheses of amide and peptide bonds[53,90,91].

Similarly, HRP was modified with activated PEG derivatives and the modified HRP was both soluble and active in water-immiscible organic solvents[72,92]. The reactivity of protease and HRP in organic solvents was also increased by modification with aliphatic acid such as acetic, octanoyl, and palmitoyl acid[72,93,94].

In this session, chemical modification of LiP of *P. chrysosporium* with PEG and aliphatic acid derivatives as a way to increase the catalytic activity at high concentrations of organic solvents was described. Additionally, the effects of hydrophobization on LiP activity are also discussed[95].

### I-3.2 Materials and methods

***Purification of LiP***

LiP was purified from cultures of *P. chrysosporium* strain ATCC 34541 as described in Section I-1.2. LiP activity and protein concentration were also measured as described in Section I-1.2. The purified LiP had a pI of 4.5 and a molecular weight of 38,000.

***Chemical modification of LiP***

Chemical modification of LiP was done at 5°C for 48 h using 1 mg of acetic and benzoic acid N-hydroxysuccinimide esters (Sigma)[96] and MPSS[97] (Sigma, MW 5,000, MPSS mol/enzyme mol=500) as modifiers in 2 ml of 0.1 M potassium phosphate buffer (pH 7.0). The reaction mixture was washed 20 times with 20 mM succinate buffer (pH 4.5) using an Amicon Centricon-10 to remove the unreacted modifier.

***Analysis of modified LiP by electrophoresis***

IEF was done with a precast gel (Phast Gel pH range 4.0–6.5, Pharmacia) on a Pharmacia LKB-Phast System. A set of standard proteins (Low pI Kit, pI 2.8–6.5, Pharmacia) were used for the calibration of pI. Active staining was done using 1.4 mM 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.3 mM $H_2O_2$. SDS-PAGE was also done with a precast gel (Phast Gel Gradient 10–15, Pharmacia) using the Phast System. Molecular weights were estimated with standard markers (Dai-ichi Kagaku). The gel was stained with Coomassie brilliant blue R-250.

***Analysis of modified LiP by high performance liquid chromatography (HPLC)***

Analysis of the LiP modified with acetic and benzoic acid derivatives was done with an anion-exchange chromatography on a Pharmacia Mono-Q column (10/10) with acetate buffer (pH 6.0) using a linear gradient of from 10 mM to 1 M. On the other hand, the increase in molecular weight of LiP modified with MPSS was investigated by GFC on Pharmacia HiLoad 16/60 Superdex 200 prep grade with 10 mM acetate buffer (pH 5.5) containing 0.1 M NaCl. In both analyses, the elution rate was 1.0 ml/min and the eluent was monitored at 407 nm, which is the maximum absorbance of hemeprotein.

***LiP activity assay in organic solvents***

In organic solvents, the activity was measured by the method described in Section I-1.2 using 14 mM 3, 3′-dimethoxybenzidine as the substrate. One unit of LiP activity in an

organic solvent was defined as the amount of enzyme that increased absorbance by one per minute.

***Measurement of viscosity of 70% organic solvents***

Flow time of 70% organic solvents was measured at 25°C using an Ostwald viscometer. The viscosity value ($\eta$) was calculated from the flow time and density of each solution using the value of water (viscosity, 0.890 ; density, 0.997 at 25°C). The flow times were measured in triplicate.

### I-3.3 Results and discussion

***Chemical modification of LiP***

The confirmation of the modification of LiP was done by IEF. As shown in Fig. 8, the pI of all modified LiPs became more acidic than the native LiP. This is thought to be caused by the decrease of p*K*a of the modified $NH_2$ group, as was discussed by Wirth *et al.*[98].

In addition, the modification of LiP was also confirmed by HPLC analyses. The analysis of the acetylated LiP (Ac-LiP) and benrylated LiP (Be-LiP) was done by anion-exchange chromatography on Mono-Q column (data not shown). The elution patterns of Ac-LiP and Be-LiP differed from that of the native LiP. Observation of two peaks in native LiP indicates that it contains two isozymes. The broad peaks observed in Ac- and Be-LiP were due to either a difference in the degree of modification between the two isozymes or to a difference in the degree of modification of the $NH_2$ groups. This result, as well as that of IEF, is thought to be caused by the decrease of the p*K*a of the modified $NH_2$ group as was discussed by Wirth *et al.*[98].

Furthermore, GFC analysis demonstrated that the molecular weight of LiP was increased from 38,000 to 320,000 by chemical modification (data not shown). LiP, however, is known to contain an N-terminal alanine and nine lysine residues[99,100] that could link to MPSS. This is not consistent with the molecular weight of 88,000 estimated on the basis of the number of $NH_2$ groups. Perhaps, aggregation of MPSS-LiP in the gel matrix

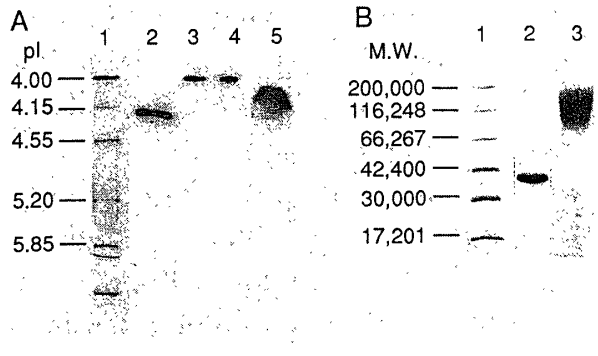


Fig. 8. Analyses of native and modified LiPs by electorophoresis. (A) IEF. Lane 1, standard marker; lane 2, native LiP; lane 3, Ac-LiP; lane 4, Be-LiP; lane 5, MPSS-LiP. (B) SDS-PAGE. Lane 1, standard markers; lane 2, native LiP; lane3, MPSS-LiP.

was thought to cause anomalous migration during GFC. In addition, the peak of the native LiP was not observed after chemical modification. Similarly, SDS-PAGE analysis also showed clearly that the molecular weight of MPSS-LiP increased from 38,000 to 86,000–170,000. This is substantially in agreement with the calculated value, indicating that MPSS linked to all $NH_2$ groups in the LiP molecule. It can be concluded from these analyses that LiP was chemically modified with MPSS and acetic and benzoic acids.

In preliminary experiments, the protein concentration of the modified LiPs was measured by the method of Bradford. The concentration was also measured by the absorbance at 407 nm using an extinction coefficient of 190.5 $mM^{-1}$ $cm^{-1}$ for the native LiP

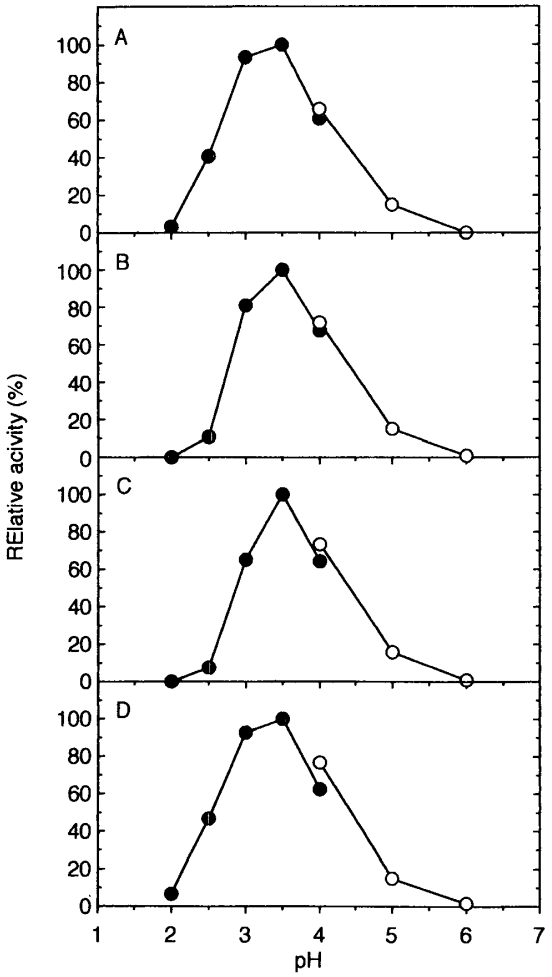


Fig. 9. Effects of pH on reaction of native and modified LiPs in aqueous solution. Activity of native LiP (A), Ac-LiP (B), Be-LiP (C), and MPSS-LiP (D) was measured using 0.8 mM veratryl alcohol as a substrate in 0.1 M Na-tartrate buffer (pH 2-4, —●—) and Na-succinate buffer (pH 4–6, —○—).

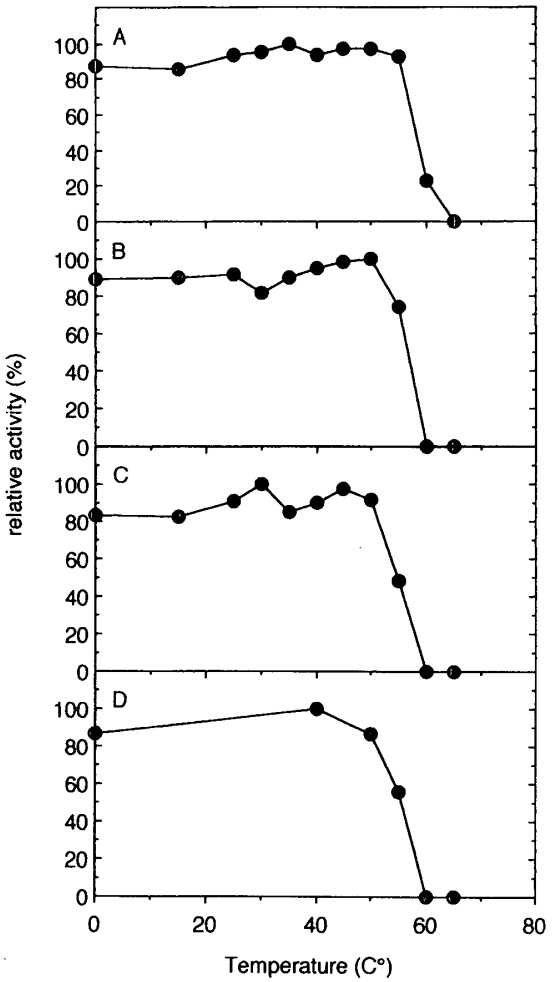


Fig. 10. Effects of temperature on stability of native and modified LiPs in aqueous solution. Native LiP (A), Ac-LiP (B), Be-LiP (C) and MPSS-LiP (D) were kept in 20 mM Na-succinate buffer (pH 4.5) for 15 min at the temperature indicated in the figure.

based on the molecular weight (38,000)[101] of the enzyme. However, no significant difference between these values was observed. Therefore, protein was measured by the method of Bradford in this study.

***Reaction of modified LiP in aqueous solution***

Enzymatic properties of the native and modified LiPs were investigated in aqueous solution. Specific activities of the native LiP, MPSS-LiP, Ac-LiP, and Be-LiP for veratryl alcohol oxidation in aqueous solution were 16,200, 16,000, 16,400, and 12,800 U/mg, respectively, suggesting that the modified LiPs retained their original activity except for Be-LiP, which showed only 80% of its original activity in aqueous solution. These results

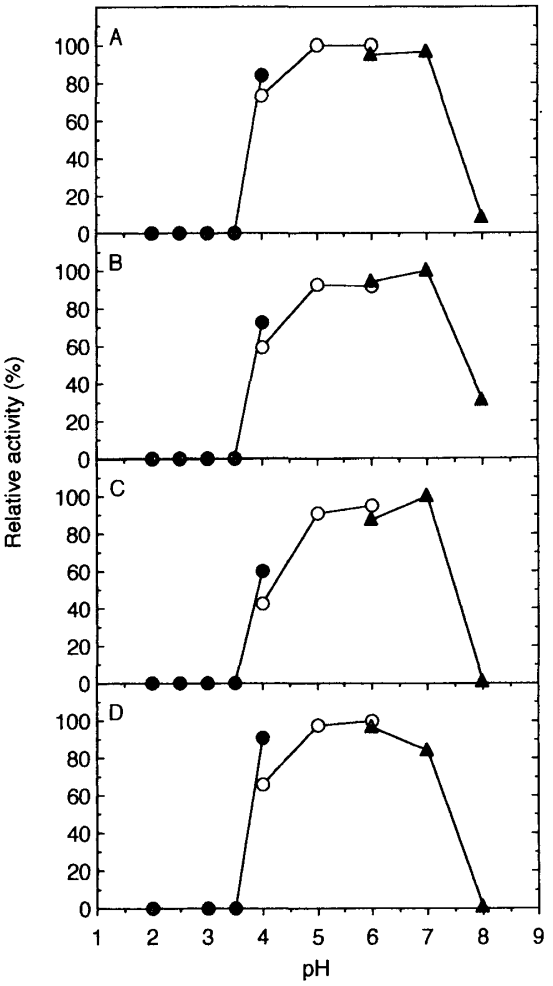


Fig. 11. Effects of pH on stability of native and modified LiPs in aqueous solution. Native LiP (A), Ac-LiP (B), Be-LiP (C) and MPSS-LiP (D) were kept at 37°C for 15 min in 0.1 M Na-tartrate buffer (pH 2–4, —●—), succinate buffer (pH 4–6, —○—), and phosphate buffer (pH 6–8, —▲—).

