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SHORT COMMUNICATION

PEROXIDASE-CATALYZED POLYMERIZATION OF *p*-CRESOL IN SUPERCRITICAL CO_2

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Abstract – The feasibility of the catalysis of horseradish peroxidase (HRP) in supercritical CO_2 was studied for the polymerization of *p*-cresol in the presence of H_2O_2. The reactions were performed at 40°C and 74.8 atm (1100 psia) above the critical conditions of CO_2. In the initial tests with 2 mM of *p*-cresol and 1 mM of H_2O_2, more oligomers of *p*-cresol were produced as more HRP was added. This indicates that HRP is active in supercritical CO_2. HRP was not completely inhibited by H_2O_2 at concentrations up to 20 mM. Increasing the initial concentrations of *p*-cresol and H_2O_2 to 20 mM, respectively, resulted in the formation of precipitates which were undissolved either in water or in dimethylforamide (DMF). The effects of adding water and/or methanol as cosolvents on the reactivity of HRP were studied subsequently. When more than 13.3 mL of water per liter of reaction volume was added, the formation of precipitates was not observed. The reactivity of HRP was sustained when up to 11.8 mL of methanol per liter of reaction volume was added. In most cases conversion of *p*-cresol was less than 50% for 5 hours of reaction time.

Key words: *Peroxidase, Supercritical, Carbon Dioxide, p-Cresol, Polymerization*

INTRODUCTION

Phenolic resins, which are produced from the condensation over acid or base catalysts of phenolic monomers functionalized by formaldehyde, have been widely used in a variety of areas including coatings, dispersions, adhesives, molding compounds, abrasives, laminates etc [Kopf, 1988]. The use of formaldehyde to produce such phenolic resins however results in the unreacted formaldehyde residing in the final products, which raises serious concerns in relation to human health due to the potential carcinogenecity of formaldehyde [Marshall, 1987]. An alternative method to make phenolic resins without using formaldehyde was developed by Dordick et al. [1987] and Pokora and Cyrus [1987], in which phenolic monomers are enzymatically oxidized by peroxidases into phenolic radicals which undergo subsequent radical transfer and coupling reactions resulting in the high molecular weight phenolic polymers. The reaction mechanism of peroxidases with a phenol and H_2O_2 is as follows [Saunders et al., 1964].

$$E + H_2O_2 \rightarrow E_I + H_2O \quad (1)$$

$$E_I + AOH \rightarrow E_{II} + AO\cdot \quad (2)$$

$$E_{II} + AOH \rightarrow E + H_2O + AO\cdot \quad (3)$$

A native peroxidase (E) is oxidized with H_2O_2 by two electrons to an enzyme intermediate called compound I (E_I) [step (1)]. Via two one-electron reduction steps, compound I is reduced to another enzyme intermediate called compound II (E_{II}), then returns to the native peroxidase [steps (2) and (3)]. During the catalytic cycle of peroxidase two molecules of phenols (AOH) are oxidized into phenolic radicals (AO·) with the concomitant reduction of H_2O_2 into water. In water, enzymatically produced phenolic radicals undergo rapid radical coupling to produce mostly phenolic dimers and low molecular weight oligomers which precipitate out of water. In organic solvents, on the other hand, the solubilities of phenolic polymers increase significantly enabling the formation of high-molecular weight phenolic polymers of molecular weights of over 10,000 [Dordick et al., 1987; Rao et al., 1993]. The enzymatic production of phenolic polymers does not require formaldehyde as a reactant, thereby, causes no environmental concerns in relation to formaldehyde. The use of organic solvents as reaction media for the peroxidase catalyzed polymerization of phenols (i.e., dioxane, ethyl acetate, ethanol, acetone, or isopropanol [Dordick et al., 1987; Pokora and Cyrus, 1987; Cyrus et al., 1992]), however, can be of potential environmental concerns.

