

DEGRADATION OF LIGNIN-CARBOHYDRATE
COMPLEX BY WOOD-ROTTING FUNGI

SHO-ICHI TSUJIYAMA

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INTRODUCTION

The biochemistry of wood degradation has been investigated for the purpose not only from the pure science but also from the applied scientific point of view, e.g. saccharification of wood polysaccharides, biological pulping, biological bleaching and bioconversion to useful products. For the industrial processing of woody materials, the biological technique will become more preferable to the chemical one in the future. The advantages of biological technique lie in lower temperature, lower atmospheric pressure, and environmentally much less toxic and more acceptable. The mechanism of biological degradation of wood needs to be clarified in detail for its utilization.

Wood biodegradation has been studied with two major types of wood-rotting fungi, white-rot and brown-rot, which are classified in Basidiomycetes and may be distinguished by the differences of decayed wood in the color, strength, dimensional stability and chemical composition [Cowling, 1961]. Clearly, these distinguishing features are the result of differences in the attack mechanisms involving cellulolytic, hemicellulolytic and ligninolytic systems.

White-rot fungi metabolize cellulose and hemicellulose and can effectively degrade lignin which covers carbohydrate portions. Previously, many investigators studied the degradation of lignin in wood by white-rot fungi and reported that the action is mainly oxidative [Ishikawa et al., 1963a,b; Hata, 1966; Kirk and Chang, 1974, 1975; Chua et al., 1982; Chen et al., 1983; Faix et al.,

1985]. In 1983, Tien and Kirk discovered that *Phanerochaete chrysosporium* produced an extracellular enzyme, lignin peroxidase, which can oxidize a non-phenolic compound, veratryl alcohol, and depolymerize ¹⁴C-methylated spruce MWL in the presence of H₂O₂ [1983]. Kuwahara et al. [1984] soon reported another peroxidase (manganese-dependent peroxidase) from *P. chrysosporium*. Since these discoveries, Higuchi and coworkers have investigated enzymic degradation systems using various lignin substructure model compounds [Higuchi, 1986, 1990; Umezawa, 1988]. However, the participation of these peroxidases in lignin degradation *in vivo* was questioned because partial polymerization also occurred during the degradation process [Haemmeri et al., 1986; Sarkanen et al., 1991], whereas the effective depolymerization by these enzymes was reported under the limited condition [Hammel and Moen, 1991; Wariishi et al., 1991]. Laccase, which was considered as a ligninolytic enzyme before these peroxidases were discovered, cannot depolymerize the lignin polymer without partial polymerization [Konishi and Inoue, 1971; Ishihara and Miyazaki, 1972; Hüttermann et al., 1980; Kawai et al., 1993].

On the other hand, a phenoloxidase-less mutant of *Sporotrichum pulverulentum* [Ander and Eriksson, 1976] or a lignin peroxidase-negative mutant of *P. chrysosporium* [Boominathan et al., 1990] had lower ability of lignin degradation than a wild strain. Addition of purified lignin peroxidase into the incubation culture enhanced the lignin mineralization rate [Kurek and Odier, 1990]. These studies suggest that phenoloxidases and peroxidases are essential for lignin

degradation.

However, the existence of an unknown lignin-degrading system of white-rot fungi was suggested to be present [Walder et al., 1988; Kurek and Odier, 1990; Sarkanen et al., 1991]. The ligninolytic actinomycete, *Streptomyces viridosporus*, was assumed to have the lignin-specific enzyme which cleaves the β -ether linkages in lignin structure [Crawford et al., 1983a; Deobald and Crawford, 1987].

The effect of the presence of carbohydrates is not clarified in lignin biodegradation by white-rot fungi. Westermarck and Eriksson [1975] reported that white-rot fungi produced cellobiose:quinone oxidoreductase and suggested the participation of carbohydrate in lignin biodegradation. Kondo et al. investigated the glycosylation of phenolics by *Coriolus versicolor* and suggested the importance of glycosylation of lignin for depolymerization by lignin peroxidase [1990].

As for the brown-rot fungi, Ishihara and Shimizu [1984] reported that *Tyromyces palustris* attacked hemicellulose more selectively than cellulose, and modified lignin slightly. Some other investigators also reported that brown-rot fungi can modify lignin in wood [Kirk, 1975; Fukuda and Haraguchi, 1984; Jin et al., 1990]. It is noteworthy that brown-rot fungi can degrade lignin model compounds and lignin component in the presence of cellulose or wood [Enoki et al., 1985]. Although brown-rot fungi don't secrete C_1 cellulase which white-rot fungi produce, generation of hydroxyl radicals initiated to degrade crystalline cellulose [Veness and Evans, 1989]. The role of oxalic acid accumulated in wood by

brown-rot fungi was studied on cellulose degradation [Schmidt et al., 1981; Akamatsu et al., 1991; Espejio and Agosin, 1991].

Thus, degradation systems of wood constituents are different between both fungi. For detection of the actions of wood-rotting fungi in wood, an obligatory condition is to work out an experiment under the co-existence of lignin and hemicellulose, but not alone. Characterization of microbial degradation of wood, however, is difficult and limited because of complexity of its structure, chemical components and insolubility in aqueous media. Especially, the mechanism of lignin biodegradation was not clarified because lignin is water-insoluble, complex and heterogeneous phenolic polymer [Nimz, 1974; Adler, 1977; Sakakibara, 1980].

From the point of view mentioned above, lignin-carbohydrate complex (LCC) is considered to be suitable for a model substrate instead of wood. LCC is the compound in which lignin and hemicellulose chemically linked with ether, ester and other bondings [Smelstorius, 1974; Košíková et al., 1979; Eriksson and Goring, 1980; Mesitsuka et al., 1982; Minor, 1982; Koshijima et al., 1984; Iversen, 1985; Watanabe et al., 1986; Takahashi and Koshijima, 1988a, b; Azuma, 1989; Watanabe, 1989]. Being water-soluble, LCC has a great potential for usage not only for investigating lignin biodegradation but also for characterizing the biodegradation of lignocellulosic materials for the following reasons : 1) Water-soluble LCC facilitates the detection of the action of the ligninolytic enzymes, because the enzyme reaction can proceed in a homogeneous aqueous solution which can overcome the possibility of

the adsorption of enzymes to the insoluble substrates which hamper the detection of enzyme activities; 2) LCC presents an excellent substrate for the investigation of the homogeneous degradation of high molecular weight lignin in co-existence with wood polysaccharides; and 3) By using LCC as a substrate the mode of participation of hemicellulases in lignin degradation can be characterized. And furthermore, 4) Since lignin in coniferous LCC consists of guaiacyl structure, any detection in structural change becomes easy.

The purpose of this thesis is to investigate degradation of a water-soluble lignin-carbohydrate complex, LCC-W, from akamatsu (*Pinus densiflora* Sieb. et. Zucc.) by the action of two species of wood-rotting fungi, *Tyromyces palustris* (Berk. et. Curr.) Murr. and *Coriolus versicolor* (L.:Fr.) Quél., and to determine the differences of degradation mode of wood constituents by both fungi.

In Chapter 1, enzyme productions of both fungi during 31 days incubation were proposed. Enzyme assays were carried out about hemicellulose- and lignin-degrading enzymes.

In Chapter 2, the structural changes of the Fraction P-1 which was insolubilized during incubation by both fungi were determined by the methods of chemical and spectroscopic analyses.

In Chapter 3, the structural analyses of the Fraction P-A, which was precipitated by acidifying the culture media of *C. versicolor* after 10 days of incubation, were carried out.

In Chapter 4, degradation experiment of LCC model compound was carried out to determine the enzymic cleavage of bondings between

lignin and hemicellulose, and release of sugars from cellulase-treated LCC-W by the action of phenol-oxidizing enzymes were detected.

In Chapter 5, newly detection method to detect the changes of wood component polymers and the decomposition of binding portions of LCC was proposed using differential scanning calorimetric (DSC) analysis.

Chapter 1 ENZYME ASSAYS OF *TYROMYCES PALUSTRIS* AND *CORIOLUS VERSICOLOR* DURING DEGRADATION OF WATER-SOLUBLE LIGNIN-CARBOHYDRATE COMPLEX

1.1 Introduction

Two kinds of wood-rotting fungi, a brown-rot fungus, *Tyromyces palustris*, and a white-rot fungus, *Coriolus versicolor*, used in this investigation are known to have different modes of degradation of wood [Cowling, 1961]. As first step, these wood-rotting fungi were incubated in liquid culture containing water-soluble lignin-carbohydrate complex (LCC-W) prepared from akamatsu (*Pinus densiflora*) as a sole carbon source, and then the difference of these fungi was investigated with respect to the kinds and orders of the produced enzymes during incubation [Tsujiyama et al., 1992].

1.2 Materials and Methods

1.2.1 Materials and microorganisms

A water-soluble LCC (LCC-W) was prepared from an extractive free and depectinated sapwood of akamatsu (*Pinus densiflora* Sieb. et. Zucc.) according to Azuma et al. [1981]. This LCC was composed of 67.8 % lignin and 32.1 % carbohydrates (compositions of D-glucose, D-galactose, D-mannose, D-xylose and L-arabinose are 15.1, 17.7, 22.7, 26.6 and 17.8 %, respectively). All other chemicals used were of reagent grade.

A brown-rot fungus, *Tyromyces palustris* (Berk. et. Curr.) Murr.

(FRI No.0507), and a white-rot fungus, *Coriolus versicolor* (L.: Fr.) Quél. (FRI No.1030), were used throughout this thesis.

1.2.2 Preparation of inoculum

The basal medium of Kirk et al. [1978] containing 1.2 mM ammonium tartrate as a nitrogen source was used to give an effective degradation of the lignin portion. It was supplemented with 2 % (w/v) LCC-W as a sole carbon source and dispensed to 100-ml Erlenmeyer flasks (20 ml per flask). The culture flasks were fitted with cotton plugs through which two small glass tubings were inserted. Oxygen gas was ventilated every 3 days through these tubes. An aliquot of fungus suspension, pre-cultured under shaking in malt-peptone-dextrose medium (glucose 2.5 %, malt extract 1 %, peptone 0.5 %, KH_2PO_4 0.3 %, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 %) at 25°C for one week, was added to a medium containing LCC-W and incubated without shaking at 28°C up to 31 days.

1.2.3 Crude enzyme preparation

The culture was centrifuged (8.0×10^4 g, 10 min) and pH value of the supernatant (S) was measured. The precipitate (P) was washed with 0.05 M sodium acetate buffer (SAB, pH 4.8) and washings were combined with the Fraction S. Ammonium sulfate was added to this solution to make 80 % saturation. The precipitate formed was separated from the supernatant by centrifugation and extracted with 70 % aqueous methanol to remove LCC fraction co-precipitated with enzymes. The residue was extracted with SAB and centrifuged to remove insoluble material. The supernatant solution was used as a crude enzyme solution (E). The fractionation procedure and

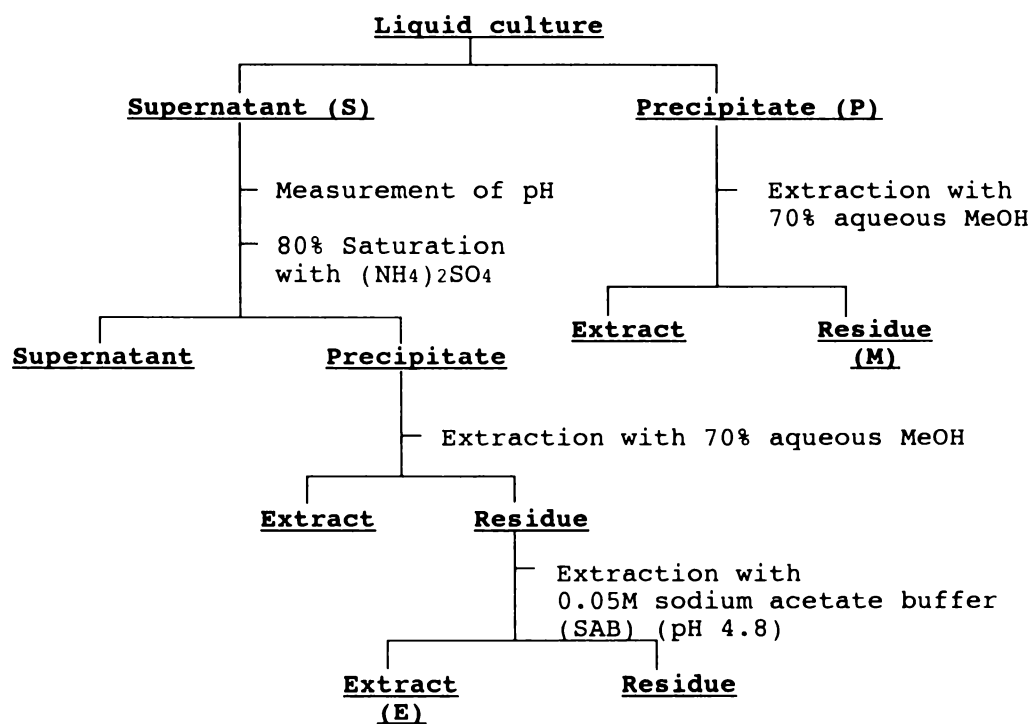


Fig. 1.1 Preparation procedure of enzyme solution.

designation of the separated fractions are shown in Fig. 1.1.

1.2.4 Determination of mycelia weight

The precipitate (P) obtained after centrifugation of the culture was extracted with 70 % aqueous methanol. The suspension was centrifuged to separate the insoluble mycelia (M) from the LCC fraction attached to the mycelia as shown in Fig. 1.1.

1.2.5 High performance liquid chromatographic (HPLC) analysis of succinic acid

Amounts of residual succinic acid in culture media was measured by high performance liquid chromatography (HPLC). HPLC conditions were as follows : apparatus, a Shimadzu LC-10AD; column, Chemco Pak NUCLEOSIL 120-10C18 (4.0 × 300 mm); column temperature, 40°C; solvent, 20 mM phosphate buffer (pH 2.8); flow rate, 1.0 ml/min; detector, Shimadzu SPD-10A (ultraviolet, 220 nm).

1.2.6 Residual ammonium ion assay

Amounts of residual ammonium ion in culture media was measured by indophenol method. One ml of appropriately diluted culture media was added with 0.5 ml of solution A (10 g of phenol and 25 mg of sodium nitropululside per 1 l) and 0.5 ml of solution B (10 ml of sodium hypochlorite solution and 15 g of sodium hydroxide per 1 l) and incubated for 60 min at 30°C. Absorbance at 640 nm was measured with Shimadzu UV-2200 using ammonium chloride solution as a standard.

1.2.7 Protein content assay

Protein content of the crude enzyme solution (E) was determined by the dye-binding assay method of BIO-RAD (Protein Assay Kit I)

using bovine gamma globulin as a standard. This method was preferred to the Lowry method [Lowry et al., 1951] because of less disturbance due to low molecular-weight phenolic degradation products from LCC.

1.2.8 Enzyme assays

Exo-glycosidase activities were assayed according to Highley [1976] by measuring the amount of liberated *p*-nitrophenol from following nine *p*-nitrophenyl glycoside substrates : *p*-nitrophenyl α,β -D-glucopyranosides, *p*-nitrophenyl α,β -D-mannopyranosides, *p*-nitrophenyl α,β -D-galactopyranosides, *p*-nitrophenyl α,β -D-xylopyranosides and *p*-nitrophenyl α -L-arabinofranoside. A reaction solution (1.0 ml), which contained 0.25 ml of 6.5 mM substrate solution, 0.1 ml of 0.5 M sodium acetate buffer (pH 4.8) and 0.1 ml of the crude enzyme solution, was incubated at 40°C for 30 min. The reaction was terminated by adding 5.0 ml of 0.6 M sodium carbonate solution. Each enzyme activity was determined by measuring absorbance at 410 nm. The reaction solution devoid of enzymes was used as a reference. One unit of enzyme was defined as the amount of enzyme that liberated 1 mmol of *p*-nitrophenol per min.

Polysaccharase activities were determined by using following eight substrates : mannan, arabinogalactan, xylan, arabinoxylan, amylose, dextran, Avicel SF (Asahi-Kasei), CMC-Na (Nakarai-tesque). A reaction solution (1.0 ml), which contained 0.25 ml of 1.0 % substrate solution, 0.1 ml of 0.5 M SAB and 0.1 ml of the crude enzyme solution, was incubated at 40°C for 2 hrs. Reducing sugar content was then determined by the 3,5-dinitrosalicylic acid method

[Millar, 1959] using glucose as a standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of glucose per hour.

Activity of laccase was measured by oxidation of syringaldazine according to Leonowicz and Grzywnowicz [1981]. Activity of peroxidase was similarly assayed by oxidation of syringaldazine in the presence of 4 mM of H_2O_2 . Manganese-dependent peroxidase activity was also measured in the presence of 4 mM of H_2O_2 and 1 mM $MnSO_4$ as described above. One unit of these enzymes was defined as the amount of enzyme that oxidized 1 mmol of syringaldazine per second.

Activity of tyrosinase was determined according to Andrawis and Kahn [1986] with D,L-Dopa (D,L-3,4-dihydroxyphenylalanine) as a substrate. One unit of enzyme activity is defined as the amount of enzyme that increases the absorbance at 480 nm per second.

Lignin peroxidase activity was measured according to Tien and Kirk [1988] using veratryl alcohol as a substrate. One unit of enzyme activity is defined as the amount of the enzyme that oxidized 1 mmol of veratryl alcohol per second.

All results were expressed as the specific activity, defined as unit per mg protein.

1.3 Results and Discussion

1.3.1 Growth of fungi

T. palustris grew as a gel-like material complexed with precipitated LCC, whereas the mycelia of *C. versicolor* floated and

spread on the surface of the medium. The color of the media cultured with *T. palustris* and *C. versicolor* changed from pale brown only to slightly pale brown and to dark brown, respectively. The weight of mycelia (M) was estimated as the residue after extraction of the solid materials with 70 % aqueous methanol from the precipitates P as shown in Fig. 1.1, weight of which is plotted against incubation time as shown in Fig. 1.2. The results indicate that both fungi can grow with water-soluble LCC as a sole carbon source, and that *T. palustris* grew much faster than *C.versicolor* and continued to grow throughout 31-days incubation time.

1.3.2 Change of pH of the culture medium

Figure 1.3 shows change of pH value of the culture media during incubation. The pH value of the culture medium of *T. palustris* gradually became lower during incubation until reaching to 2.9 after 31-days incubation. This may be caused by an accumulation of oxalic acid as reported in the case of degradation of wood [Shimazono, 1952]. Recently, Akamatsu et al. showed *T. palustris* formed oxalic acid from oxaloacetic acid and glyoxylic acid enzymically [Akamatsu et al., 1991; Akamatsu, 1993]. The pH value of *C. versicolor*, on the other hand, slightly lowered initially, but abruptly became higher after 10-days incubation. This observation was in agreement with the results of Morohoshi et al. [1987] Initial decrease can be explained by that *C. versicolor* has an oxaloacetase activity and produces oxalic acid [Akamatsu et al., 1993; Dutton et al., 1993]. But, a white-rot fungus *C. versicolor* can inhibit the drop of pH enzymically different from *T. palustris*. Shimazono reported that the

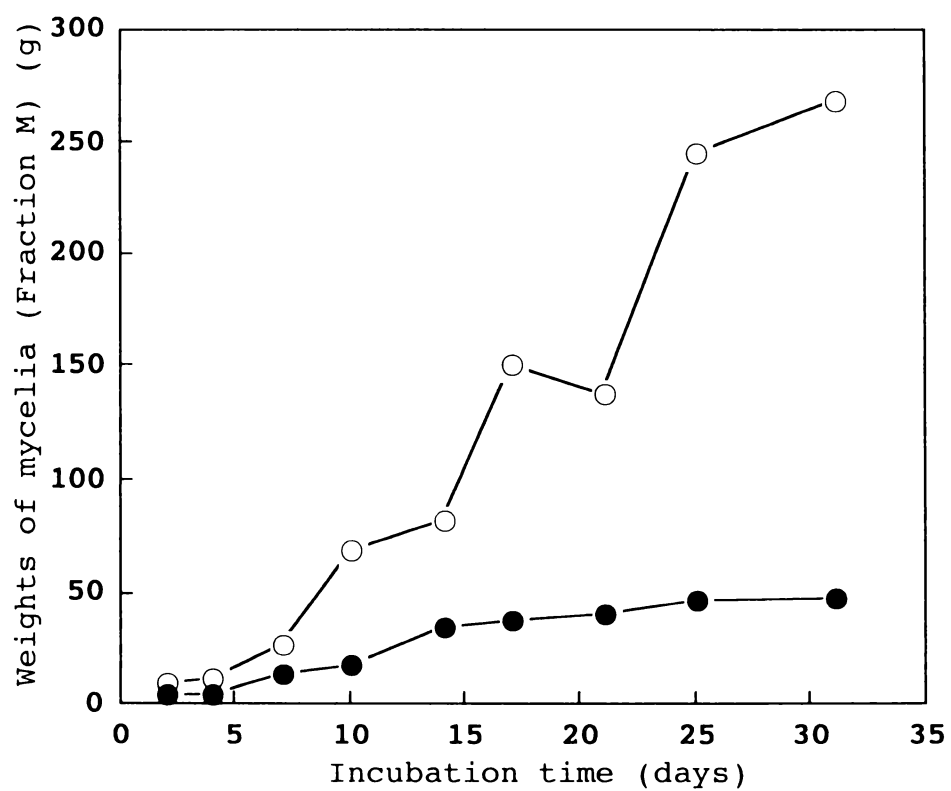


Fig. 1.2 Growths of the mycelia (Fraction M).
 O: *T. palustris*, ●: *C. versicolor*.

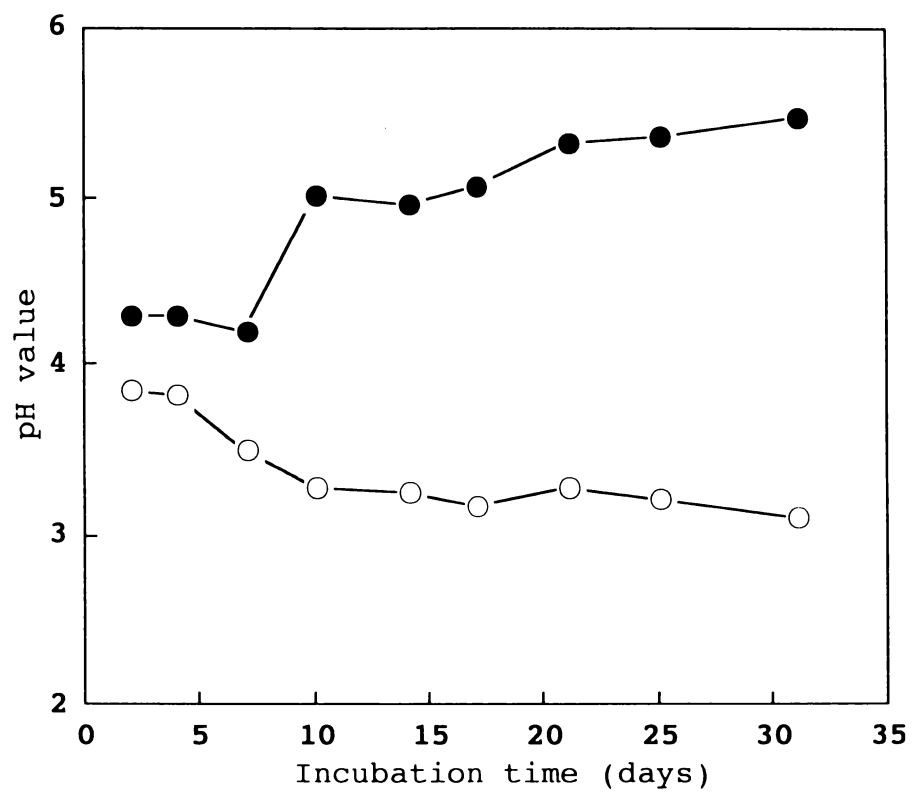


Fig. 1.3 Changes of pH values of the culture media.
O: *T. palustris*, ●: *C. versicolor*.

oxalic acid decarboxylase was produced from white-rot fungi but not from brown-rot fungi [1955]. Recently, *Phanerochaete crysospolrium* has been shown to metabolize oxalic acid by lignin peroxidase [Akamatsu, 1990]. Therefore, pH drop was concluded to be inhibited by metabolizing oxalic acid. But, this reason can not explain the rise of pH. In this culture condition, pH value was supposed to be controlled with succinate buffer (pH 4.65). The succinate must be metabolized during incubation. By HPLC analysis, succinic acid decreased initially from 0.01 mM to 0.001 mM within 31 days. This indicates that pH value rose by metabolizing succinate.

1.3.3 Polysaccharase production

The polysaccharase productions from *T. palustris* and *C. versicolor* were shown in Fig.1.4. In the case of *T. palustris*, mannanase was produced constantly during 31-days incubation with a peak between 14- to 17-days incubation. Arabinogalactanase was reduced at the middle stage, while xylanase appeared in the later stage. In the case of *C. versicolor*, mannanase appeared in the middle stage, while arabinogalactanase was prominent in the later stage. Two kinds of cellulase, CMCase and Avicelase, were detected only in the case of *C. versicolor*, but their activities were not so remarkable and the result is in good agreement of the report that cellulase was an enzyme induced by cellulose [Johansson, 1966] which was absent in the present LCC.