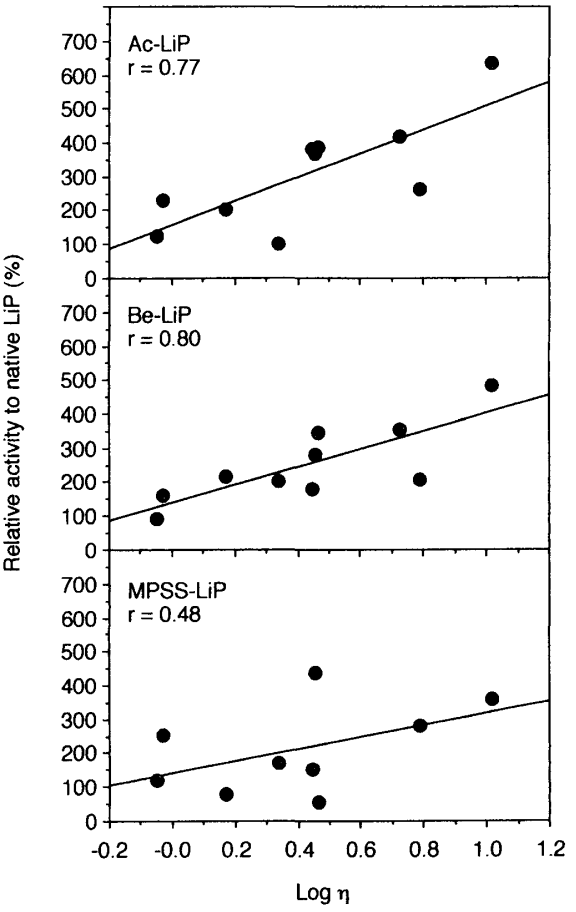


Fig. 12. Effects of viscosity of water-miscible organic solvents on the reaction of modified LiPs. Lines in the figure were drawn by the method of least-squares. The results represent the means of three independent experiments. Viscosity was measured by the method described in the Materials and Methods. These values are listed in Table 5.

indicates that the inactivation did not occur during the modification process. In addition, native and modified LiPs had the same optimal pH of 3.5 (Fig. 9), and the same stability at temperature below 40°C (Fig. 10) and in the pH range 4–7 (Fig. 11). Therefore, it was concluded that there was no significant differences in the enzymatic properties of native and modified LiPs in aqueous solution.

***Reaction of modified LiP in the presence of organic solvents***

The activity of native and modified LiP in organic solvents was investigated. The best catalytic activity of the native LiP was obtained using 3, 3′-dimethoxybenzidine as a substrate in a reaction mixture containing 70% ethylene glycol[63]. Hence, the reaction of these modified LiPs was compared with that of the native LiP using 3, 3′-dimethoxybenzidine in the reaction mixture containing 70% of various organic solvents. As summarized in Table 5, both native and modified LiPs oxidized 3, 3′-dimethoxybenzidine in 70% aqueous solutions of water-miscible organic solvents including ethylene glycol and

Table 5 Activity of native and modified LiPs in organic solvents.

| Solvents | $\eta$ (25°C) | Specific activity (U/mg) | | | |
|---|---|---|---|---|---|
| | | Native LiP | Modified LiP | | |
| | | | Ac-LiP | Be-LiP | MPSS-LiP |
| Water | 0.89 | 159 | 199 | 149 | 188 |
| 70% Acetone | 0.94 | 37 | 86 | 60 | 94 |
| 70% Dioxane | 1.90 | 0 | 23 | 19 | 33 |
| 70% DMF | 2.35 | 0 | 0 | 0 | 0 |
| 70% Ethylene glycol | 6.14 | 591 | 1,540 | 1,237 | 1,669 |
| 70% Methylcellosolve | 2.86 | 52 | 191 | 146 | 227 |
| 70% 1, 2-Dimethoxyethane | 1.48 | 12 | 24 | 126 | 10 |
| 70% THF | 1.30 | 0 | 0 | 1 | 7 |
| 70% Diethylene glycol | 10.5 | 118 | 749 | 571 | 425 |
| 70% DEGME | 5.30 | 21 | 88 | 74 | 0 |
| 70% DEGDE | 2.79 | 5 | 19 | 9 | 8 |
| 70% Pyridine | 2.18 | 3 | 3 | 6 | 5 |
| 70% Methanol | 1.30 | 0 | 0 | 0 | 0 |
| 70% 2-Propanol | 2.92 | 7 | 27 | 24 | 4 |
| Benzene | | 1 | 2 | 2 | 0 |
| Toluene | | 0 | 1 | 0 | 0 |
| Ethylacetate | | 0 | 0 | 0 | 0 |
| Chloroform | | 1 | 2 | 3 | 0 |

Viscosity of water-miscible organic solvents was measured as described in the Materials and Methods. LiP activity was measured by the method described in Materials and Methods using 3, 3′-dimethoxybenzidine dihydrochloride and 3, 3′-dimethoxybenzidine as substrates in water and in organic solvents, respectively. One unit of enzimes produces an absorbance of 1.0 after 1 minute. Protein contents of native LiP, Ac-LiP, and MPSS-LiP were 1.0, 0.7, 0.7, and 0.4 mg, respectively.

methylcellosolve, diethylene glycol, and acetone. In particular, the introduction of these modifiers onto LiP improved the activity in a system containing water-miscible organic solvents. HRP modified with PEG was shown to effectively oxidize the phenolics and aromatic amines in water-immiscible organic solvents including diethylether, benzene, and toluene[72,92]. MPSS-LiP was inactive in these organic solvents. This result is thought to be caused by the structural differences between two peroxidases such as the amino acid sequence and carbohydrate content[99,100,102,103]. However, the relationship between the difference of the reaction of both peroxidases in organic solvents and their structural differences is not still understood. Further studies on these relationships are now in progress.

The specific activities of Ac-LiP, Be-LiP, and MPSS-LiP were 2.6, 2.1, and 2.8 times higher, respectively, than that of native LiP in aqueous 70% ethylene glycol. In addition, the activity of LiP was also observed in the reaction mixture containing 70% acetone, and was also increased by modification with these modifiers.

Furthermore, the activity of these modified LiPs in 70% aqueous diethylene glycol medium, which has a higher viscosity than ethylene glycol, was increased by the chemical modification, in particular, Ac-LiP showed 6 times higher activity than native LiP. HRP are known to be solubilized in water-immiscible organic solvents by the chemical modification, thereby being active in these solvents[72,92]. However, the mechanism of the increase of the modified LiP activity in water-miscible organic solvents is not understood. These results obviously imply that viscosity of organic solvents is an important factor in the reaction of modified LiP.

Therefore, to measure the effects of viscosity on the activity of modified LiPs, the relative activity of modified LiP to that of native LiP in each water-miscible organic solvent was plotted *vs* viscosity of water-miscible organic solvents. As can be seen from Fig. 12, the reaction of Ac-LiP and Be-LiP in water-miscible organic solvents showed a viscosity dependence while that of LiP-MPSS did not. In general, the superficial hydrophobicity of the enzyme was increased by the modification with aliphatic acid and PEG derivatives[72]. Kawasaki *et al.* proposed that the addition of hydrophobicity to an enzyme molecule by chemical modification contributes to reduction of the attractive forces between enzyme and solvent molecules[94]. Similarly, addition of hydrophobicity to the LiP is thought to decrease interaction between the enzyme and high viscosity solvent molecules. Therefore, the modified LiP having a high hydrophobicity can move easily in high-viscosity organic solvents, so that the modification of LiP may increase the affinity of enzyme to a substrate dissolved in high-viscosity organic solvents. In addition, the increase of molecular weight of LiP by the chemical modification may decrease the velocity of diffusion in high viscosity organic solvents. Apparently, MPSS-LiP has a higher molecular weight than Ac-LiP and Be-LiP. Therefore, viscosity dependence was thought not to be observed in the case of the

reaction of MPSS-LiP.

The *K*m of the reaction of LiP in the presence of 70% ethylene glycol could not be defined because the plot of velocity against substrate concentration showed a sigmoid curve (data not shown). Kinetics of the reaction is now under investigation.

From this discussion, the chemical modification with acetic and benzoic acid derivatives, which have a lower molecular weight than MPSS, seems to promote the diffusibility of the enzyme in high viscosity organic solvents and to increase the affinity of the enzyme for substrates. In conclusion, these results show that the modification of LiP with aliphatic acid was very effective for increasing of the activity in water-miscible organic solvents. In this study, the effects of viscosity on the reaction of chemically modified LiPs were discussed. Effects of the other parameters of organic solvents on the reaction of LiP are now under investigation.

## I-4 Degradation of lignin by manganese peroxidase of *Bjerkandera adusta* in the reaction mixtures containing water-miscible organic solvents

### I-4.1 Introduction

Development of an enzyme reaction system using organic solvents as the reaction media is necessary for the degradation and transformation of water-insoluble lignin. Many studies of incubations of lignin preparations with ligninolytic enzymes have been reported previously[52,104–110]. Degradation of lignin in mixtures of organic solvents and buffers using different peroxidases should be investigated in detail. Dordick *et al.* used organic solvent for depolymerization of dehydrogenation polymer (DHP), milled wood lignin (MWL) and kraft lignin by HRP[111]. However, Lewis *et al.* could not reconfirm these promising results[112]. On the other hand, Hammel *et al.* reported that addition of organic solvents capable of dispersal of the polymer to the reaction medium is important for the ligninolysis by enzymes[52]. Thus, the actions of enzyme systems on the degradation of lignin polymers remain to be investigated further.

In this session, the depolymerization of DHP by MnP of *B. adusta* in organic solvents is discussed[113].

### I-4.2 Materials and methods

#### *Preparation of MnP of B. adusta*

*B. adusta* (K-2679) was grown statically at 30°C in 200-ml Erlenmeyer flasks with 20 ml of culture medium containing 2% glucose, 0.5% polypeptone, 0.2% yeast extract, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ as described by Kimura *et al.*[16]. MnP activity was determined by the method of Kofujita *et al.* using a reaction mixture containing 0.4 mM guaiacol, 50 mM Na-lactate buffer (pH 4.5), 0.2 mM $MnSO_4$ and 0.1 mM $H_2O_2$ in a total volume of 1 ml[17]. The reaction was monitored by measuring the increase in absorbance of the reaction product at 465 nm. MnP activity was calculated by subtracting the activity

determined in the absence from that in the presence of Mn (II). One unit of MnP was defined as the amount of enzyme that increased the absorbance at 465 nm by 1.0 per min. Protein was determined by the method of Bradford[65]. MnP was purified from 13-days-old cultures by the method of Kirk *et al.* on a DEAE-Sepharose CL-6B and a Pharmacia Mono-Q column (10/10)[66]. The purified MnP had a pI of 4.5 and a molecular weight of 40,000.

***MnP activity assay in organic solvents***

In water-miscible organic solvents, the activity was determined spectrophotometrically using reaction mixtures containing 14 mM substrate, 50 mM lactate buffer (pH 4.5), 0.2 mM $MnSO_4$, 0.25 mM $H_2O_2$, 70% organic solvent and the enzyme solution in a final volume of 1 ml. One unit of MnP activity in organic solvent was defined as the amount of enzyme which increased one absorbance per 1 minute.

***Preparation of DHP***

Coniferyl and sinapyl alcohols were synthesized by the method of Freudenberg and Hübner[114]. Guaiacyl type DHP was prepared with HRP/$H_2O_2$ from coniferyl alcohol by the Zutropfverfahren[115]. Syrigyl type DHP was prepared from sinapyl alcohol by the dialysis tube method[116].

***Enzyme reactions***

*In vitro* reactions were carried out at 37°C in 2.0 ml reaction mixtures containing 70% organic solvents ; 50 mM Na-lactate (pH 4.5), 13.6 $\mu$g of MnP, 2.0 mg of DHP (added as a stock solution in 200 $\mu$l of methylcellosolve solution), 10% methylcellosolve (added with DHP as the stock solution), 0.2 mM $MnSO_4$, 0.1 mM $H_2O_2$ and 60% acetone. Additional MnP and $H_2O_2$ were added at various intervals. After incubation for the desired period, the reaction mixtures were evaporated to dryness and dried under high vacuum.

***Analysis of molecular weight distribution by gel-permeation chromatography***

Samples were dissolved in 200 $\mu$l of DMF, and insoluble material was removed by centrifugation at 4,000 rpm for 15 min at room temperature. The supernatant was applied to a Sephadex LH-60 column (1.5×41 cm) equilibrated in DMF containing 0.1 M LiCl. The column was eluted with the same solvent, and 1.5 ml fractions were collected. Absorbance of fractions at 280 nm was measured. The column was calibrated with standard model compounds and polystyrene standard.