Supercritical CO_2 has intermediate properties between liquid and gas thus retains many advantages including high diffusivity of solutes, easy control of solvent properties by changing pressure, and increased solubility of hydrophobic compounds. Supercritical CO_2 has proven to be an acceptable reaction medium for various enzymes [Randolph et al., 1988; Marty et al., 1992; Kamat et al., 1993]. The most valuable feature of supercritical CO_2 is that it causes no environmental problems. After reactions CO_2 can simply be vaporized out by depressurizing reactors, thereby leaving products, unreacted reactants, and enzymes which can be separated further from each other by using minimum amount of aqueous and organic solvents. The feasibility of peroxidase catalyzed polymerization of phenols in supercritical CO_2 has not been examined so far and is of major concern for our research. Among various peroxidases used for the production of phenolic polymers [Cyrus et al., 1992] the most readily available peroxidase, HRP, was used for this work with *p*-cresol as a model phenolic compound.

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EXPERIMENTAL

1. Materials

Pure HRP (Type II) was purchased from Sigma Chemical Co. (USA) and used without further purification. *p*-Cresol was purchased from Aldrich Chemical Co. (USA). All other chemicals were purchased from Sigma Chemical Co. and used without further purification.

2. Reaction of *p*-Cresol by HRP

A whole experimental system is depicted in Fig. 1. The reactor and the injector were enclosed in a constant temperature chamber. *p*-Cresol as solid and HRP dissolved in a predetermined amount of an aqueous buffer (pH 7, 100 mM KH_2PO_4) were added into a cylinderical reactor of internal volume of 85 mL. Supercritical CO_2 stored in a reservoir at 40°C under a pressure higher than 200 atm was supplied rapidly into the reactor. After the pressure and temperature inside the reactor reached to and equilibrated at 74.8 atm and 40°C, respectively, predetermined amount of H_2O_2 [as a 35% (v/v) solution] was added through an injection port using a needle syringe. Reactions were usually continued for two hours with agitation using a magnetic stirrer. Reactions were stopped by depressurizing the reactor by venting CO_2 into atmosphere through a chloroform trap. The unreacted monomer and products were separated from HRP through the recovery steps described in the followings.

3. Recovery and Fractionation of Products

Chloroform was used to wash out unreacted *p*-cresol and products from the reactor. This chloroform solution was gathered together with the chloroform solution from the CO_2 trap. Precipitates of HRP and high molecular weight phenolic polymers which are insoluble in chloroform were recovered by scraping them out from the reactor wall and by filtration of the above chloroform solution. The filtered chloroform solution was vacuum dried in a rotary evaporator and dissolved in a small volume of DMF. Precipitates formed in the DMF solution were recovered by filtration and pooled with the previous precipitates recovered from the chloroform solutions. The precipitates were washed with fresh DMF then with distilled water to dissolve off HRP. Final precipitates were dried in a vacuum oven for weighing.

4. Analyses

To determine the conversion of *p*-cresol, the final DMF solution containing uncreacted *p*-cresol monomer and low molecular weight oligomers was analysed by reverse phase HPLC. A HPLC system (Waters Co., USA) with a C^{18} reverse phase column and a UV detector was used. A methanol/water (50/50) mixture was used as an eluent at 1 mL/min. Monomer and oligomers of *p*-cresol were detected at 280 nm. The molecular weight distribution of DMF soluble fraction was determined by GPC (System-II, Shodex Co., Japan) using KF-801, 802, 804, and 80GL columns in series at 45°C and a refractive index detector (RI-71, Shodex Co., Japan). THF was used as an eluent at 1.0 mL/min.

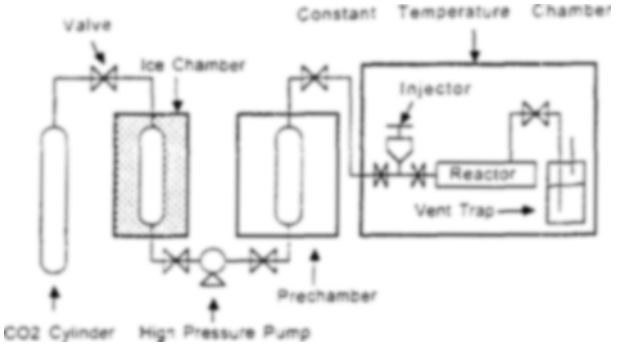

Fig. 1. A schematic diagram of the whole experimental system.