1.3.4 Exo-glycosidase production

The exo-glycosidase activities of *T. palustris* and *C. versicolor* are shown in Fig. 1.5. The results indicate that both fungi

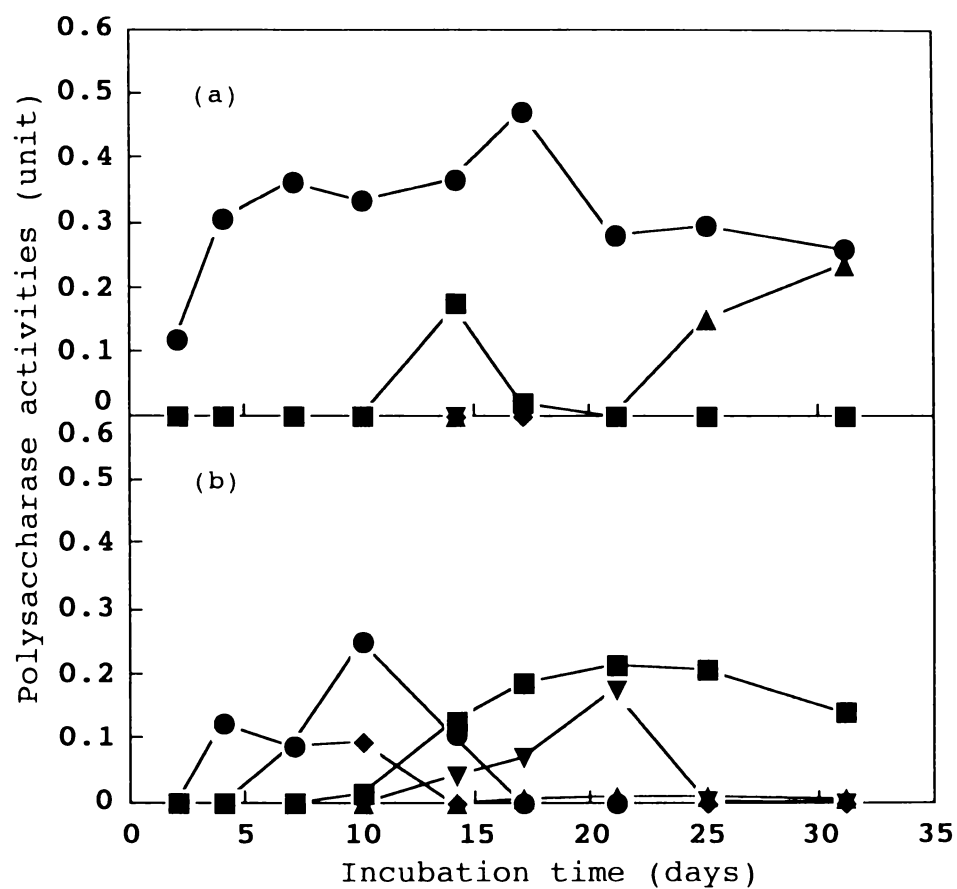


Fig. 1.4 Productions of polysaccharases during incubation.
 (a) *T. palustris*, (b) *C. versicolor*.
 ●:mannanase, ▲:arabinogalactanase, ■:xylanase,
 ◆:CMCase, ▼:Avicelase.

secreted various kinds of exo-glycosidases in the media. They produced highly active α -galactosidases and α -arabinosidases differing in order, but the other exo-enzyme activities were kept low throughout the experiment. All enzyme activities became higher at the later stage. In the case of β -glycosidases, both fungi produced almost equal amounts of β -glucosidase and β -mannosidase, but *T. palustris* produced 2 to 7 times more active β -galactosidase than did *C. versicolor*, and its activity becoming higher with progress of incubation days. A substantial amount of β -xylosidase was produced only by *T. palustris*, although its activity was not so high as the other β -glycosidases.

1.3.5 Phenoloxidase production

The activities of two kinds of phenoloxidases and three kinds of peroxidases are shown in Fig. 1.6. These enzyme activities could be noticed only in the case of *C. versicolor* after 7 days. Most of ammonium ion has already metabolized within 7 days (2.4 mM to 0.031 mM). Keyser et al. showed that nitrogen starvation initiated the ligninolytic system of *Phanerochaete chrysosporium* [1978]. In this culture condition, *C. versicolor* did not produce phenoloxidases and peroxidases until nitrogen metabolism was over.

First, the activity of lignin peroxidase was detected during 7- to 14- days incubation, and after that it was not noticeable. The activities of two phenoloxidases, laccase and tyrosinase, appeared after 10 days and had maximum 21 and 17 days, respectively. The activities of peroxidase and Mn-dependent peroxidase were detected after 14-days incubation. From this result, *C. versicolor* produced

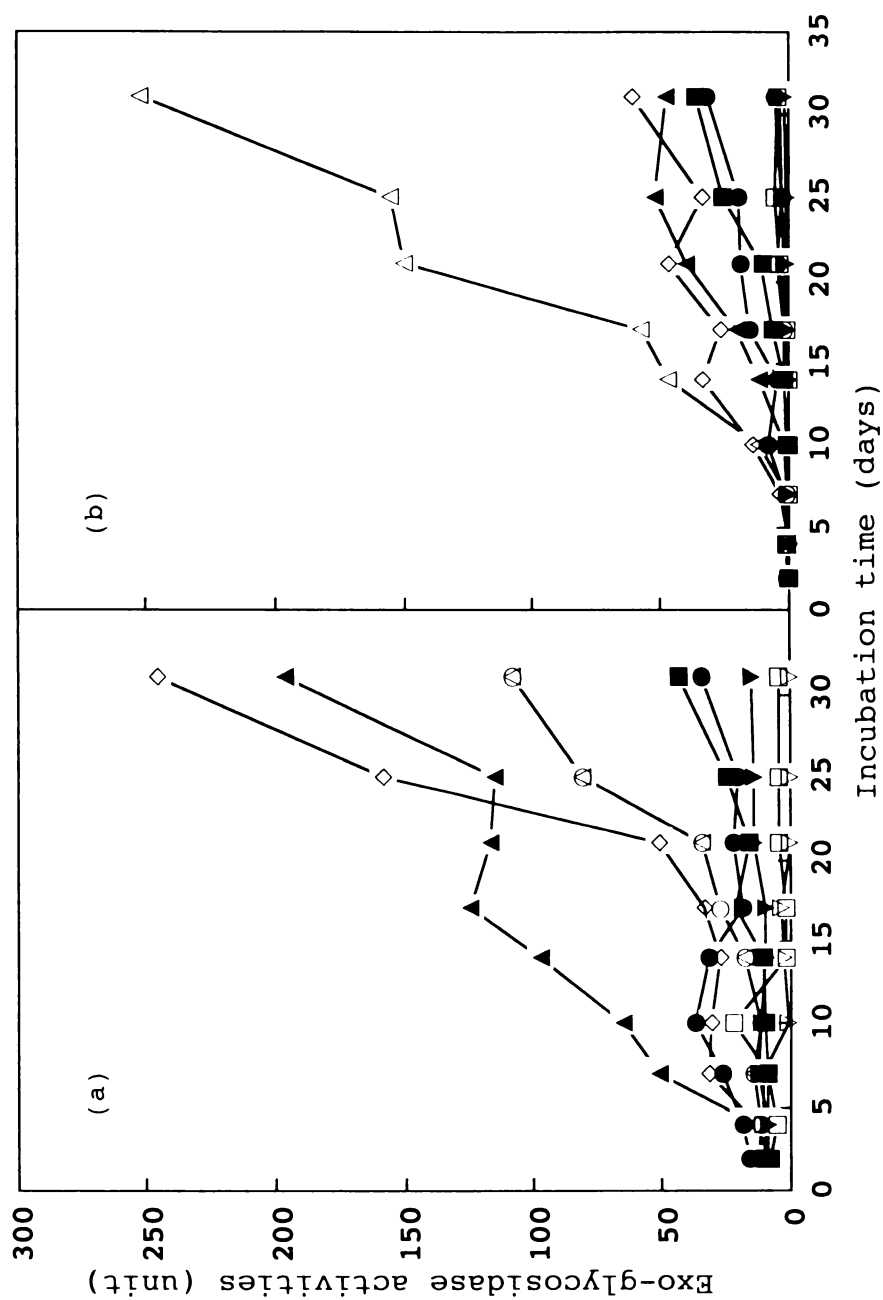


Fig. 1.5 Productions of exo-glycosidases during incubation.
 (a) *T. palustris*, (b) *C. versicolor*.
 $\circ$: α -Glucosidase, $\bullet$: β -Glucosidase, $\triangle$: α -Galactosidase,
 $\blacktriangle$: β -Galactosidase, $\square$: α -Mannosidase, $\blacksquare$: β -Mannosidase,
 ∇ : α -Xylosidase, $\diamond$: α -Arabinosidase.

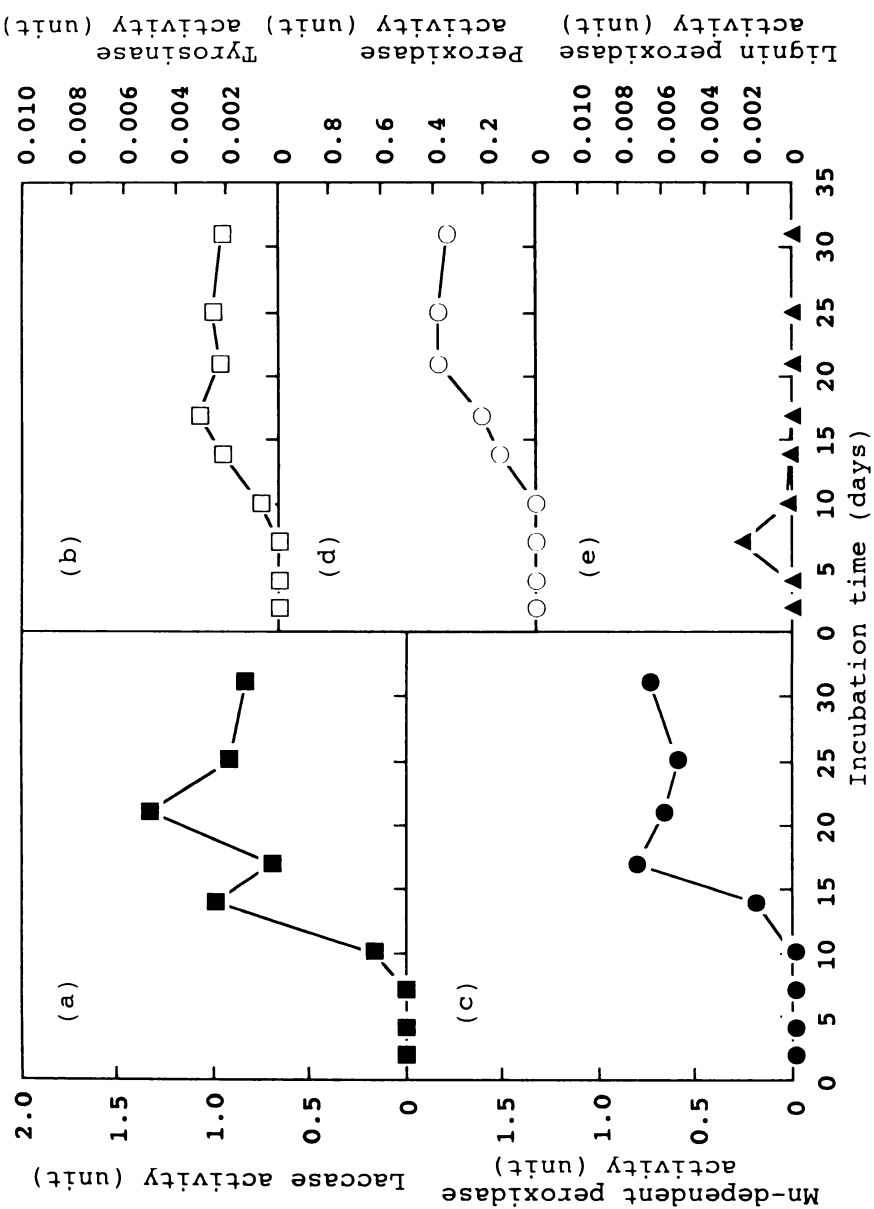


Fig. 1.6 Productions of phenoloxidas and peroxidases of *C.versicolor*.
 (a) Laccase, (b) Tyrosinase, (c) Mn-dependent peroxidase,
 (d) Peroxidase, (e) Lignin peroxidase.

high active laccase and Mn-dependent peroxidase extracellularly in this culture condition after 10 days.

1.4 Summary

Two types of wood-rotting fungi, *Tyromyces palustris* and *Coriolus versicolor*, were incubated in liquid culture containing water-soluble lignin-carbohydrate complex (LCC-W) prepared from akamatsu (*Pinus densiflora*) as a sole carbon source. The differences between both fungi were examined with respect to the enzyme productions during incubation. *T. palustris* produced larger amounts and higher active hemicellulose-degrading enzymes than did *C. versicolor*. *C. versicolor* produced phenoloxidases and peroxidases after 7 days of incubation when ammonium ion used as a nitrogen source was metabolized. First, lignin peroxidase activity appeared prior to others, although its activity was low. As lignin peroxidase disappeared, other phenoloxidases and peroxidases were determined. Among two kinds of phenoloxidases and three kinds of peroxidases, *C. versicolor* produced highly active laccase and Mn-dependent peroxidase.

Chapter 2 STRUCTURAL ANALYSIS OF THE WATER-INSOLUBLE FRACTION

2.1 Introduction

A part of water-soluble LCC (LCC-W) was precipitated during incubation by the actions of *Tyromyces palustris* and *Coriolus versicolor*. This phenomenon was observed in both fungi. This insolubilized fraction was easily prepared without contamination of salts or another products from fungi, and expected to have distinguishable changes by the actions of both fungi.

In this chapter, chemical and spectroscopic analyses of this fraction named P-1 were made with emphasis on the differences in the decomposition systems of two fungi [Tsujiyama et al., 1992, 1993a].

2.2 Materials and Methods

2.2.1 Inoculation and organisms

Two kinds of wood-rotting fungi, *T. palustris* and *C. versicolor*, were incubated for 31-days in the culture condition as described in Chapter 1.

2.2.2. Preparation of the water-insoluble fraction (P-1)

The water-insolubilized LCC fraction during incubation from culture media was extracted with 70 % aqueous methanol from the precipitates P as shown in Fig. 1.1, and evaporated under reducing pressure.

2.2.3 General analytical methods

Lignin and uronic acid contents were determined according to van Zyl [1978] and Wardi [1974], respectively. Infrared (IR) spectra were measured as KBr discs using a Shimadzu FT-IR 4000 spectrophotometer. Ultra-violet (UV) spectra were measured in 50 % aqueous dioxane with a Shimadzu UV-365 spectrophotometer. Gas-liquid chromatography (GC) was carried out with a Shimadzu GC-15 gas chromatograph equipped with flame ionization detectors, and peaks were integrated with a Shimadzu Chromatopac C-R6A. Carbon-13 nuclear magnetic resonance (^{13}C -NMR) spectra were recorded with a Varian VXR-200 spectrometer (75 MHz for ^{13}C) in deuterated dimethylsulfoxide ($\text{DMSO}-d_6$) at 70°C. Chemical shifts were nomarized in ppm from tetramethylsilane by taking the center peak of $\text{DMSO}-d_6$ as 39.5 ppm.

2.2.4 Molecular weight distribution

Molecular weight distribution was determined by gel-permeation chromatography (GPC) on a column (1.0 × 100 cm) of TOYOPEAL HW-55F equilibrated with *N,N*-dimethylformamide (DMF) containing 0.1 M LiCl [Brown et al., 1968; Connors, 1980]. One mg of the Fraction P-1 was dissolved in 1 ml of the equilibration solvent, applied to the column and eluted with the same solvent at a flow rate of 1 ml/min. Elution was monitored by both a UV detector (Waters Lambda MAX-481) at 280 nm and a refractive index (RI) detector (TOSO RI-8). In addition, 2 ml of fractions were collected by an Advantec Toyo SF-60L fraction collector, and the carbohydrate content in each

fraction was monitored by measuring absorbance at 490 nm after development of color by the phenol-sulfuric acid method [Dubois et al., 1956]. The column was calibrated against polystyrenes having known molecular weights.

2.2.5 Neutral sugar composition analysis

Three mg of the sample was hydrolyzed in a sealed glass tube with 3 ml of 1 N sulfuric acid at 100°C for 6 hrs. Neutral sugars were analyzed as alditol acetates by GC on a column (0.25 mm × 25 m) of DB-225 using inositol as an internal standard [Borchardt and Piper, 1970].

2.2.6 Alkaline nitrobenzene oxidation

Three mg of the sample was oxidatively degraded with alkaline nitrobenzene at 160°C for 2 hrs according to Pepper and Siddiqueullah [1961]. The reaction mixture was extracted with ether to remove unreacted nitrobenzene. The aqueous layer was acidified with dilute hydrochloric acid solution and extracted with ether. After removing ether by evaporation, the oxidation products were trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide and analyzed by using GC on a capillary column (0.25 mm × 25 m) of CBP-1 using acetoguaiacone as an internal standard. The contents of vanillin and vanillic acid were evaluated as % weight basis of the lignin content.

2.2.7 Functional group determination

Phenolic hydroxyl and α -carbonyl contents were estimated by measuring the alkaline-neutral difference ($\Delta\epsilon_1$) and the reduction

difference ($\Delta\epsilon_r$) spectra, respectively, according to Kirk and Chang [1975]. The values were expressed as % weight basis of the lignin content. Methoxyl content was measured by the method of Japan Industrial Standard (JIS) P8013. Total carboxyl group content was measured by titration with a 0.01 N NaOH solution.

2.3 Results and Discussion

2.3.1 Yields of Fraction P-1

Yield change of the water-insolubilized fraction (P-1) shown in Fig. 2.1 is different between both fungi. The value of the Fraction P-1 from *T. palustris* increased drastically within 7 days and then kept almost constant. The amount of P-1 in the case of *C. versicolor* was less than that of *T. palustris* (below 45 % of that) and its change is quite different : it initially increased but turned to decrease after 10-days incubation. This indicates that the degradation of the hydrophilic carbohydrate portion in LCC took place first and the insolubilized LCC was gradually re-solubilized accompanied by the degradation of lignin in the case of *C. versicolor*. The coincidence of the production time of phenoloxidases and peroxidases (Chapter 1) and the re-solubilization time of the Fraction P-1 indicates that these enzymes might relate to solubilization of water-soluble fraction P-1.

By multiplying the yields with the Fraction P-1 by their lignin contents, changes of amounts of lignin and hemicellulose in the Fraction P-1 were calculated and shown in Fig. 2.2. This shows

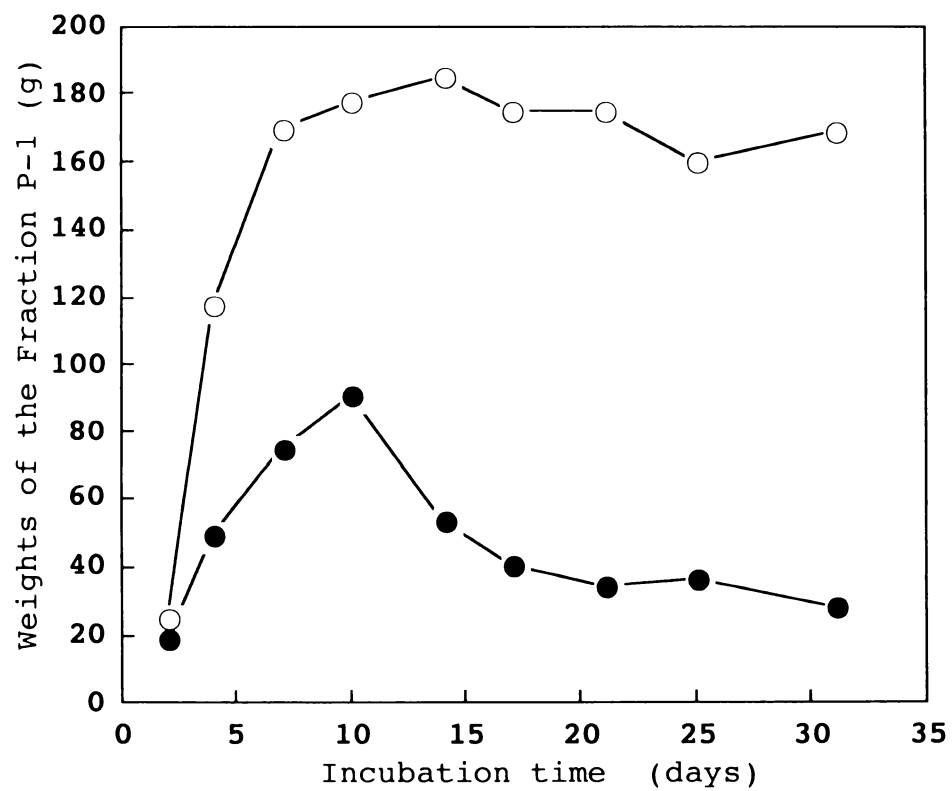


Fig. 2.1 Changes of yields of the Fraction P-1.
 ○: *T. palustris*, ●: *C. versicolor*.

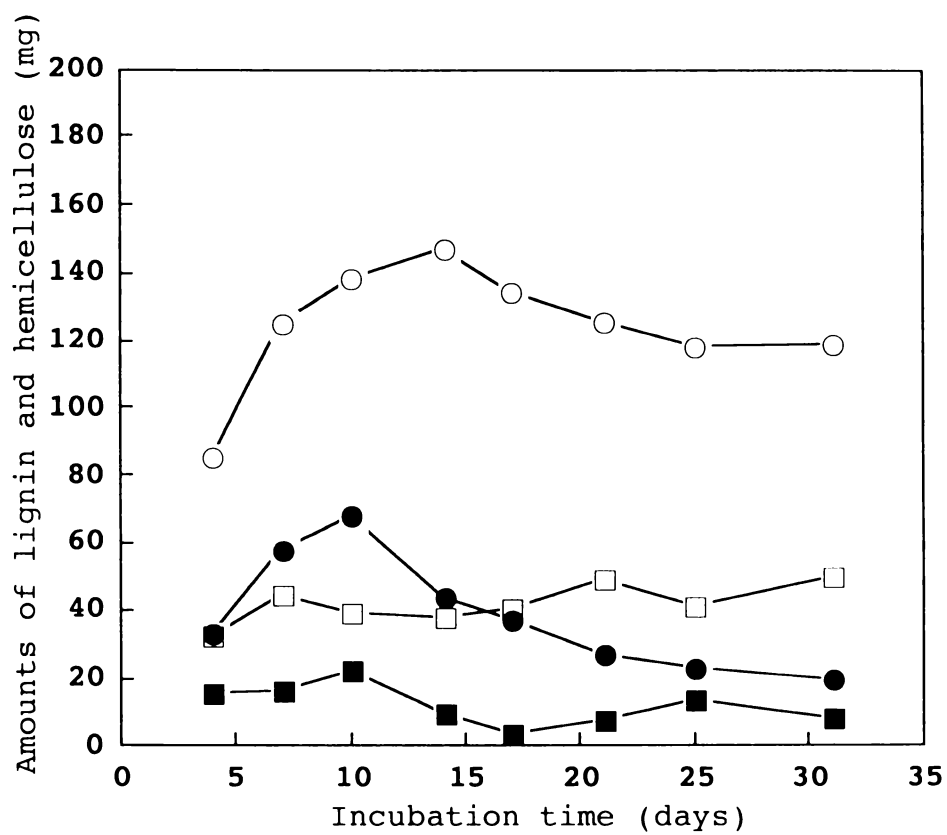


Fig. 2.2 Amounts of lignin and hemicellulose of the Fraction P-1.

○, □: *T. palustris*, ●, ■: *C. versicolor*.

○, ●: Lignin, □, ■: Hemicellulose.

clearly that, in the case of *C. versicolor*, amounts of both lignin and hemicellulose increased until 10 days as similar to *T. palustris* but lignin amount decreased after that, while in the case of *T. palustris*, amounts of the two kept almost constant after 10 days.

2.3.2 Molecular weight distribution

The GPC elution profiles of the Fraction P-1 are shown in Fig. 2.3, and the changes in its weight-average molecular weight estimated at the peak top are shown in Fig. 2.4. In the case of *T. palustris* (a), all the fractions appeared as a single peak, and the peak due to lignin closely overlapped with that of hemicellulose. It is noteworthy that the weight-average molecular weight of the Fraction P-1 obtained at 4-days incubation (3,900) was almost constant between 7- to 14-days incubation, then became smaller to be 2,900 at 31-days incubation (shown in Fig. 2.4). This strongly indicates that the depolymerization of the Fraction P-1 began after 14-days incubation when a large amount of the hemicellulose degrading enzymes starts to be produced as described in Chapter 1.