### I-4.3 Results and discussion

***Activity of MnP of B. adusta in organic solvents***

MnP activity in water-miscible organic solvents was measured using guaiacol and 2, 6-dimethoxyphenol as guaiacyl and syringyl type model substrates, respectively. Lignin has been reported to be completely or partially soluble in the solvents used in this study[117]. As summarized in Table 6, MnP oxidized both substrates in 70% water-miscible organic solvents including acetonitrile, acetone, dioxane, ethylene glycol and methylcellosolve. Guaiacol was not oxidized by MnP in reaction mixtures containing 70% ethanol and

Table 6 Activity of MnP of *B. adusta* in organic solvents.

| Solvents | Relative activity (%) | |
|---|---|---|
| | Guaiacol (%) | 2, 6-Dimethoxyphenol (%) |
| Water[a)] | 100 | 100 |
| 70% Acetonitrile | 35 | 77 |
| 70% Acetone | 26 | 73 |
| 70% Dioxane | 20 | 23 |
| 70% Ethyleneglycol | 17 | 31 |
| 70% Methylcellosolve | 11 | 16 |
| 70% Ethanol | 0 | 20 |
| 70% Methanol | 0 | 1 |
| 70% DMF | 0 | 0 |
| 70% DMSO | 0 | 0 |

DMF: *N*, *N*-Dimethylformamide; DMSO: Dimethyl sulfoxide. a: In water, specific activity of MnP for guaiacol and 2, 6-dimethoxyphenol were 231 and 1,065 U/mg, respectively.

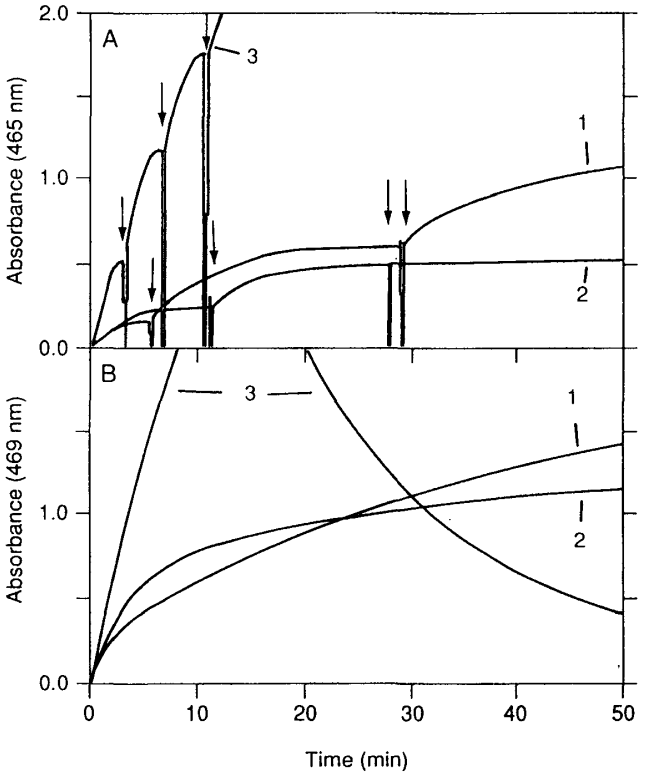


Fig. 13. Oxidation of phenolics by MnP of *B. adusta* in aqueous 70% acetonitrile and acetone media. Arrows show the time points at which 50 $\mu$l of 50 mM $H_2O_2$ was added to the solutions. The oxidation of guaiacol (A) and 2, 6-dimethoxyphenol (B) was carried out using reaction mixtures containing 70% acetone (1) and acetonitrile (2). Oxidation of both substrates was also carried out in the absence of co-solvents (3).

methanol. However, 2, 6-dimethoxyphenol was slightly oxidized in both solutions. DMF and dimethyl sulfoxide (DMSO) completely inhibited the oxidation of both substrates by MnP.

MnP activity retained 77% and 73% of the original activity in reaction mixtures containing 70% acetonitrile and acetone, respectively. Thus, these appeared to be effective solvents to use for the reaction of MnP with lignin. To investigate the stability of MnP in both solvents, the increases in absorbance by oxidation of both substrates were monitored for 50 min. As shown in Fig. 13, increases in absorbance were observed for both substrates for 50 min in reaction mixtures containing 70% acetone. Initial oxidation rates in 70% acetonitrile solution were higher than those in acetone, but the increases in absorbance reached maximum values after 10 min. In addition, the oxidation of guaiacol by MnP was restarted by further addition of $H_2O_2$, suggesting that MnP was not disturbed in 70% acetone solution. The oxidation rates of both substrates were higher than in the absence than in the presence of acetone and acetonitrile. Furthermore, decreases in absorbance were observed using 2, 6-dimethoxyphenol as the substrate. The reason for this is not yet clear. However, as it is effective for depolymerization of lignin to decrease the concentration of lignin phenoxy radicals in reaction mixture[52], the oxidation rate of the substrate in water is thought to be too fast to depolymerize lignin. In addition, the importance of the presence of co-solvents has also been reported by Hammel *et al.*[52]. Therefore, acetone was used in the following depolymerization experiments.

***Reaction of MnP with DHP in reaction mixtures containing acetone***

The c- and s-DHPs were incubated with MnP for 4 hrs in reaction mixtures containing

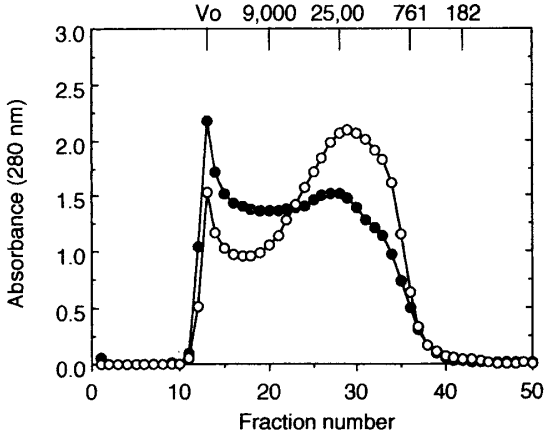


Fig. 14. Gel-permeation chromatograms of c-DHP incubated with MnP of *B. adusta* in reaction mixtures containing both 60% acetone and 10% methylcellosolve (—●—: complete reaction; —○—: MnP was omitted). DHP was analyzed by GPC on a Sephadex LH-60 column using DMF containing 0.1 M LiCl as an eluent. Fractions of 1.5 ml were collected and absorbance at 280 nm was measured. Experimental details are given in Materials and Methods.

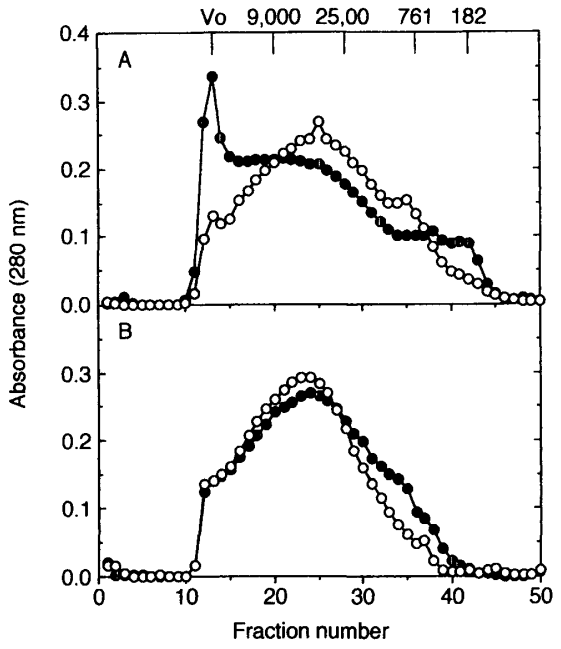


Fig. 15. Gel-permeation chromatograms of s-DHP incubated with MnP of *B. adusta* and Mn (III) in reaction mixtures containing both 60% acetone and 10% methylcellosolve. DHPs incubated with MnP/$H_2O_2$ (A, —●—: complete reaction; —○—: MnP was omitted) and Mn (III) (B, —●—: complete, —○—: Mn (III) was omitted) were analyzed by GPC on a Sephadex LH-60 column using DMF containing 0.1 M LiCl as an eluent. Fractions of 1.5 ml were collected and absorbance at 280 nm was measured. Experimental details are given in Materials and Methods.

10% methylcellosolve added with DHP as a stock solution, and 60% acetone. Additional MnP and $H_2O_2$ were added at 1 h intervals. Control experiments were carried out with omission of MnP or $H_2O_2$. A GPC chromatogram of c-DHP is shown in Fig. 14. The molecular weight of c-DHP increased and no depolymerization products was observed. No reaction was observed in control experiments. In contrast, s-DHP was depolymerized by MnP in reaction mixtures containing acetone with small amounts of polymerized compounds (Fig. 15A). Depolymerized fraction of s-DHP had MWs of 180–400. Depolymerized products were checked by thin-layer chromatography using *n*-hexane/ethyl acetate (2/1) as the developing solvents. Syringaldehyde was detected by UV irradiation, suggesting that $C_\alpha$-$C_\beta$ cleavage of s-DHP was induced by MnP[108]. Depolymerization also occurred when Mn (III) was used instead of MnP/$H_2O_2$ in the same solution (Fig. 15B). The difference in the behavior of MnP against c- and s-DHPs is thought to be due to the chemical structures of the substrates. c-DHP contains a greater proportion of unsubstituted aromatic 5-positions than s-DHP[6], and consequently would be more susceptible to further polymerization or repolymerization[107,108].

***Suppression of repolymerization of s-DHP by MnP in reaction mixtures containing organic solvents***

Previous studies have demonstrated has been shown the repolymerization of DHP by ligninolytic enzymes *in vitro*[52,107,108,118]. Although MnP, like LiP, both polymerized and depolymerized lignin *in vitro*, MnP resulted in more polymerization and less depolymerization than LiP[52]. In this study, repolymerization of c- and s-DHPs by MnP was also observed. Depolymerization is probably initiated by oxidation of the free phenolic group to form a phenoxy radical, as previously shown for the degradation of phenolic diarylpropane model compound by MnP[119]. High transient concentration of lignin phenoxy radicals is thought to facilitate repolymerization rather than depolymerization as described by Hammel *et al.*[52]. Therefore, to retain the concentration of phenoxy radicals at low revel, additional enzyme and $H_2O_2$ were added to the reaction mixture at various intervals for a total reaction time of 28 hrs. Incubation of s-DHP with MnP was initiated with 13.6 $\mu$g of MnP and 0.1 mM $H_2O_2$, and was continued with further addition of the same amounts of MnP and $H_2O_2$ after 2.5 and 19 hrs of incubation. $H_2O_2$ was also added after 5.5 and 26 hrs of incubation. The molecular weight of s-DHP decreased using this reaction system,

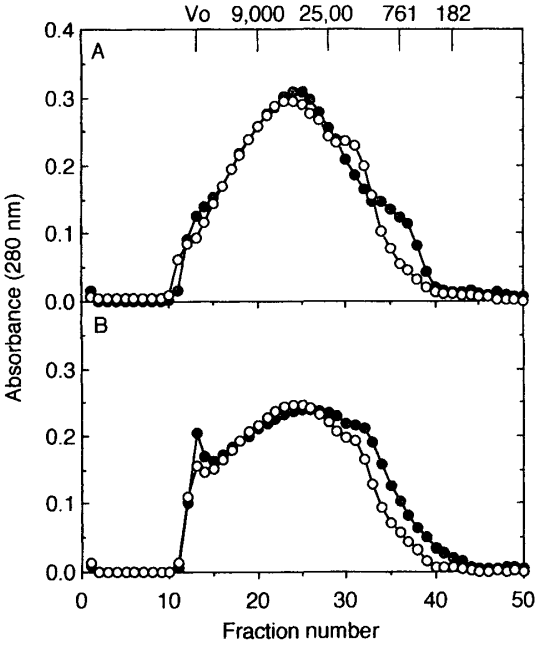


Fig. 16. Gel-permeation chromatograms of s-DHP incubated with MnP of *B. adusta* in reaction mixtures containing both 60% acetone and 10% methylcellosolve under various conditions. DHPs incubated for 28 hrs (A) and incubated in the presence of 0.1 mM ascorbic acids (B) were analyzed by GPC on a Sephadex LH-60 column using DMF containing 10 mM LiCl as an eluent (—●—: complete reaction; —○—: MnP was omitted). Fractions of 1.5 ml were collected and absorbance at 280 nm was measured. Experimental details are given in Materials and Methods.

suggesting that polymerization of lignin was suppressed (Fig. 16A).

Radical concentrations can be decreased by addition of radical scavengers such as ascorbic acid, tocopherols and several phenols [120]. Oxidation of 2, 6-dimethoxyphenol by MnP was decreased to 1/1470 of the original activity in 70% acetone by addition of 10 mM ascorbic acid (data not shown). Therefore, s-DHP was incubated with MnP in aqueous 70% acetone medium containing 10 mM ascorbic acid. As shown in Fig. 16B, repolymerization was suppressed by the addition of ascorbic acid. These reaction conditions were thus effective for the depolymerization of DHP. Reaction mixtures containing high concentrations of lignin without co-solvents led to poor dispersal of the polymer[52]. The influence of dispersion of lignin on enzymatic degradability was also investigated[105,121,122]. High concentrations of $H_2O_2$ supported high transient concentrations of lignin phenoxy radicals and resulted in the polymerization of lignin. Addition of 70% acetone to the reaction mixture improved the dispersion of lignin.