RESULTS AND DISCUSSION

1. The Reactivity of HRP in Supercritical CO_2

At first, the formation of precipitates of high molecular weight products was suppressed by using a low concentration of *p*-cresol of 2 mM. The initial concentration of H_2O_2 was 1 mM which is the stoichiometric amount required for the complete conversion of *p*-cresol. This enabled the accurate observation of the effects of HRP on the formation of oligomers of *p*-cresol. HRP dissolved in 1 mL of the aqueous buffer was used. As shown in Fig. 2-(a) no formation of products was detected by

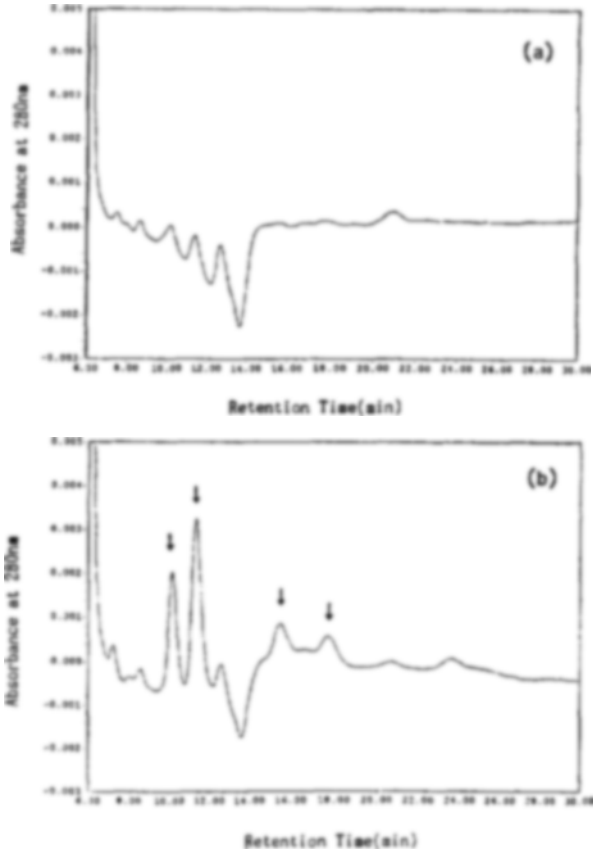

Fig. 2. The chromatogram from the reverse phase HPLC analysis of the *p*-cresol oligomers dissolved in DMF when 0 mg (a) or 20 mg (b) of HRP dissolved in 1 mL of a buffer (100 mM KH_2PO_4, pH 7) was used. *p*-Cresol monomer eluted at 5.8 min, therefore, is not shown in this figure. Arrows (↓) indicate the product peaks. Reaction conditions are explained in the text.

Table 1. Effects of the content of an aqueous buffer on the conversion of *p*-cresol and the formation of precipitates catalyzed by HRP in supercritical CO_2

Buffer added (μL)	Conversion of *p*-cresol (%)	Precipitates recovered (mg)
0	22.5	-
100	25.7	-
500	25.1	2.6
1000	41.5	22.4
1500	40.1	-

Reaction conditions were [HRP]=20 mg/85 mL of reactor volume, [*p*-cresol]=20 mM, [H_2O_2]=20 mM. Reactions were performed at 40°C and 74.8 atm (1100 psia). Reaction time was 2 hours and the aqueous buffer solution was 100 mM KH_2PO_4 at pH 7. The precipitates were not dissolving either in water or DMF.