In contrast, the Fraction P-1 from *C. versicolor* gave a remarkable change during incubation, i.e. the peak due to hemicellulose started to separate from that of lignin, indicating that the linkages between lignin and hemicellulose were split by *C. versicolor*. This phenomenon is observed accompanied by the productions of phenoloxidases and peroxidases (after 14 days), suggesting the participation of these enzymes. Average molecular weight of the lignin and hemicellulose portions gradually became

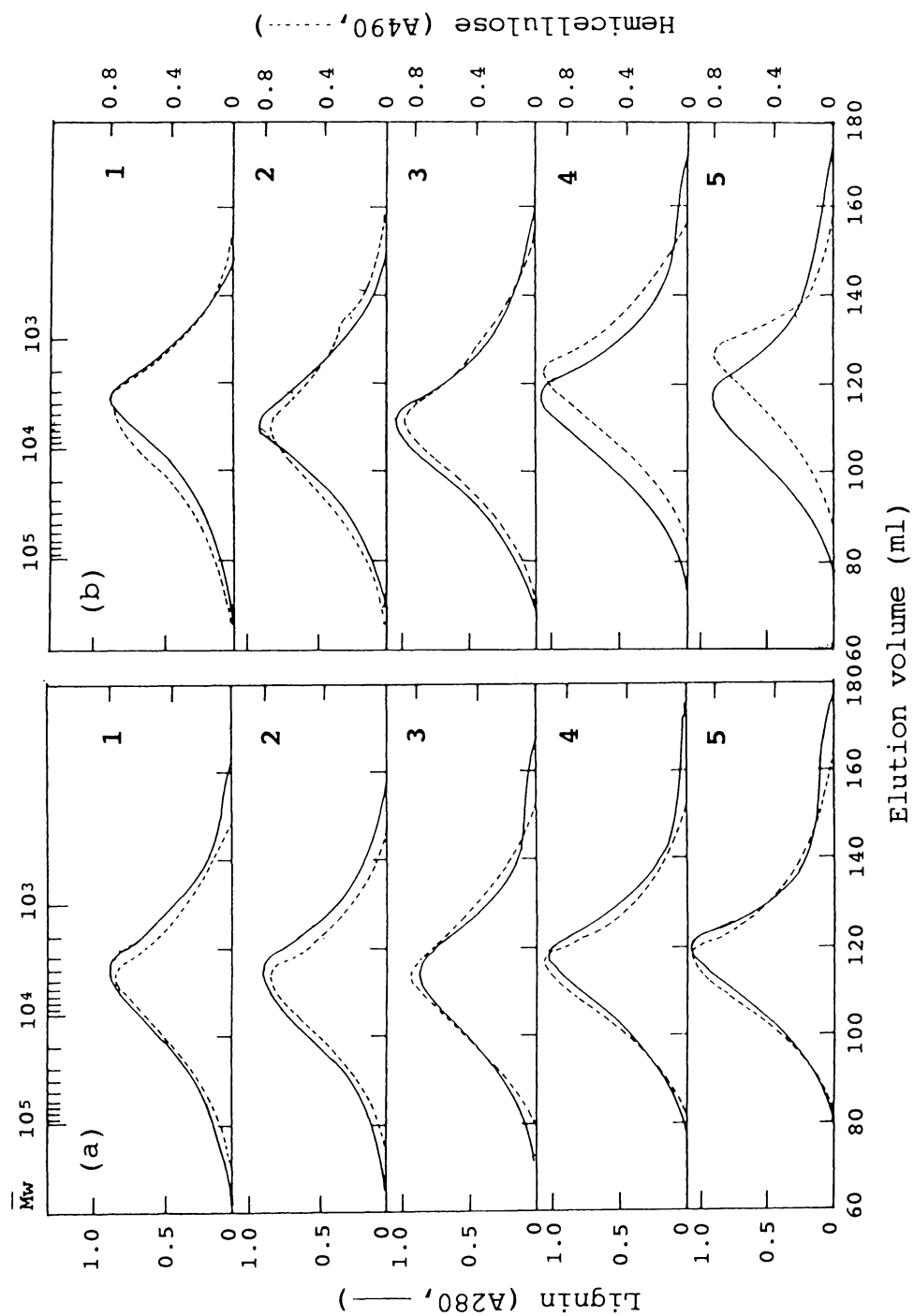


Fig. 2.3 GPC elution profiles of the Fraction P-1.
 (a) *T. palustris*, (b) *C. versicolor*.
 1:4 days, 2:10 days, 3:14 days, 4:21 days, 5:31 days.

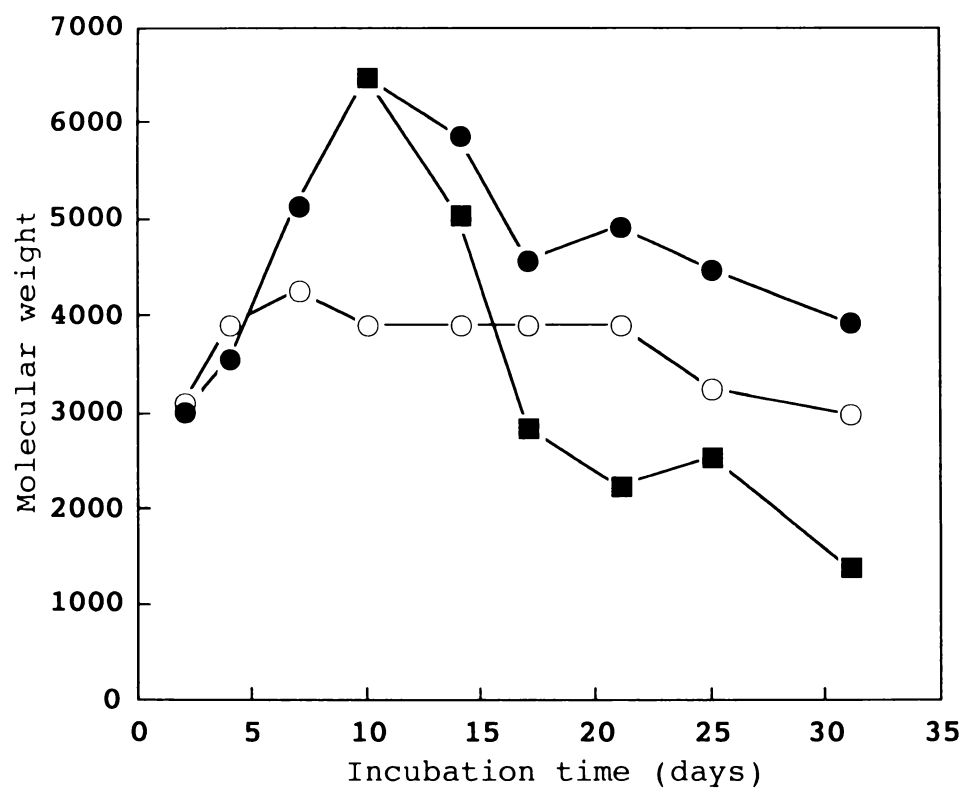


Fig. 2.4 Molecular weight distribution of the Fraction P-1.
 O: Fraction P-1 of *T.palustris*, ●: lignin portion in
 Fraction P-1 of *C.versicolor*, ■: hemicellulose
 portion in the Fraction P-1 of *C.versicolor*.
 note : molecular weight is calibrated by polystyrene
 standard.

smaller from 3,600 at 4 days to 6,400 (maximum) at 10 days. This indicates the occurrence of polymerization of the lignin portion in the LCC until 10 days of incubation. However, the molecular weights of the split lignin and hemicellulose became smaller to 3,900 and 1,150, respectively, with the progress of incubation (Fig. 2.4).

2.3.3 Spectroscopic analyses

Figure 2.5 shows the change of absorptivity at 280 nm monitored by UV spectrophotometer. In the case of *T. palustris*, the value decreased slightly at early stage of incubation, and was almost constant after 10-days incubation as observed in α -carbonyl content (Fig. 2.12). Kirk reported a remarkable increase in UV absorbance of the heavily brown-rotted lignin due to aromatic carboxyl groups [Kirk, 1975]. Fukuda and Haraguchi [1984] also noticed a slight increase in UV absorbance of lignins decayed by *T. palustris*. The discrepancy of the present results may be ascribed to the decrease in the carbonyl group conjugated with aromatic nuclei resulting in good co-relationship between profiles of α -carbonyl content and UV absorption in the present case.

In the case of *C. versicolor*, the decrease in absorptivity was more prominent than that of *T. palustris*, and became constant after 14-days incubation quite similar to the variations of α -carbonyl content and vanillin yield. These phenomena may be resulted from the side-chain modification as discussed in 2.3.6.

The IR spectra of the P-1 fractions (Fig. 2.6) were similar to that of the milled akamatsu wood lignin, but the absorption bands were heavily overlapped with spectra of hemicellulose which made a

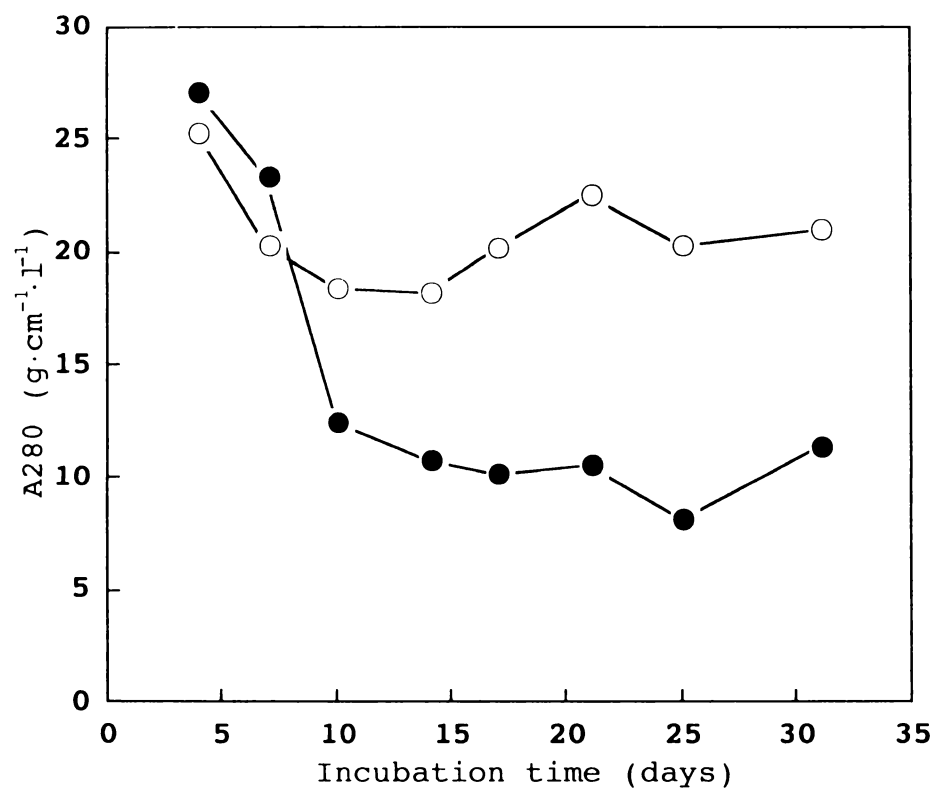


Fig. 2.5 Changes of absorptivity (at 280 nm) of the Fraction P-1.
 ○: *T. palustris*, ●: *C. versicolor*.

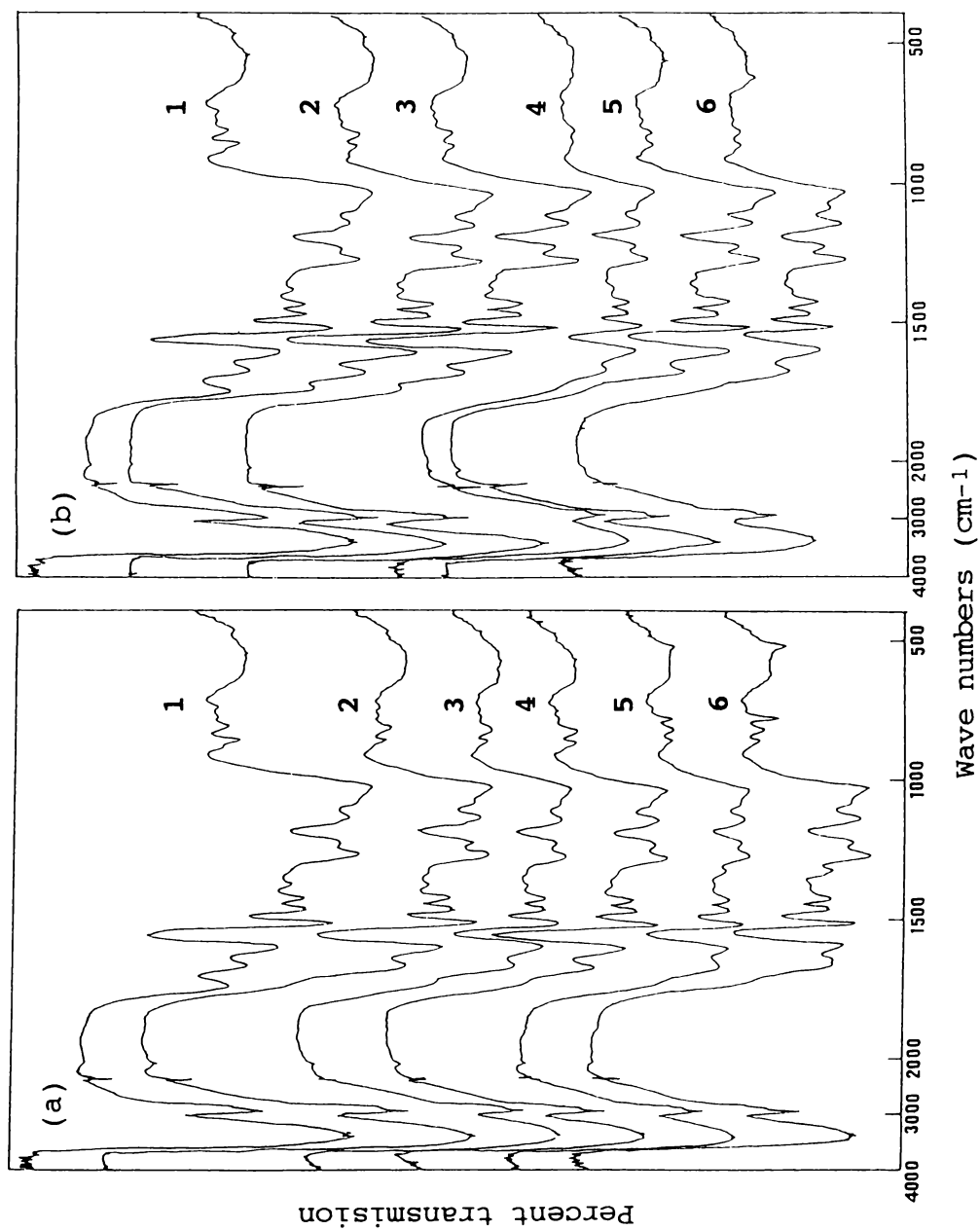


Fig. 2.6 Infrared spectra of the Fraction P-1.
 (a) *T. palustris*, (b) *C. versicolor*.
 1: LCC-W, 2: 2:2 days, 3: 3:7 days, 4: 4:14 days, 5: 5:21 days, 6: 6:31 days.

quantitative analysis difficult. The most prominent change during the incubation was noticed in decrease of absorbance at 1,738 cm^{-1} which was due to acetyl carbonyl groups esterified to the hemicellulose portion of LCC. This absorption band diminished and only remained as a shoulder of the strong absorption at 1,660 cm^{-1} at early stage of incubation (2-days incubation) in the case of *T. palustris*, but it took over 7 days in the case of *C. versicolor* to complete this diminution. The present result supports that *T. palustris* can decompose the hemicellulose portion more rapidly than *C. versicolor*.

Figure 2.7 shows ^{13}C -NMR spectra of the P-1 fractions from 31-days culture together with the original LCC-W. The signals were assigned according to the literature [Gorin, 1980; Robert and Chen, 1989] and listed in Table 2.1. Many signals due to hemicellulose appearing at two regions of 73-78 ppm and 100-110 ppm in the original LCC diminished remarkably in both fungi-degraded P-1 fractions, indicating an intensive fungal depolymerization of hemicellulose portion. The remaining of β -O-4 and condensed structures as well as a remarkable reduction of acetyl groups could be easily demonstrated. The major difference between two fungi-degraded P-1 fractions was in the carbonyl region. In the case of *C. versicolor*, the signal due to aldehyde or α -carboxyl carbon (193.9 ppm) diminished, and two new signals due to carboxyl carbon (172.1 and 167.2 ppm) appeared, confirming an occurrence of oxidative modification at the side-chain of lignin as presented above. In the case of *T. palustris*, however, aldehyde and

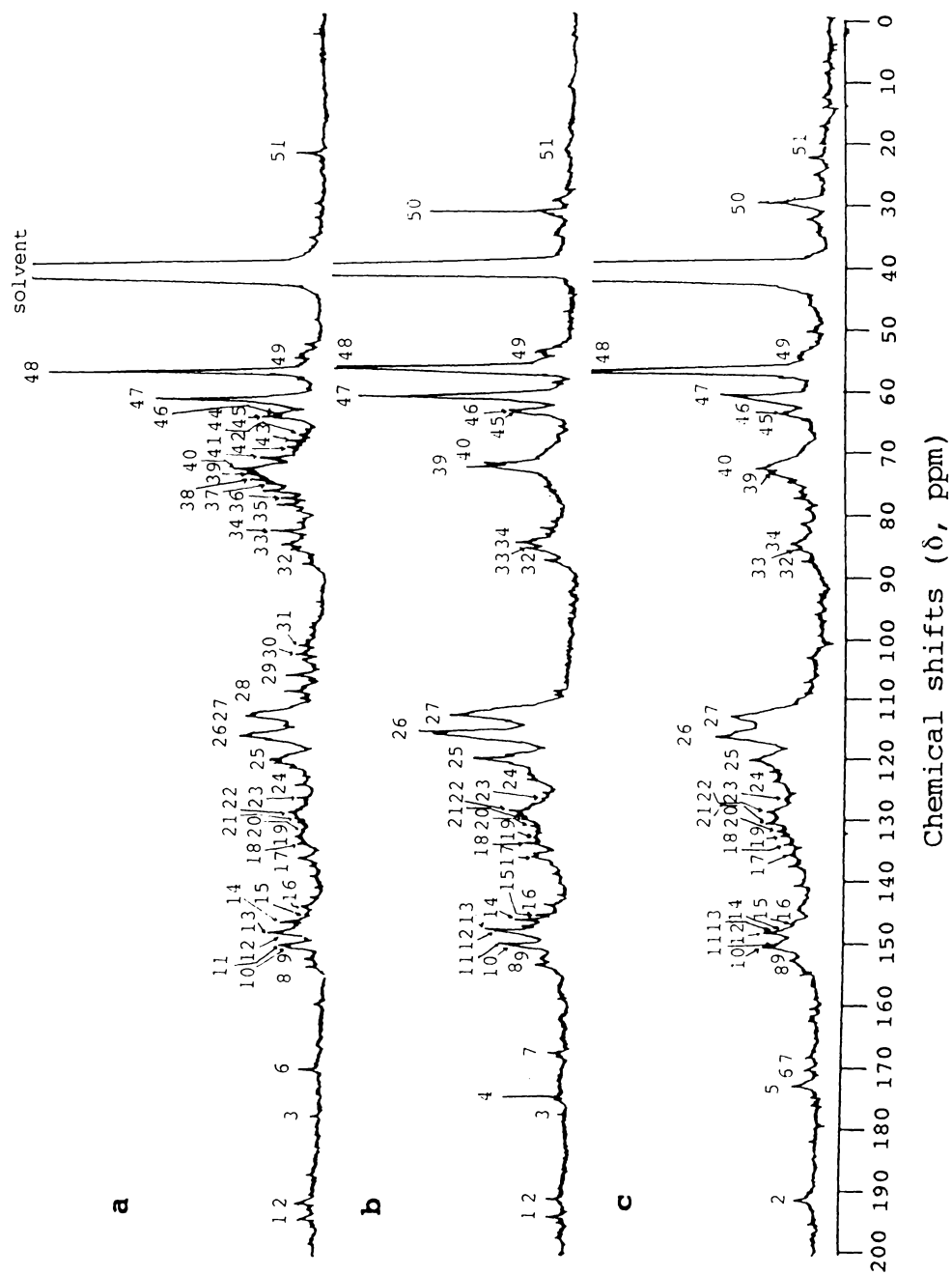


Fig. 2.7 Carbon-13 NMR spectra of original LCC-W and the Fraction P-1 after 31 days of incubation.
a:LCC-W, **b:***T. palustris*, **c:***C. versicolor*.

Table 2.1 Chemical shifts and signal assignments for the native LCC and the P-1 fractions obtained by *T.palustris* and *C.versicolor* after 31-days incubation.

Signal number	Chemical shifts (δ , ppm)	Intensities*)			Assignments**)
		LCC-W	Fraction P-1		
			<i>T. palustris</i>	<i>C. versicolor</i>	
1	193.9	w	w	-	C=O in Ar-CH=CH-CHO C=O in Ar-CO-CH(-Ar)-C-O
2	190.9	w	w	w	C=O in Ar-CHO
3	177.1	vw	w	-	C=O in 4-O- methylglucuronic acid
4	174.1	-	m	-	C=O in -C-C(-OH)-COOH
5	172.1	-	-	w	C=O in R-O-CH ₂ -CH ₂ COOH
6	169.6	w	-	vw	Ester C=O in R-O-CH ₂ -COOH
7	167.2	-	vw	vw	C=O in Ar-COOH
8	152.9	vw	w	vw	C-3/C-3' in etherified G-units with C- β in Ar-CH=CH-CHO units
9	151.6	vw	vw	-	C-3 in etherified G-units α -C=O group
10	149.8	s	s	s	C-3 in etherified G-units
11	149.2	s	s	s	C-3 in etherified G-units
12	147.3	s	s	s	C-4 in etherified G-units
13	147.1	s	s	s	C-3 in nonetherified G-units
14	145.5	m	m	w	C-4 in nonetherified G-units
15	144.7	vw	w	vw	C-4/C-4' in etherified 5-5' units
16	143.5	vw	w	w	C-4/C-4' in non- etherified 5-5' units
17	135.1	w	w	vw	C-1 in etherified G-units
18	132.7	vw	vw	vw	C-5/C-5' in etherified 5-5' units
19	132.0	vw	vw	vw	Unknown
20	131.1	vw	vw	w	C-1 in nonetherified G-units
21	129.4	vw	vw	w	C- α and C- β in Ar-CH=CH-CH ₂ OH units
22	127.7	vw	w	w	C- β in Ar-CH=CH-CH ₂ OH units
23	125.8	vw	w	w	C-5/C-5' in etherified 5-5' units
24	123.0	w	w	w	C-1 in 4-O-alkyl-vanillic acid units C-6 in G-units with -C=O group
25	119.2	s	s	s	C-6 in G-units
26	115.2	s	s	s	C-5 in G-units

*) : vw = very weak; w = weak; m = moderate; s = strong.

**) : G = guaiacylpropane.

Table 2.1 Chemical shifts and signal assignments for the native LCC and the P-1 fractions obtained by *T.palustris* and *C.versicolor* after 31-days incubation. (continued)

Signal number	Chemical shifts (δ , ppm)	Intensities*)			Assignments**)
		LCC-W	Fraction P-1		
			<i>T.palustris</i>	<i>C.versicolor</i>	
27	112.8	s	s	s	C-2 in G-units
28	108.0	m			Carbohydrates
29	105.2	m			Carbohydrates
30	101.7	w			Carbohydrates
31	100.2	w			Carbohydrates
32	87.0	w	w	vw	C- β in G-type β -O-4 units (threo)
33	84.0	m	s	m	C- β in G-type β -O-4 units (erithro)
34	81.7	m	w	w	C- β in G-CO-CH(-OAr)-CH ₂ OH
35	77.6	w			Carbohydrates
36	76.7	w			Carbohydrates
37	75.4	m			Carbohydrates
38	73.6	m			Carbohydrates
39	71.8	s	s	s	C- α in G-type β -O-4 units (threo)
40	71.3	s	s	s	C- α in G-type β -O-4 units (erithro)
41	69.7	m			-OCH ₂ - in Ar-O-CH ₂ -CH ₂ OH units
42	68.4	w			-OCH ₂ - in R-O-CH ₂ -COOH
43	67.1	w			Unknown
44	66.3	w			Unknown
45	63.1	m	m	m	-CH ₂ -OH in Ar-CH ₂ OH units
46	62.8	m	m	m	C- γ in G-type β -5 and β -1 units
47	60.3	s	s	s	C- γ in G-type β -O-4 units
48	55.8	s	s	s	-CH ₃ in Ar-OCH ₃
49	55.6	w	w	w	C- β in β - β units or β -5 units
50	29.6	-	s	s	-CH ₂ - in saturated <i>n</i> -Alkyl group
51	20.6	m	vw	vw	-CH ₃ in acetyl units

*) : vw = very weak; w = weak; m = moderate; s = strong.

**) : G = guaiacylpropane.

α -carbonyl carbon signals still remained and a new sharp signal (174.1 ppm), due to aliphatic carboxyl group probably at the γ -positions, appeared together with a sharp methylene signal at 30.4 ppm. The presence of uronic acid and aromatic carboxyl carbon signals was also noticed. It is assumed that the aromatic carboxylic acids were produced from the $C\alpha$ - $C\beta$ cleavage followed by oxidative reaction [Dodson et al., 1987; Kawai et al., 1987; Kawai et al., 1990a]. The participation of α -carbonyl group in the $C\alpha$ - $C\beta$ cleavage has been suggested by some other investigators [Konishi et al., 1974; Kawai et al., 1990b].

2.3.4 Neutral sugar composition

The change of neutral sugar composition of the Fraction P-1 was analyzed and the result is shown in Fig. 2.8. In both fungi, five different neutral sugars were evenly detected with a few exceptions. In the case of *T. palustris* (Fig. 2.8a), the proportions of mannose and xylose slightly decreased. This seems reasonable because *T. palustris* produces high content of mannan- and xylan-degrading enzymes as described in Chapter 1. The result of *C. versicolor* shown in Fig. 2.8b is characteristic that arabinose proportion had a tendency to decrease because of the production of highly active α -arabinofuranosidase as described in Chapter 1.

