

## Effects of Hydrogen Peroxide Concentration on the Polymerization of *p*-Phenylphenol in Organic Solvent by Peroxidase

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In horseradish peroxidase-catalyzing polymerization of phenol under the water/dioxane solvent system, the optimal concentration of hydrogen peroxide was found to be 10 mmol/l. Feeding of hydrogen peroxide at its optimal concentration improved the polymerization performance by reducing reaction time and increasing molecular weights. Monomer conversions and the molecular weights of the enzymatically produced polymer were in the ranges of 83.1~94.2% and 58000~68000, respectively.

Horseradish peroxidase (HRP, hydrogen peroxide-oxidoreductase, EC 1.11.1.7) catalyzes the oxidation-reduction of numerous phenolic compounds and aromatic amines using hydrogen peroxide as an oxidant(3). Among peroxidase-catalyzing reactions, the peroxidatic reaction has been known to produce polymerized compounds from various phenols (5).

Recent research reported on the enzymatic polymerizations of phenolic compounds using HRP in water/water-miscible organic solvent systems (2). According to this research, the phenol polymers produced under nonaqueous media had a higher degree of polymerization than that obtained by conventional aqueous enzymatic reactions. Also, among the phenolic polymers synthesized under the nonaqueous solvent systems, poly(*p*-phenylphenol) exhibited higher melting points than that of phenolic resins produced by conventional chemical methods. Rationale for the employment of such nonaqueous media was to increase the solubility of water-insoluble or slightly-soluble phenolic substrates and then to enhance the performance of enzymatic reactions (8).

Horseradish peroxidase is inhibited with higher concentrations of hydrogen peroxide (3) and also can be deactivated by direct contact with hydrophilic organic solvents present in the water/organic miscible solvent system (2). It is, therefore, important to shorten the reaction time of polymerization when HRP is employed in the organic solvent system.

In the present work, we found the optimal concentration of hydrogen peroxide in the water/organic miscible solvent system, and investigated the feeding effects of optimal concentrations of hydrogen peroxide as oxidant on the reaction time and properties of the enzymatically formed polymer.

### MATERIALS AND METHODS

#### Materials

Horseradish peroxidase and *p*-phenylphenol were purchased from Sigma Chemical Co.; hydrogen peroxide (30% solution in water) was purchased from Junsei Chemical Co.; polystyrene standards were from Aldrich Chemical Co.. All other chemicals and solvents, used in the present study, were of reagent grades.

#### Activity Assay of HRP

Activity of HRP was defined as follows: 1 unit of HRP can form 1  $\mu$ mole of purpurogallin from pyrogallol in 1 minute at 25°C and in potassium phosphate buffer (0.1mol/l, pH 6.0); specific activity of HRP was defined as units per mg enzyme solid. Concentrations of purpurogallin produced were measured from absorbances at 420 nm using a spectrophotometer (Kontron, UVIKON 930). The specific activity of HRP, according to the present definition of the enzyme unit, was measured as 220 units/mg solid.

#### H<sub>2</sub>O<sub>2</sub> Concentration on Initial Activities of HRP

Effects of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration on the initial activities of HRP were examined in 15% aqueous acetate buffer (10 mmol/l, pH 5.0)/85% dioxane system. Pyrogallol was used as a substrate instead of

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*p*-phenylphenol, since the products from *p*-phenylphenol are polymerized products and therefore could not be detected by spectrophotometer. Purpurogallin produced from pyrogallol by the enzymatic reaction can be easily detected at the wavelength of 420 nm.

#### Polymerization System

Enzymatic polymerizations were carried out in an 100ml round-bottomed flask as working volume of 50 ml at 25°C with vigorous stirring using a magnetic bar. Fifteen percent aqueous acetate buffer (10 mmol/l, pH 5.0)/85% dioxane solvent system was used as the reaction medium. Concentrations of HRP and *p*-phenylphenol were 1 mg/ml and 10 mmol/l, respectively. Reactions were initiated by adding 10 mmol/l of hydrogen peroxide to the reaction mixture. In all reactions, the total amount of hydrogen peroxide added was 100 mmol/l.