## Chapter II Synthesis of Dehydrogenation Polymer of Monolignols by Ligninolytic Enzymes in Organic Solvents

Many different preparations, such as Klason, kraft, enzymatically liberated, acidolysis, Brawn's native, cellulase, milled wood and synthetic lignins and lignosulfonates have been used to study the reactions of lignin. The advantages and disadvantages of all these different lignin preparations have been discussed so far[123–125]. It was concluded that MWL is the best lignin preparation, and that prepared lignin is good for general use. Synthetic lignins are synthesized by oxidative polymerization of the *p*-hydroxycinnamyl alcohols, such as coniferyl, sinapyl and *p*-coumaryl alcohols, with peroxidase/$H_2O_2$ or $FeCl_3$ as oxidants and are, therefore, known as the dehydrogenation polymers (DHP) of monolignols. To examine lignin polymerization *in vitro*, dehydrogenation experiments with lignin precursors have also been performed.

DHP can be prepared in three different ways: Zulauf, Zutropf, and dialysis tube methods. In the Zulaufverfahren[4], coniferyl alcohol solution is added all at once to the peroxidase/$H_2O_2$ solution, and this produced DHP with a molecular weight of 1,000–1,200[1,125]. If the coniferyl alcohol is added dropwise (Zutropf) over a long period, DHP with molecular weights ranging from 1,270 to 2,940 is obtained[126]. High molecular weight DHP was synthesized by immersion of a dialysis tube containing peroxidase in a solution of $H_2O_2$ and coniferyl alcohol. When the latter two components diffused into the dialysis tube, polymerization occurred, giving a DHP with MW of about 6,000[127].

However, the chemical structure and molecular weight of conventionally prepared DHP are different from those of native lignin. The characteristics of DHP vary with reaction conditions[116].

The effects of various factors on the dehydrogenative polymerization of monolignols by HRP and $FeCl_3$ are described in Section II-1.

Section II-2 describes the synthesis and characterization of DHP using MnP of *B. adusta* in organic solvents.

## II-1 Effect of organic solvents and pH of reaction medium on dehydrogenative polymerization of sinapyl alcohol

### II-1.1 Introduction

Lignin is produced by random polymerization following enzymatic dehydrogenation of a mixture of coniferyl, sinapyl and *p*-coumaryl alcohols whose proportions vary with plant species. In vitro, the biosynthesis of lignin can be mimicked by dehydrogenative polymerizations of these cinnamyl alcohols using $FeCl_3$ or peroxidase/$H_2O_2$ as the oxidants. The study of DHP has contributed to the explanation of the structure of a native lignin.

By the Zulaufverfaharen with HRP/$H_2O_2$, a large amount of syringaresinol and a small amount of syringylglycerol-$\beta$-sinapyl alcohol ether ($\beta$-*O*-4) were produced from sinapyl alcohol[128,129]. Even in the Zutropfverfahren, syringaresinol with rather a small amount of polymerized materials was mainly produced[116,129,130]. Because syringyl lignin in a hardwood consists of a large amount of $\beta$-*O*-4 structure and the amount of resinol structure is less than 5%, conventionally prepared DHP is different from a native syringyl lignin in the chemical structure.

On the other hand, the characteristics of DHP are varied with the reactive conditions. For example, Tanahashi and Higuchi reported that a large amount of $\beta$-*O*-4 type dimer and a small amount of syringaresinol were produced from sinapyl alcohol in a non-polar solvent[116]. The result shows that the $\beta$-*O*-4 linkage in the cell wall may be generated mainly in the hydrophobic regions of hemicelluloses[131]. Additionally, Uchida and Terashima reported that the pH in the circumstances of the reaction was important for lignification[132]. The results shows that $\beta$-5 type dimer was mainly produced from coniferyl alcohol with an increase of the acidity of the solution. Therefore, the investigation of the effect of the reaction medium, such as polarity and pH, on the dehydrogenative polymerization of monolignols is thought to be very important[133].

As reported here, to investigate the effects of reaction medium on the dehydrogenative polymerization of monolignols, sinapyl alcohol was reacted under various pHs and polarities of the solvent by the Zulaufverfaharen with $FeCl_3$ or HRP/$H_2O_2$ as an oxidant. The chemical structures of dimerized products were analyzed by nuclear magnetic resonance (NMR) and mass-spectrometry (MS)[134,135].

### II-1.2 Materials and methods

***Dehydrogenative polymerization of sinapyl alcohol by $FeCl_3$***

Sinapyl alcohol was synthesized by the method of Freudenberg and Hübner[114] and 25

mg of it was dissolved in 1 ml of 1, 4-dioxane/water solution; the mixing ratios of the solutions were varied, 1/0, 1/1, and 0/1 (v/v). After stirring for 30 min, 0.9 ml of 2% $FeCl_3$ aqueous solution was added as an oxidant to the solutions. The dehydrogenative reaction was conducted for 2 hrs under nitrogen atmospheres and light shieldings at room temperatures. For the system of various pHs, sinapyl alcohol was dissolved in 0.1 M KCl-HCl buffer (pH 2.0), 0.1 M acetate buffer (pH 4.0), or 0.1 M phosphate buffer solutions (pH 6.5 and 8.0) without 1, 4-dioxane, and used for the reactions.

Reaction products were extracted with ethyl acetate three times and washed with saturated brine. After drying with anhydrous sodium sulfate, ethyl acetate was evaporated under reduced pressure. The reaction products were separated by a preparative thin-layer chromatography (PTLC) and HPLC. The chemical structures and the ratios of obtained products were identified from proton nuclear magnetic resonance ($^1$H-NMR) spectra measured by using a JEOL FT-NMR JMN GX-270 spectrometer in deuterated chloroform ($CDCl_3$) containing 0.03% of tetramethyl silane (TMS).

Syringaresinol $^1$H-NMR ($CDCl_3$) (ppm) : 3.10 (2H, multiplet, $H_\beta$), 3.90 (12H, singlet, -$OCH_3$), 3.92 (2H, multiplet, $H_{\gamma 2}$), 4.92 (2H, dd, $J$=6.8 Hz, 2.14 Hz, $H_{\gamma 1}$), 4.73 (2H, d, $J$=4.2 Hz, $H_\alpha$), 5.51 (2H, s, phenol-OH), 6.59 (4H, s, aromatic).

Syringylglycerol-$\beta$-sinapyl alcohol ether (*erythro*) $^1$H-NMR ($CDCl_3$) (ppm) : 3.89 (6H, s, -$OCH_3$), 3.92 (6H, s, -$OCH_3$), 4.13 (1H, multiplet, $H_\beta$), 4.37 (2H, d, $J$=5.6 Hz, $H_{\gamma'}$), 4.99 (1H, d, $J$=3.7 Hz, $H_\alpha$), 5.46 (2H, broad, phenol-OH), 6.36 (1H, dt, $J$=13.2 Hz, 5.6 Hz, $H_{\beta'}$), 6.60 (2H, s, aromatic), 6.62 (1H, d, $J$=13.2 Hz, $H_{\alpha'}$), 6.70 (2H, s, aromatic).

Syringylglycerol-$\beta$-sinapyl alcohol ether (*threo*) $^1$H-NMR ($CDCl_3$) (ppm) : 3.90 (6H, s, -$OCH_3$), 3.93 (6H, s, -$OCH_3$), 4.34 (2H, d, $J$=6.1 Hz, $H_{\gamma'}$), 5.03 (1H, d, $J$=8.8 Hz, $H_\alpha$), 5.40 (2H, broad, phenol-OH), 6.33 (1H, dt, $J$=15.1 Hz, 6.13 Hz, $H_{\beta'}$), 6.56 (1H, d, $J$=15.1 Hz, $H_{\alpha'}$), 6.68 (2H, s, aromatic), 6.71 (2H, s, aromatic).

***Dehydrogenative polymerization by HRP/$H_2O_2$***

Sinapyl alcohol (10 mg) was dissolved in 1 ml of dioxane/water=1/1 and 4/1 (v/v). Forty nine $\mu$l of 20 mg/ml HRP (Toyobo, 128 U/mg from horseradish) solution and 50 $\mu$l of 9.5% $H_2O_2$ solution were added to the solution. Then, the solution was stirred for 10 min under a nitrogen atmosphere and light shielding. For the experiment of pH effect, sinapyl alcohol was dissolved in 0.1 M acetate buffer (pH 4.0) or 0.1 M phosphate buffer (pH 6.5 and 8.0), and reacted in the same manner as described above. Separation and $^1$H-NMR analyses of the products were conducted by the same method described in the case of $FeCl_3$.

***Dehydrogenative polymerization in the presence of pectin***

Pectin from citrus and HRP (No. p-8000, 33 purpurogallin unit/mg) were purchased from Sigma Co. Ltd. Sinapyl alcohol was polymerized in the presence of pectin by the Zulaufverfahren as follows. Sinapyl alcohol (100 mg) was dissolved in 10 ml of 0.1 M

phosphate buffer (pH 6.5) containing 800 mg of pectin. After replacing the air by nitrogen, the solution was stirred for 24 hrs under light shielding. Then, 0.49 ml of a 0.2 mg/ml HRP solution and 0.5 ml of a 0.5% $H_2O_2$ solution were added. This mixture was stirred for 10 min at room temperature. The reaction products were extracted with ethyl acetate three times, and the combined ethyl acetate solutions were washed with saturated brine. After drying with anhydrous sodium sulfate, the solvent was evaporated, and the products were isolated from the residue and analyzed as described below.

***Isolation of products***

The products were separated by PTLC (20 cm×20 cm×2 mm) with methanol/chloroform (1/20, v/v) and *n*-hexane/ethyl acetate (1/4,v/v), successively, for developing solvents. The separated crude products were acetylated with acetic anhydride/pyridine (1/1, v/v) at room temperature for 24 hrs. The acetylated products were separated again by PTLC with *n*-hexane/ethyl acetate (1/2, v/v), and the purified product (acetylated compound-X) was obtained in an oily state. The yield was 10.8 mg. $^1$H-NMR, $^{13}$C-NMR (completely decoupling and off-resonance) and 2D-NMR ($^1$H-COSY) of products were measured in 0.5 ml of $CDCl_3$ containing 0.03% of TMS.

$^1$H-NMR ($CDCl_3$) of acetylated compound-X : 2.07 ppm (2H, s, alcoholic -$OA_c$), 2.09 ppm (3H, s, alcoholic -$OA_c$), 2.20 ppm (2H, multiplet, $H_{\delta'}$), 2.33 ppm (6H, s, phenolic -$OA_c$), 2.3–2.4 ppm (1H, multiplet, $H_\beta$), 3.83 ppm (12H, s, -$OCH_3$), 4.15 ppm (1H, dd, $J$=11.4 Hz, 4.0 Hz, $H_{\gamma 1}$), 4.32 ppm (1H, dd, $J$=11.4 Hz, 5.0 Hz, $H_{\gamma 2}$), 5.77 ppm (1H, d, $J$=15.8 Hz, $H_\alpha$), 5.96–6.07 ppm (1H, multiplet, $H_{\beta'}$), 6.22 ppm (1H, d, $J$=15.8 Hz, $H_{\alpha'}$), 6.54 ppm (2H, s, aromatic), 6.58 ppm (2H, s, aromatic).

$^{13}$C-NMR ($CDCl_3$) of acetylated compound-X : 18.8–22.2 ppm (multiplet, $CH_3$-CO-), 31.5 ppm (t, $C_{\gamma'}$), 43.3 ppm (d, $C_\beta$), 56.2 ppm (q, -$OCH_3$), 62.5 ppm (t, $C_\gamma$), 75.2 ppm (d, $C_\alpha$), 102.7 ppm (d, aromatic 2,6), 103.8 ppm (d, aromatic 2′, 6′), 127.0 ppm (d, $C_{\delta'}$), 128.1 ppm (s, aromatic 1 or 1′), 128.5 ppm (s, aromatic 1 or 1′), 132.6 ppm (d, $C_{\alpha'}$), 135.6 ppm (s, aromatic 4, 4′), 137.0 ppm (s, aromatic 3, 5), 168.6–171.1 ppm ($CH_3$-CO-).

MS spectra of samples were measured by using a Shimadzu GCMS-QP1000 with a direct injection method and 70 eV of electron intensity for the ionization.

MS m/z (relative intensity %) of acetylated compound-X : 588 ($M^+$, 7.9), 546 ($M^+$-42, 100.0), 504 (546-42, 23.9), 486 (546-60, 11.5), 444 (504-60 or 486-42, 21.0), 426 (486-60, 6.8), 384 (444-60 or 426-42, 61.0).

***Hydrogenation of compound-X***

A 10 mg sample was hydrogenated with 10 mg of palladium charcoal (Pd-C, Pd 5%) and hydrogen in 1 ml of methanol for 24 hrs. The solution was filtrated, and the filtrate was evaporated under reduced pressure. Then, the residue was analyzed by $^1$H-NMR spectrometry as described above.