2.3.5 Alkaline nitrobenzene oxidation

Figure 2.9 shows the yields of vanillin and vanillic acid after alkaline nitrobenzene oxidation. The results indicate a considerable reduction of vanillin in both cases, being more prominent in the case of *C. versicolor*. The yields of vanillic acid

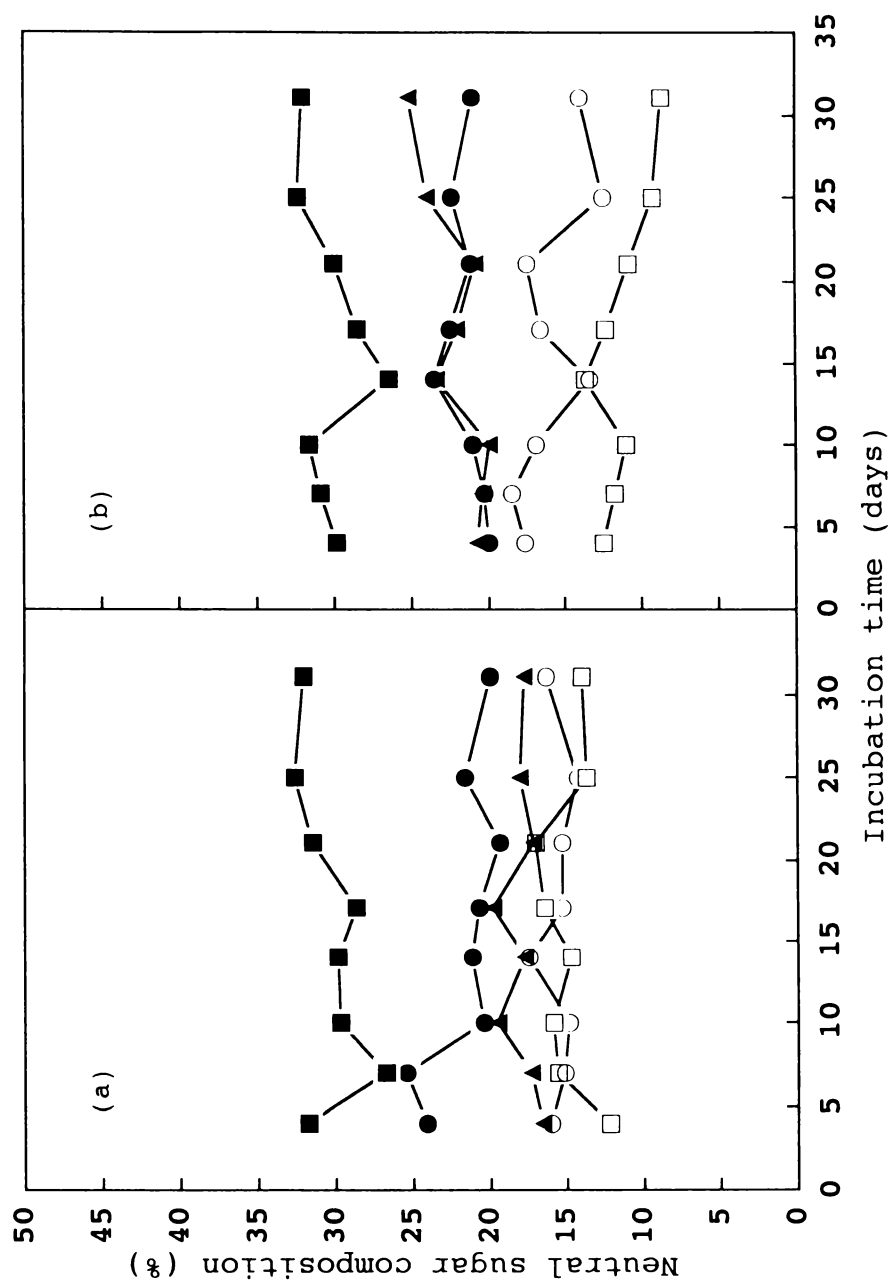


Fig. 2.8 Neutral sugar composition of the Fraction P-1.
 (a) *T. palustris*, (b) *C. versicolor*.
 ○: Xylose, □: Arabinose, ●: Mannose,
 ■: Galactose, ▲: Glucose.

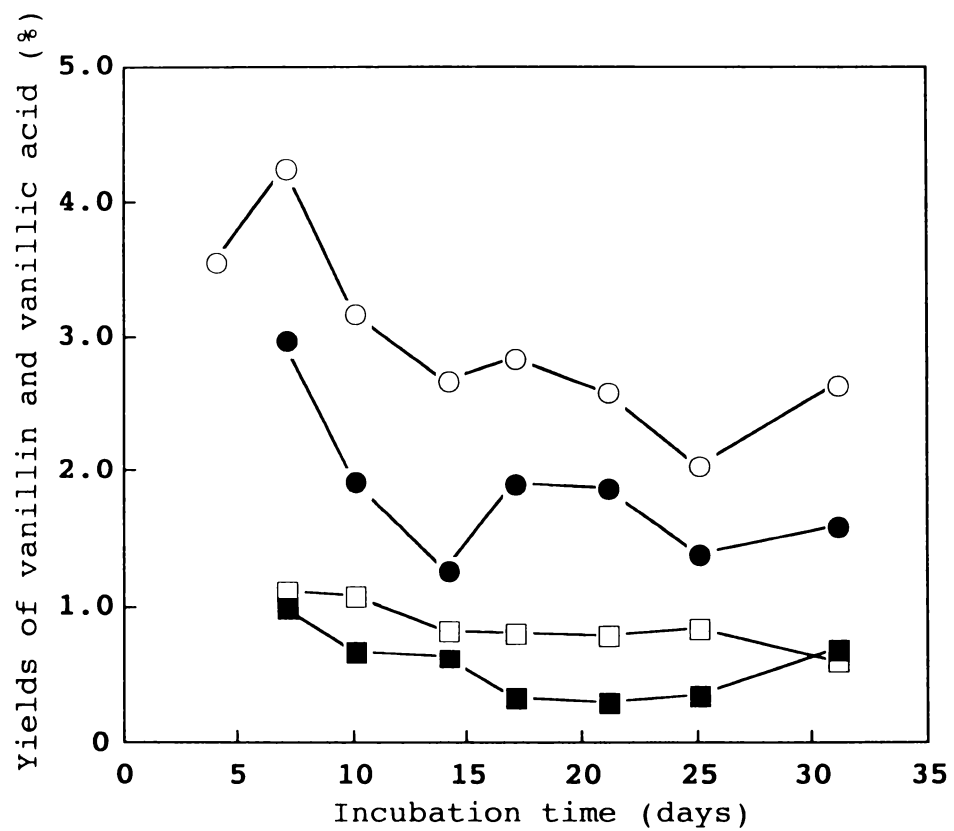


Fig. 2.9 Yields of vanillin and vanillic acid by alkaline nitrobenzene oxidation from the Fraction P-1.
 O, □: *T. palustris*, ●, ■: *C. versicolor*.
 ○, ●: Vanillin, □, ■: Vanillic acid.

were also slightly smaller in both fungi. Previously, some investigators also reported that the yield of vanillin was reduced in the brown-rotted lignin [Higuchi et al., 1955; Ishikawa et al., 1963a; Jin et al., 1990]. In the case of *C. versicolor*, Konishi et al. [1972] ascribed this as the result of a partial conversion of non-condensed units of lignin into condensed units by the action of laccase. The present results confirmed this and could be ascribed to the side chain modification by both fungi.

2.3.6 Functional group analyses

Changes in phenolic hydroxyl, α -carbonyl, methoxyl and carboxyl contents with progress of incubation are shown in Figs. 2.10-2.15. In the case of *T. palustris*, both non-conjugated and conjugated phenolic hydroxyl groups kept almost constant (Figs. 2.10 and 2.11). A slight decrease in α -carbonyl content (Fig. 2.12) suggests the occurrence of a side chain modification. As for carboxyl group (Fig. 2.13), which increased during incubation, the uronic acid content (Fig. 2.14) and ^{13}C -NMR spectroscopic analysis (Fig. 2.7) indicate a complicated contribution due to uronic, aliphatic and aromatic acids.

In the case of *C. versicolor*, non-conjugated phenolic hydroxyl group content, most of the time, stayed constant within 21 days, after that, however, the value gradually increased (Fig. 2.10). In contrast, conjugated phenolic hydroxyl content initially decreased but turned to increase after 21-days incubation (Fig. 2.11). Alpha-carbonyl group content generally increased in the white-rotted lignin [Ishikawa, 1963; Hata, 1966; Kirk and Chang, 1975], but that

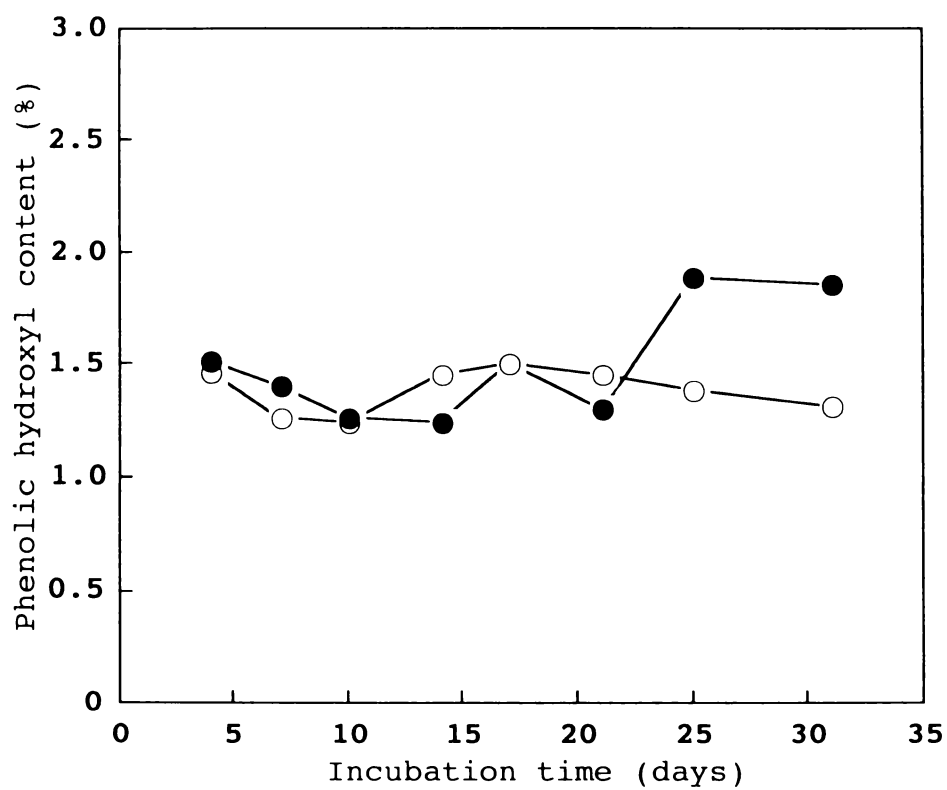


Fig. 2.10 Phenolic hydroxyl group content of the Fraction P-1.

○: *T. palustris*, ●: *C. versicolor*.

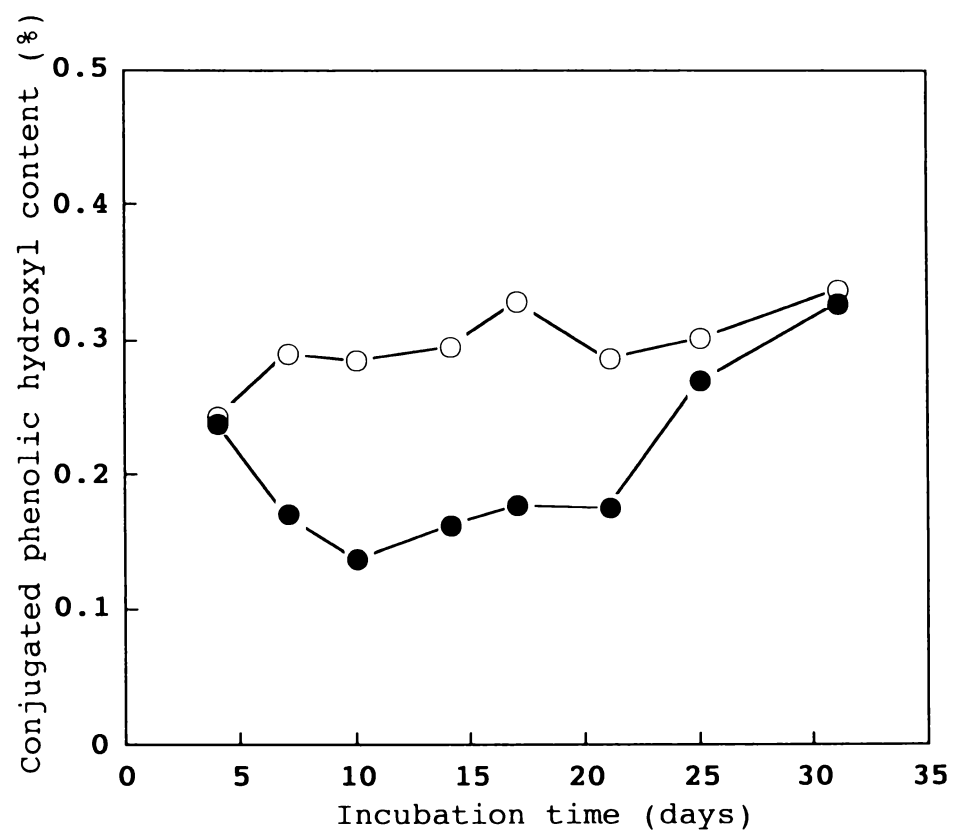


Fig. 2.11 Conjugated phenolic hydroxyl group content of the Fraction P-1.
 ○: *T. palustris*, ●: *C. versicolor*.

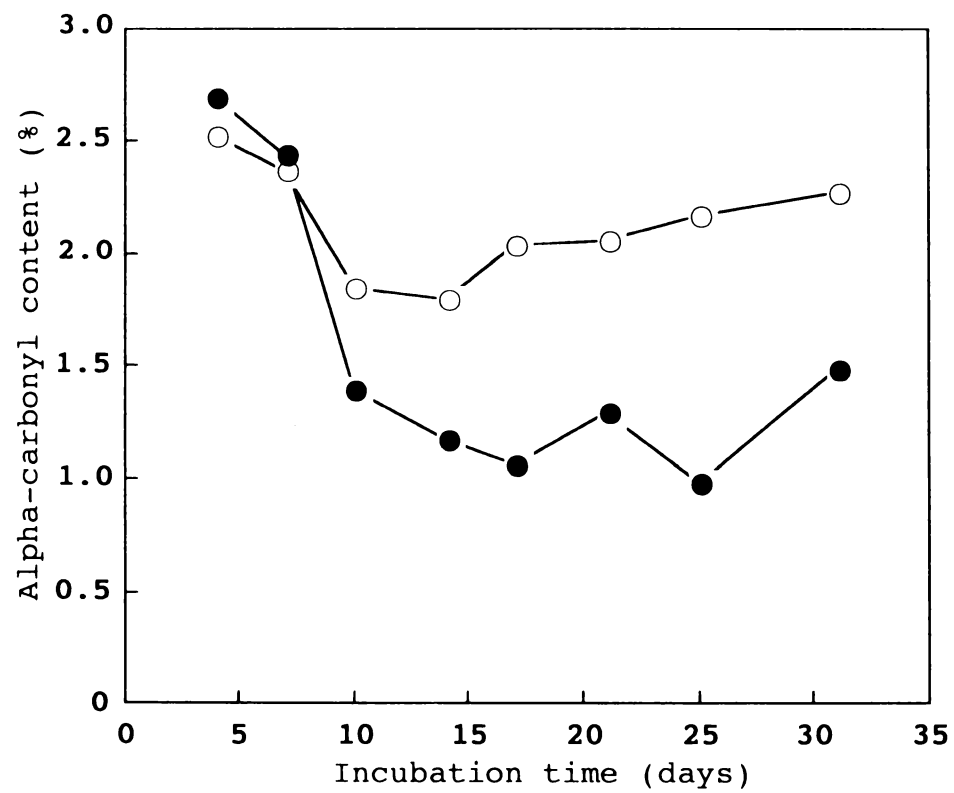


Fig. 2.12 Alpha-carbonyl group content of the Fraction P-1.
 ○: *T. palustris*, ●: *C. versicolor*.

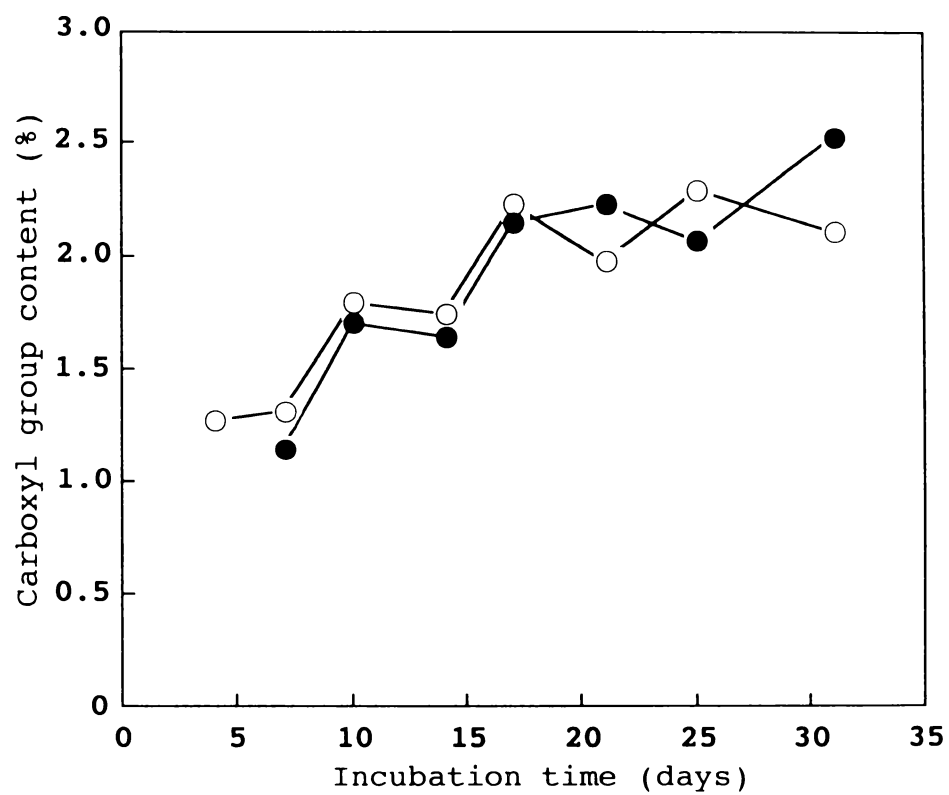


Fig. 2.13 Carboxyl group content of the Fraction P-1.
○: *T. palustris*, ●: *C. versicolor*.

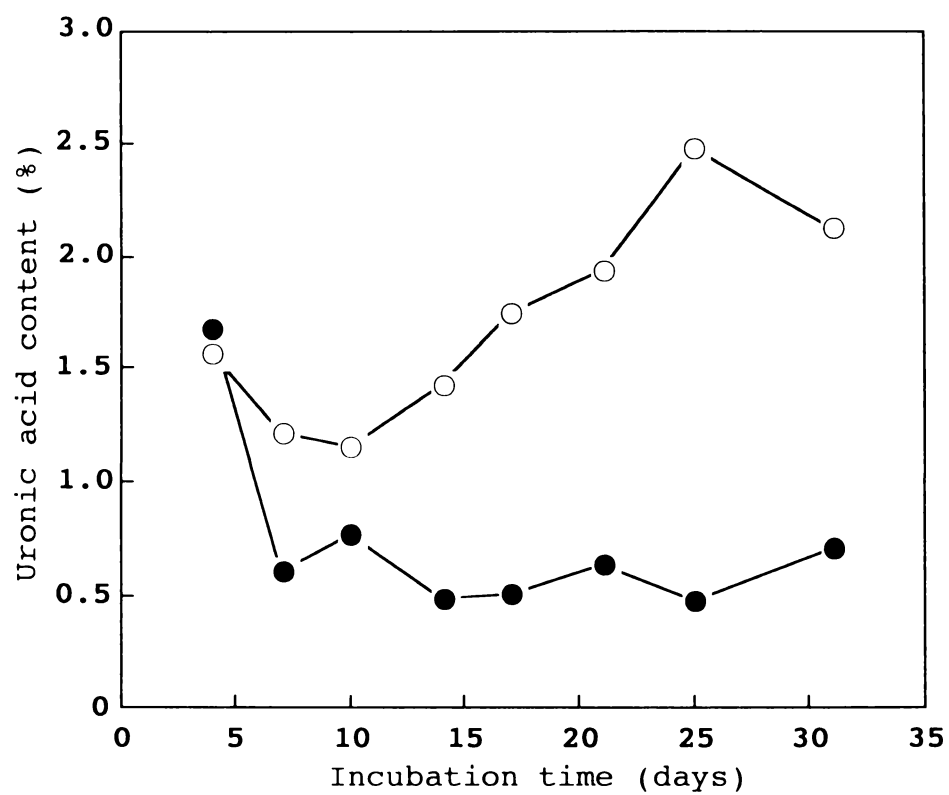


Fig. 2.14 Uronic acid content of the Fraction P-1.
○: *T. palustris*, ●: *C. versicolor*.

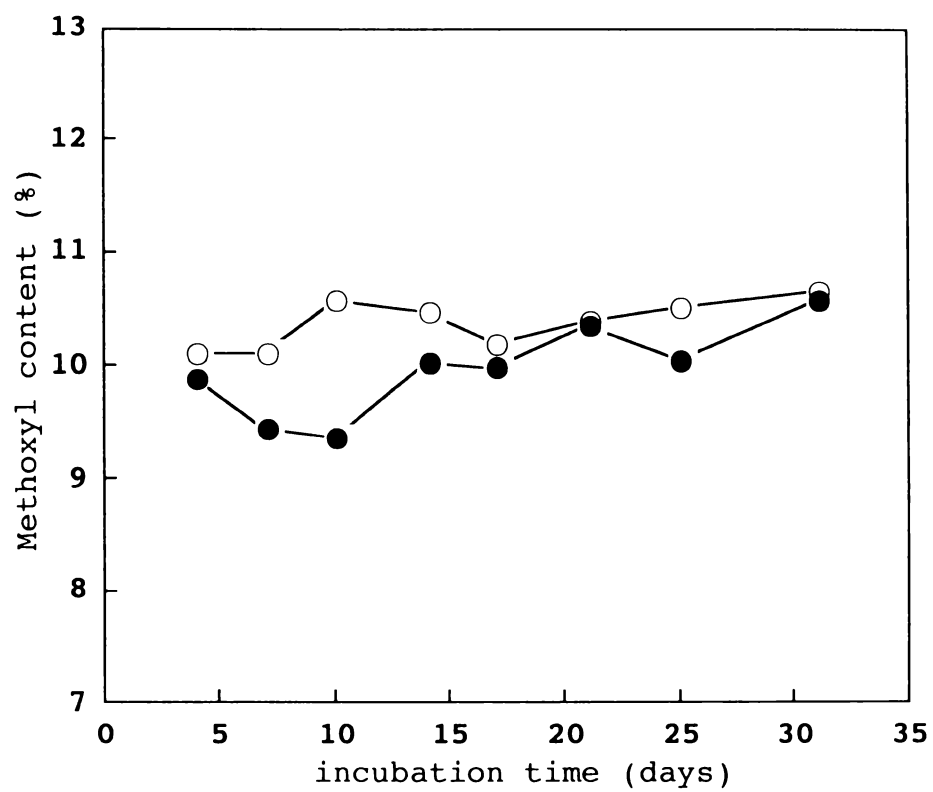


Fig. 2.15 O-Methoxyl group content of the Fraction P-1.
○: *T. palustris*, ●: *C. versicolor*.

in this fraction, as shown in Fig. 2.12, did not follow the tendency and decreased in the early stage of the incubation. The methoxyl content seemed to keep almost constant (Fig. 2.15), which indicates that demeth(ox)ylation did not occur.

The results of functional group analyses indicate that the degradation of lignin by *C. versicolor* progressed in two different steps : modification of side chains at early stage of incubation followed by increase of phenolic hydroxyl group contents after 21-days incubation. There are two interpretations to explain this degradation mode : 1) a cleavage of the side chains with α -carbonyl groups by oxidases such as laccase [Kawai et al., 1990], or 2) a reduction of α -carbonyl group [Chua et al., 1982]. Since phenoloxidases and peroxidases appeared after 7-days incubation as described in Chapter 1 and the decrease of α -carbonyl content (Fig. 2.12) coincided with the decrease of conjugated phenolic hydroxyl groups (Fig. 2.11), the first interpretation seems to be more reasonable. This is supported by the change of carboxylic acid content increased after 10-days incubation with decrease of uronic acid content in contrast to the case of *T. palustris*, indicating the formation of carboxylic acid in the side chains of lignin which supports the mechanism postulated by Konishi et al. [1972] The appearance of several types of carboxylic carbonyl signals in ^{13}C -NMR spectrum of this fraction obtained after 31-days incubation (Fig. 2.7, Table 2.1) further supports this interpretation.

2.4 Summary

Water-insoluble fraction P-1 was formed during incubation of *Tyromyces palustris* and *Coriolus versicolor* using water-soluble LCC (LCC-W) as a sole carbon source. Its yield, in the case of *C. versicolor*, increased until 14 days and then turned to decrease, while that in the case of *T. palustris* increased initially and kept almost constant. Re-solubilization of the Fraction P-1 in the case of *C. versicolor* is caused by the degradation of the lignin portion accompanied by the productions of phenoloxidases and peroxidases.

The structural changes of the Fraction P-1 by each fungi were characterized by the chemical and spectral analyses. Although the neutral sugar compositions of the Fraction P-1 from both fungi did not show characteristic changes during incubation except for a little discrepancy, remarkable differences were found in the lignin portion. In the case of *T. palustris*, the results of functional and spectral analyses indicated the occurrence of a slight modification of the lignin portion, and the molecular weight of the Fraction P-1 decreased from about 4,000 to 2,900.

On the other hand, an attack on the lignin portion by *C. versicolor* was found to occur in at least two steps. The first was initiated after about 7 days of incubation. In this step, the contents of phenolic hydroxyl and α -carbonyl groups decreased, whereas an increase of carboxylic acid and an increase in molecular weight from 3,600 to 6,400 was observed, indicating the occurrence of a side-chain modification and the polymerization of the lignin portion. The second step was initiated after about 21 days of

incubation. At this step, an increase in phenolic hydroxyl group content was noticed, and carbon-13 nuclear magnetic resonance (^{13}C -NMR) spectroscopic and gel-permeation chromatographic (GPC) analyses suggest the occurrence of cleavage of the aryl-ether linkages of lignin, leading to the decrease in its molecular weight. In addition, only in the case of *C. versicolor*, bondings between lignin and hemicellulose were cleaved accompanied by the productions of phenoloxidases and peroxidases, suggesting that these enzymes participated in this reaction.