#### Determination of *p*-Phenylphenol Concentration

Concentrations of *p*-phenylphenol were analyzed by HPLC (Waters Associates, Model 510). The column used was a silica gel normal phase one (pore size 5  $\mu$ m, 4.6  $\times$  250 mm). The eluent consisted of 94%(v/v) chloroform and 6% methanol. Aliquots of 100  $\mu$ l were withdrawn from the reaction mixture, and were rapidly mixed with 0.1 mol/l trichloroacetic acid solution to stop the reaction. Three microliters of the diluted aliquot (below  $10^{-4}$  mol/l) was injected, and the concentration of *p*-phenylphenol was determined at 280 nm using a UV detector (Lambda-Max, Model 481).

#### Intrinsic Viscosity and Molecular Weights of the Polymer

The polymer synthesized enzymatically was separated and purified by the following procedure: 1) after the reaction was stopped, the reaction mixture was centrifuged at 13000 rpm for 10 minutes; 2) the precipitate was thoroughly washed with water under vigorous stirring to remove the enzyme; 3) the remaining solid was washed with dioxane to remove monomer and oligomers; 4) the remaining polymer was washed with petroleum ether and dried at 50°C for 10 hours.

Intrinsic viscosities of the polymer solution were measured using a capillary viscometer (Schott Ger te, Ubbelohde type 52503) at 30°C. Solvent system was made up of 80% dimethylformamide and 20% methanol. The efflux time of the solute was measured five times and its averaged value was taken. The intrinsic viscosities were obtained for various polymer concentrations and by extrapolating the relative viscosities to zero polymer concentration (4).

Molecular weights of the polymer were measured by GPC (Waters Associates, Millipore). Two columns( $\mu$ -styragel 10<sup>3</sup> Å and  $\mu$ -styragel 500 Å) in series were used. Dimethylformamide was run as an eluent at a flow rate of 0.9 ml/min. Polystyrene standards were used to obtain

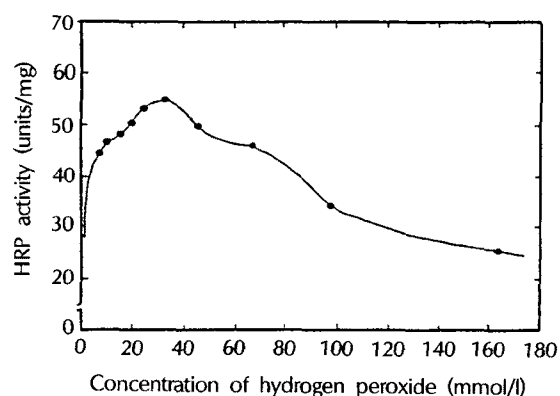
the calibration curve. All polymer samples were filtered by 5 micron filter papers before injection. Eluted samples were detected using a refractive index detector (Waters Associates, Model R401).

## RESULTS AND DISCUSSION

As shown Fig. 1, initial activities of HRP for various concentrations of hydrogen peroxide were measured in the 85% dioxane/15% aqueous acetate buffer (10 mmol/l, pH 5.0). HRP activity was highest at the concentration of hydrogen peroxide of 30 mmol/l when pyrogallol was used as a substrate.

