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RESEARCH ARTICLE

Copolymerization of lignin with cresol catalyzed by peroxidase in reversed micellar systems

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Peroxidase catalysed copolymerization of lignin with cresol in a reversed micellar system was performed successfully. The molecular weight of the copolymer was controlled by adjusting the surfactant concentration and a maximal mean molecular weight of 1890 kDa was obtained.

Lignin is the second most abundant biopolymer on earth, after cellulose. The use of this raw material as a template for phenolic polymer synthesis has potential applications in the production of polymeric dispersants, soil conditioning agents, phenolic resins or adhesives, and laminates, among others. Several methods have been developed for the production of lignin containing phenolic resins (Tock et al, 1987; Matt and Doucet, 1988), many of which employ formaldehyde to hydroxymethylate lignin and /or phenol in order to crosslink the polymer. However, these lignin phenol resins are as toxic as formaldehyde itself. The enzymatic polymerisation of phenols has proven to be an attractive alternative for the synthesis of phenolic resins. In the presence of H_2O_2, peroxidases catalyse the oxidation of phenols that eventually give rise to higher molecular weight polymers (Xu et al, 1995; Nicel and Wright, 1997). The copolymerization of phenols with Kraft lignin has been performed in aqueous-organic solvent mixtures catalysed by horseradish peroxidase (Blinkovsky and Dorkick, 1993; Popp et al, 1993). The enzyme horseradish peroxidase, when encapsulated in reversed micelles, was able to catalyse the polymerisation of phenolic compounds and aromatic amines (Rao et al, 1993).

This article deals with the enzymatic copolymerization of lignin with cresol in revered micelles. The objective here is not so much to examine the potential application of the polymer, but rather to assess the feasibility of the reaction in terms of kinetics and monomer conversions and to measure the gross characteristics of the polymer, especially its molecular weight and distribution.

Experimental

Materials

Horseradish peroxidase (EC 1.11.1.7) type II (HRP) was purchased from Shanghai Lizu Orient Technology Corp. Ltd., with activity of 250 units/mg, assuming an enzyme molecular weight of 40,000. Other chemicals used in this work were of the highest purity commercially available. Polystyrene molecular weight standards were obtained from Polysciences, Inc. (Warington, PA). The lignin used in this work was the straw-pulp-lignin obtained from black liquor or alkali liquor.

Analytical Methods

For soluble dimethylformamide fractions, the molecular weights of the resulting polymer and lignin were determined by gel permeation chromatography (GPC, C-R7A, Warters, Milford, MA). The concentration of unreacted phenols was measured by HPLC. Fourier Transfer Infra-Red spectra were performed using a Nicolet Magna-IR 750 instrument with the sample as KBr pellet. Thermal analyses were conducted using a differential scanning calorimeter (DSC) SR-1 (Beijing Analysis instrument Factory).

Copolymerization procedure

Enzymatic polymerisation was carried out in reversed

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micelles and a typical reaction was carried out as follows. First, phenol and the solution of enzyme-in-buffer were added to a solution of lignin-reversed micelles, and the solution so obtained was incubated in a constant temperature bath for 10-20 min at a defined temperature, e.g., 25°C. Then, certain amount of hydrogen peroxide, e.g., 200μm, was microinjected to initiate the copolymerization of lignin with phenol. The range of enzyme concentration was from $1.0025x10^{-7}$ mol/L to $8.7525x10^{-7}$ mol/L; the range of water to surfactant molar ratio (W/O) was from 29.06 to 90.94; the range of phenol concentration was from 62.04 to 141.05 mg/mL; the range of lignin concentration was from 46.4 to 135.6 mg/mL; the range of surfactant concentration (CTAB) was from 0.863 to 1.637 mol/L; the ratio of n-butanol to isooctane was from 2.0 to 4.0.

We have found that the enzymatic copolymerization of lignin with p-cresol in reversed micelles was extremely fast and occurred through the formation of a dark precipitate within a minute of reaction. Accordingly, the precipitate was easily recovered from the supernatant by centrifugation or filtration, washed with water to remove any traces of enzyme, and washed repeatedly with isooctane to remove any residual surfactant. The clean precipitate was dried free of both solvent and water at 60°C. and stored for subsequent polymer characterisation. Micellar integrity was retained subsequent to reaction, and the filtrate containing the reversed micelles could be reused because the enzyme activity was retained.