Chapter 3 STRUCTURAL ANALYSIS OF THE FRACTION PRECIPITATED BY ACIDIFYING LIQUID CULTURE OF *CORIOLUS VERSICOLOR*

3.1 Introduction

During incubation of *Coriolus versicolor* with LCC-W, the fraction precipitated by acidifying the culture media was formed after 10 days. Crawford et al. [1983a] have already reported that the ligninolytic actinomycetes *Streptomyces viridosporus* generated acid-precipitable polymeric lignin (APPL) as an intermediate during lignin degradation in the solid-state lignocellulose fermentation. As for white-rot fungi, *Phanerochaete chrysosporium* and *C. versicolor* also generated APPL but its amount is quite small and less than that of *Streptomyces* [Crawford et al., 1983b]. But, during degradation of LCC-W, the amount of the fraction precipitated by acidification was major and larger than that of the Fraction P-1.

In this chapter, this fraction (named P-A) was isolated at various incubation times of up to 31 days, and its chemical and structural changes were characterized to estimate the action by *C. versicolor* [Tsujiyama et al., 1993b].

3.2 Materials and Methods

3.2.1 Incubation of mycelia and preparation of the fraction (P-A) precipitated by acidification of the culture medium

C. versicolor was incubated for 31 days in the liquid culture containing LCC-W as a sole carbon source as described in Chapter 1.

After an appropriate incubation time interval, the culture was centrifuged to remove insoluble materials, mycelia and water-insoluble degraded LCC fraction (P-1). The supernatant was taken out and its pH value was determined. The supernatant was then acidified with dilute aqueous hydrogen chloride solution to pH 2-3, and 5 ml of dichloromethane was added to this solution. The mixture was shaken and centrifuged (8×10^4 g, 10 min) to remove organic layer which contained the lower molecular weight degraded products. The extraction with dichloromethane was repeated twice. The insolubilized material floating between organic and water layers was recovered as a precipitate by washing several times with distilled water. The precipitate was lyophilized and termed Fraction P-A.

3.2.2 General chemical and analytical methods

Chemical and analytical methods, gel-permeation chromatography (GPC) and instrumentations used were the same as those described in Chapter 2.

3.3 Results and Discussion

3.3.1 Preparation of the Fraction P-A and pH change

Yield change of the Fraction P-A was shown in Fig. 3.1. Note that no insolubilized material was formed by acidifying the culture medium when the LCC was substituted for by glucose as well as the intact culture media. In addition, the brown-rot fungus,

T. palustris, did not produce any acid-precipitable materials during incubation. Therefore, the Fraction P-A was deemed characteristic of *C. versicolor* and would be formed by modification and/or degradation by its ligninolytic system.

Figure 3.1 shows that the Fraction P-A was formed after 10 days of incubation. The yield of the Fraction P-A increased gradually during 10 to 17 days, and then decreased slightly. As described in Chapter 2, the decrease of the water-insoluble Fraction P-1 obtained from *C. versicolor* culture occurred after 10 days of incubation by re-solubilization of the fraction into the culture medium caused by a modification of the lignin portion. Therefore, the increment of the Fraction P-A between 10 and 17 days may include the solubilized portion of the Fraction P-1. In the case of *Streptomyces* sp., APPL production and peroxidase activity are correlated positively [Pasti et al., 1991]. It is certain that yield of the Fraction P-A increased as the productions of phenoloxidases and peroxidases increased between 10-17 days, but the increase of the Fraction P-A seems to cease when oxidase productions achieved maximum.

By multiplying the yields with lignin content, the amount changes of lignin and hemicellulose in the Fraction P-A can be calculated and the values are shown in Fig. 3.2. The amount of lignin in the Fraction P-A increased gradually until 17 days of incubation and after that remarkably decreased, while the amount of hemicellulose was constant until 17 days and after that increased.

The pH change of the culture media occurred after 10 days of incubation as described in Chapter 1. Since the Fraction P-A was

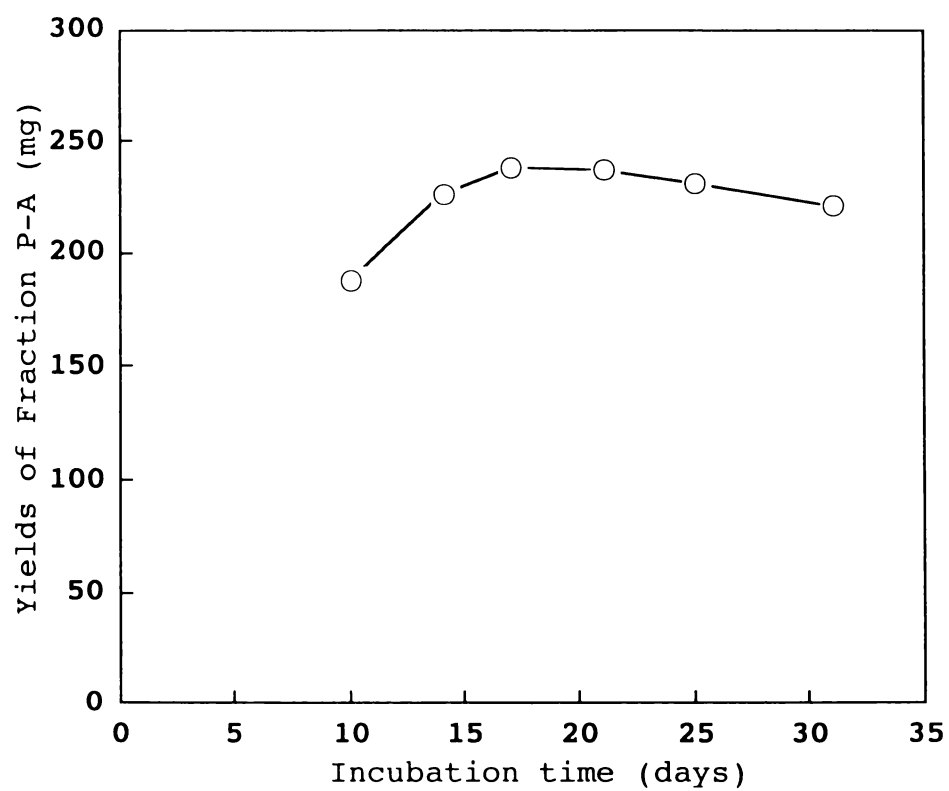


Fig. 3.1 Changes of yield of the Fraction P-A.

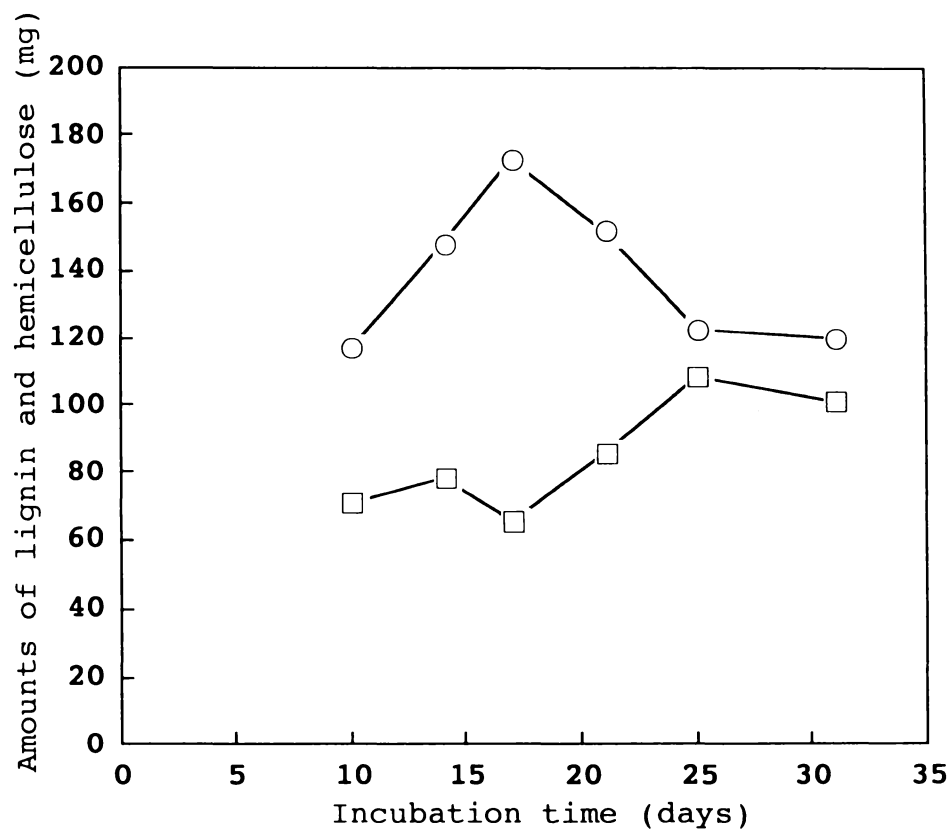


Fig. 3.2 Amounts of lignin and hemicellulose of the Fraction P-A.
○:Lignin, □:Hemicellulose.

recovered as an insoluble material after acidification, the formation of alkaline salt of weak acids, such as aromatic or phenolic acids, might take place to keep this fraction soluble in the aqueous medium. Although acid-precipitable polymeric lignin (APPL) was generated by *Streptomyces* sp. during the solid-state lignocellulose fermentation, its production was promoted at neutral to alkaline pH [Pometto and Crawford, 1986]. In this result, the Fraction P-A started to be formed after pH turned to rise, indicating that pH value might affect the formation of this fraction.

3.3.2 Molecular weight distribution

Figure 3.3 shows a GPC profile on TOYO PEARL HW55-F of the Fraction P-A after 31 days of incubation. As the incubation progressed, the hemicellulose portion was separated into several peaks which did not overlapped with ultraviolet (UV) absorption. The molecular weight of the major fraction having the largest UV absorption was estimated to be 77 % of the original LCC-W, indicating that the LCC was slightly degraded by *C. versicolor*.

3.3.3 Spectroscopic analyses

The change of absorptivity at 280 nm is shown in Fig. 3.4. The absorptivity increased drastically during 14-21 days of incubation and decreased gradually, similar to the α -carbonyl and carboxyl group contents (Figs. 3.10 and 3.11).

Figure 3.5 shows ^{13}C -NMR spectra of the Fraction P-A from a 31-days culture. Signal assignments were made according to the literature [Chua et al., 1982; Robert and Chen, 1989] and the

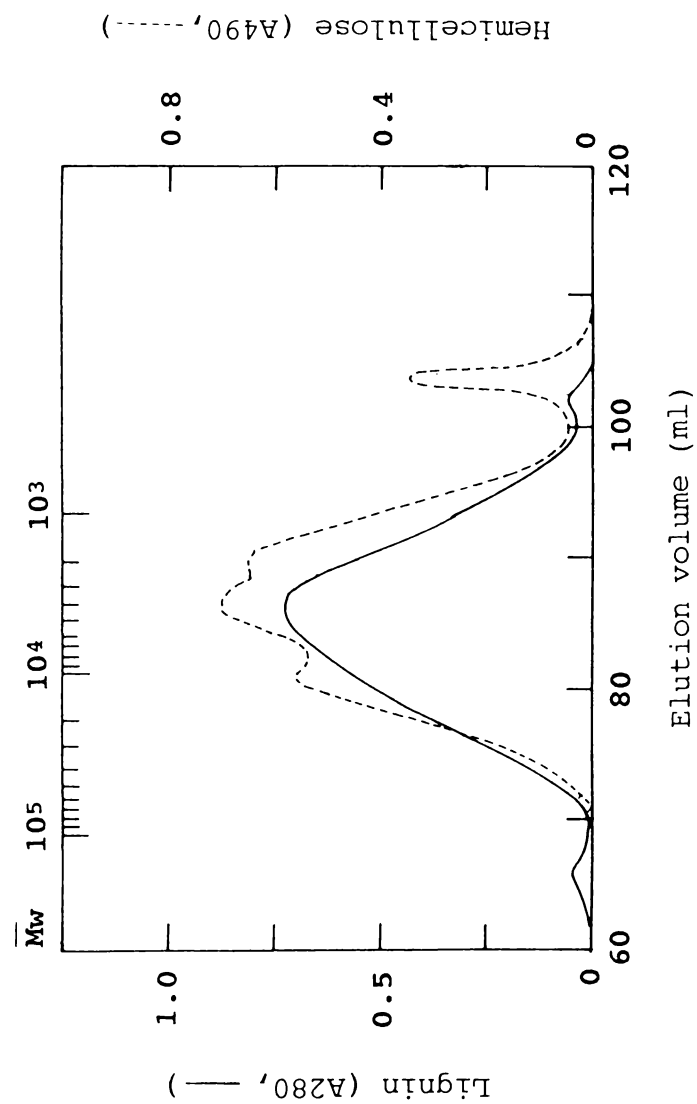


Fig. 3.3 GPC elution profiles of the Fraction P-A after 31 days of incubation.

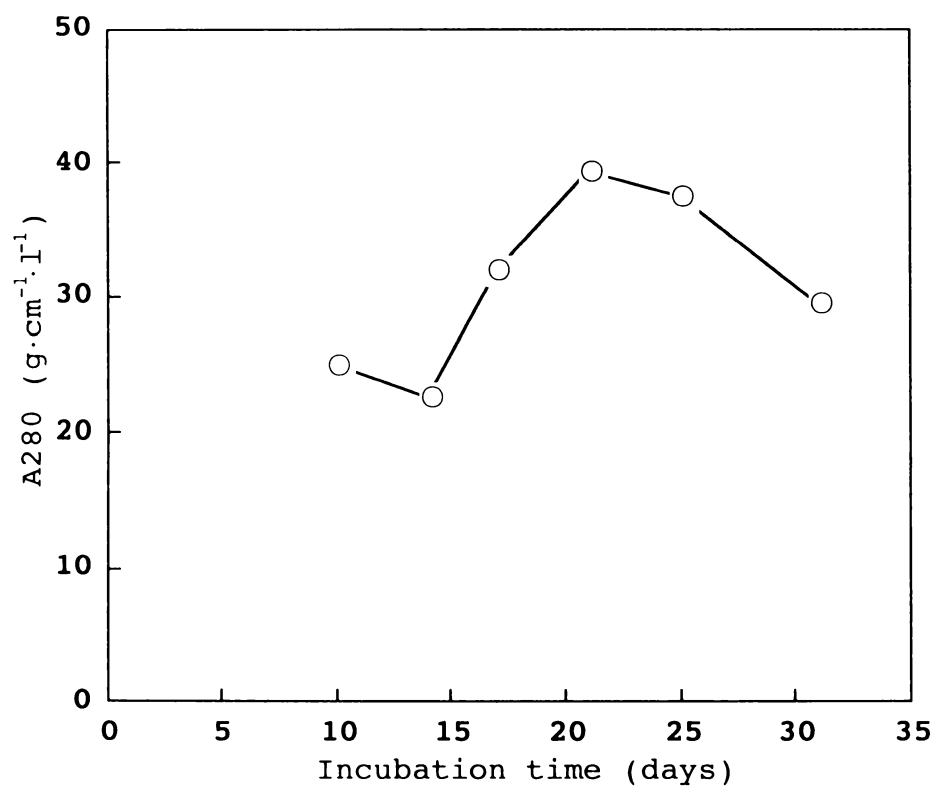


Fig.3.4 Change of absorptivity (at 280 nm) of the Fraction P-A.

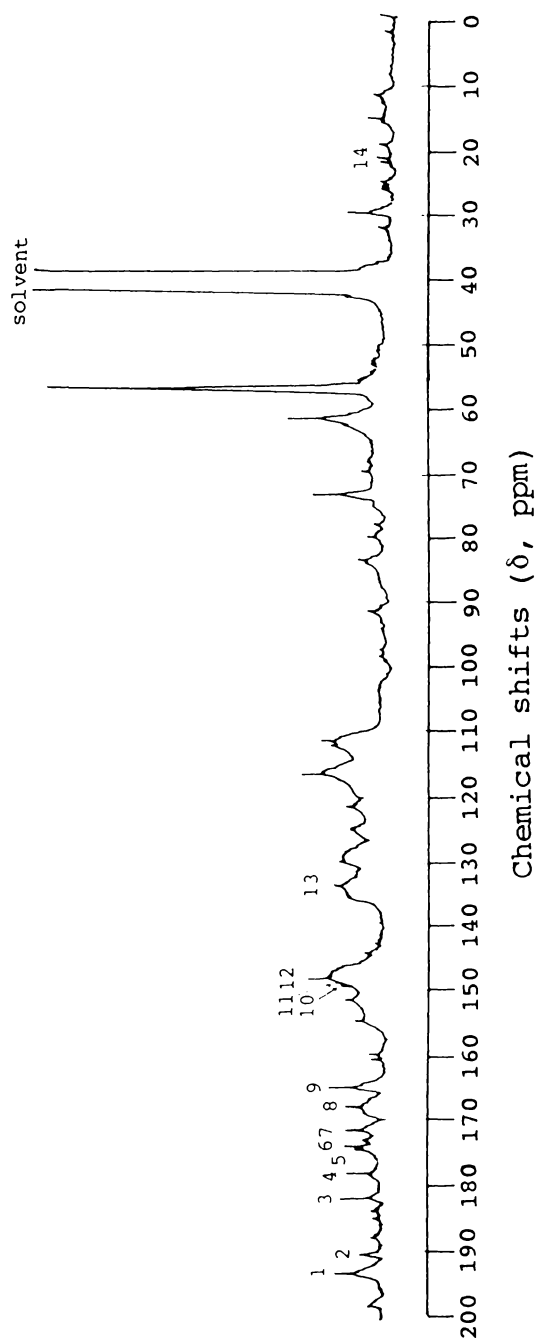


Fig. 3.5 Carbon-13 NMR spectrum of the Fraction P-A after 31 days of incubation.

Table 3.1 Chemical shifts and signal assignments for the native LCC and the P-A fractions obtained by *C.versicolor* after 31-days incubation.

Signal number	Chemical shifts (δ , ppm)	Intensities ^{*)}		Assignments ^{**)}
		LCC-W	Fraction P-A (<i>C.versicolor</i>)	
1	194.3	m	-	C=O in Ar-CH=CH-CHO C=O in Ar-CO-CH(-Ar)-C-
2	191.5	w	w	C=O in Ar-CHO
3	183.8	w	-	Unknown
4	177.1	w	-	C=O in 4-O-methylglucuronic acid
5	174.8		w	C=O in -C-C(-OH)-COOH
6	174.1	vw	vw	C=O in -C-C(-OH)-COOH
7	171.9	w	vw	C=O in R-O-CH ₂ -CH ₂ COOH
8	167.2	m	vw	C=O in Ar-COOH
9	165.6	m	-	Unknown
10	149.8	m	s	C-3 in etherified G-units
11	149.2	m	s	C-3 in etherified G-units
12	147.7	s	s	C-4 in etherified G-units
13	133.1	m	s	C-5/C-5' in etherified 5-5' units
	110-100		w	Carbohydrates
	78-70		vw	Carbohydrates
14	20.6	vw	w	CH ₃ in acetyl groups

^{*)} : vw = very weak; w = weak; m = moderate; s = strong.

^{**)}: G = guaiacylpropane.

results of major peaks were listed in Table 3.1. The characteristic signals of the Fraction P-A existed in the carbonyl region (160-195 ppm), including the signals which are due to aliphatic carboxylic acid and α -carbonyl groups. The signals due to aromatic carboxylic acids (167.2 ppm) also appeared with great intensities, indicating that the C α -C β cleavage seemed to be remarkable in this fraction. From these results, together with those of the alkaline nitrobenzene oxidation, a conjugated structure such as α -carbonyl was suggested to be formed in the middle to later period of incubation.

The signal of carbonyl carbon due to uronic acid (177.1 ppm) was also detected clearly. In addition, intensities of the signals at 149.8 and 149.2 ppm assigned as the aromatic carbons decreased, indicating an occurrence of structural changes in the aromatic moiety of the lignin portion as suggested by the action of phenoloxidases [Nakamura et al., 1989]. A decrease in absorptivity at 280 nm at the later period of incubation (Fig. 3.4) supports this interpretation. On the other hand, the signals due to hemicellulose (100-110 and 70-78 ppm) did not diminish so clearly as observed in the Fraction P-1. The disappearance of acetyl methyl signal at about 20.6 ppm and ester carboxyl band at 1735 cm⁻¹ in IR spectrum, however, indicates a removal of esterified acetyl groups during incubation.

3.3.4 Neutral sugar composition

The result of neutral sugar composition analysis of the Fraction P-A is shown in Fig. 3.6. It is clear that both galactose and mannose contents were large, more prominent with the former (about

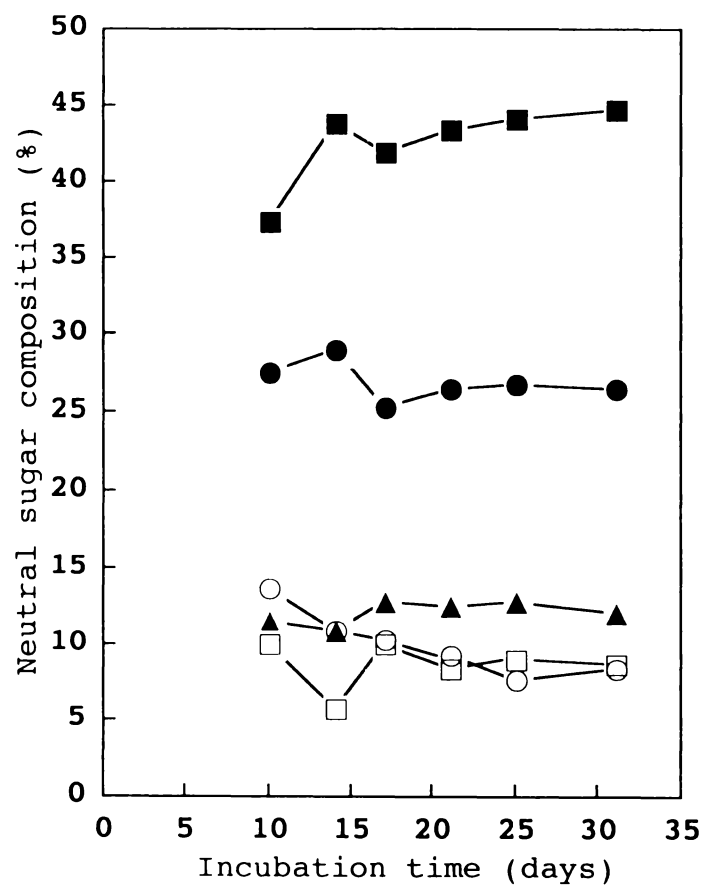


Fig. 3.6 Neutral sugar composition of the Fraction P-A.
 ○:Xylose, □:Arabinose, ●:Mannose,
 ■:Galactose, ▲:Glucose.

1.7-times compared to the original LCC-W at 31 days of incubation). The difficulty in the release of galactose may be due to the linkage to lignin in akamatsu LCC-W. By using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), Azuma showed that 27.5% of galactose was linked to lignin at the 6-position in akamatsu LCC-W [1989]. In contrast, the arabinose content decreased gradually, and both xylose and glucose contents were kept small. These results indicate that large amounts of galactosylated mannose residues originated from galactoglucomannan in LCC remained soluble in the culture medium for the Fraction P-A.

3.3.5 Alkaline nitrobenzene oxidation

The yields of the nitrobenzene oxidation products from the Fraction P-A, vanillin and vanillic acid, are plotted against incubation times as shown in Fig. 3.7. In early periods, the yield of vanillin decreased similar to the previous result of the Fraction P-1 as described in Chapter 2, but it began to increase after 17 days of incubation. On the other hand, the yield of vanillic acid increased slightly during incubation. These results were similar to the previous results given in the case of white-rotted lignin [Ishikawa et al., 1963a; Hata, 1966] and suggest the occurrence of a slight condensation of the lignin portion of the Fraction P-A in the early stage and shift to oxidation reactions such as C α -C β cleavage and C α -oxidation after about 17 days of incubation, confirming the results described in Chapter 2.

3.3.6 Functional group analysis of the Fraction P-A

The contents of two types of phenolic hydroxy group, conjugated

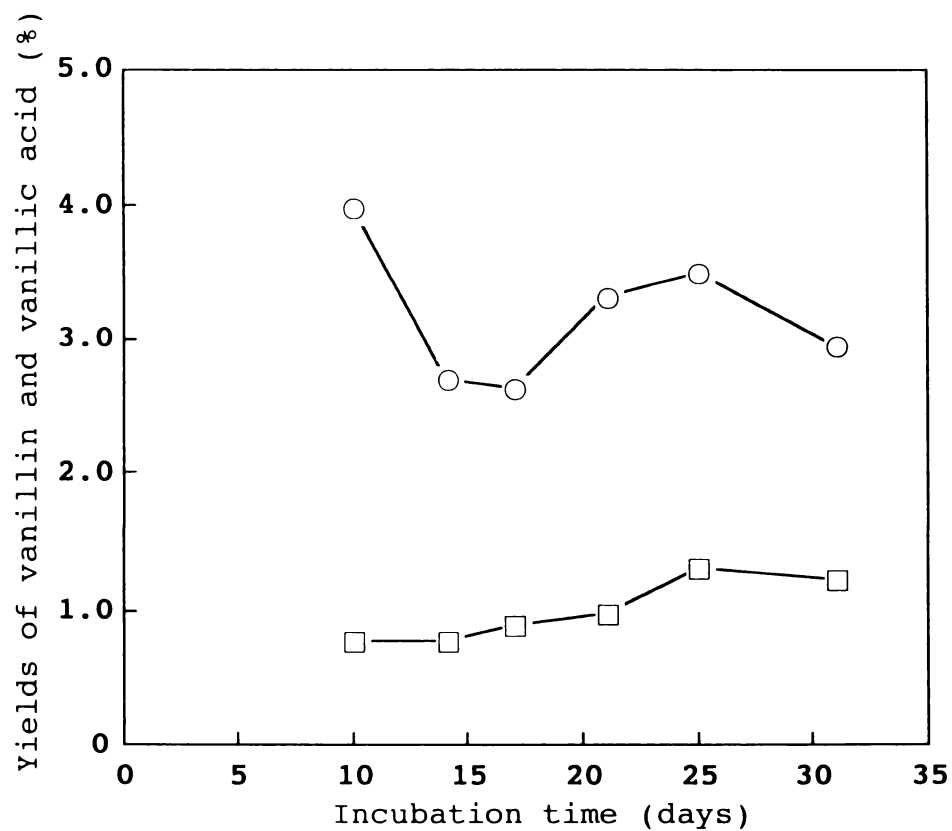


Fig. 3.7 Yields of vanillin and vanillic acid from the Fraction P-A by alkaline nitrobenzene oxidation.
O:Vanillin, □:Vanillic acid.

and non-conjugated, are shown in Figs. 3.8 and 3.9. Both phenolic hydroxyl group contents increased gradually to 2-2.5 times over 25 days. Note that the contents of both phenolic hydroxyl groups were about three times larger than those of the water-insolubilized Fraction P-1 as described in Chapter 2.