$^1$H-NMR ($CDCl_3$) of hydrogenated acetylated compound-X : 1.50–1.80 ppm (2H,

multiplet, $H_{\gamma'}$), 1.50–1.80 ppm (1H, multiplet, $H_{\beta'}$), 2.02 ppm (3H, s, alcoholic -$OA_c$), 2.07 ppm (3H, s, alcoholic -$OA_c$), 2.17 ppm (1H, multiplet, $H_{\beta}$), 2.32 ppm (3H, s, phenolic -$OA_c$), 2.33 ppm (3H, s, phenolic -$OA_c$), 2.50 ppm (2H, multiplet, $H_{\alpha'}$), 3.79 ppm (6H, s, -$OCH_3$), 3.82 ppm (6H, s, -$OCH_3$), 4.12 ppm (1H, dd, $J$=13.5 Hz, 4.4 Hz, $H_{\gamma 1}$), 4.28 ppm (1H, dd, $J$=13.5 Hz, 4.8 Hz, $H_{\gamma 2}$), 5.73 ppm (1H, d, $J$=8.1 Hz, Ha), 6.37 ppm (2H, s, aromatic), 6.55 ppm (2H, s, aromatic).

***Reaction without HRP/$H_2O_2$***

Under the same conditions as described in Section of dehydrogenative polymerization in the presence of pectin, sinapyl alcohol was reacted in 0.1 M citric buffer (pH 3.2) without HRP/$H_2O_2$ and pectin. The reaction mixture was extracted with ethyl acetate, and the products were analyzed by $^1$H-NMR.

## II-1.3 Results and discussion

***Dehydrogenative polymerization by $FeCl_3$***

Figure 17 shows the $^1$H-NMR spectra of reaction products by the dehydrogenative polymerization of sinapyl alcohol with $FeCl_3$ in various mixed solvents. The whole spectrum shows the products obtained in aqueous solutions without dioxane, and the partial spectra show those in mixed solutions of dioxane/water (1/1, v/v) and in dioxane. Two dimers were obtained, that is, syringylglycerol-$\beta$-sinapyl alcohol ether and *d, l*-

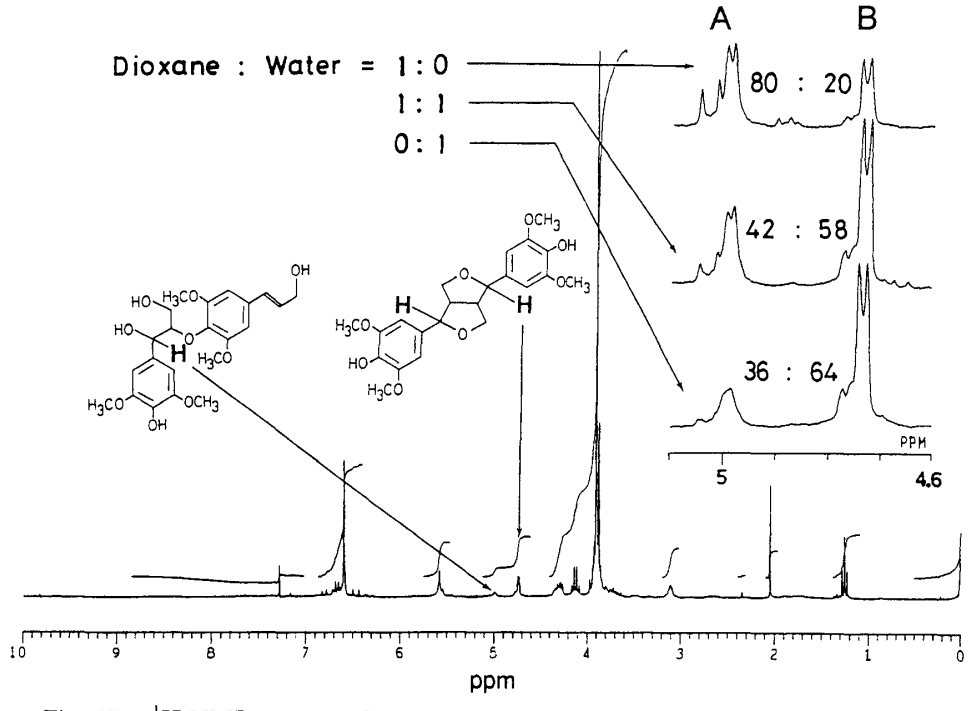


Fig. 17. $^1$H-NMR spectra of reaction products from sinapyl alcohol by $FeCl_3$ in dioxane/water solution. Syringylglycerol-$\beta$-sinapyl alcohol ether (A) was predominantly produced in dioxane, whereas syringaresinol (B) was mainly produced in water.

syringaresinol. Two $\alpha$-methine protons of syringaresinol appeared at 4.73 ppm as one doublet peak, and one proton of syringylglycerol-$\beta$-sinapyl alcohol ether did so at 4.99 ppm and 5.03 ppm as two doublet peaks which consisted of *erythro* and *threo* forms. The yields of these dimeric compounds were determined by the areas of their $\alpha$-methine peaks[136]. Syringylglycerol-$\beta$-sinapyl alcohol ether was produced in an 80% yield in dioxane whereas it was only 36% in water. Therefore, the proportion of $\beta$-ether type dimer was increased with increases of the dioxane content in the solvent. Under any conditions, the *erythro* isomer of the $\beta$-*O*-4 type dimer was produced more than the *threo* isomer.

Figure 18 shows the effect of pH of the reaction medium. The $\beta$-*O*-4 type dimer was produced at 13, 22, 47, and 54% at pH=8.0, 6.5, 4.0, and 2.0, respectively. This indicates that the ratio of $\beta$-ether increased with decreases of the pH of the reaction medium.

From the results of the experiments in various reaction media, it was found that the polarity and acidity affected the radical coupling of sinapyl alcohol. Most effective factor was thought to be the electronic effects of the substituent groups, especially the methoxyl groups. In a polar solvent (water), the electron density at the $\beta$-position carbon ($C_\beta$) of the sinapyl alcohol radical was increased by the E-effect (electron release) of the methoxyl groups, and the radicals were coupled to give *d*, *l*-syringaresinol. On the other hand, in a

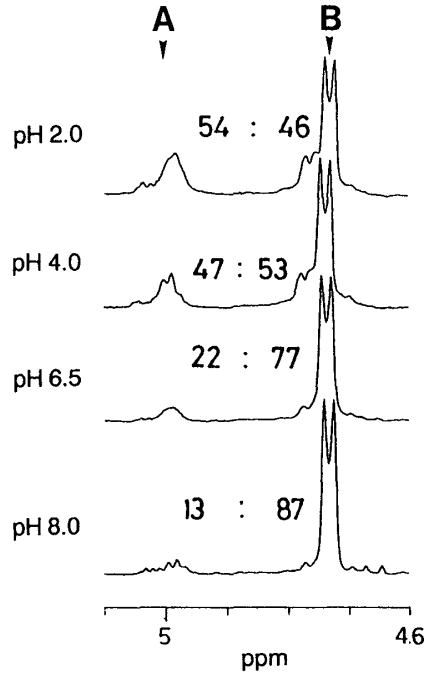


Fig. 18. $^1$H-NMR spectra of reaction products from sinapyl alcohol by $FeCl_3$ in various pH solutions. $\alpha$-Methine peaks of *erythro* and *threo* forms of syringylglycerol-$\beta$-sinapyl alcohol ether appeared at 4.99 ppm and 5.03 ppm, respectively (A) ; an $\alpha$-methine proton of syringaresinol gave a doublet peak at 4.73 ppm (B). The yield of syringylglycerol-$\beta$-sinapyl alcohol ether was increased with increases of the acidity of the solution.

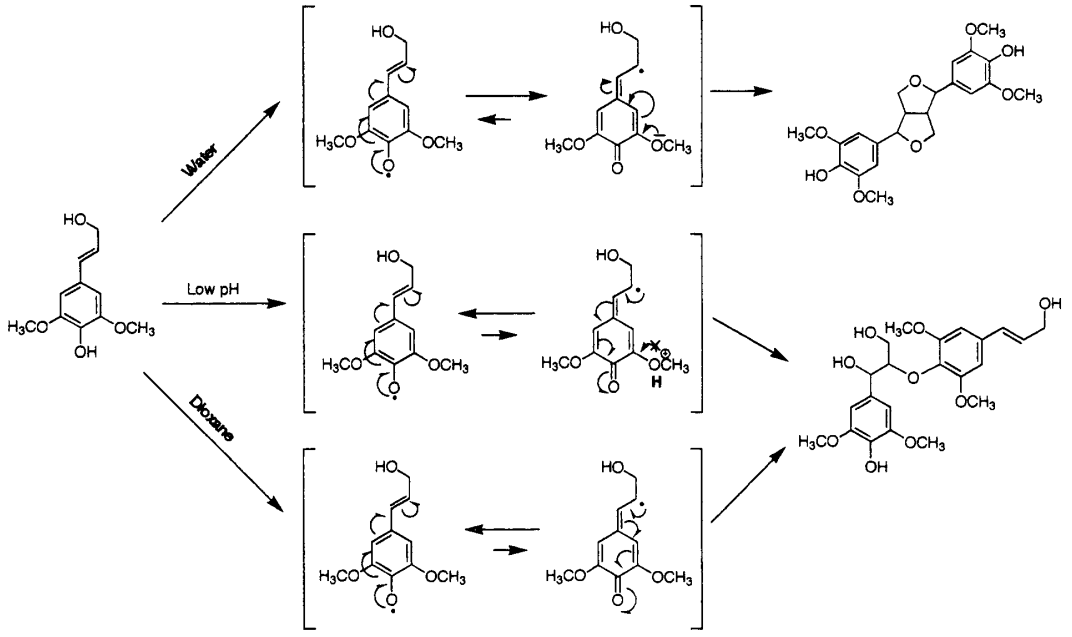


Fig. 19. Reaction mechanism of sinapyl alcohol radicals in various solutions. In water, the $\beta$-radical is stabilized by the E-effect (electron release) of methoxyl groups. In an acidic solution, the E-effect is surpressed by the protonation of a methoxyl group, which results in a stabilization of the phenoxy radical. The phenoxy radical is also stabilized by the I-effect (electron induction) in an non-polar solvent.

non-polar solvent (dioxane), the mesomeric effect of lone pairs of methoxyl groups were limited and the E-effect was suppressed by the I-effect (electron attracting) of the methoxyl groups resulting in a decrease of the electron density of $C_\beta$. Therefore, the electron density of phenoxyl oxygen was more than that of $C_\beta$, the yield of the $\beta$-*O*-4 type dimer increased in a non-polar solvent. Furthermore, the E-effect was also suppressed by the addition of a proton to a lone pair of the methoxyl oxygen at a low pH, and the $\beta$-*O*-4 type dimer was produced predominantly by the same effect in dioxane solution. The schematic reaction diagram is shown in Fig. 19.

***Difference between enzymatic and inorganic oxidants***

Sinapyl alcohol was reacted by HRP/$H_2O_2$ in a dioxane/water solution in the same manner as $FeCl_3$. The NMR spectra of products are shown in Fig. 20. Because HRP was inactive in 100% dioxane, the reaction was conducted in dioxane/water (1/1 and 4/1, v/v) solutions. As a results, syringaresinol was mainly produced in both reaction media. Furthermore, only syringaresinol was produced at any pH (Fig. 21). Therefore, in a HRP/$H_2O_2$ system, the sinapyl alcohol radical was hardly effected by the electron effect of the solvent used, and a $\beta$-$\beta$ coupling preferentially occurred because of the greater electron density at the $C_\beta$-position of the radical by the E-effects of methoxyl groups.

These results suggested that the oxidation mechanism of sinapyl alcohol is different between enzymatic and inorganic oxidants. The hydration shell of the enzyme molecule is

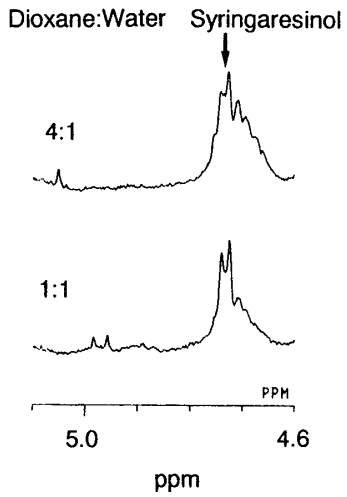


Fig. 20. $^1$H-NMR spectra of reaction products from sinapyl alcohol by peroxidase/ $H_2O_2$ in 1, 4-dioxane/water.

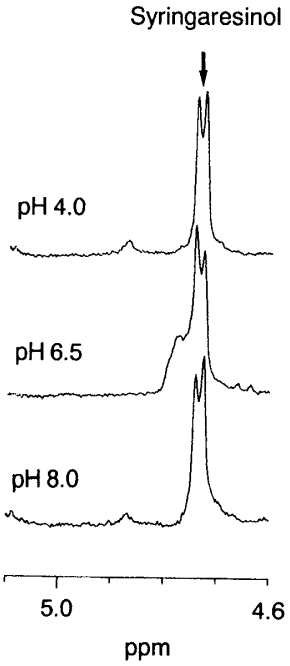


Fig. 21. $^1$H-NMR spectra of reaction products from sinapyl alcohol by peroxidase/ $H_2O_2$ in various pHs.

necessary for maintaining the native conformation in organic solvents. The catalytic properties of an enzyme are retained if the hydrated enzyme and the organic solvents have separated locations in the reaction system, that is, direct contact between them is prevented[53]. Therefore, the reaction was considered to proceed in the hydration shell of the peroxidase molecule when the HRP/$H_2O_2$ was used as an oxidant in dioxane. As a result, dioxane did not affect the methoxyl groups of sinapyl alcohol, and then mainly syringaresinol was produced. On the other hand, dioxane was considered to affect the methoxyl groups of sinapyl alcohol when $FeCl_3$ was used as an oxidant, because the hydration shell was not necessary for its catalytic reaction.