Results and Discussion

The oxidation of phenols by peroxidase in the presence of H_2O_2 produces phenoxy radicals that are subjected to a number of different non-enzymatic reactions. In the absence of other phenols or other hydrogen donors, phenoxy radicals can undergo radical transfer or termination (coupling) reactions to give phenolic homopolymers. In the presence of other phenols, however, radical transfer and coupling reactions can occur between different phenolic groups. One can expect three possible reactions to be catalysed by peroxidase in the presence of a monomeric phenol (Blinkovsky and Dorkick, 1993): (1) phenol-phenol interactions; (2) lignin-phenol interactions; (3) lignin-lignin interactions. The first case is undesirable in view of modifying lignin properties. The second is the most desirable. The third is less likely to occur, as peroxidase prefers low molecular weight substrates (Saunders et al, 1964), although in the absence of phenols, the enzyme will catalyse lignin oxidation (Kaplan, 1979). The goal of our research was to gain an insight in the way of controlling molecular weight and distribution of copolymer resulting from the enzymatic reaction with reversed micelles, in other words, to optimise the enzymatic copolymerization of lignin with monomeric phenols.

Rate of copolymerization

The data of copolymerization rates are given in Table 1. Before the data was collected, some experiments had to be repeated to determine the range of experimental error

Table 1. rate data of copolymerization

Run No.	Reaction Condition	R1	R2	R3	R4	time	R5	R6
1								
	RT =20° C	200	2.72	2.90	0.50	0.00	376.00	0.0
	CE = $5x10^{-7}$	100	0.83	2.94	1.00	150	88.20	76.5
	Mol/L	100	0.91	3.01	1.00	300	30.27	91.9
11								
	RT = 35°C	20	0.87	4.19	1.0	60	41.3	0.0[a]
	CE = $5x10^{-7}$	70	0.88	2.63	1.0	120	23.4	43.3
	mol/L	100	0.84	4.27	1.0	610	19.7	52.3
35								
	RT= 35°C	80	9.50	2.0	1.0	0.0	380.00	0.0
	CE =$1.33x10^{-7}$	100	1.10	1.6	1.0	30	68.75	81.9
	Mol/L	100	0.70	3.3	1.0	60	21.2	94.4

Where R1 is the size of internal standard, microlitre; R2 is the height of p-cresol which is a height of a peak in HPLC, centimetre; R3 is the height of phenyl standard which is also a height of a peak in HPLC, centimetre; R4 is the size of sample, millilitre; R5 is the relative concentration of unreacted p-cresol; R6 is the conversion of p-cresol, %; time is sampling time, i.e., the reaction time, second; RT represents reaction temperature, and CE, concentration of enzyme HRP, mol/L.

(±11%). The concentration of p-cresol is not an absolute value; however, it can be used to estimate conversion Conversion of p-cresol was 94.5% at 60 second in run No. 35 with an enzyme concentration of $1.33x10^{-7}$ mol/L. Comparison of run 35 with run 1, one can find that effect of temperature on reaction rate is significant, at 60 seconds run 35 has a conversion of 94.5% with a higher temperature, 35°C, it is much higher than 88.2% of run 1 at 150 second with a lower temperature, 20°C, although the enzyme of Run 35 is much lower than that of Run 1.

Copolymerization did not occur when the enzyme concentration was reduced to $2.5x10^{-8}$ mol/L; a possible explanation is inhibition by hydrogen peroxide. Very high reaction rates as obtained has also been claimed by Rao et al (1993) in the enzymatic polymerisation of phenols in reversed micellar system of dioctyl sodium sulfosuccinate (AOT)-water-isooctane, a significant difference being observed between polymerisation in reversed micelles and in other monophasic organic systems. The fact that enzyme molecule occupancy per micelle is seldom greater than unity indicates that the catalyst dispersion is extremely high, leading to efficient utilisation. The reversed micellar system may sustain the growing chain in solution very well, and it has a significant effect on keeping the growing chain in solution and in promoting continued polymerisation. In addition to provide a high degree of dispersion to the enzyme, the surfactant does appear to interact with the monomer and polymeric species, promoting the reaction. Perhaps this is a consequence of the surfactant (CTAB) induced positioning of the substrate (cresol) at the interface and the easier accessibility to the enzyme active site.