Figure 3.10 shows change of α -carbonyl group content in the Fraction P-A. The value was about 3 % at the beginning, almost the same as that of the original LCC (2.9 %), but increased drastically during 14 to 21 days. The increase in α -carbonyl groups was in good agreement with the previous results [Kirk and Chang, 1975; Chua et al., 1982], as was the increase in the lignin model compounds by the action of laccase secreted by *C. versicolor* [Kawai et al., 1988]. Because the Fraction P-A was water-soluble, the oxidation effect of laccase or other peroxidases was much more remarkable than on the insoluble Fraction P-1.

Figures 3.11 and 3.12 show the changes of total carboxyl group and uronic acid contents in the Fraction P-A, respectively. The former profile was similar to that of α -carbonyl group content (Fig. 3.10), and included values due to uronic acid and carboxylic acids such as aromatic and aliphatic carboxylic acids. The increase of the total carboxylic acid that started at 14 days of incubation may mainly be ascribed to the formation of the latter acids formed probably by the oxidation reaction. Konishi and Inoue [1971] reported that carboxylic acid content in the solubilized lignin increased by treatment with laccase of *C. versicolor*. Because the results described in Chapter 2 indicated that the productions of

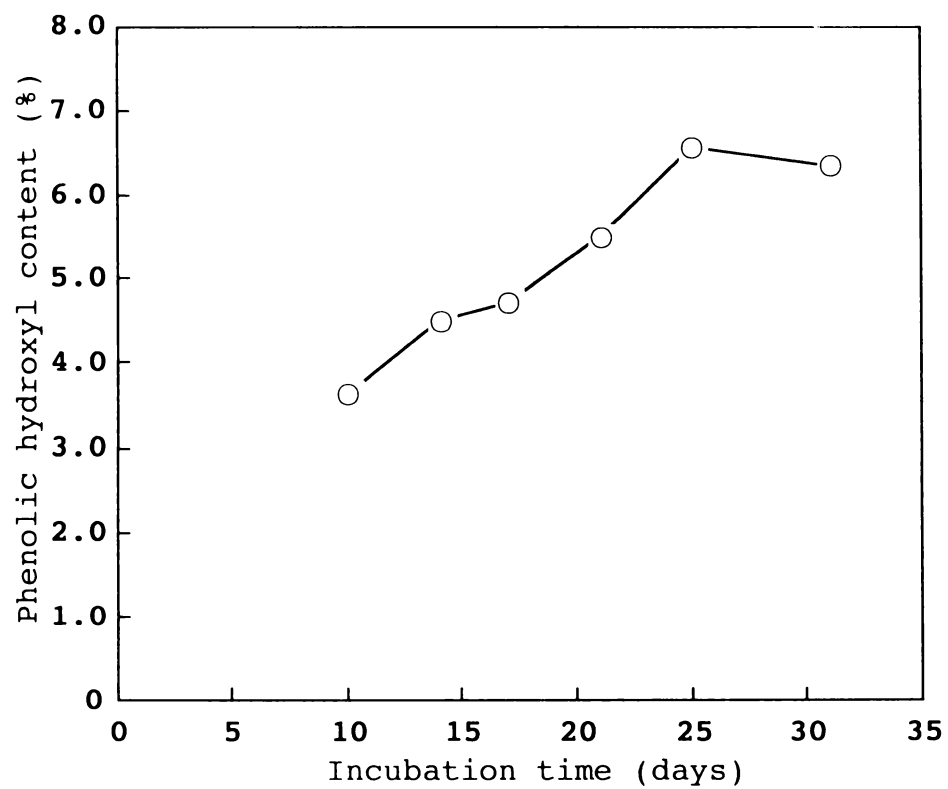


Fig. 3.8 Phenolic hydroxyl group content of the Fraction P-A.

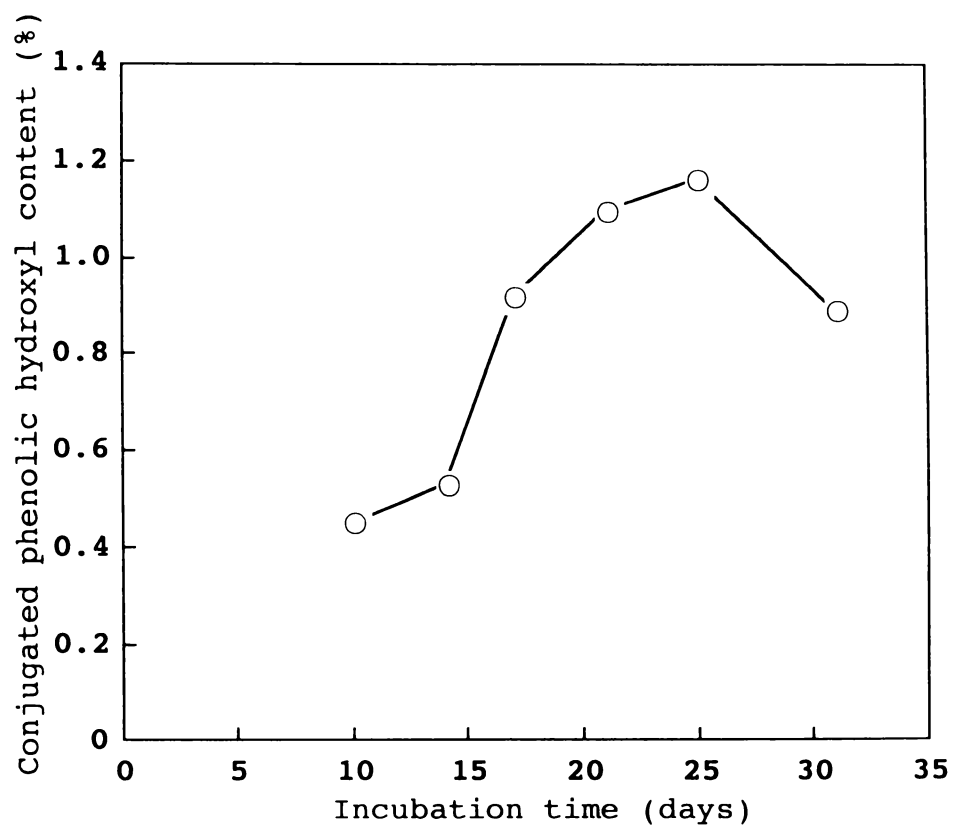


Fig. 3.9 Conjugated phenolic hydroxyl group content of the Fraction P-A.

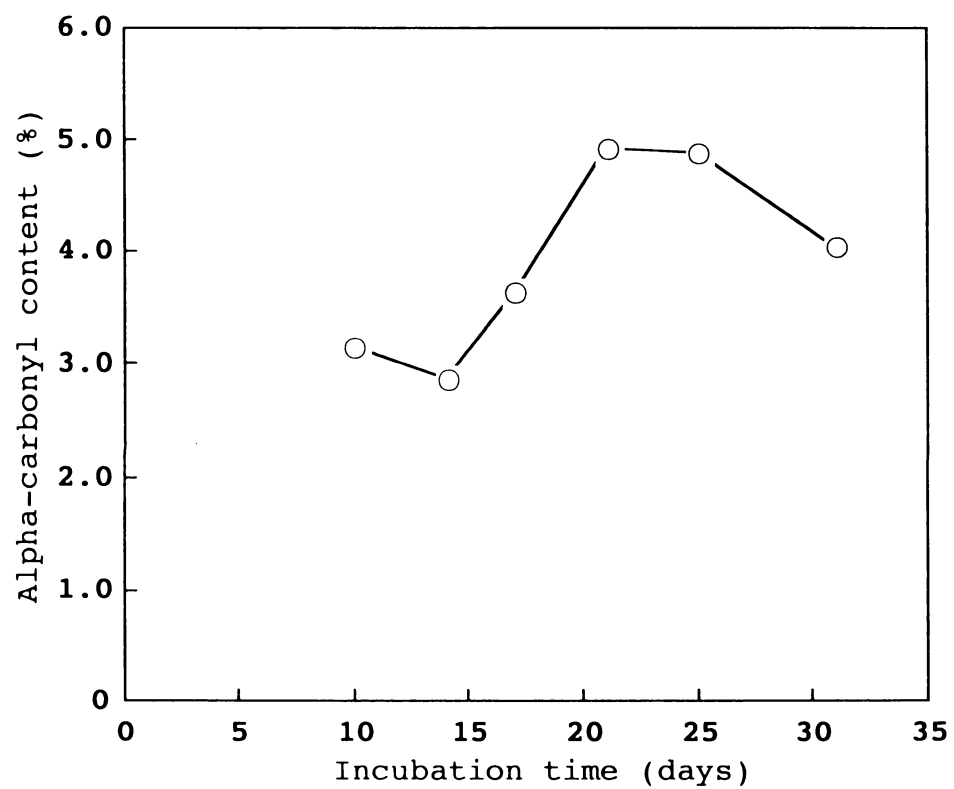


Fig. 3.10 Alpha-carbonyl group content of the Fraction P-A.

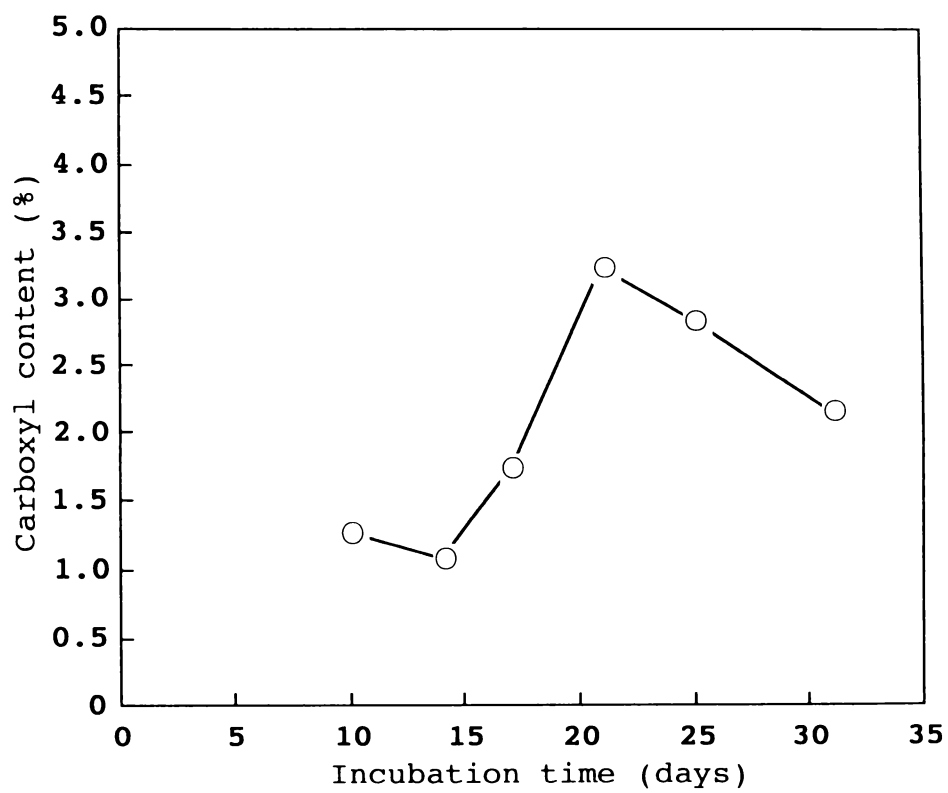


Fig. 3.11 Carboxyl group content of the Fraction P-A.

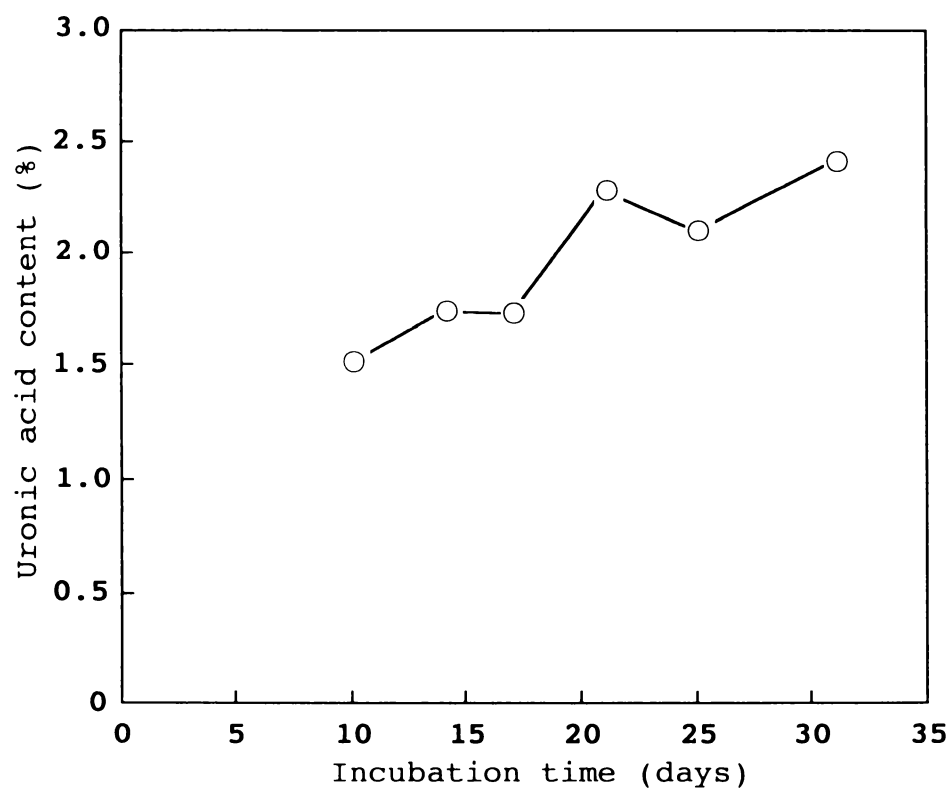


Fig. 3.12 Uronic acid content of the Fraction P-A.

phenoloxidasases and peroxidasases were prominent after 14 days of incubation, the increase of carboxylic acid content after 21 days was suggested to have been caused by the oxidation reaction due to phenoloxidasases. Considering the results of α -carbonyl group content, the oxidation effect was prominent in the Fraction P-A at the later stage of incubation.

Figure 3.13 shows the changes of methoxyl group content which gradually increased during incubation. However, the increment was as small as 2 %. From the change of uronic acid content (Fig. 3.12), about 50 % of the increase probably was due to the increasing proportion of 4-O-methyl glucuronic acid. It seems, therefore, that the increment of phenolic hydroxyl group observed in Figs. 3.8 and 3.9 was not formed by demethylation.

From functional group analysis, the degradation of the lignin portion in LCC by *C. versicolor* to form the acid-precipitated Fraction P-A was concluded to proceed by the initial formation of phenolic hydroxyl groups followed by the oxidation reaction to form new α -carbonyl and carboxyl groups. Formation of new phenolic hydroxyl groups may introduce the hydrophilicity to lignin and make the action of phenol-oxidizing enzymes easy, leading to the effective decomposition of lignin. Kurek et al. [1990, 1993] proposed that colloidal lignin, which is highly dispersed in liquid culture, was easily oxidized and modified by lignin peroxidase and horseradish peroxidase. Thus, the following oxidation reaction was remarkable more than that in the case of the Fraction P-1. This oxidation reaction is considered to be caused by phenoloxidasases

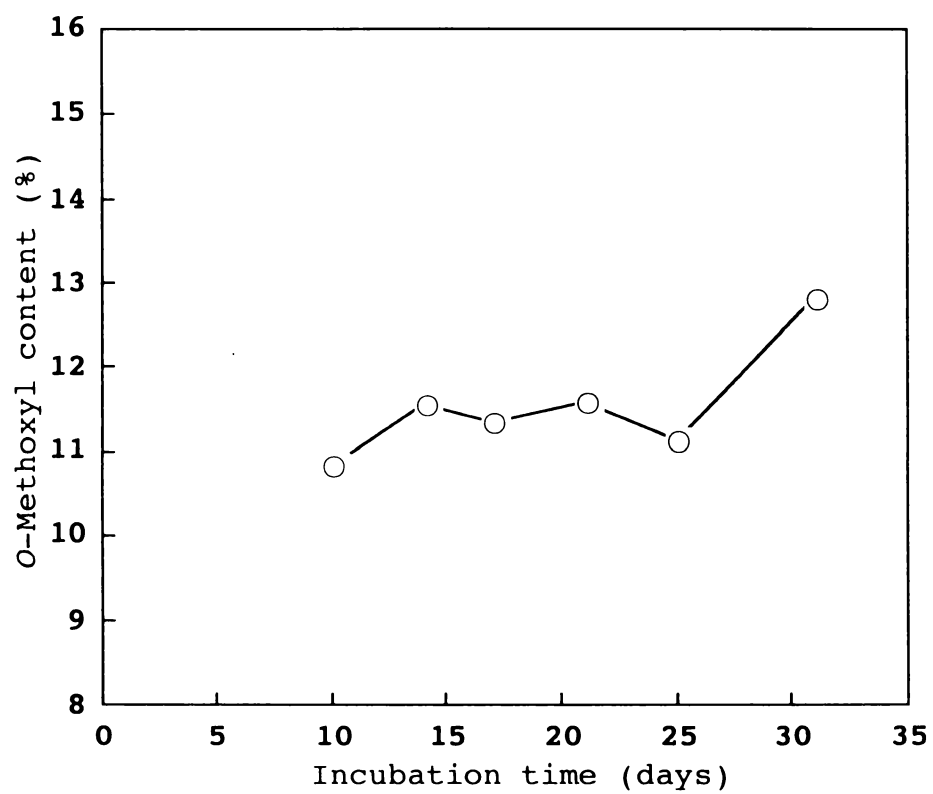


Fig. 3.13 O-Methoxyl group content of the Fraction P-A.

because it was prominent after 14 days of incubation when substantial secretions of phenoloxidases and peroxidases could be detected (Chapter 1).

In contrast, however, it was difficult to interpret the initial reaction to the increase in phenolic hydroxyl groups at the beginning of the Fraction P-A formation (10 days of incubation). Although secretion of laccase started in this stage of incubation (Chapter 1), participation of this enzyme was excluded, as Konishi and Inoue explained that the phenolic hydroxyl group content in milled wood lignin diminished by the action of laccase [1971]. As for lignin peroxidase, which was detected at the time of the P-A fraction formation, it has been reported that lignin peroxidase from *Phanerochaete chrysosporium* can cleave β -O-4 linkage of lignin model compound via an initial single-electron oxidation reaction [Umezawa, 1988] to form phenolic hydroxyl groups which were subjected, however, to secondary transformation into quinones, leading to unexpectedly small yields of phenolic compounds [Yokota et al., 1988]. Even if this actually occurred in the present case, an oxidative change such as C α -oxidation or aromatic acid formation should be detected in the Fraction P-A in the same period of the new phenolic hydroxyl group formation. However, as shown in Figs. 3.10 and 3.11, these results did not fit this observation. Thus, lignin peroxidase was also excluded. The possibility of the participation of Mn-dependent peroxidase, which is considered to be one of the ligninolytic enzymes, also can be excluded because of the lack of secretion at the time of the initial increase in phenolic hydroxyl

groups as described in Chapter 1. Therefore, the increase of the phenolic hydroxyl group content seemed to be caused by neither phenoloxidases nor peroxidases. The quinone reductases might be considered to disturb the conversions of the phenolic hydroxyl groups to the quinone structures formed by phenolic hydroxyl groups. Westermarck and Eriksson [1974] discovered a quinone reducing enzyme, cellobiose:quinone oxidoreductase (CBQase), which utilized reaction products of laccase to form phenols coupled with the oxidation of cellobiose. This enzyme was produced with cellulolytic enzymes, induced by cellulose and had a great specificity for cellobiose. LCC-W was free of cellulose, and Avicelase was produced within 7-10 days of incubation but not so much. CMCase was produced after 7-10 days of incubation but not as much of Avicelase. Beta-glucosidase also was produced, but in not so large amount. Therefore, the possibility of the participation of CBQase is considered to be quite small, although participation of other quinone reducing enzymes cannot be excluded.

Therefore, it is assumed that *C. versicolor* has an uncharacterized lignin-degrading system which can form new phenolic hydroxyl groups prior to the oxidation reaction. Beta-etherase seems to be a key enzyme of this system. Fukuzumi et al. reported the cleavage of the aryl-ether bond by wood-rotting fungi [Fukuzumi, 1960; Fukuzumi et al., 1969] and by bacteria [Fukuzumi and Katayama, 1977; Katayama and Fukuzumi, 1979], and β -etherase was detected in bacterium *Pseudomonas pausimobilis* [Masai et al., 1989]. Sarkanen et al. [1991] proposed the presence of an unknown lignin-

depolymerization system like β -etherase by *Phanerochaete chrysosporium*. Crawford and co-workers supposed that the enrichment of phenolic hydroxyl groups in APPL generated by *S. viridosporus*, similar to the Fraction P-A, was caused by the cleavage of the para-ether linkages and the demethylation [Crawford et al., 1983a] and that the formation of APPL and the solubilization of lignin were caused by uncharacterized lignin-specific enzymes, such as β -ether cleaving enzyme system [Deobald and Crawford, 1987].

Therefore, re-solubilization of insolubilized LCC is the important function of degradation by *C. versicolor*, and new phenolic hydroxyl groups would be formed by an uncharacterized degradation system. This reaction system might cause re-solubilization of the water-insolubilized fraction P-1 by releasing the hydrophilic moieties of lignin which involves large amounts of phenolic hydroxyl groups, while the moieties which contained low content of phenolic hydroxyl groups were still precipitated as the Fraction P-1.

3.4 Summary

During incubation of *Coriolus versicolor* with water-soluble lignin-carbohydrate complex (LCC-W), the formation of the fraction precipitated by acidifying (pH 2-3) the culture medium was found to start after 10 days of incubation. This fraction named P-A was prepared with various incubation times up to 31 days, and its structural changes were analyzed by chemical and spectroscopic methods. Based on the time course of structural changes of the lignin portion, the initial attack by *C. versicolor* was the

formation of new phenolic hydroxyl groups, which probably was followed by an oxidation reaction to form new α -carbonyl and carboxyl groups. Although the latter reaction could be explained by the co-operative actions of laccase and other peroxidases, the former reaction cannot be interpreted fully by the other preceeding systems. Therefore, an uncharacterized lignin-degrading system to participate in the lignin degradation is suggested to be present, such as an aryl-ether cleaving system.

Chapter 4 ENZYMIC SPLITTING OF BONDINGS BETWEEN LIGNIN AND HEMICELLULOSE VIA QUINONE METHIDE BY *CORIOLUS VERSICOLOR*

4.1 Introduction

In Chapter 2, gel-permeation chromatographic analysis of the degraded LCC fraction P-1 indicated that the peaks due to hemicellulose and lignin were separated by the actions of *C. versicolor*, suggesting the splitting of bondings between lignin and hemicellulose, while no indication was observed in the case of *T. palustris* (Fig. 2.13). Considering the results of enzyme analyses shown in Chapter 1, separation of the peaks seemed to be accompanied by the productions of phenoloxidases and peroxidases.

Sugars in LCC were confirmed to link to lignin at α -position with ether-linkages by treatments with alkaline borohydride [Eriksson and Goring, 1980; Iversen, 1985] and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone [Azuma, 1989; Watanabe, 1989]. Especially, benzyl ethers which have free phenolic hydroxyl groups at *para*-positions are unstable and easily cleaved by alkaline treatment via quinone methide [Gierer and Noren, 1962; Larsson and Lindberg, 1962]. Phenol-oxidizing enzymes, such as laccase and horseradish peroxidase, can form phenoxy radicals from phenolic compounds [Nakamura, 1960; Caldwell and Steelink, 1969; Young and Steelink, 1973; Higuchi, 1986], which mediate the benzyl ether cleavage reaction as shown in Fig. 4.1

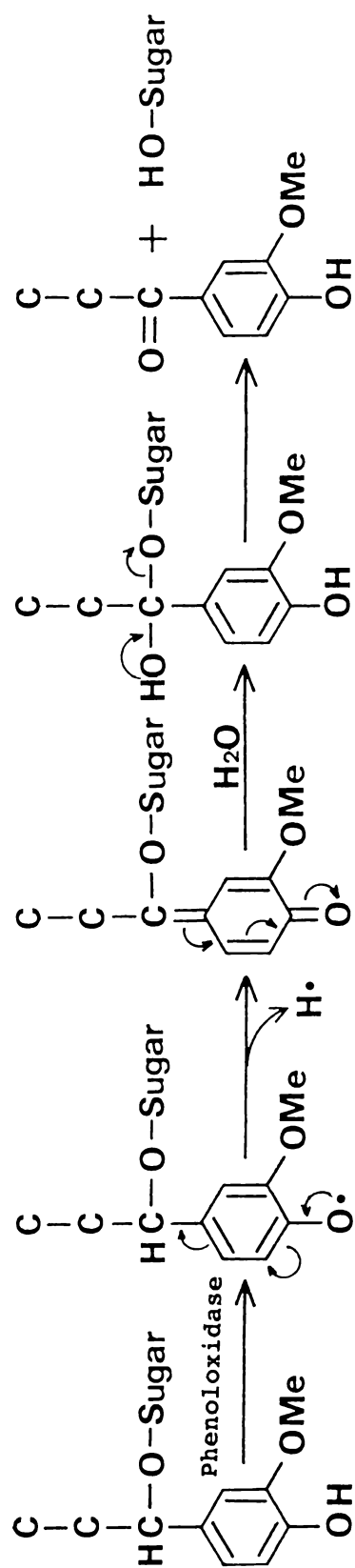


Fig. 4.1 Reaction scheme of splitting bondings between lignin and hemicellulose by phenoloxidase treatment.