HPLC for the blank reaction in the absence of HRP. The formation of oligomers of *p*-cresol was detected when 2 mg of HRP was used. The final DMF solution which contained unreacted *p*-cresol and its oligomers showed yellow color which is the characteristics of the oligomers of *p*-cresol as also observed by Dordick et al. [1987] and Rao et al. [1993]. In order to conform that the appearance of yellow color is due to the formation of oligomers of *p*-cresol, we added 1 mg of HRP and 1.8 mg of *p*-cresol into 10 mL of fresh DMF. In this DMF solution, no yellow color was developed and HRP was precipitated. As illustrated in Fig. 2-(b), when 20 mg of HRP was used, the chromatogram obtained from the HPLC analysis of the final DMF solution showed two major peaks of products at the retention times of 10.5 min and 11.5 min, respectively. In all cases, the degree of conversion of *p*-cresol was not significant, possibly due to the slow reaction rate and/or deactivation of the enzyme. All these results, however, demonstrate that HRP can function in supercritical CO_2.

Next, we tested the inhibition of HRP by H_2O_2. The initial concentration of *p*-cresol was increased to 10 mM while that of H_2O_2 was varied between 5 mM and 20 mM. The amount of enzyme used was 20 mg. Supprisingly the formation of *p*-cresol oligomers was observed with as high as 20 mM of H_2O_2, which indicated that HRP was not completely inhibited. This is in contrast to the cases in aqueous buffers in which the activity of dissolved HRP was inhibited by above 0.2 mM of H_2O_2 [Ryu and Dordick, 1989; 1992]. The causes of stabilization of HRP against high concentrations of H_2O_2 in supercritical CO_2 can be deduced from two factors. One is that HRP forms aggregates in supercritical CO_2. Generally, enzymes in a solution are more stable in heterogeneous states than in homogeneous states against inactivations. This is due to the stronger intermolecular interactions inside the aggregates. The other is the existence of external and/or internal mass transfer limitations for the enzyme aggregates, which reduce the H_2O_2 concentration at the surface of enzyme molecules much lower than that in a bulk phase.

2. The Effects of Cosolvents

One of the advantages of using supercritical fluid is that the properties of solvent, i.e. polarity, can be readily adjusted by adding small amount of various cosolvents [Randolph et al., 1987]. We first investigated the effects of adding an aqueous buffer (pH 7, 100 mM KH_2PO_4) as a cosolvent on the HRP's reaction with *p*-cresol. The initial concentration of *p*-cresol was increased to 20 mM. After adding 20 mg of HRP dissolved in various amount of the aqueous buffer reactions were started by injecting 10 mM of H_2O_2. The same amount of H_2O_2 was injected again after 30 min thereby total concentration of H_2O_2 added became 20 mM. Table 1 lists the conversion of *p*-cresol and the weight of precipitates recovered at various content of the aqueous buffer from zero to 1.5 mL per 85 mL of reaction volume. The solubility of water in supercritical CO_2 is known to increase between 1.5 and 2.2 g/l as temperature or pressure increases [Marty et al., 1992]. In our experiments, the content of water that was added to the reactor through 35% H_2O_2 solution (200 μL per 85 mL reaction volume) was approximately 1.5 g/l which is close to the saturation content of water in supercritical CO_2 as found by Marty et al. [1992]. Until additional 1.5 mL of the aqueous buffer was added into the reaction mixture, however, no separate aqueous phase was observed as inspected visually through a sapphire window of the reactor. Excess water above the saturation level could be distributed among supercritical CO_2, enzyme aggregates, and the reactor wall to such a degree that a separate aqueous phase was not visually discernable. The precipitates which were insoluble either in water or in DMF were formed only when 0.5 mL or 1 mL of the aqueous buffer was added. These precipitates were presumed to be the high molecular weight polymers of *p*-cresol (possible molecular weight>10,000) as determined by Dordick et al. [1987]. No precipitates were formed when 1.5 mL of the aqueous buffer was added. This indicates the presence of a separate aqueous phase in which dissolved HRP catalyze the formation of oligomers of *p*-cresol only. The maximum recovered weight of precipitates was 22.4 mg (yield was approximately 11% based on the initial amount of *p*-cresol) when 1 mL of the aqueous buffer was added. Not all the precipitates was fully recovered due to the stickiness of the precipitates onto the reactor wall.