Infrared spectroscopy

Figure 1 shows the FTIR spectra of the free (or native) lignin and lignin-p-cresol resin. As shown, strong differences were observed in the 470 to 3300 cm^{-1} range. For example, there is a strong absorbance peak at 2850cm^{-1} in (B), but the same one does not appear in (A). The peak at 2850 $cm^{-1}$is benzene CH_3 asymmetric stretch; the peak at 2925 cm^{-1} in (B) becomes bigger than that in (A), this peak being a benzene ring CH_3 symmetric stretch. Finally, in (B), sample exhibits significant differences in peaks at 1593, 1506, 1463, 1218, 1126, 1101,1035 and 470 cm^{-1}. The peak at 3400 cm^{-1} shifts to lower wave number; it might be a distribution of the hydrogen bonds between polycresol. Hence, significant differences between the lignin-cresol copolymer and free lignin are most likely due to the distributions of incorporation of cresol into lignin or/and polycresol segments in the copolymer. Blinkovsky et al (1993) observed that the FTIR spectrum of lignin-cresol is strongly different from that of polycresol obtained in aqueous-organic solvent mixtures, but not in reversed micelles.

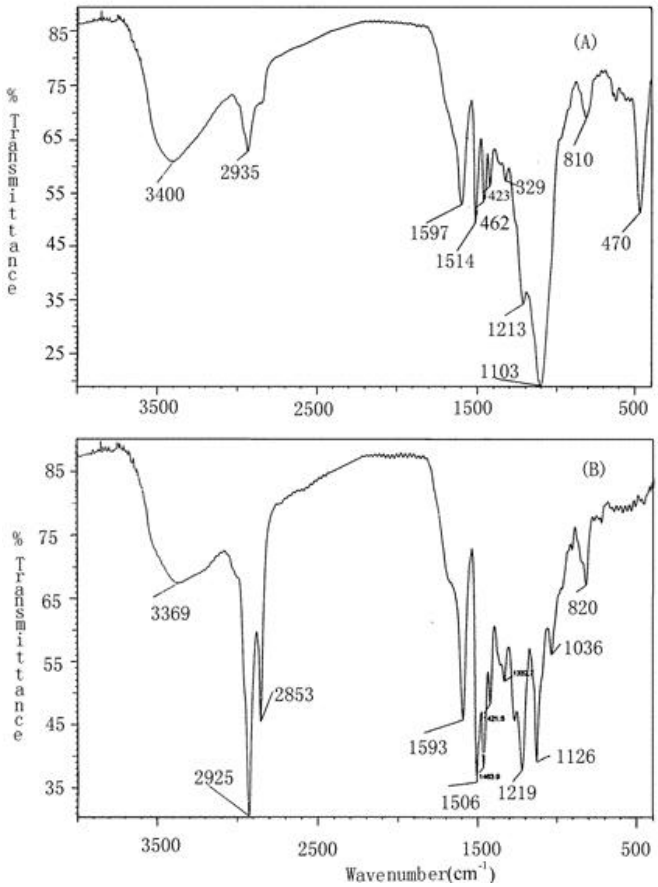

Figure 1. FTIR spectra , (A) FTIR spectrum of copolymer,(B) FTIR spectrum of lignin vertical coordinate: Raletive transmittance abscissa: Wavenumber(cm^{-1})