In this chapter, it was tested whether benzyl ether bondings in LCC model compounds are cleaved by the crude enzyme solution from *C. versicolor*, and compared the reactions of laccase prepared from the culture media of *C. versicolor* and horseradish peroxidase against cellulase-treated akamatsu LCC with the result of alkaline treatment [Tsujiyama et al., 1993c].

4.2 Materials and Methods

4.2.1 Synthesis of LCC model compounds

Two kinds of LCC model compounds (I and II) were prepared (Fig. 4.2).

Methyl 6-*O*-vanillyl- β -D-glucopyranoside (I) was synthesized using quinone methide according to Ralph and Young [1983] : one mmol of vanillyl alcohol was dissolved into 10 ml of distilled chloroform. Bromination was taken place by adding 1 g of trimethyl bromosilane to chloroform solution at 4°C. After confirming the disappearance of a spot due to vanillyl alcohol on TLC plate (R_f = 0.37, solvent; chloroform : methanol = 10 : 1 (v/v)), reaction solution was poured into saturated sodium carbonate solution, and the mixture was shaken vigorously. Then organic layer was recovered and dried with anhydrous sodium sulfate. This quinone methide solution was poured into 10 ml of dimethylsulfoxide solution containing 10 mmol of methyl β -D-glucopyranoside and left over night with stirring. Model compound I was purified by dry column chromatography (solvent; hexane : ethyl acetate = 20 : 1 (v/v)) and high performance liquid

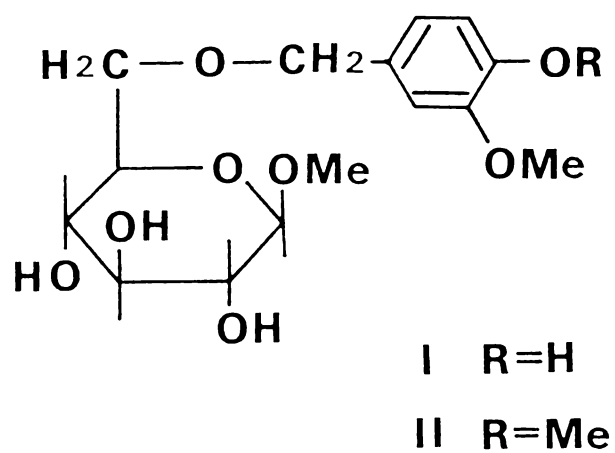


Fig. 4.2 LCC model compounds.

chromatography (HPLC) using KNAUER HPLC PUMP 64 with ODS No.201549 (YMC) (solvent; acetonitrile : H₂O = 15 : 85 (v/v), flow rate ; 4.0 ml/min). Compound I : ¹³C-NMR (methanol-*d*₄) : δ 105.3 (C-1), 74.9 (C-2), 78.0 (C-3), 71.8 (C-4), 76.8 (C-5), 70.4 (C-6), 57.4 (glucosidic CH₃), 56.4 (vanillyl CH₃) and 70.4 (CH₂) ; ¹H-NMR (methanol-*d*₄) : δ 4.15 (1H, d, H-1), 3.15 (1H, dd, H-2), 3.31 (1H, t, H-3), 3.29 (1H, d, H-4) 3.37 (1H, m, H-5), 3.37 (1H, dd, H-6), 3.61 (1H, dd, H-6'), 3.51 (3H, s, glucosidic CH₃), 3.84 (3H, s, vanillyl OCH₃), 4.48 (2H, s, benzyl CH₂).

Methyl 6-*O*-veratyl β-D-glucopyranoside (II) was synthesized from compound I by methylation of phenolic hydroxyl group with diazomethane. Model compound II was purified by HPLC with Asahi pak NH₂ P-90 (solvent ; acetonitrile : H₂O = 90 : 10 (v/v), flow rate ; 4.0 ml/min). Compound II : ¹H-NMR (methanol-*d*₄) : δ 4.15 (1H, d, H-1), 3.18 (1H, dd, H-2), 3.32 (1H, t, H-3), 3.50 (1H, t, H-4), 3.35 (1H, m, H-5), 3.63 (1H, dd, H-6), 3.62 (1H, dd, H-6'), 3.50 (3H, s, glucosidic CH₃), 3.80 (3H, s, veratryl OCH₃), 3.82 (3H, s, veratryl OCH₃), 4.51 (2H, s, benzyl CH₂).

4.2.2 Enzyme treatment of LCC model compounds

Crude enzyme solution from 14-days incubation culture media of *C. versicolor* was prepared following the procedure described in Chapter 1.

Reaction solution was contained 0.25 mmol of substrate (compound I or II), 0.25 mmol of inositol, 50 μM of glucono-1,5-lactone,

inhibitor of β -glucosidase which can cleave glucosidic linkage of the substrate, 0.1 ml of 0.1 M sodium acetate buffer (pH 5.3) and 0.1 ml of crude enzyme solution. Total volume was adjusted to 0.5 ml by addition of distilled water. The effects of peroxidase on the reaction was analyzed by addition of 0.3 μ mol of H_2O_2 in the reaction mixture. Inhibition test of phenoloxidas and peroxidas was carried out by the addition of 10 μ mol of sodium azide into the reaction solution. After incubation at 36°C for 1 hr, the reaction solution was desalted by passage through ion-exchange resins (Dowex 50 $\times$ 1 (H^+) and Dowex 1 $\times$ 8 (AcO^-)). Eluent was concentrated to dryness and acetylated. The amount of methyl β -D-glucopyranoside was analyzed as acetate, as described in Chapter 2.

4.2.3 Preparation of cellulase-treated LCC

Akamatsu LCC-W was treated with Dricelase (Kyowahakko, Co., Ltd.) at 36°C. Substrate and enzyme concentrations were 1.0 and 0.2 %, respectively. After incubation for 24 hrs, equivalent amount of the enzyme was added into the reaction mixture, and incubation was further continued for 24 hrs. The precipitate was recovered by centrifugation, washed with water, and lyophilized. This precipitate was further treated with cellulase Onozuka R-10 as described above. The insoluble material was recovered by centrifugation, and then dissolved in 90% aqueous acetone and centrifuged to remove insoluble materials. Acetone-soluble material was evaporated under reduced pressure. The materials precipitated during removing acetone was recovered by centrifugation, washed with McIlvane buffer (pH 7.0) to remove residual enzyme following by

water, and lyophilized.

4.2.4 Preparation of laccase

C. versicolor was incubated in the liquid culture of Kirk's basal media [Kirk et al, 1978] containing 1.2 mM of ammonium tartrate, 0.1 % of glucose and 0.01 % vanillin at 28°C for 7 days without agitation.

Crude laccase was prepared according to Fåhræus and Reinhammer [1967]. The culture media was centrifuged to remove insoluble materials and ammonium sulfate was added to 80 % saturation. Precipitate was dissolved in a small amount of distilled water, and this solution was desalted by gel-filtration on a column (550 × 10 mm) of Sephadex G-15. The fractions containing laccase activity was applied into a column (200 × 10 mm) of DEAE Sephadex A-50 preequilibrated and eluted with 0.1 M sodium phosphate buffer (pH 6.0), and the eluents were monitored by measuring absorbances at 250 and 280 nm using a Shimadzu spectrophotometer UV-365. The fractions having laccase activity were desalted again by gel-filtration on Sephadex G-15 column and lyophilized. Laccase activity was measured spectrophotometrically by measuring absorbance at 525 nm using syringaldazine as a substrate [Leonowicz and Grzyhowicz, 1981].

4.2.5 Enzymic treatment of cellulase-treated LCC

Horseradish peroxidase (HRP) was purchased from Wako Chem. Co., Ltd.

Enzyme solution was prepared by mixing, i.e. 0.10 ml of crude laccase solution (17.1 μ unit) or 0.15 ml of HRP solution (14.8 μ unit) (Note that one unit of enzymes was defined as the amount of

enzyme in enzyme solution that oxidized 1 mmol of syringaldazine per second) ; 0.05 ml of 30 mM H₂O₂ solution in the case of HRP; 0.10 ml of 0.5 M sodium acetate buffer (pH 5.3). Total volume was adjusted to 0.8 ml by addition of distilled water. This solution pre-incubated at 36°C was added 0.2 ml of 96 % dioxane solution containing 5 mg of cellulase-treated LCC, and incubated at 36°C for 24 hrs. After incubation, 0.5 ml of acetic acid was added and the reaction solution was filled up to 10 ml with distilled water containing inositol as an internal standard for GC analysis. After removal of insoluble materials by filtration, the filtrate was evaporated, re-dissolved in small amount of distilled water and applied into ion-exchange resins (Dowex 50 × 1 (H⁺) and 1 × 8 (AcO⁻) and then evaporated again. The neutral monosaccharides released by the enzyme reaction were then analyzed by GC as alditol acetates.

Inhibition tests of both enzymes were carried out by addition of 0.1 mmol of sodium azide in the reaction mixture as described above.

4.2.6 Alkaline borohydride treatment of cellulase-treated LCC

Five mg of cellulase-treated LCC was dissolved into 0.5 ml of 1 N sodium hydroxide solution containing 0.1 mol sodium borohydride and incubated at 20°C for 2 hrs. After incubation, 0.5 ml of acetic acid was added to terminate the reaction and 9.0 ml of distilled water containing inositol as an internal standard for GC analysis was added. After removal of insoluble materials by filtration, filtrate containing released sugars was evaporated and analyzed as described as above.

4.3 Results and Discussion

4.3.1 Enzymic cleavage of benzyl ether bondings in LCC model compounds

The amounts of methyl β -D-glucopyranoside released from LCC model compounds by crude enzyme from *C. versicolor* are shown in Table 4.1. Under the presence and absence of H_2O_2 , 24.1 and 39.0 % of methyl β -D-glucopyranoside released, respectively, from the model compound I. However, the crude enzyme did not have an ability to release methyl β -D-glucopyranoside from the model compound II which has no phenolic hydroxyl group. Addition of 10 μ mol sodium azide, an inhibitor of laccase and peroxidase, completely inhibited the release of methyl β -D-glucopyranoside from the model compound I. These results suggest that methyl β -D-glucopyranoside was released resulting from cleavage of unstable benzyl ether having free phenolic hydroxyl group at *para*-position by the action of phenol-oxidizing enzymes (laccase and peroxidase).

4.3.2 Enzymic release of sugars from cellulase-treated LCC

The amounts of neutral monosaccharide released from the cellulase-treated LCC by laccase treatment are shown in Table 4.2 (column 1). This indicates that arabinose, xylose and glucose were released by laccase treatment. In the presence of an inhibitor of laccase, only trace amounts of arabinose and xylose were detected. The result obtained by HRP treatment is shown in column 2 of Table 4.2. Arabinose, xylose and glucose released similar to the case of laccase treatment. Addition of sodium azide, which can also inhibit the action of HRP, suppressed the release of sugars and only trace

Table 4.1 Amounts of methyl β -D-glucopyranoside released from model compounds by crude enzyme from *Coriolus versicolor* (%)*)

	Absence of H ₂ O ₂	Presence of H ₂ O ₂
Compound I		
enzyme	39.0	24.1
enzyme + NaN ₃	0	0
Compound II		
enzyme	0	1.1
enzyme + NaN ₃	0	2.1

*) : molar percent of methyl β -D-glucopyranoside released from model compounds.

amount of glucose could be detected. Both laccase and HRP belong to phenol-oxidizing enzyme and can form phenoxy radicals at free phenolic hydroxyl groups in lignin. If phenoxy radicals would propagate, quinone methide would be formed and sugars bonding to α -position with ether linkages would be released following the Fig. 4.1.

In Table 4.2 (column 3), the amounts of sugars released by alkaline borohydride treatment are shown. It was found that almost the same amounts and compositions of neutral sugars were released by three different treatments : laccase, HRP and alkaline treatments. Because alkaline treatment is known to release sugars linking to α -position of phenylpropane having free phenolic hydroxyl groups at p -position, the present results indicate that arabinose, xylose, and glucose listed in Table 4.2 are linked to α -position. In the presence of base, phenolic hydroxyl groups are converted to phenoxide ions and easily transferred to quinones. Then the sugars bonding to α -position with ether linkages are easily split via quinone methide following Fig. 4.3. It is suggested that sugars linked at benzyl position could be released by enzyme and alkaline treatments following the similar reaction pathway having quinone methide as an intermediate.

The possibility of participation of different oxidases from *C. versicolor* in the cleavage of benzyl ether linkages still remains : namely, lignin peroxidase and Mn-dependent peroxidase. Lignin peroxidase can attack non-phenolic compounds as well as phenolic compounds, and it forms phenoxy radicals from phenolic

Table 4.2 Amounts of monosugars released from cellulase-treated LCC by enzyme and alkaline treatment ($\mu\text{g/g}$)

1. Laccase treatment		
	Absence of NaN_3	Presence of NaN_3
Arabinose	$19.7 \pm 3.9^*)$	$t^{***})$
Xylose	26.1 ± 5.4	t
Galactose	$-^{**})$	-
Glucose	21.8 ± 4.9	-
Mannose	-	-
2. HRP treatment		
	Absence of NaN_3	Presence of NaN_3
Arabinose	20.5 ± 3.9	-
Xylose	32.7 ± 9.5	-
Galactose	-	-
Glucose	24.0 ± 6.6	t
Mannose	t	-
3. Alkaline treatment		
Arabinose	24.5 ± 6.5	
Xylose	26.8 ± 6.4	
Galactose	-	
Glucose	20.2 ± 5.7	
Mannose	-	

*) : mean values are expressed with standard deviations at the 95 % confidence level

**) : not detected.

***) : trace.

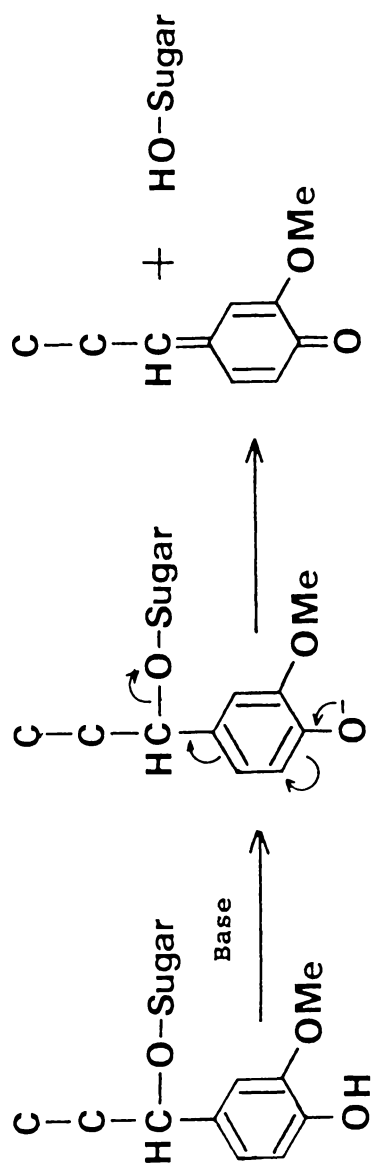


Fig. 4.3 Reaction scheme of splitting bondings between lignin and hemicellulose by alkaline treatment.

compounds [Odier, et al., 1988]. Its action on free phenolic compounds is similar to that of HRP [Sarkanen et al., 1991]. Thus, lignin peroxidase is suggested to cleave the bondings between lignin and hemicellulose in LCC, although its participation in the cleavage reaction is small because of secretion of quite a small amount of extracellular lignin peroxidase from *C. versicolor* under the present culture condition as described in Chapter 1. Manganese-dependent peroxidase can attack free phenolic hydroxyl groups similarly to laccase and HRP. In fact, a crude enzyme solution from *C.versicolor* released 22.2 % of methyl β -D-glucopyranoside from the LCC model compound I (0.0 % from model compound II) in the presence of 0.1 mmol MnSO_4 and 0.3 μmol of H_2O_2 . Note that this reaction was also inhibited perfectly by the addition of sodium azide. Therefore, both Mn-dependent peroxidase and lignin peroxidase could act on phenolic hydroxyl groups in LCC, and cleave the unstable benzyl-ether bondings between lignin and carbohydrates similar to laccase and HRP.

Based on above results, *C. versicolor* can split the benzyl ether linkages having phenolic hydroxyl groups at *p*-position in LCC by phenol-oxidizing enzymes. This phenomenon is significant in hemicellulolytic systems. Amounts of the bondings between lignin and hemicellulose are expected to be greater than those in isolated LCC, because ball-milling treatment for extraction of LCC is thought to split the bondings. The portions of sugars binding to lignin would inhibit the actions of hemicellulolytic enzymes. Therefore, splitting of the bondings is essential for the effective degradation

of hemicellulose in wood. The results in this chapter indicate that *C. versicolor* makes the hemicellulose degradation easy by splitting the bondings between lignin and hemicellulose.

4.4 Summary

Crude enzyme from liquid media of *Coriolus versicolor* released methyl β -D-glucopyranoside from a LCC model compound, methyl 6-O-vanillyl- β -D-glucopyranoside, but not from a compound methyl 6-O-veratryl- β -D-glucopyranoside. Methyl β -D-glucopyranoside was released from the compound having a free phenolic hydroxyl group, suggesting that phenol-oxidizing enzymes secreted by *C. versicolor* might participate this reaction. Phenol-oxidizing enzyme (laccase from *C. versicolor* and horseradish peroxidase) treatments to cellulase-treated LCC were compared to an alkaline treatment, resulting in that the similar amounts and composition of monosaccharides were released. These results suggest that enzymic cleavage of benzyl ether bonding between lignin and hemicellulose by *C. versicolor* occurred via quinone methide by phenol-oxidizing enzymes : laccase and peroxidase.

Chapter 5 DIFFERENTIAL SCANNING CALORIMETRIC ANALYSIS OF LCC FRACTIONS DEGRADED BY WOOD-ROTTING FUNGI

5.1 Introduction

In the previous chapters, the differences of wood degradation mode between two kinds of fungi, *Tyromyces palustris* and *Coriolus versicolor*, are clearly shown in terms of the ability of lignin modification : while *T. palustris* can cause only a slight change of the lignin portion, *C. versicolor* can attack the lignin portion by oxidation and another action, and split the bondings between lignin and hemicellulose.

In order to study the physical changes of the two kinds of polymers in LCC, lignin and hemicellulose, by degradation reaction of fungi, thermal analysis is intended to be applied. Lignin and hemicellulose consist of phenylpropane unit and carbohydrate residue, respectively, resulting in the differences of their thermal properties [Goring, 1963; Sander mann and Augustin, 1963; Beall, 1971; Arima, 1973; Back and Salmén, 1982]. As for thermal properties of LCC, some investigators reported that it was characteristic because lignin and hemicellulose are complexed [Nassar and Mackay, 1984; Shigematsu et al., 1991]. Shigematsu et al. proposed the difference of thermal properties of lignin, hemicellulose and LCC, and analyzed the miscibility of lignin and hemicellulose with LCC by measuring glass transition temperature (T_g) with differential scanning calorimeter (DSC) [1991]. Their

result indicates that hardwood LCC has three Tg peaks due to lignin, hemicellulose (glucuronoxylan) and the binding portions of the two. This indicates that the LCC portions show a distinctly different thermal behavior from lignin or hemicellulose alone. Therefore, individual changes of three portions in degraded LCC can be detected by DSC analysis [Tsujiyama].

In this chapter, thermal analysis is applied to detect the physical property change of wood component polymers by the attacks of two wood-rotting fungi, *T. palustris* and *C. versicolor*.

5.2 Materials and Methods

5.2.1 Incubation of mycelia and preparation of the degraded fractions

Two kinds of wood-rotting fungi, *T. palustris* and *C. versicolor*, were incubated in liquid medium containing LCC-W from akamatsu as a sole carbon source as described in Chapter 1.

The fractions insolubilized during incubation (P-1) were prepared from the media of both fungi as described in Chapter 2. The fraction precipitated by acidifying the culture media (P-A) was prepared from only the liquid media of *C. versicolor* after 10 days of incubation as described in Chapter 3.

5.2.2 DSC analysis of the fractions degraded by wood-rotting fungi

A differential scanning calorimetric (DSC) analysis was carried out as follows : one mg of finely grounded sample was placed on the aluminum pan and dried up under reduced pressure. Glass transition

temperatures (Tgs) were measured by using a Seiko Industry SSC-5200 at a constant heating rate of 20°C/min. The results were displayed as differential-DSC (D-DSC) curves, which indicated the distinct differences.

5.2.3 Alkaline treatment of LCC-W

In order to split the bondings between lignin and hemicellulose, alkaline treatment was carried out : 5 mg of LCC-W was incubated with 0.5 ml of 1.0 N NaOH solution containing 1.0 M of sodium borohydride at 20°C for 2 hrs. After the treatment, reaction mixture was added 0.5 ml of acetic acid to neutralize, diluted with 9 ml of distilled water, dialyzed and evaporated under reducing pressure at 40°C. This sample was also analyzed with DSC as described above.

5.2.4 Cellulase treatment of LCC-W and the Fraction P-A of *C.versicolor*

In order to find the change in D-DSC chart when the hemicellulose portion is removed, LCC-W and the Fraction P-A were treated with cellulase. The sample and enzyme (CELLULYSIN; *Tricoderma viride*, Calbiochem Co.) concentrations were 1.0 and 0.2 %, respectively. After incubation for 24 hrs, an equivalent amount of the enzyme was added into the reaction mixture, and incubation was further continued for 24 hrs. Precipitates were recovered by centrifugation. In the case of LCC-W, the sample was extracted with 70 % aqueous methanol from precipitates followed by the preparation procedure of the Fraction P-1, to compare with the Fraction P-1. Soluble parts, which consist of almost all of the

precipitates, were obtained by evaporation under the reduced pressure. In the case of the Fraction P-A, precipitates were washed with water and lyophilized. DSC analysis was carried out as described above.

5.3 Results and Discussion

5.3.1 Glass transition temperature (T_g) of LCC-W

Figure 5.1 shows that the D-DSC curve of akamatsu LCC-W. In D-DSC charts, endothermal transition (T_g) appeared as the convex peak. LCC-W prepared from akamatsu indicates at least five T_g peaks between 100 and 250°C, due to lignin, hemicelluloses and their binding portions. T_g of lignin appeared at around 140°C and peaks of hemicelluloses, galactoglucomannan and arabinoglucuronoxylan, are characterized at 180 and 205°C, respectively, by comparing with the D-DSC curves of standard samples isolated from akamatsu.

As for binding portions, two peaks seemed to appear at around 160-175°C, similarly as reported by Shigematsu et al. [1991]. In Fig.5.1b, these two peaks disappeared after alkaline treatment to split the alkaline-instable linkages between lignin and hemicelluloses, and two T_g peaks due to lignin and hemicelluloses remained clearly. Cellulase-treated LCC-W was shown in Fig. 5.1c. In this profile, three T_g peaks were clearly observed but the peaks due to hemicelluloses diminished. Two peaks between 160-170°C remained, indicating that these are due to the binding portions, because bonding to lignin caused the difficulty of cellulase attack.

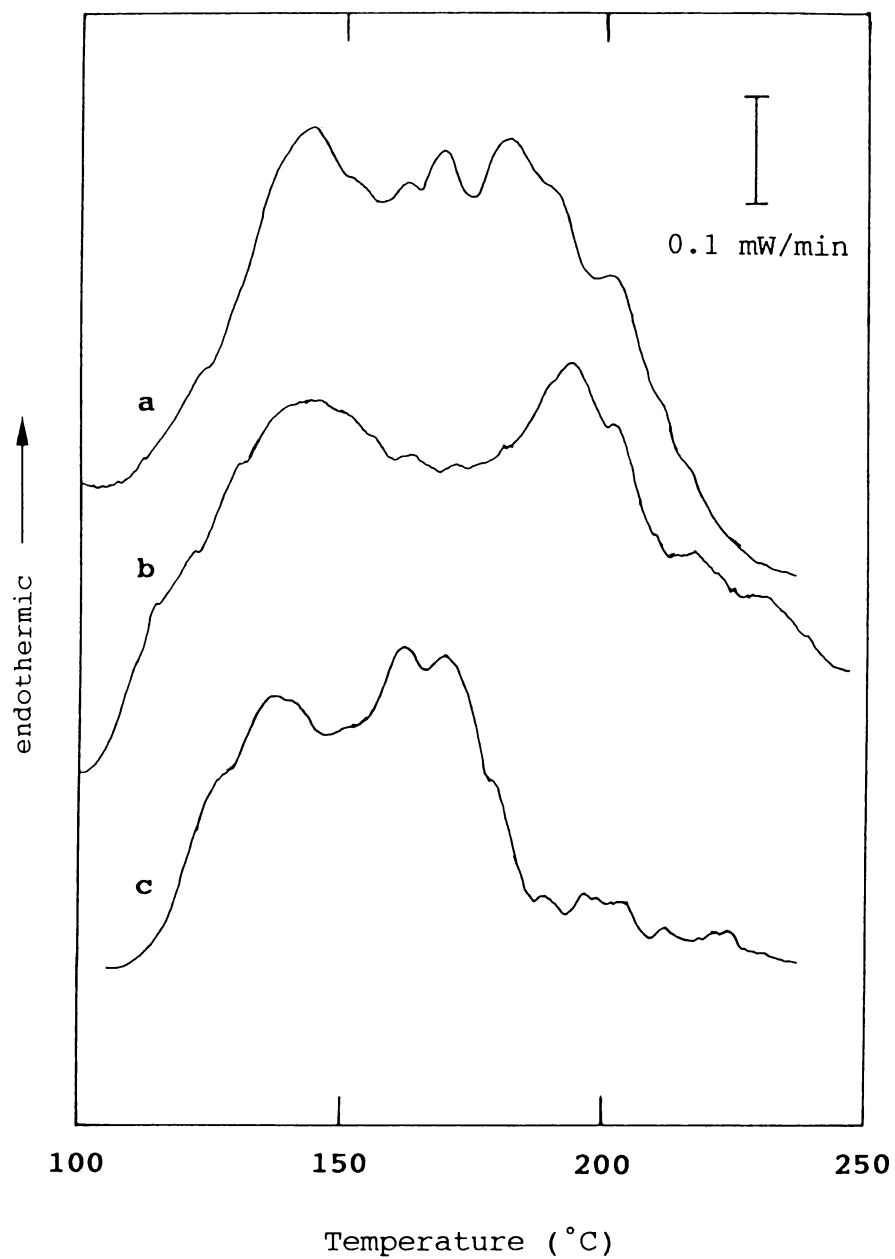


Fig. 5.1 D-DSC curves of akamatsu LCC-W and treated-LCC.
a: original LCC-W, **b:** alkaline treated LCC-W,
c: cellulase treated LCC-W.