Table 2. Effects of methanol on the conversion of *p*-cresol and the formation of precipitates catalyzed by HRP in supercritical CO_2

Methanol added (μL)	Conversion of *p*-cresol (%)	Precipitates recovered (mg)
200	36.0	9.0
500	34.8	16.5
1000	35.0	29.8

Reaction conditions were the same as for Table 1 except reaction time was increased to 5 hours. HRP dissolved in the aqueous buffer (20 mg/1 mL) was used.

The effects of adding methanol as another cosolvent on the conversion of *p*-cresol and the formation of precipitates were studied and results are shown in Table 2. The reaction time was increased to 5 hours. HRP dissolved in the aqueous buffer (20 mg/1 mL aqueous buffer) was used. As methanol content increased up to 1 mL per 85 mL of reaction volume, the formation of the precipitates increased while the conversion of *p*-cresol did not change significantly. When more than 1 mL of methanol was added precipitates were not formed. Fig. 3 shows the chromatogram from the HPLC analysis of the oli-

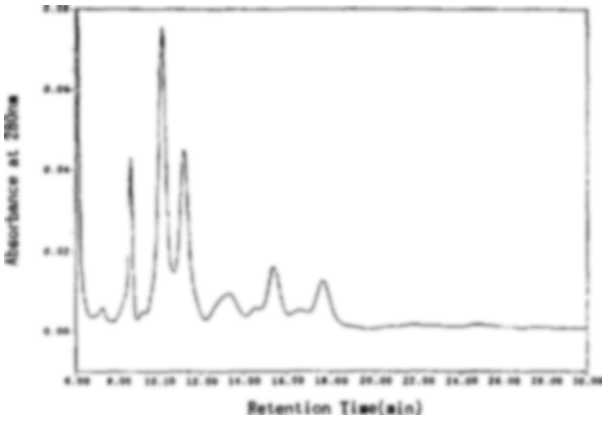

Fig. 3. The chromatogram from the reverse phase HPLC analysis of the *p*-cresol oligomers dissolved in DMF when 1 mL of methanol was used as a cosolvent. *p*-Cresol monomer eluted at 5.8 min, therefore, is not shown in this figure.

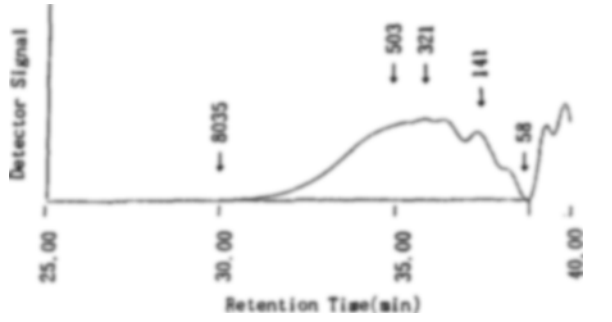

Fig. 4. The molecular weight distribution of *p*-cresol oligomers dissolved in DMF as determined by GPC analysis when 1 mL of methanol was used as a cosolvent. Numbers in the figure are the molecular weights corresponding to the retention times.

gomeric products dissolved in DMF when 1 mL of methanol was added. Fig. 3 indicates that at least five oligomeric products were formed. The molecular weight distribution of *p*-cresol oligomers dissolved in DMF was measured by GPC as shown in Fig. 4. The maximum molecular weight was 8,000. The molecular weight of the major oligomer was 321 which corresponds to the molecular weight of *p*-cresol trimer. In all cases, the conversion of *p*-cresol was rather low under 40%, possibly due to the deactivation of HRP in supercritical CO_2 or the diffusional limitation caused by the aggregation of HRP. The use of properly immobilized HRP can alleviate these problems.

In summary, HRP catalyzed polymerization of *p*-cresol was feasible in supercritical CO_2 in the presence of water and/or methanol as cosolvents. The analysis of precipitates including the molecular weight distributions and solubilities in other organic solvents and the investigation about the influence of other cosolvents such as ethanol and acetone on the reactivity of HRP in supercritical CO_2 are of future concerns.

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NOMENCLATURE

DMF: dimethylformamide
HRP : horseradish peroxidase

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