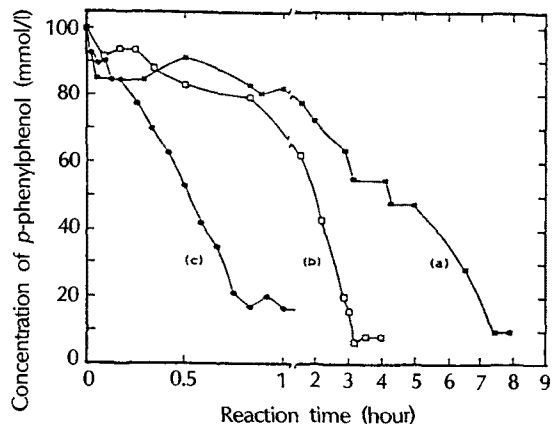
In the polymerizations using *p*-phenylphenol as a monomer, 10 mmol/l hydrogen peroxide was fed into the reaction mixture, because *p*-phenylphenol has one hydroxyl group per molecule whereas pyrogallol has three hydroxyl groups per molecule. In all reactions, hydrogen peroxide at its optimal concentration was fed periodically to avoid substrate inhibition by the oxidant. As depicted in Fig. 2, when 10 mmol/l hydrogen peroxide was added intermittently every 50 minutes, the conversion of *p*-phenylphenol reached 90% in 7.5 hours (Fig. 2-(a)). When 10 mmol/l H<sub>2</sub>O<sub>2</sub> was added intermittently at 20-minute intervals, the reaction time was shortened to about 3 hours and the conversion was 94.2%(Fig. 2-(b)). Furthermore, when 10 mmol/l H<sub>2</sub>O<sub>2</sub> was added every 5 minutes, the reaction time was greatly shortened to 50 minutes but the conversion was 83.1% (Fig. 2.(c)).

There were almost no differences, however, in final conversions regardless of the feeding intervals of H<sub>2</sub>O<sub>2</sub>. So it was deduced that the reactions progressed in proportion to the amount of H<sub>2</sub>O<sub>2</sub> added. Therefore reaction time can be further shortened if H<sub>2</sub>O<sub>2</sub> is fed so as to maintain its optimal concentration in the reaction medium.



**Fig. 1.** Effect of hydrogen peroxide concentration on the activities of horse radish peroxidase.

Conditions: 85% dioxane/15% aqueous acetate buffer(10 mmol/l, pH 5.0, 25°C), 0.075 mg/ml HRP, 7.5 mmol/l pyrogallol.



**Fig. 2.** Changes of *p*-phenylphenol concentration with reaction time when the feeding periods of hydrogen peroxide were (a) 50min, (b) 20min, and (c) 5min.

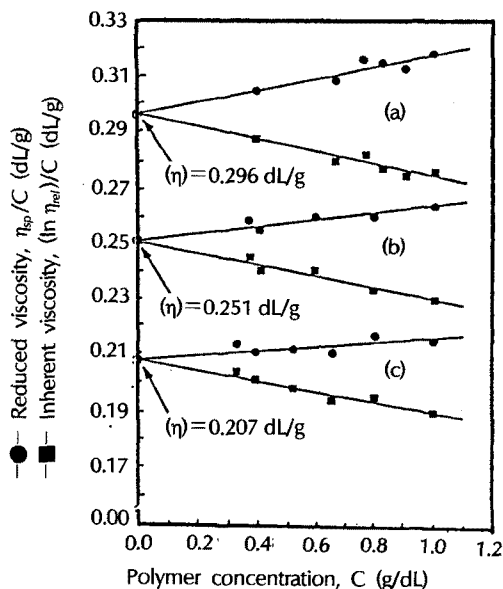
Conditions: 85%(v/v) dioxane/15% aqueous acetate buffer(10mmol/l, pH5.0, 25°C), 1 mg/ml HRP, 100 mmol/l *p*-phenylphenol.

Color changes of the reaction mixture were also observed during polymerizations. As soon as  $H_2O_2$  was added, the reaction mixture turned black. It seems that free radicals caused the reaction mixture to be black, and hence the black color increased absorbances in the overall scanning region (data not shown). In the peroxidatic reactions by HRP, free radicals are known to be formed by oxidation of phenolic compounds (6, 7). As the reaction progressed, the color of the reaction mixture changed from black to reddish brown. This change, black to reddish brown, continued periodically whenever  $H_2O_2$  was added to the reaction mixture.

Enzymatically formed polymer was a greenish gray solid after separation. After stirring vigorously for 1 hour at 100°C, about 70% of the polymer was dissolved in dimethylformamide. Solution obtained after filtering out the undissolved solids was used to measure intrinsic viscosities and molecular weights by gel permeation chromatography (GPC).