Furthermore, it was considered that the dehydrogenative polymerizations of monolignols proceeded in the same manner as in the case of $FeCl_3$ because lignin in the plant cell-walls consists of large amounts of the *β-O*-4 linkages. Westermark has hypothesized that the enzyme catalyzed an oxidation of a small molecule which could act as a radical carrier, and the radical carrier could move in the cell-wall and then oxidize monolignols[137]. Because, it is difficult to imagine that an enzyme whose diameter is 5.5–6.0 nm can penetrate into the thin lamella of cell-walls, a phenol-oxidizing enzyme, such as peroxidase, was generally assumed to require direct contact with substrates. If her hypothesis should be correct and the radical carrier should exist, the yield of *β-O*-4 dimer would be increased by the effect of the reaction medium on the dehydrogenative polymerization. In the case of using $FeCl_3$ as an oxidant, Fe (III) would work as a radical carrier, and the *β-O*-4 type

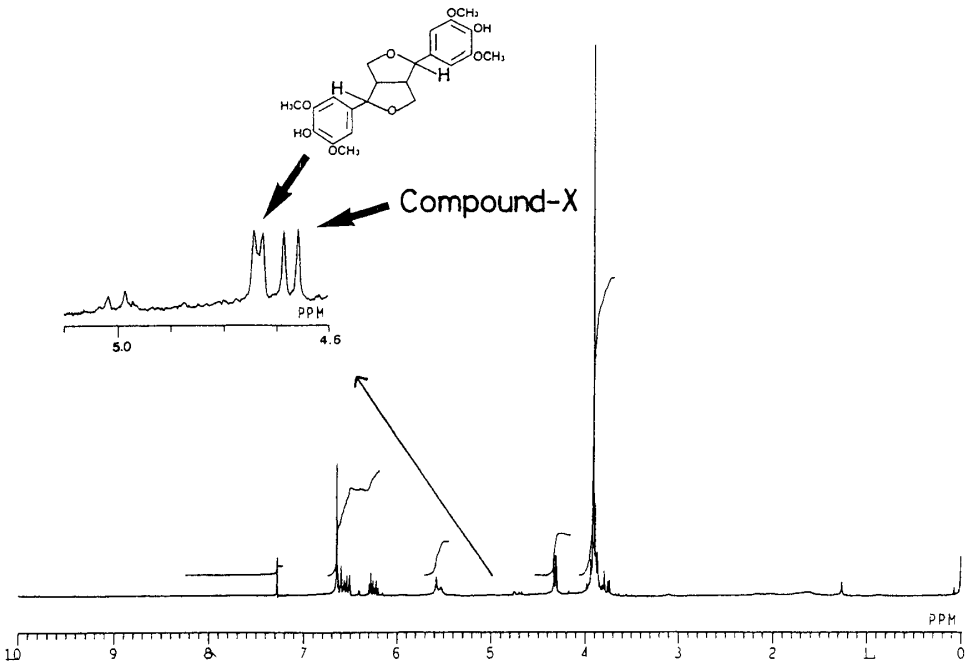


Fig. 22. $^{1}$H-NMR spectrum of reaction mixture reacted in the presence of pectin.

dimer mainly would be generated because Fe (III) freely can approach sinapyl alcohol molecules.

***Reaction products in the presence of pectin***

Figure 22 shows the $^{1}$H-NMR spectrum of the reaction mixture from sinapyl alcohol dehydrogenated in the presence of pectin (pH 6.5). $\alpha$-Methine protons appeared at around 4.75 ppm and 4.65 ppm as doublet peaks. The former was due to syringaresinol and corresponded to two protons (2H), while the latter was due to an unknown compound. The unknown compound was designated as compound-X in this article. The yield of compound-X could not be neglected because of approximately the same area of $\alpha$-methine peaks of both compounds.

Identification of compound-X (acetate) was carried out using $^{1}$H-NMR (Fig. 23), 2D-NMR ($^{1}$H-COSY), $^{13}$C-NMR and MS spectroscopy (data not shown). The analysis was also carried out using hydogenated acetylated compound-X. These results indicated that the compound-X is a $\beta$-$\gamma$ type dimer whose chemical structure is 1, 5-di (3, 5-dimethoxy-4-hydroxyphenyl)-4-hydroxymethyl-5-hydroxy-1-pentene (Fig. 23).

***Construction mechanism of $\beta$-$\gamma$ type dimer***

To reveal the construction mechanism of the $\beta$-$\gamma$ type dimer in the presence of pectin, sinapyl alcohol was reacted without HRP/$H_2O_2$ in the presence of pectin, and without both HRP/$H_2O_2$ and pectin. As shown in Fig. 24, sinapyl alcohol gave $\beta$-$\gamma$ type dimer in the presence of pectin without HRP/$H_2O_2$, and a very small amount of syringaresinol without both HRP/$H_2O_2$ and pectin. Furthermore, the $\beta$-$\gamma$ type dimer was constructed in a buffer

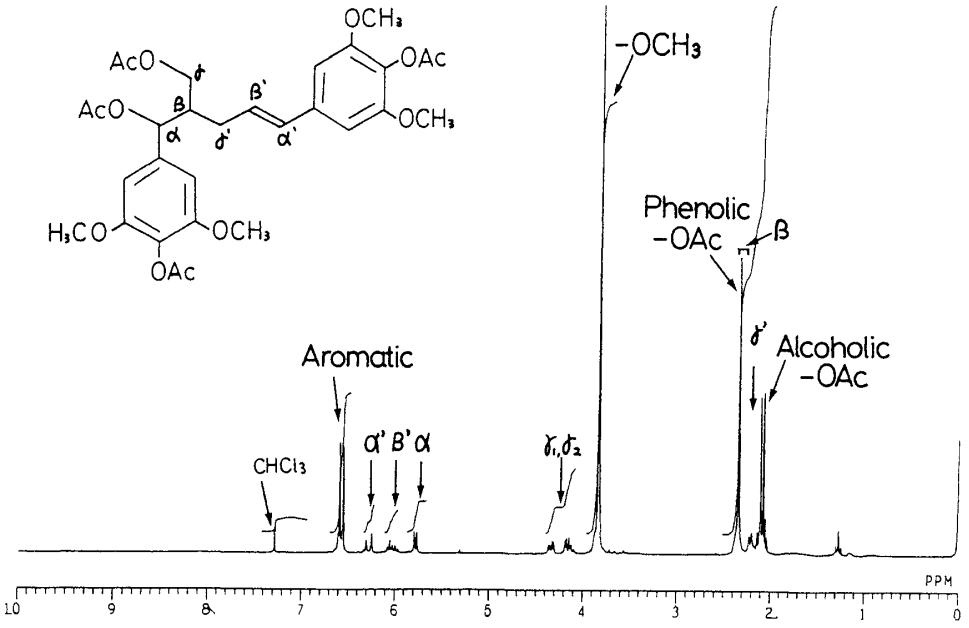


Fig. 23. $^1$H-NMR spectrum of acetylated compound-X.

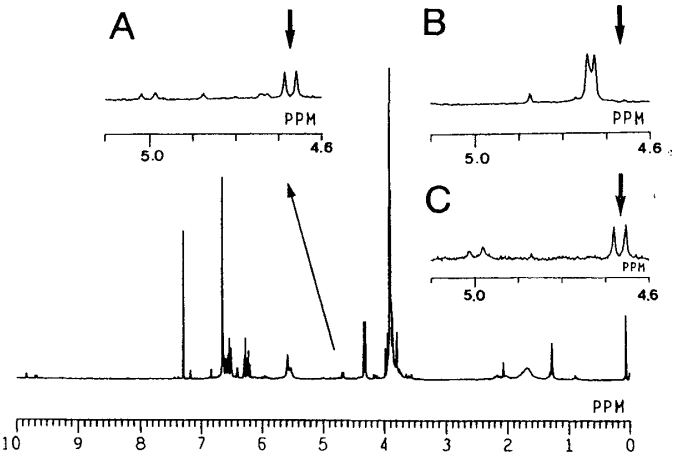


Fig. 24. $^1$H-NMR spectrum of the reaction mixture reacted in the presence of pectin without addition of peroxidase/$H_2O_2$. A, zoomed spectrum from 4.6 to 5.1 ppm ; B, zoomed spectrum of reaction products reacted without addition of both peroxidase/$H_2O_2$ and pectin ; C, zoomed spectrum of reaction products reacted in acidic solution (pH 3.2).

solution (pH 3.2) without both HRP/$H_2O_2$ and pectin. These results indicate that the enzyme did not participated in the formation of the $\beta$-$\gamma$ type dimer.

Aminoff and others reported that coniferyl alcohol, which is one of the lignin degradation products in kraft cooking, was reacted to guaiacyl type $\beta$-$\gamma$ dimer in an ionic reaction in alkali *via* the extended-quinonemethide intermediate[138]. Similarly, Lee and others reported that the $\beta$-$\gamma$ type dimer was produced from coniferyl alcohol in the mechanochemical reaction[139]. Therefore, in the pectin solution reported herein, it was

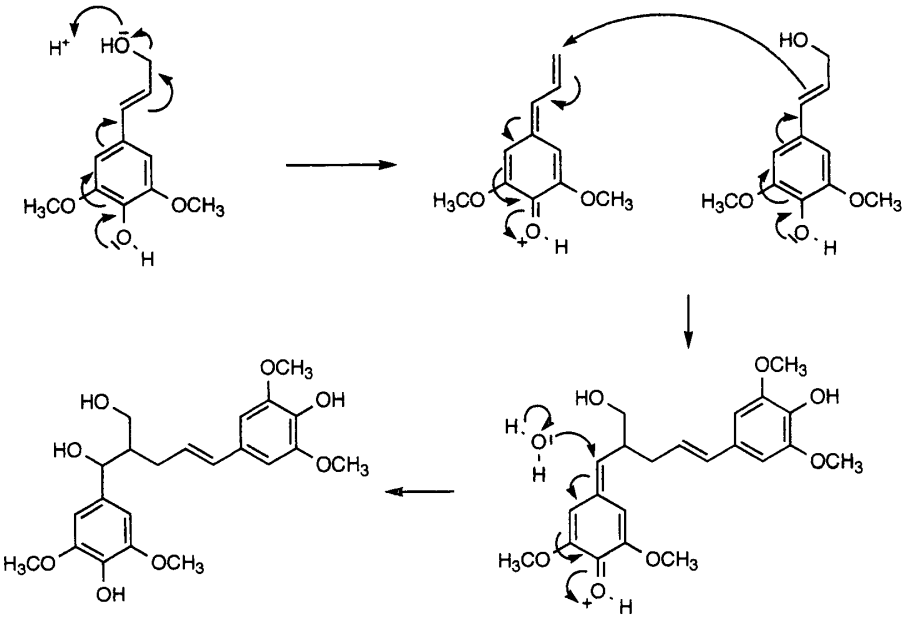

Fig. 25. Assumed reaction mechanism of sinapyl alcohol in a pectin solution.

assumed that the $\beta$-$\gamma$ type dimer was generated *via* the extended-quinonemethide which was produced by dehydroxylation from the $\gamma$-position of sinapyl alcohol in the acidic solution. The assumed schematic diagram of the reaction mechanism is shown in Fig. 25.

It is widely known that monolignols are polymerized by the enzymatic coupling and the non-enzymatic addition to quinonemethide intermediates in carbohydrate matrix gel[133]. Because a pectin solution is very viscous due to the high-molecular weight, sinapyl alcohol seems to be hardly in contact with the enzyme and $H_2O_2$, and to be hardly polymerized in a pectin solution. In such a solution, an ionic reaction would be preferred to a radical reaction. Consequently, the $\beta$-$\gamma$ type dimer was more producible than the syringaresinol produced by a radical coupling.

Aminoff *et al.* and Lee *et al.* have suggested that the $\beta$-$\gamma$ type dimer was an artifact of lignin degradation products[138,139]. However, the existence of the $\beta$-$\gamma$ type structure in a native lignin cannot be disclaimed, because, as demonstrated in this work, the $\beta$-$\gamma$ type dimer was produced predominantly from sinapyl alcohol during dehydrogenative polymerization in the pectin solution which was used as a model for the middle lamellae of a cell wall. If the $\beta$-$\gamma$ type structure exists in a native lignin, the possibility cannot be denied that the compound is not only produced as an artifact but also remains as an unchanged structure in the lignin degradation progress. Therefore, it is necessary to confirm the existence of a $\beta$-$\gamma$ type linkage in a native lignin by an analysis of milled wood lignin, especially that from middle lamella.

Furthermore, a doublet peak appeared at around 5.0 ppm in the spectrum (Fig. 22). Generally, $\alpha$-methine peaks of *erythro* and *threo* isomers of $\beta$-*O*-4 type dimer appear at around

4.99 ppm ($J$=3.7 Hz) and 5.03 ppm ($J$=8.8 Hz), respectively, as a doublet peak corresponded to 1H. However, this peak may not be due to $\beta$-$O$-4 type dimer because the chemical shift and coupling constant of this peak are different from those of syringylglycerol-$\beta$-sinapyl alcohol ether (5.00 ppm, $J$=8.8 Hz). Regretfully, this unknown compound (compound-Y) could not be isolated and identified. We will report the chemical structure of compound-Y in the next paper.

