Transactions of the Materials Research Society of Japan. Vol. 20

Synthesis of phenolic lignin derivatives by phase-separation process and their functions for protein immobilization

Masamitsu Funaoka, Hiroyuki Ioka and Norio Seki

Chemical Wood Technology Lab, Faculty of Bioresources, Mie University
1515 Kamihama, Tsu, Mie 514, Japan

Highly phenolic lignin derivatives (lignophenol derivatives) with different phenolic functionalities were synthesized directly from native lignins, through the phase-separation process using phenol derivatives and concentrated acid. The relationships between the structures of lignophenol derivatives and their functions for proteins were discussed.

Lignophenol derivatives had much higher affinities for proteins, compared with conventional lignins: -10 and -100 times higher in lignomonophenols and lignopolyphenols, respectively. The protein affinity was significantly affected by the substitution pattern of phenolic hydroxyl groups and was decreased by the introduction of hydrophobic alkyl substituents. The protein adsorbing capacities of lignophenol derivatives differed with the species from which they were derived. The maximum protein adsorption was achieved at the isoelectric points of used proteins. The lignophenol derivative-protein complex was rapidly and perfectly separated into lignin and protein moieties in aprotic solvents, although it was very stable at any pH in aqueous solution. The enzymes immobilized on lignophenol derivatives retained high activity: especially an enzyme on lignocresol comparable to a native one. The activity patterns had good correlations between native and immobilized enzymes.

1. INTRODUCTION

Lignin is an amorphous, aromatic network polymer, which is contained in the amount of 3 x 10^{11} tons in the biosphere with an annual biosynthesis rate of ca. 2 x 10^{10} tons [1]. Lignin, unlike synthetic polymers, is perfectly biodegraded in nature, giving no undesirable influence to the biocycle, although it is one of the most durable biopolymers. Because of this unique function, there has been a great interest in its degradation and potential application in the manufacture of various chemicals and other products. However, the utilization of lignin has not yet been successfully achieved. The main reason for this is the fact that lignin molecules lack stereoregularity, and repeating units in its molecule are too heterogeneous and complex. In addition, nonselective modifications during isolation make lignin molecules much more heterogeneous.

We have designed a new process (phase-separation process) for synthesizing phenolic lignin derivatives (lignophenol derivatives), retaining principal interunit linkages of native lignins [2, 3]. In the present paper, lignophenol derivatives having different phenolic functionalities were synthesized directly from various native lignins, and their functions for proteins were investigated. The potential application for the bioreactor system was discussed.

2. EXPERIMENTAL

2.1. Synthesis of lignophenol derivatives

One step process:

Phenol derivatives were added to extractive-free wood meals with stirring. After 10 min, 72% sulfuric acid was added to the mixture and the vigorous stirring was continued at room temperature for 20 min. The reaction mixture was rapidly separated into organic and aqueous phases by stopping the stirring. The organic phase was taken up and added dropwise to an excess amount of ethyl ether with vigorous stirring. The precipitates were dissolved in acetone and

insoluble materials were removed by centrifugation. The acetone solution was then concentrated under reduced pressure and added dropwise to an excess amount of ethyl ether with stirring. The precipitated lignin (lignophenol derivative) was collected by centrifugation and dried over P_2O_5 after evaporating the solvent.

Two step process:

Phenol derivatives were dissolved in ethanol and added to extractive-free wood meals, and the mixture was stirred for several hours. Ethanol and excess phenol derivatives were removed by filtration. Seventy-two percent sulfuric acid was added to the phenol derivative-sorpted wood meals and the mixture was vigorously stirred at room temperature for 20 min. The phenol-benzene solution was added to the mixture with stirring to extract the lignin derivatives (lignophenol derivatives). By stopping the stirring, the reaction mixture was separated into two phases. The isolation and purification of lignophenol derivatives from the organic phase were carried out in the same manner as one step process.

2.2 Structural analysis of lignocresols

The amounts of combined cresol were calculated based on the signal intensity of its methyl protons on ^{1}H-NMR spectra of lignocresols. The hydroxyl group contents were determined from acetoxyl proton signals on ^{1}H-NMR spectra of acetylated lignocresols. *p*-Nitrobenzaldehyde was used as the internal reference for the determination. The combination modes of cresolic nuclei were analyzed by combined nucleus exchange and $NaIO_4$ oxidation techniques [3]. The molecular weight ($\overline{M}w$) were calculated from gel permeation chromatograms using Shodex columns, KF802 and KF804.

2.3. Measurement of protein - adsorbing capacities of lignin preparations

The protein was dissolved in buffer solutions in the range of pH2-10, which was added to lignin preparations. The mixture was shaken at 20 °C for 60 min. After the centrifugation of the mixture followed by the filtration of supernatant, the free protein in the resulting filtrate was determined by the Bradford's method [4] using Coomassie Brilliant Blue G-250. The amount of protein adsorbed on lignin preparations was calculated from the difference between the amount of initially charged protein and that of residual protein in the filtrate.