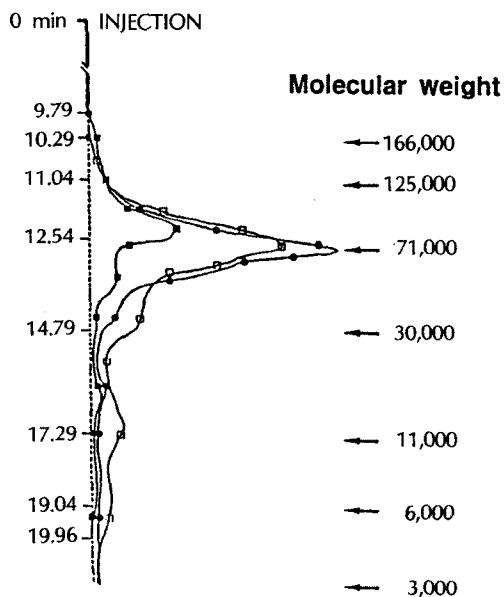
Intrinsic viscosity of polymer solution is basically a measure of the size or extension in space of polymer molecules (1). The used solvent system was 80% dimethylformamide/20% (v/v) methanol, and the efflux time of the cosolvent was 292.2 seconds in the viscometry. As shown in Fig. 3, the intrinsic viscosities of the polymer solutions obtained from the three reactions were 0.296, 0.251, and 0.207 dL/g, respectively.

Molecular weights were also measured using gel permeation chromatography. As depicted in Fig. 4, similar patterns in the molecular weight distribution were observed for the three cases. The weight-averaged molecular weights from the three reactions were 68000, 58000 and 66000, respectively. Polydispersities, defined as the ratio of weight-averaged molecular weight to number-



**Fig. 3.** Reduced and inherent viscosities-concentration curves for the polymer produced from (a) the first, (b) the second, and (c) the third reactions of Fig. 2.

Conditions: 80% (v/v) dimethylformamide/20% methanol (30°C).



**Fig. 4.** Molecular weight distributions of the polymers produced from the three reactions of Fig. 2:  $\blacksquare$ , from the first reaction;  $\square$ , from the second reaction;  $\bullet$ , from the third reaction.

Conditions: eluent (dimethylformamide) flow rate 0.9 ml/min at 30°C (arrows in the right side point out its molecular weights).

averaged molecular weight, were 1.19, 1.72 and 1.25, respectively. The molecular weights were higher than that previously reported by Dordick et al. (2).

The values of intrinsic viscosities were not in direct proportion to that of the averaged molecular weights.

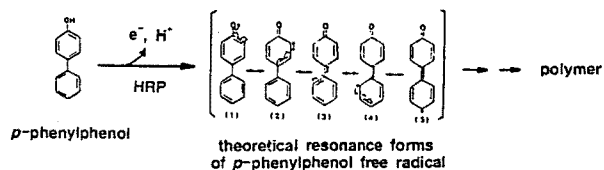


Fig. 5. Schematic representation of HRP-catalyzing polymerization from *p*-phenylphenol.

This is thought to result from the effects of branched or cross-linked portions of the polymer mixture (7). In general, the common step in the peroxidase-catalyzing polymerizations is the deprotonation from the hydroxyl groups of phenolic monomers, and the generated phenolic free radicals usually react with little site-selectivity (7). For *p*-cresol, various polymer products have been reported to be formed by the peroxidase-H<sub>2</sub>O<sub>2</sub> polymerization system. Also, previous works (2) have shown that only 1-naphthol and *p*-phenylphenol, among the many types of phenolic monomers, exhibited higher averaged molecular weights (26000 for *p*-phenylphenol). So it can be deduced that phenolic monomers with more theoretical resonance forms of their free radicals have more chances of coupling; give higher molecular weights; then, by the random couplings of free radicals, branched or cross-linked forms, as well as linear forms, can be produced from *p*-phenylphenol (Fig. 5).

## CONCLUSION

The peroxidase-catalyzing polymerizations in organic solvent environments confirmed that the optimal feeding strategy of H<sub>2</sub>O<sub>2</sub> was important to reduce the reaction

time and to achieve high molecular weights of the produced polymer.

## Acknowledgement

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