## II-2 Preparation of synthetic lignin by ligninolytic enzyme in organic solvents

### II-2.1 Introduction

In reaction systems containing organic solvents using Fe (III) as an oxidant, sinapyl alcohol was oxidized to $\beta$-$O$-4 type dimer, whereas only syringaresinol was detected in reaction mixtures using HRP/$H_2O_2$ instead of Fe (III), as described in Section II-1. This indicated that there are differences between oxidation of sinapyl alcohol with enzymatic and inorganic oxidants. As $\beta$-$O$-4 type linkage is predominant in native lignin, the dehydrogenative polymerization of monolignols was considered to proceed in the same manner as that catalyzed by Fe (III). However, polymerization of lignin precursors to lignin has been shown to be catalyzed by peroxidase/$H_2O_2$ in plant cell walls[140]. Therefore, a radical carrier which functions similarly to Fe (III) may function in the dehydrogenative polymerization catalyzed by peroxidase/$H_2O_2$.

MnP is known to oxidize Mn (II) to Mn (III) which acts as a mediator and further oxidizes aromatic substrates[141]. It is, therefore, reasonable to use MnP for the study of dehydrogenative polymerization to prepare $\beta$-$O$-4 rich DHP which more closely approximates the structure of native lignin.

This session describes synthesis and characterization of DHP using MnP of *B. adusta* in reaction mixtures containing organic solvents[142].

### II-2.2 Materials and methods

***Production and purification of MnP***

MnP of *B. adusta* was purified as described in Section I-4.2. The purified MnP had a pI of 4.1 and a molecular weight of 40,000.

***Measurement of MnP and HRP activity in organic solvents***

The activities of MnP and HRP (Peroxidase 1-C, Toyobo) in organic solvents were measured using guaiacol and 2, 6-dimethoxyphenol as substrates as described in Section I-4.2. In the experiment using HRP, $MnSO_4$ was omitted from reaction mixtures.

***Preparation of DHP from monolignols***

Coniferyl and sinapyl alcohols were synthesized by $LiAlH_4$ reduction of acetyl ethylferulate and ethylsinapate, respectively, by the method of Freudenberg and Hübner[114], and crystallized from *n*-hexane/ether solution.

DHP was prepared by the Zutropfverfahren. Under a nitrogen atmosphere, 20 ml of

70% acetone solution containing 20 mg of monolignol and 0.03% of $H_2O_2$ solution in 70% acetone solution were added dropwise to 20 ml of a solution containing 0.1 mg of MnP or HRP, 50 mM lactate buffer (pH 4.5), 0.2 mM $MnSO_4$ and 70% acetone over a period of 20 hrs and the reaction was continued more for a further 5 hrs. In the experiment using HRP, $MnSO_4$ was omitted from reaction mixtures. MnP or HRP (0.1 mg) was added every 12 hrs. After 25 hrs, acetone was removed by evaporation under reduced pressure. The residue was suspended in 50 ml of water and centrifuged at 3,600 rpm at room temperature for 15 min. The precipitate was resuspended in water and centrifuged under the same conditions. This operation was repeated five times. The precipitate thus obtained was dissolved in DMF and centrifuged at 3,600 rpm at room temperature for 20 min to remove the insoluble DHP. The DMF-soluble fraction was used for the subsequent experiments. The water-soluble fraction was extracted with ethyl acetate five times. The ethyl acetate solution wded in 50 ml of water and centrifuged at 3,600 rpm at room temperature for 15 min. The precipitate was resuspended in water and centrifuged under the same conditions. This operation was repeated five times. The precipitate thus obtained was dissolved in DMF and centrifuged at 3,600 rpm at room temperature for 20 min to remove the insoluble DHP. The DMF-soluble fraction was used for the subsequent experiments. The water-soluble fraction was extracted with ethyl acetate five times. The ethyl acetate solution was washed with saturated NaCl, dried over anhydrous $Na_2SO_4$ and evaporated to dryness.

Preparation of DHP was also carried out using distilled water instead of 70% acetone.

***Gel-permeation chromatography***

Gel-permeation chromatography was performed at 50°C using a Shodex GPC KD-803 column. Elution was carried out at a flow rate of 0.5 ml/min using DMF containing 0.05 M LiCl as the eluent was monitored by determining UV absorption at 280 nm. Calibration curves were obtained using polystyrene standards.

Table 7. Yield of DHP formed from monoliggnols by MnP of *B. adusta*.

| Enzyme | DHP | Synthesis medium | Monomer[a] conversion (%) | DMF-soluble (mg) | DMF-insoluble (mg) |
|---|---|---|---|---|---|
| MnP | c-DHP | Acetone | 90.0 | 17.7 ( 98.3%) | 0.3 ( 1.7%) |
| | | Water | 99.5 | 18.9 ( 99.5%) | 0.1 ( 0.5%) |
| | s-DHP | Acetone | 49.0 | 8.7 ( 88.8%) | 1.1 (11.2%) |
| | | Water | 29.5 | 5.9 (100.0%) | 0.0 ( 0.0%) |
| HRP | c-DHP | Acetone | 38.1 | 7.3 ( 94.8%) | 0.4 ( 5.2%) |
| | | Water | 65.2 | 13.9 (100.0%) | 0.0 ( 0.0%) |
| | s-DHP | Acetone | 0.0 | 0.0 ( 0.0%) | 0.0 ( 0.0%) |
| | | Water | 16.0 | 1.9 (57.6%) | 1.4 (42.4%) |

a) Calculated as water-insoluble fraction.

***Analysis by NMR spectrometry***

$^1$H-NMR spectra of acetylated DMF soluble fraction were measured using a JEOL FT-NMR JNM-LA 400 MK spectrometer (400 MHz) with TMS as an internal standard. Acetylated DMF-soluble DHP was prepared by removal of DMF from the DMF-soluble fraction under high vacuum, and the residue was acetylated with acetic anhydride and pyridine (1/1, v/v) for 24 hrs at room temperature.

**II-2.3 Results and discussion**

***Dehydrogenative polymerization of coniferyl and sinapyl alcohols by MnP or HRP***

Yields of DHP formed from monolignols by MnP of *B. adusta* and HRP are shown in Table 7. In dehydrogenative polymerization of coniferyl alcohol by MnP in a solution without acetone, the monomer conversion was greater than 99%. In 70% acetone solution, 90% of coniferyl alcohol was converted to water-insoluble DHP. Coniferyl alcohol-DHP (c-DHP) prepared by MnP in water did not completely dissolve in DMF, and the yield of DMF-insoluble polymer was estimated as 0.5%. Similarly, the DMF-insoluble part of c-DHP prepared in 70% acetone solution was obtained in a 1.7% yield.

Coniferyl alcohol was also polymerized by HRP in the media with and without acetone. However, yield of water-insoluble polymer prepared HRP was lower than that prepared by MnP.

Forty-nine and 30% of sinapyl alcohol were converted to DHP in the solutions with and without acetone, respectively. Sinapyl alcohol-DHP (s-DHP) prepared by MnP in the solution without acetone dissolved completely in DMF, while a small amount of precipitate was observed in the case of s-DHP prepared in 70% acetone solution (11.2%). However, no water-insoluble product was obtained from sinapyl alcohol by the action of HRP in reaction mixtures containing 70% acetone. Even in water, only 16% water-insoluble material was obtained from sinapyl alcohol by HRP. Control reactions lacking MnP produced no water-insoluble material from coniferyl or sinapyl alcohols.

Specific activities of MnP and HRP in the presence of 70% acetone were investigated using guaiacol and 2, 6-dimethoxyphenol as substrates. The activities of HRP for both substrates were marked decreased in aqueous 70% acetone medium. However, MnP retained 26% and 73% of the original for guaiacol and 2, 6-dimethoxyphenol oxidation activities, respectively, in the presence of 70% acetone (Table 8).

Water-soluble products of DHPs were analyzed by $^1$H-NMR spectroscopy. In the spectrum of the water-soluble fraction prepared from sinapyl alcohol by MnP, the $\alpha$-methine proton of syringylglycerol-$\beta$-sinapyl alcohol ether (4.97 ppm) and *d, l*-syringaresinol (4.74 ppm) were observed. Production of 2, 6-dimethoxy-*p*-benzoquinone was also observed (3.82 ppm, -$OCH_3$ ; 5.86 ppm, aromatic H). The ratios of these three compounds were 37, 24 and 39%, respectively, in the product prepared in reaction mixtures containing 70% acetone, whereas 2, 6-dimethoxy-*p*-benzoquinone was mainly detected in that from solutions

Table 8. Activity of HRP and MnP of *B. adusta*.

| Reaction media | Specific activity (U/mg) | | | |
|---|---|---|---|---|
| | HRP | | MnP | |
| | Guaiacol | 2, 6-DMP | Gualacol | 2, 6-DMP |
| Buffer | 2,894 | 4,628 | 231 | 1,065 |
| 70% Acetone | 21 | 13 | 61 | 779 |

2, 6-DMP: 2, 6-dimethoxyphenol.

without acetone. The water-soluble fraction obtained from coniferyl alcohol by MnP in both water and aqueous 70% acetone medium was not investigated because yields of the both recovered water-soluble materials were less than 10%. Three $\alpha$-methine protons of phenylcoumaran (5.58 ppm), guaiacylglycerol-$\beta$-coniferyl alcohol ether (4.96 ppm) and *d*, *l*-pinoresinol (4.73 ppm) were detected in the water-soluble fraction prepared from coniferyl alcohol by HRP in aqueous 70% acetone medium. The yields of these three compounds were 48, 38 and 14%, respectively. Similarly, these compounds were also detected in the

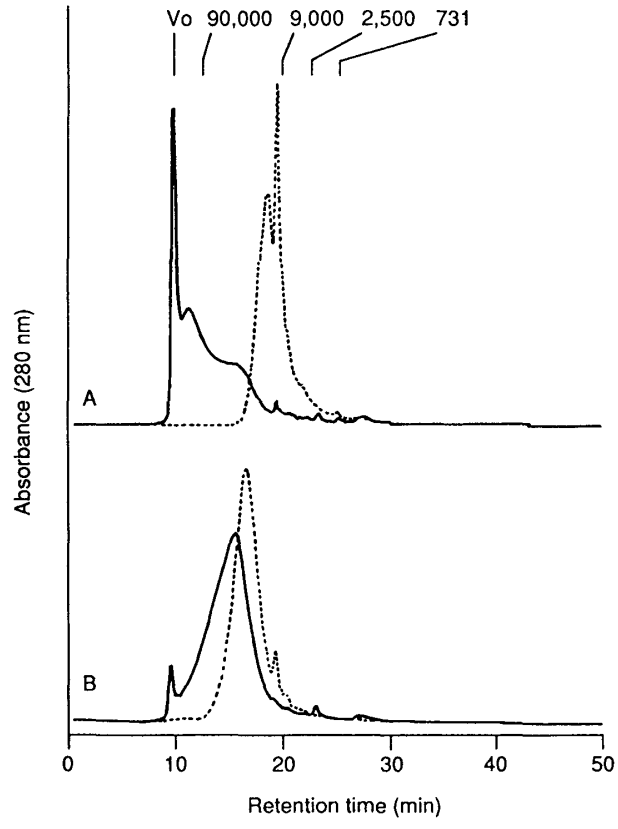


Fig. 26. Gel-permeation chromatograms of c-DHP prepared by MnP of *B. adusta* and HRP. The c-DHPs prepared by MnP (——) and HRP (·····) in aqueous 70% acetone medium (A) and in water (B) were analyzed on a Shodex GPC KD-803 column at 50°C. Elution was carried out at a flow rate of 0.5 ml/min using DMF containing 0.05 M LiCl as an eluent and monitored at 280 nm.

product prepared in water, and the yields of these three dimers were 38, 34 and 28%, respectively. However, coniferyl alcohol was not detected in this fraction, indicating that the reaction was completed. Sinapyl alcohol yielded syringaresinol (68%) and 2,6-dimethoxy-*p*-benzoquinone (32%) as water-soluble materials following reaction with HRP in water, whereas sinapyl alcohol and trace syringaresinol was observed in the production prepared with HRP in 70% acetone solution.

***Molecular weight of DHP***

Gel-permeation chromatograms to determine the molecular weight of DMF-soluble c- and s-DHPs are shown in Fig. 26 and 27, respectively. Preliminary experiments showed that Sephadex LH-60 is not suitable for measurement of the molecular weights of these DHPs, because all DHPs eluted with the void volume. Therefore, a Shodex GPC KD-803 column was used in this study. Molecular weights of c- and s-DHPs formed by MnP were higher than those by HRP. c-DHP prepared by MnP in 70% acetone solution was eluted with the void volume, indicating a molecular weight of >90,000. Both c- and s-DHPs prepared by MnP in acetone solution had higher molecular weights than those in water.