The desorption of proteins from the lignin-protein complexes was carried out by shaking the complexes with different buffer solutions in the range of pH2-10.

2.4. Preparation of lignin - β-glucosidase complex and its activity assay

A lignophenol derivative was suspended in β-glucosidase solution (pH5). The suspension was shaken at 25 °C for 60 min. The resulting lignin-enzyme complex was thoroughly washed with pH5 buffer solution. The substrate solution (*p*-nitrophenyl -β-D- glucopyranoside) was added and the mixture was incubated at 40°C. After centrifugation, *p*-nitrophenol released in the supernatant was determined by UV (405nm).

The amount of enzyme adsorbed was determined from the difference between the enzymic activity in initially charged solution and that in the supernatant after the suspension with lignin preparation.

3. RESULTS AND DISCUSSION

3.1. Structural features of lignocresols

Lignocresol derived from native lignin had few conjugated systems, being pinkish white, its brightness comparable to milled wood lignin. Spruce lignocresol included 0.64 mol/C_9 of cresolic nuclei in the molecule (0.9mol/C_9 in birch lignocresol), 77% of which were linked to lignin C_α-positions through carbon-carbon linkages, 16% possibly to C_γ-positions, and the remaining 7% etherified to lignin side chains through its phenolic hydroxyl groups. The molecular weight ($\overline{M}w$) of lignocresol was ca.3500 in spruce, lower in birch. Most of the β- and γ -positions in the side chains of C_9 units remained intact, except the coniferyl alcohol and aldehyde units. There were few variations in these structural features among softwood and hardwood species, respectively.

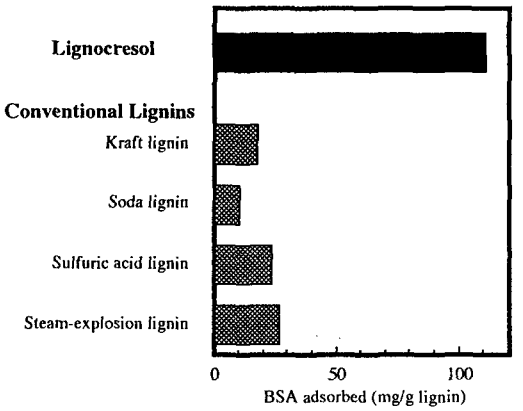

Fig. 1. Affinity of conventional lignins and spruce lignocresol for bovine serum albumin (BSA).

3.2. Protein affinity of lignocresol

Fig. 1 shows the affinity of spruce lignocresol for bovine serum albumin (BSA) in comparison with conventional lignin preparations. The BSA affinity of spruce lignocresol was 5-10 times higher than conventional lignins. Lignocresol had almost the same affinity also for other proteins such as γ-globulin, hemoglobin and β-glucosidase. The maximum protein adsorption was achieved at the isoelectric points of used proteins. These facts indicate that the affinity between lignocresol and proteins is not due to the biological complementarity.

However, the protein adsorbing capacities of lignocresols differed with the species from which they were derived (Table 1). As mentioned above, there were few variations in the structural features of lignocresols among the wood species. Therefore, the high protein affinity of lignocresol is not attributed simply to increased phenolic functionality due to the introduction of cresolic nuclei, but its network structure, and the frequency, orientation and distribution of substructures and functional groups significantly participate in the development of protein affinity.

3.3. Effect of phenolic functionality of introduced phenol derivatives

Lignomonophenols had almost the same BSA adsorbing capacities, independent of the side chain structures of phenol derivatives (Table 2). On the other hand, the maximum BSA adsorptions of lignopolyphenols were much higher, compared with lignomonophenols: especially the affinity of lignophenol derivative having catechol, resorcinol, or pyrogallol nucleus was more than 5 times higher. The protein affinity of lignopolyphenols was significantly affected by the substitution pattern of phenolic hydroxyl groups (*O-* > *m-* > *p-*) and was decreased by the introduction of hydrophobic alkyl substituents.

3.4. Stability of lignophenol derivative-protein complex

The lignomonophenol-protein complex was very stable at any pH in aqueous solutions, whereas it was perfectly separated into lignin and protein moieties in aprotic solvents. This indicates that the hydrophobic interaction is an important factor for the formation of complex. On the other hand, in the case of lignopolyphenols having high protein affinity (more than 500 mg/g lignin), a part of immobilized protein was released from the complex by the change of pH. This

Table 1 BSA-adsorbing capacities of lignocresols.