Therefore, Tgs of the binding portions between lignin and hemicelluloses appear at around 160-175°C and have distinct Tg values from lignin and hemicelluloses alone.

5.3.2 Glass transition temperatures (Tgs) of the Fraction P-1

Figures 5.2 and 5.3 show D-DSC curves of the water-insoluble P-1 Fractions from *T. palustris* and *C. versicolor*, respectively. In the case of *T. palustris* (Fig. 5.2), the peaks due to hemicelluloses at around 180 and 205°C diminished within 7 days, while the peak of lignin and binding portions still remained clearly at 31 days.

In the case of *C. versicolor*, as shown in Fig. 5.3, the peak due to arabinoxylan diminished gradually but the peak due to galactoglucomannan remained even at 31 days. As shown in Fig. 2.15 (Chapter 2), xylose and arabinose contents in the Fraction P-1 decreased during incubation. This result supports that *C. versicolor* preferentially degraded arabinoxylan. Furthermore, the peak at around 165°C due to the binding portions between lignin and hemicellulose became smaller in comparison with the peak due to lignin. Based on gel-permeation chromatographic (GPC) analysis (Chapter 2) and enzymic degradation test using LCC model compound (Chapter 4), it has been proposed the splitting of the bondings between lignin and hemicelluloses. DSC analysis suggests a modification of the binding portions by *C. versicolor*, such as the splitting of bondings between lignin and hemicelluloses.

Tg changes of the lignin portion were shown in Fig. 5.4. This reveals that Tgs of lignin in the case of *T. palustris* did not

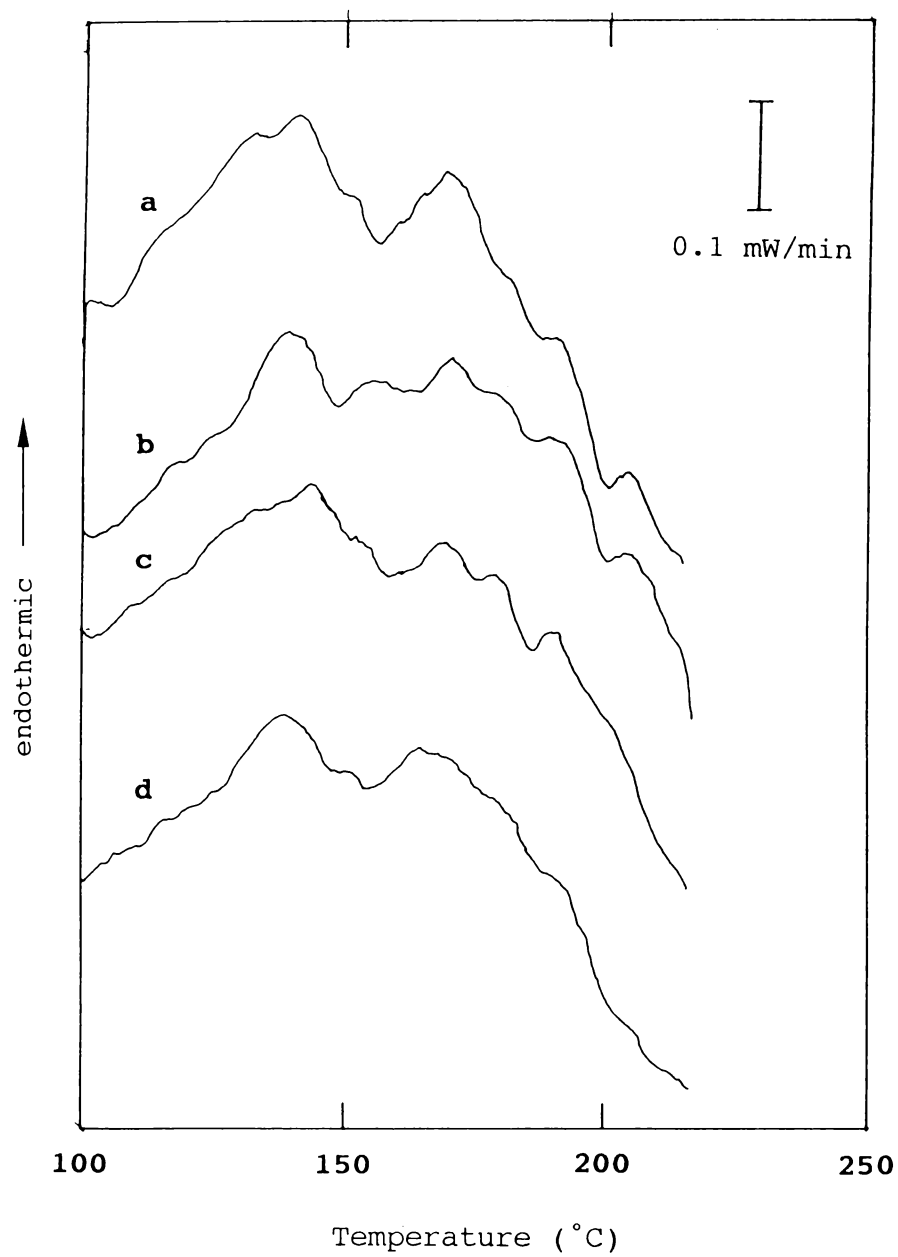


Fig. 5.2 D-DSC curves of the Fraction P-1 (*T. palustris*).
a: 7 days, b: 14 days, c: 21 days, d: 31 days.

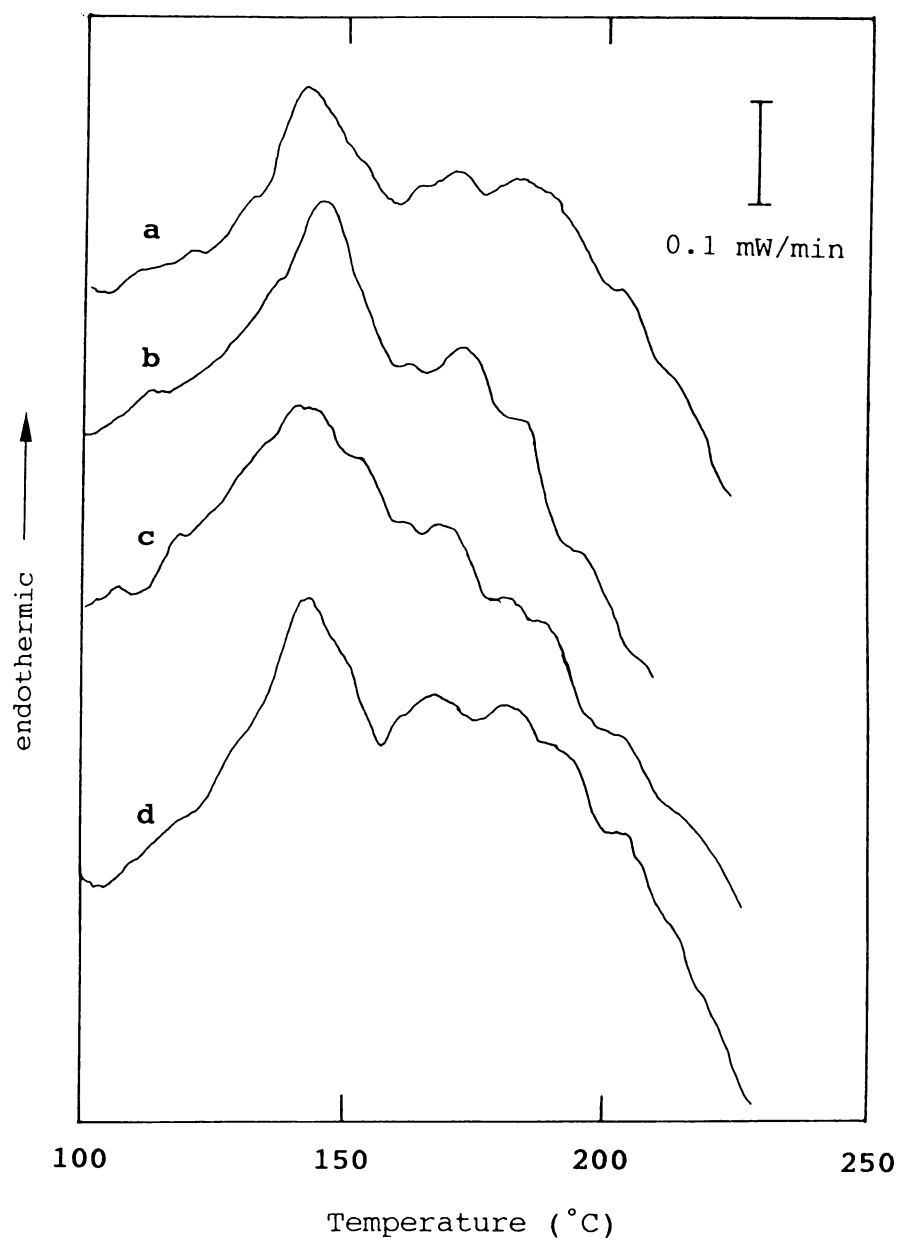


Fig. 5.3 D-DSC curves of the Fraction P-1 (*C. versicolor*).
a:7 days, b:14 days, c:21 days, d:31 days.

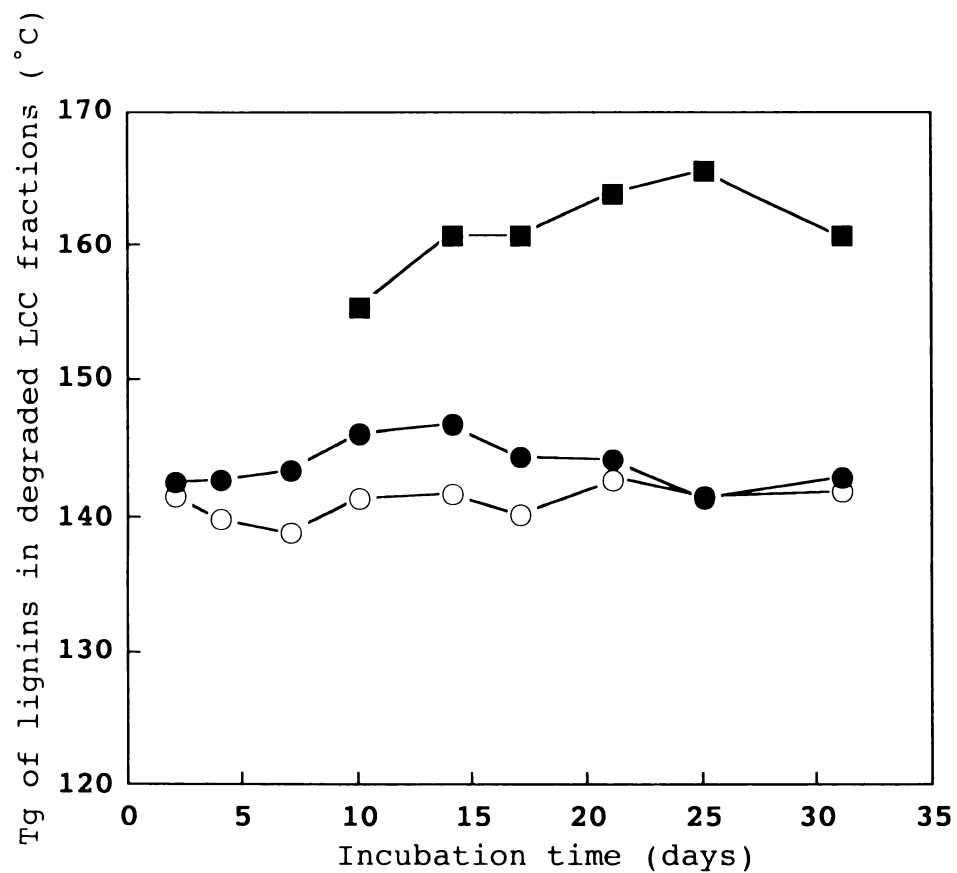


Fig. 5.4 Glass-transition temperatures (Tgs) of the lignin portions in the degraded LCC fractions (P-1 and P-A). ○:the Fraction P-1 of *T. palustris*, ●:the Fraction P-1 of *C. versicolor*, ■:the Fraction P-A of *C. versicolor*.

change so much, indicating that *T. palustris* did not modify the lignin portion drastically. Although some investigators reported that brown-rot fungi attack lignin oxidatively [Kirk, 1975; Jin et al., 1990] and the result shown in Chapter 2 indicated that *T. palustris* could cause the structural changes in lignin, DSC analysis indicates that *T. palustris* scarcely caused the changes in thermal property of lignin polymer. On the other hand, in the case of *C. versicolor*, Tgs of lignin rose up until 10 days and then gradually lowered after that. This phenomenon seems to be caused by the change of molecular weight. As shown in Chapter 2, molecular weight of lignin in the Fraction P-1 of *C. versicolor* became larger in this periods and smaller after that. Therefore, this result suggests the existence of the relationship between molecular weight and Tg in lignin, as Hatakeyama et al. reported [1975].

5.3.3 Glass transition temperatures (Tgs) of the Fraction P-A

Figure 5.5 indicates the D-DSC curves of the Fraction P-A obtained from the culture media of *C. versicolor*. In the profiles of the Fraction P-A, two main peaks appeared at around 160 and 185°C. The latter is due to galactoglucomannan. Together with the result of sugar composition analysis, this indicates that the Fraction P-A contained large amounts of galactoglucomannan fragments (Chapter 3). On the other hand, the peaks at 160°C and at around 125-140°C are not identified. In order to find which of these are due to lignin or hemicellulose, cellulase-treatment was applied to the Fraction P-A. The result shown in Fig. 5.5e indicates that the

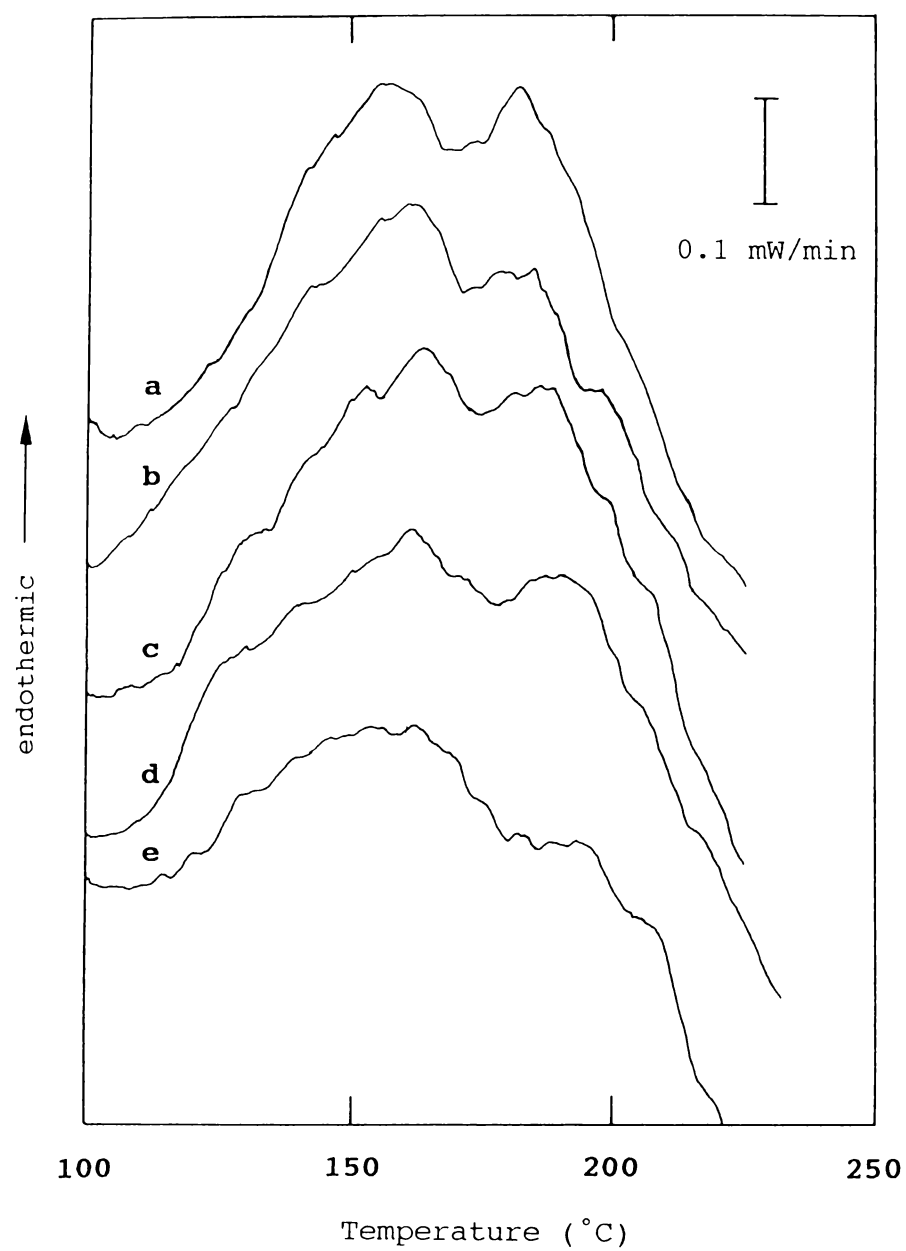


Fig. 5.5 D-DSC curves of the Fraction P-A (*C. versicolor*).
a:10 days, b:14 days, c:21 days, d:31 days,
e:31 days (cellulase-treated sample).

peak due to galactoglucomannan diminished but the peak at around 160°C and shoulder peaks at around 125-140°C did not change. Therefore, these must be due to modified lignin polymers. The peaks and shoulders at lower temperature range were remarkably determined in the later period of incubation, when large amounts of phenoloxidasases and peroxidases were produced (Chapter 1) and the changes of lignin by oxidation were detected substantially in the Fraction P-A (Chapter 3). Considering that the Fraction P-A was dissolved in the culture media and could be easily attacked by oxidases, these peaks between 125-140°C might be due to the lignin fragments formed by the oxidation reactions.

In Fig. 5.4, it is clear that the peak of lignin are higher temperature range in comparison with that in the Fraction P-1. Considering the structural changes of lignin, which contains larger amounts of hydrophilic functional groups, phenolic hydroxyl groups and carboxyl groups, than those in the Fraction P-1 (Chapters 2 and 3), two reasons are conceivable. One is that the hydrogen bonding interactions of hydrophilic functional groups cause higher Tg values of lignin, as Hatakeyama et al. reported [1981]. Another is that the higher hydrophilicity of lignin enhanced the interaction against the binding portions containing carbohydrates, resulting in an appearance of one broad peak at around 160°C between the peaks of lignin and binding portions. It is noteworthy that Tgs of lignin in the Fraction P-A gradually increased until 25 days accompanied by the changes of phenolic hydroxyl contents (in Figs. 3.4 and 3.5), indicating that hydrophilic functional groups affect Tgs.

5.4 Summary

Differential scanning calorimetric (DSC) analysis was applied to the LCC fractions degraded by wood-rotting fungi, *Tyromyces palustris* and *Coriolus versicolor*, to detect the changes in glass transition temperatures (T_g) of lignin, hemicelluloses and the binding portions of the two. In the case of *C. versicolor*, T_g of the lignin portion in LCC rose, and the small peaks and shoulders of T_g due to lignin appeared in the lower temperature range. Furthermore, intensities of T_gs due to the binding portions reduced, indicating that *C. versicolor* can decompose the bondings between lignin and hemicellulose in the polymeric states. On the other hand, in the case of *T. palustris*, only the intensities of T_g peaks due to hemicelluloses reduced. DSC analysis indicates that *C. versicolor* can change the thermal property of polymeric lignin and decompose the binding portions between lignin and hemicellulose, while *T. palustris* cannot.

CONCLUSION

To study the differences of wood degradation mechanisms by two types of wood-rotting fungi, a brown-rot fungus *Tyromyces palustris* (Berk. et. Curr.) Murr. and a white-rot fungus *Coriolus versicolor* (L.:Fr.) Quél., an incubation experiment was carried out using a water-soluble lignin-carbohydrate complex (LCC-W) prepared from akamatsu (*Pinus densiflora* Sieb. et. Zucc.) as a sole carbon source because of its potential advantages.

In Chapter 1, the differences between *T. palustris* and *C. versicolor*, were examined with respect to the enzyme productions during incubation. *T. palustris* produced larger amounts and higher active hemicellulose-degrading enzymes than *C. versicolor*. *C. versicolor* produced phenoloxidases and peroxidases after 7 days of incubation when ammonium ion as a nitrogen source was metabolized. Lignin peroxidase activity was detected prior to other phenoloxidases and peroxidases, and disappeared when others were produced actively, suggesting that this might participate initial lignin degradation. Among two kinds of phenoloxidases and three kinds of peroxidases, *C. versicolor* produced highly active laccase and Mn-dependent peroxidase.

In Chapter 2, the water-insoluble fraction P-1, which was formed during incubation of *T. palustris* and *C. versicolor* in culture media, was prepared and its structural changes were examined. Its yield change was different between both fungi. Re-solubilization of the Fraction P-1, only in the case of *C. versicolor*, was observed

accompanied by the start of productions of phenoloxidases and peroxidases and suggested to be caused by the degradation of the lignin portion.

The structural changes of the Fraction P-1 by each fungi were characterized by the chemical and spectroscopic analyses. The remarkable differences were found in the lignin portion, but not in the hemicellulose portion. In the case of *T. palustris*, the occurrence of only a slight modification of the lignin portion was observed. On the other hand, an attack on the lignin portion by *C. versicolor* was found to occur in at least two steps. The first was initiated after about 7 days of incubation. In this step, the occurrence of a side-chain modification and the polymerization of the lignin portion were observed by the functional analyses and gel-permeation chromatographic (GPC) analysis. At the second step (at about 21 days of incubation), the occurrence of cleavage of the aryl-ether linkages of lignin was suggested from the results of the increase in phenolic hydroxyl group content. This phenomenon was estimated by the carbon-13 nuclear magnetic resonance (^{13}C -NMR) spectroscopic and GPC analyses. In addition, only in the case of *C. versicolor*, the cleavage of the bondings between lignin and hemicellulose started accompanied by the productions of phenoloxidase and peroxidases.

In Chapter 3, during incubation of *C. versicolor*, the formation of the fraction (P-A) precipitated by acidifying (pH 2-3) the culture medium was found to start after 10 days of incubation. Based on the time course of structural changes of the lignin

portion, the initial attack by *C. versicolor* was the formation of new phenolic hydroxyl groups, which probably was followed by an oxidation reaction to form new α -carbonyl and carboxyl groups. Although the latter reaction could be explained by the co-operative actions of oxidases such as laccase, Mn-dependent peroxidase and others, the former reaction was suggested to be caused by an uncharacterized lignin-degrading system. This reaction system might cause re-solubilization of the water-insolubilized fraction P-1 by splitting the hydrophilic moieties of lignin which involves large amounts of phenolic hydroxyl groups.

In Chapter 4, an enzymic cleavage of the bondings between lignin and hemicellulose was examined. Crude enzyme prepared from liquid media of *Coriolus versicolor* could release methyl β -D-glucopyranoside from a LCC model compound, methyl 6-O-vanillyl- β -D-glucopyranoside, but not from a compound methyl 6-O-veratryl- β -D-glucopyranoside. From this result, methyl β -D-glucopyranoside was released from the compound having a free phenolic hydroxyl group, suggesting that phenol-oxidizing enzymes secreted by *C. versicolor* might participate this reaction. Treatments by phenol-oxidizing enzymes (laccase from *C. versicolor* and horseradish peroxidase) to cellulase-treated LCC-W were compared to an alkaline treatment, resulting in that the similar amounts and composition of monosaccharides were released. These results suggest that enzymic cleavage of benzyl ether bonding having phenolic hydroxyl group at *para*-position between lignin and hemicellulose occurred via quinone methide by phenol-oxidizing enzymes : laccase and peroxidase.

In Chapter 5, a new detection method of the changes of wood component polymers, lignin, hemicelluloses and the binding portions of the two, was applied to the degraded LCC fractions by wood-rotting fungi, *T. palustris* and *C. versicolor*, by measuring the glass transition temperatures (T_g) using differential scanning calorimeter (DSC). In the case of *C. versicolor*, T_g of the lignin portion in LCC rose and the small peaks and shoulders of T_g due to lignin appeared in the lower temperature range. Furthermore, intensities of T_gs due to the binding portions reduced, indicating that *C. versicolor* can decompose the bondings between lignin and hemicellulose. On the other hand, in the case of *T. palustris*, only the intensities of the T_g peaks due to hemicelluloses reduced. DSC analysis indicates that *C. versicolor* can change the thermal property of polymeric lignin and decompose the binding portions between lignin and hemicellulose, while *T. palustris* cannot.

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ERRATA

	error	correction
p.12 line 17 :	veratrylalcohol	veratryl alcohol
p.14 Y-axis :	(g)	(mg)
p.14 Y-axis :	(g)	(mg)
P.30 line 2 :	in Fraction P-1	in the Fraction P-1
p.38 line 9 :	ther	×(DELETE)