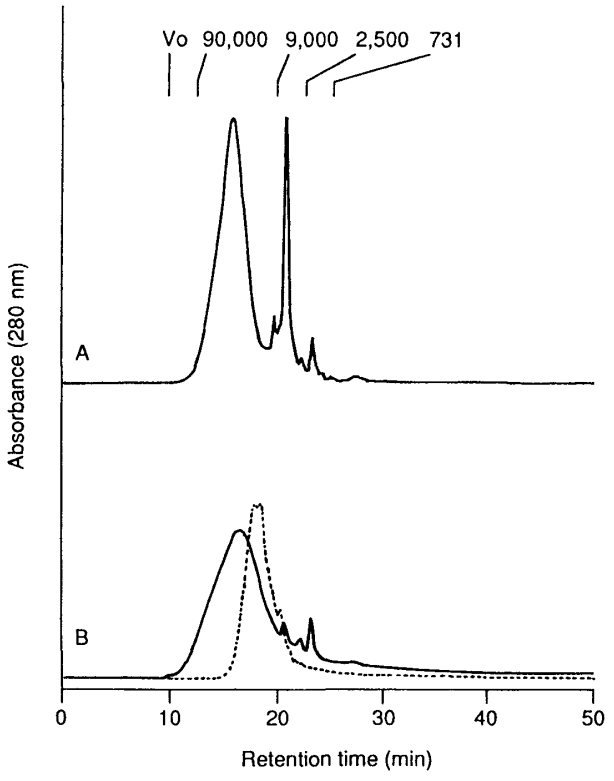


Fig. 27. Gel-permeation chromatograms of s-DHP prepared by MnP of *B. adusta* and HRP. The s-DHPs prepared by MnP (——) and HRP (·····) in aqueous 70% acetone medium (A) and in water (B) were analyzed on a Shodex GPC KD-803 column at 50°C. Elution was carried out at a flow rate of 0.5 ml/min using DMF containing 0.05 M LiCl as an eluent and monitored at 280 nm.

However, the opposite results were observed with HRP as an oxidant. This was thought to be due to the decrease in activity of HRP in the reaction mixtures containing acetone. The stability of MnP in 70% acetone solution in addition to the increase in solubility of DHP in the presence of acetone are thought to be important factors to promoting further polymerization.

***Chemical structure of DHP***

The structures of DHPs were examined as acetate derivatives in $CDCl_3$ solution by $^1$H-NMR spectroscopy (Fig. 28, 29). The peaks in the spectra were assigned by comparison with data for DHP and milled wood lignin (MWL)[143]. Each spectrum is similar to those of DHP and MWL reported previously. In the spectrum of c-DHP prepared by MnP in solution without acetone, the peak at 5.48 ppm was due to Ha in the *β*-5 structure (Fig. 28B). However, this signal was weak in the spectrum of c-DHP prepared in reaction mixtures containing 70% acetone, indicating that the contents of *β*-5 type structure is low (Fig. 28A). Signals which could be attributed to *β*-*O*-4 structures were detected at around

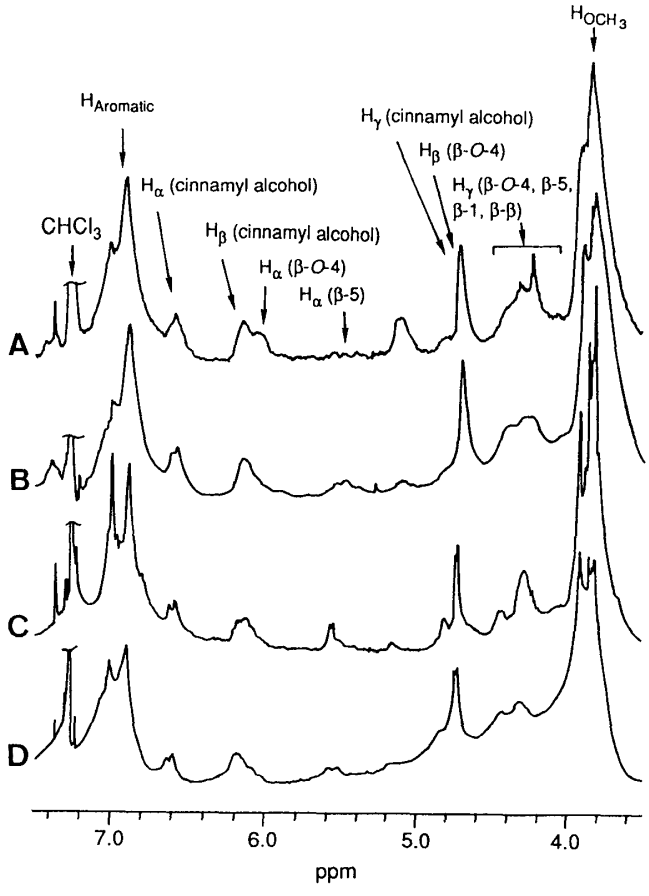


Fig. 28. $^1$H-NMR spectra of acetylated c-DHP prepared by MnP of *B. adusta* and HRP. A, c-DHP prepared by MnP in aqueous 70% acetone medium; B, c-DHP prepared by MnP in water; C, c-DHP prepared by HRP in aqueous 70% acetone medium; D, c-DHP prepared by HRP in water.

6.05 ppm as a shoulder peak. This signal was also intense in the spectrum of c-DHP formed in acetone solution (Fig. 26A). In spruce lignin, the contents of $\beta$-5 type structure have been estimated to be ca. 10%[6], whereas conventional prepared c-DHP contains 45% of $\beta$-5 and 27% of $\beta$-*O*-4 structures[144]. These results suggested that DHP formed by MnP in the presence of organic solvents is similar to the native lignin. Similarly, intense $\beta$-*O*-4 signals were also observed in the spectrum of s-DHP prepared by MnP in 70% acetone solution (Fig. 29A). However, the signal around 6.00 ppm was weak in the spectrum of s-DHP prepared by HRP in water, indicating that the $\beta$-*O*-4 content of this DHP is low (Fig. 29C).

MnP is known to oxidize Mn (II) to Mn (III) which catalyze the further oxidation of aromatic compounds[141]. Oxidation of monolignols mediated by Mn (III) probably leads to the formation of $\beta$-*O*-4 structures in the presence of organic solvents, as discussed

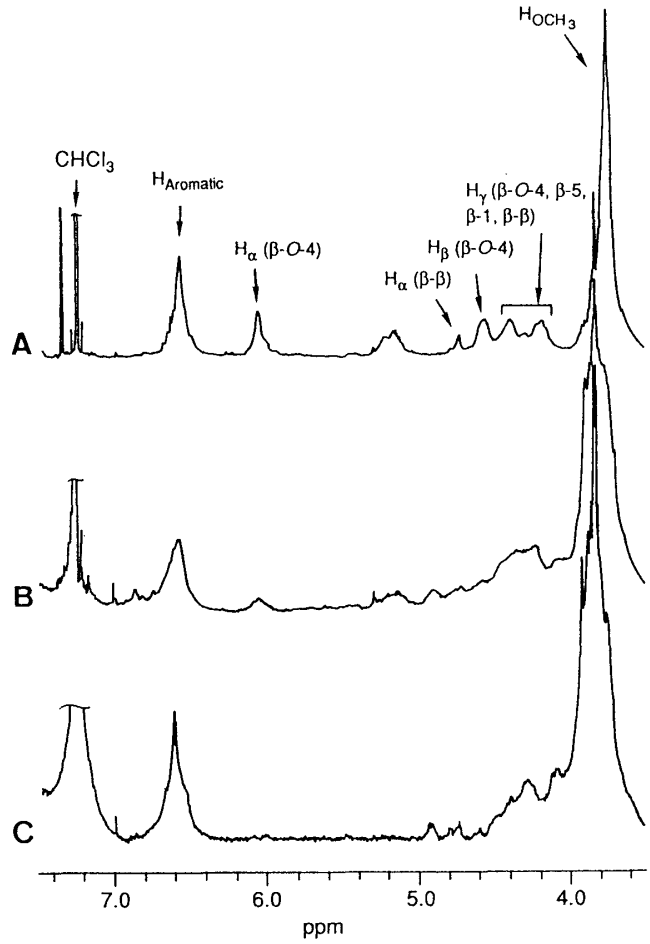


Fig. 29. $^1$H-NMR spectra of acetylated s-DHP prepared by MnP of *B. adusta* and HRP. A, s-DHP prepared by MnP in aqueous 70% acetone medium; B, s-DHP prepared by MnP in water; C, s-DHP prepared by HRP in water. Water-insoluble polymer was not formed by HRP in aqueous 70% acetone medium.

previously[116,129,135]. In addition, the presence of organic solvent improving poor solubility of the formed oligomer lignin would result in further polymerization, as discussed for polymerization of phenols by HRP[58]. Thus, DHP with high molecular weight and *β-O*-4 content could be prepared by MnP in medium containing 70% acetone.

## Conclusions

For various applications of the ligninolytic enzymes produced by white-rot fungi, the characterization of these enzymes and the development of new reaction systems, especially their reactions in organic solvents and large-scale production for industrial applications were investigated. This study focused on the development of reaction syntems for the transformation and preparation of lignin and its related compounds by the ligninolytic enzymes of white-rot fungi.

In Section I, the reaction of the ligninolytic enzymes in organic solvents was attempted to degrade lignin in the reaction mixture containing organic solvents. LiP of *P. chrysosporium* catalyzed the oxidation various phenolics and aromatic amines in the reaction mixture containing water-miscible organic solvents such as ethylene glycol, methylcellosolve, diethylene glycol and acetone. Especially, LiP exhibited high 3, 3′-dimethoxybenzidine oxidation activity in 70% aqueous ethylene glycol medium. In addition, this effective oxidation of 3, 3′-dimethoxybenzidine by LiP was suggested to due to differences between the oxidation intermediates in water and in 70% aqueous ethylene glycol medium.

Effects of water-miscible organic solvents on the reaction of LiP were also investigated to elucidate the optimum reaction condition. Interestingly, LiP activity in water-miscible organic solvents was correlated with the Dimroth-Reichardt parameter ($E_T$ (30)) of the solvents, which is directly related to free energy of the solvation process. In addition, absorption spectra of LiP in several glycols were similar to those in succinate buffer. These results suggest that these glycols do not disturb the conformation around the active center, heme, of LiP. Furthermore, LiP was found to effectively oxidize the aromatic compounds which have low ionization potential and high hydrophobicity.

In addition, chemical modification of LiP was attempted to increase the activity of LiP in organic solvents. The modified LiPs with acetic and benzoic acids and MPSS had higher 3, 3′-dimethoxybenzidine oxidation activity than the native LiP in aqueous 70% water-miscible organic solutions including ethylene glycol and diethylene glycol. Furthermore, the activity of LiP modified with aliphatic acid was found to depend on the viscosity of the reaction system containing 70% water-miscible organic solvents, suggesting that the viscosity of the reaction system containing water-miscible organic solvents is important for the activity of LiP modified with aliphatic acids.

Finally, degradation of water-insoluble highly polymerized synthetic lignin by the

ligninolytic enzymes was examined using reaction systems containing organic solvents. MnP of *B. adusta* catalyzed the oxidation of the synthetic lignin in reaction mixtures containing both 60% acetone and 10% methylcellosolve in the presence of $H_2O_2$. GPC analysis showed that the molecular weight of c-DHP increased whereas that of s-DHP decreased with a small amount of polymerized compounds. Furthermore, the repolymerization of s-DHP was suppressed by the addition of $H_2O_2$ and MnP at long intervals. Similarly, addition of ascorbic acid to the reaction mixture containing acetone was also found to result in preferential depolymerization of s-DHP.

In Section II, synthesis of lignin by the ligninolytic enzymes in organic solvents was investigated to development the tequnics for the industrial synthesis reaction. At first, effects of polarity or pH of the reaction medium on the synthesis of lignin were studied. Sinapyl alcohol gave syringylglycerol-$\beta$-sinapyl alcohol ether in the reaction mixture containing high concentration of organic solvent whereas syringaresinol was mainly produced in water. Similar results were obtained in the low pH reaction medium. Furthermore, a new compound, 1, 5-di (3, 5-dimethoxy-4-hydroxyphenyl)-4-hydroxymethyl-5-hydroxy-1-pentene ($\beta$-$\gamma$ type dimer), was also isolated from reaction mixtures in the presence of pectin. These results clearly showed that proper design of the reaction medium, especially the presence of organic solvents, is important to control the polymer molecular weight, polydispersity and chemical structure.

To use the ligninolytic enzymes for the industrial synthesis reaction, the synthesis of highly polymeried model lignin from monolignols including coniferyl and sinapyl alcohols was attempted. As the results, high molecular weight lignin was obtained from monolignols by Zutropfverfahren using MnP. Addition of water-miscible organic solvents to the reaction mixture resulted in marked increase in the molecular weight of the lignins formed. Furthermore, the chemical structure was shown to be similar to that of native lignin.

In this study, the ligninolytic enzymes were shown to catalyze both the degradation of highly polymerized lignin and polymerization of monolignols in the reaction mixture containing organic solvents. Therefore, the reactions of the ligninolytic enzymes in the reaction mixture containing organic solvents are applicapable for the various reactions and are though to be effective methods for the conversion of lignin and its related compounds to useful products. Furthermore, the results obtained in this study suggests that these enzymes may also be useful for development of recycling systems such as transformation of waste aromatic polymers to new highly polymerized aromatic polymer.

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