Thermal properties

Figure 2 shows differential calorimetric scans for lignin-cresol resin and free lignin, respectively. An exotherm is also observed for lignin-cresol copolymer in (A) of figure 2 at about 275°C, which indicates that some crosslinking and /or branching is taking place at a higher temperature. This heat flow is irreversible as indicated by the absence of heat flow in a second heating (data not shown). However, no exotherms appear up to about 400 °C for straw-pulp-lignin in (b), and this is different from Kraft lignin. In (a) an endotherm at 96°C is shown that may indicate a glass transition. Generally, straw-pulp-lignins from different sources have different glass transition temperatures, in the range from 127 to 193°C; however, in our sample of straw-pulp-lignin no glass transition temperature was observed and the reason for this is unclear. Thus, copolymerization of lignin with cresol lowers the endothermic glass transition temperature. This is consistent with published reports performed in non-aqueous media (Blinkovsky and Dordick, (1993). Therefore, the incorporation of phenol into lignin does not appear to destroy the native structure of the lignin;

albeit bound phenols can radically alter its thermal properties.

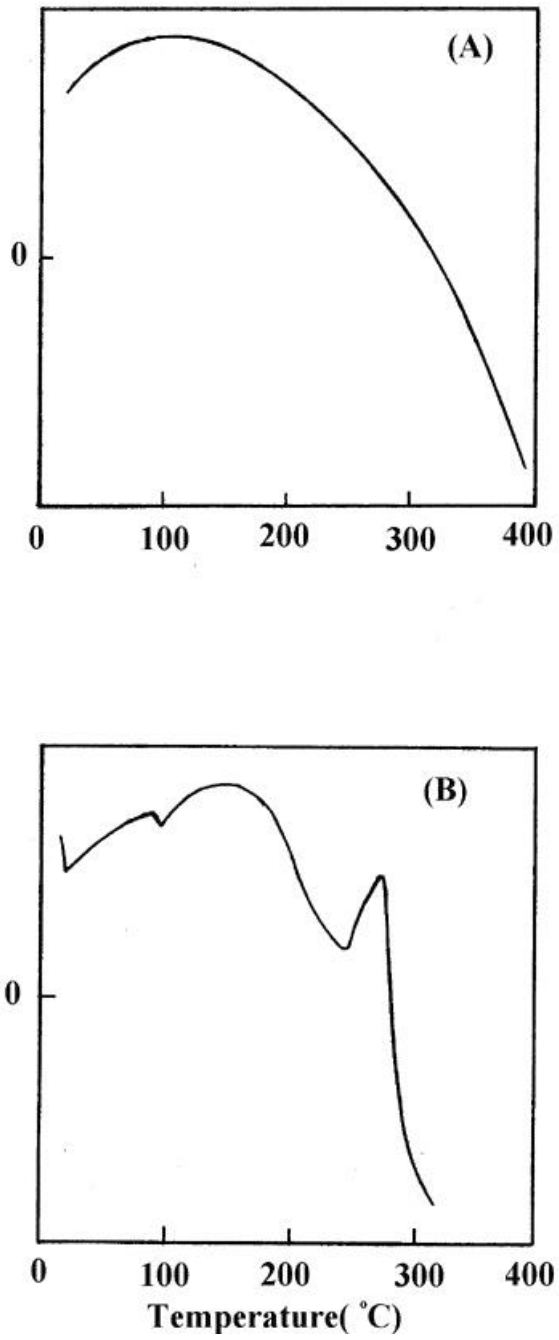

Figure 2. DSC profile,(A) lignin , (B) copolymer vertical coordinate: Heat flow, ?,>0 abscissa: Temperature(°C)

The drop in glass transition temperature for the resin with p-cresol relative to free lignin may reflect the increased flexibility afforded by phenolic groups added to a relatively rigid lignin structure (Odian, 1991). Besides, the addition of phenols to the lignin can alter the balance of internal hydrogen bonds that control the thermal properties of Kraft lignin (Falkehag, 1975).

A mechanism of copolymerization of lignin with cresol (Popp et al, 1991) is seen in Figure 3, where p-cresol and units of lignin are oxidised by HRP/H_2O_2 to phenoxy radicals that undergo subsequent radical coupling and postulated radical transfer. However, cresol is a better substrate for HRP than is lignin, because of its size and phenolic hydroxyl accessibility, and is more readily

oxidised. Thus, it is possible that phenoxy radicals of cresol also oxidise lignin subunits via a radical transfer reaction.The grafting of p-cresol onto lignin is not complete.