	Origin of lignocresol		Maximum BSA adsorption (mg/g lignocresol)
Softwood	Norway spruce	(*Picea abies*)	100.0
	Yezo spruce	(*Picea jezoensis*)	105.4
	Loblolly pine	(*Pinus taeda*)	95.5
	Slash pine	(*Pinus elliottii*)	133.5
	Japanese ceder	(*Cryptomeria japonica*)	120.8
Hardwood	Sweet gum	(*Liquidambar styraciflua*)	43.1
	White birch	(*Betula papyrifera*)	41.5
	Japanese birch	(*Betula platyphylla*)	55.3
	Japanese oak	(*Quercus mongolica*)	76.1
	Camphor tree	(*Cinnamomum camphora*)	54.8
	Kapur	(*Dryobalanops aromatica*)	125.6
	Ramin	(*Gonystylus bancanus*)	101.5
	Apitong	(*Dipterocarpus grandiflorus*)	102.1
	Para-rubber tree	(*Hevea brasiliensis*)	63.8
	Perupok	(*Lophopetalum torricellense*)	54.0
Grass	Wheat straw	(*Triticum aestivum*)	104.4
	Rice straw	(*Oryza sativa*)	80.0
	Kenaf	(*Hibiscus cannabinus*) bastfiber	83.7
	Reed	(*Phragmites longivalvis*)	100.8
	Manila hemp	(*Musa textilis*)	68.9

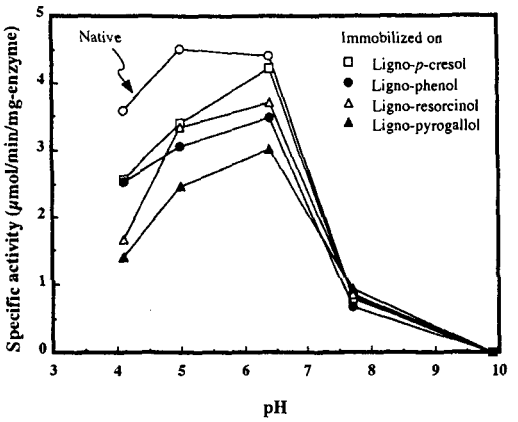

Fig. 2. pH-Activity profiles of β-glucosidase immobilized on spruce lignophenol derivatives.
Substrate: *p*-nitrophenyl-β-D-glucopyranoside

means that in addition to the hydrophobic interaction, the electrostatic interaction also participates partly in the protein affinity of lignopolyphenols.

3.5. Activity of immobilized enzyme

In general, the immobilization of enzyme on insoluble supports results in the decrease of its activity, due mainly to the change of conformation, the block of catalytic domains or the decrease of accessibility with the substrates. The activity of β-glucosidase immobilized on lignophenol derivatives is shown in Fig. 2. The activity of immobilized β-glucosidase was very high, and its pH-activity profile had good correlation with that of free enzyme. This implies that the enzyme immobilized on lignophenol derivatives retains the high mobility and functions comparable to free enzyme. This would be due to much lower molecular weights of lignophenol derivatives, compared to enzymes (Fig. 3).

The lignophenol derivative-enzyme complex was very stable in aqueous media, whereas it was separated perfectly into lignin and protein moieties in aprotic solvents.

These unique features of lignophenol derivatives, easy immobilization of enzyme, high stability of the complex, high activity of immobilized enzymes, perfect separation of the complex, would be useful not only for the bioreactor system, but for the protein purification and separation or the affinity chromatography.

Table 2 BSA-adsorbing capacities of spruce lignophenol derivatives.

Phenol derivative	BSA-adsorbing capacity (mg/g lignophenol der.)
[Monophenol]	
Phenol	87
p-Cresol	99
m-Cresol	84
o-Cresol	84
2,6-Xylenol	80
2,3,5-Trimethylphenol	90
p-Ethylphenol	81
p-n-Propylphenol	66
p-iso-Propylphenol	80
p-Chlorophenol	35
β-Naphthol	95
[Diphenol]	
Catechol	635
4-Methylcatechol	149
Resorcinol	478
2-Methylresorcinol	85
Hydroquinone	164
Methylhydroquinone	80
[Triphenol]	
Pyrogallol	547

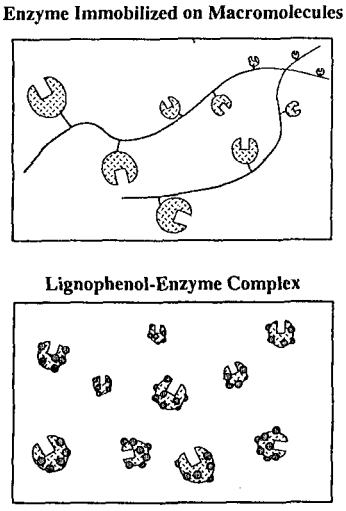

Fig. 3. Schematic illustrations for immobilized enzymes.

REFERENCES

1. H. Sandermann, D. Scheel and T. Van der Trenck, J. Appl. Polym. Sci., 37 (1983) 407.
2. M. Funaoka, M. Matsubara, H. Seki and S. Fukatsu, Biotech. Bioeng., 46 (1995) 545.
3. M. Funaoka and S. Fukatsu, Holzforschung, 50 (1996) 245.
4. M. M. Bradford, Anal. Biochem., 72 (1976) 248.