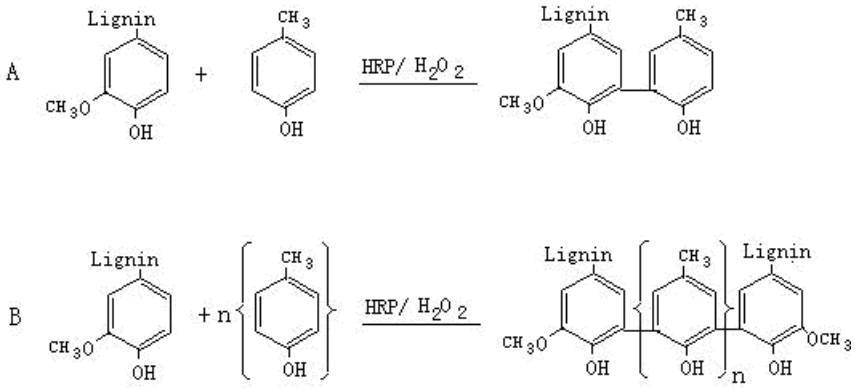

Figure 3. Reaction for HRP catalyzed copolymerization of lignin with cresol

Another possible structure of the copolymers is phenol/polyphenol bridges that form between individual lignin molecules (Blinkovsky and Dordick, 1993). These bridges would provide more rotational freedom to the copolymer as compared to native lignin, or lignin-lignin polymer. Further reaction between different phenolic groups in the lignin and phenol results in additional bridges and a crosslinked lignin polymer. It is probable that both intralignin and interlignin bridges are formed. Either would be expected to modify lignin sufficiently to alter its solubility and thermal properties.

Molecular weight and distribution of copolymer

To our knowledge, no published reports exist on the control of molecular weight and distribution of lignin-phenol copolymer in the enzymatic reversed micellar system. In this system, both the molecular weight and distribution may be related to the concentration of surfactant, enzyme, cresol and lignin, and the ratio of alcohol to hydrocarbon in organic phase. Hence, a quadratic regression orthogonal design with five factors was used for the experimental design of control of molecular weight and distribution. We will briefly discuss here these experimental results, and the detail will be given in other article.

The experimental results for the control of molecular weight and distribution show that the molecular weight changes from 2066 Da to 1.89×10^7Da, certainly a very big change. This indicates that the molecular weight of copolymer can be controlled by changing the concentration of surfactant, enzyme, cresol and lignin, and the ratio of alcohol to hydrocarbon in organic phase. These factors can be controlled easily; thus, enzymatic copolymerization of phenol with lignin appears to be feasible in reversed micellar system. The molecular weight obtained was 1.89×10^7Da, which is very high when compared to about 10000 Da as obtained in the synthesis in aqueous-organic solvent mixtures. The observation appears to validate the hypothesis that chain growth in reversed micelles is strongly influenced by the ability of the surfactant to sustain the growing chain in solution and that there is a rather sharp loss of solubility leading to growth cut-off (Rao et al, 1993).

The importance of the above observation from a design point of view is the fact that surfactant concentration can be used to control the copolymer molecular weight. This is a much more tractable design variable than the choice of solvent in organic solvent system. In such systems, the solvent has to be optimised not only to sustain chain growth but also to maintain enzyme activity.

Conclusions

(1) The peroxidase-catalyzed copolymerization of straw-pulp-lignin with cresol in reversed micellar systems is attractive because it is possible to use the surfactant, as design variable in manipulating the mean molecular weight of the copolymer. In other words, enzymatic copolymerization of lignin with cresol appears to be feasible in reversed micellar systems.

(2) The copolymer has quite different properties than native lignin, which include a lower glass transition temperature and higher curing exotherm. Potential applications of these materials include adhesives,

bonding agents and laminates, and polymeric dispersants. The potential as thermosetting resins may also be examined in future works.

(3) The surfactant sustains remarkably chain solubility and promotes copolymerization, e. g.; the maximal molecular weight is up to 1890 kDa. The reaction is very fast.

Acknowledgement

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