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Phenol Conversion and Dimeric Intermediates in Horseradish Peroxidase-Catalyzed Phenol Removal from Water

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Phenol was removed from water by horseradish peroxidase-catalyzed polymerization. Five dimeric and one trimeric products from the reaction were identified in the aqueous solution. The trimer had a structure of 4-(4-phenoxyphenoxy)phenol (**VI**) determined from its NMR spectrum. The dimers such as *p,p'*-biphenol (**I**), *o,o'*-biphenol (**II**), and *p*-phenoxyphenol (**III**) were the reaction intermediates. With more than 95% phenol removal from an initial phenol concentration of 188 mg/L, the final concentrations of the three dimers were each below 1 mg/L. About 7% of the precipitate mass was attributed to the three dimers (**I, II, III**), and the rest consisted mainly of the compounds of higher hydrophobicity and molecular mass. With an equimolar ratio of phenol to hydrogen peroxide, the phenol conversion behaved as a first-order reaction with respect to phenol concentration. A peroxidase inactivation model for the reaction in the presence of poly(ethylene glycol) (PEG) was proposed, and the inactivation rate constant was found to have a logarithmic relationship with the ratio of PEG to enzyme doses. The three dimers were the substrates of peroxidase, and their conversion could also be depicted with a first-order model with respect to the dimer concentrations. A comparison of the specific reaction rates indicated that *p*-phenoxyphenol was the best substrate of peroxidase (2.172 nM^{-1} min^{-1}) followed by *p,p'*-biphenol (0.671 nM^{-1} min^{-1}), phenol (0.0105 nM^{-1} min^{-1}), and *o,o'*-biphenol (0.00453 nM^{-1} min^{-1}). Therefore, the predominant polymerization bonds in the products may be the oxygen–para connection whereas the ortho–ortho connection would hardly be found in the higher oligomers.

Introduction

Phenols are priority pollutants (*1*) that are found in various industrial wastewaters, e.g., petroleum refinery, resin manufacture, and coal processing. Phenol removal through enzyme-catalyzed polymerization has recently been investigated as an alternate detoxification method (*2–6*). The oxidoreductive enzymes investigated include tyrosinase (*2*), laccase (*3*), and peroxidase (*4–6*). Horseradish peroxidase (HRP) has stable activity in the temperature range of 5–55 °C (*7*) and low substrate specificity (*8*). With hydrogen peroxide, peroxidase catalyzes the oxidation of phenols to phenoxy radicals that undergo further nonenzymic reactions generating various oligomers and polymers (*4*). These products have low solubility in water and can be removed by sedimentation and filtration.

A major concern with this enzyme technology is the nature and fate of the reaction products. The pioneering work (*4*) did not identify specific products but noted that the products were insoluble polymers of high molecular mass. The products from 2,4-dichlorophenol polymerization were found to contain two major fractions, a highly polar fraction and a high molecular mass nonpolar fraction (*5*). It was recently suggested (*9*) that, due to the extreme multitude and diversity of the products formed, quantitation of the initial products such as dimers would enable the determination of the prevailing coupling routes. Biphenols (dihydroxybiphenyls) are generated from the coupling of two phenoxy radicals (*10, 11*). Danner and co-workers (*12*) reported that the only product of phenol oxidation by HRP was *o,o'*-biphenol, which could act as a substrate for HRP. Another dimer product, *p,p'*-biphenol was also detected in the reaction of HRP-mediated phenol coupling and shown to be further oxidized to *p*-diphenoquinone (*13*). Catalyzed by HRP, 2-*tert*-butyl-4-methoxyphenol yielded a dimer that was oxidized again yielding a dimeric phenoxy radical (*14*). It was reported that the formation of dimeric products accounted for 3% of the initial amount of phenol in an HRP-catalyzed phenol coupling reaction (*15*).

Kinetic analysis of the formation and conversion of the dimers may provide valuable information on the prefered reaction pathways and, hence, the structure of the reaction products of higher molecular mass. On the other hand, since the dimeric products are the most soluble products from the reaction, their formation and conversion can determine the final degree of phenol removal from water. Recent progress on increasing HRPs turnover in the presence of poly(ethylene glycol) (PEG) has significantly reduced the peroxidase dosage requirement (*16, 17*) and made this enzymatic process economically competitive with other treatment processes (*16*). Kinetic data on phenol conversion and enzyme inactivation in the presence of PEG are essential to the process design.

This paper reports the identification of soluble products in the aqueous phase from phenol polymerization and gives quantification of the phenol conversion, the enzyme inactivation, and the oxidation of three dimeric products, *p,p'*-biphenol (**I**), *o,o'*-biphenol (**II**), and *p*-phenoxyphenol (**III**) in the presence of PEG. The distribution of the three dimers in the final reaction solution and in the precipitates is also described.

Materials and Methods

Materials. Crystalline horseradish peroxidase (EC 1.11.1.7, Grad I, Boehringer Mannheim GmbH, Germany) was dissolved in 50 mM phosphate buffer (pH 7.0) as a peroxidase stock solution (0.8 g/L). Phenol, *p,p'*-biphenol (**I**), *o,o'*-biphenol (**II**), and poly(ethylene glycol) (PEG, approximate molecular mass 3350) were purchased from Sigma Chemical Co. (St. Louis, MO); *p*-phenoxyphenol (**III**) was purchased from ICN Pharmaceuticals Inc. (Plainview, NY); and hydrogen peroxide (30% analytical

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reagent) was purchased from BDH Inc. (Toronto, ON). The stock solutions of these chemicals were kept at 0–4 °C. All other chemicals and solvents were of highest commercially available quality.

Reaction and Sample Preparation. Phenol polymerization was carried out in a batch reactor agitated with a Teflon-coated magnetic bar at room temperature (around 25 °C) and neutral pH. The initial reaction solution contained 50 mM phosphate buffer (pH 7) and 2 mM phenol for all the experiments while HRP had a range from 1.52 to 6.89 nM and PEG had a range from 30 to 100 mg/L. The reaction was initiated by adding peroxide stock solution to give equimolar peroxide and phenol in the initial reaction solution. A 2-mL aliquot of the reaction solution was mixed immediately with 0.1 mL of 10% phosphoric acid to stop the reaction (pH <2.0). The acidified sample was then filtered through a 0.2-μm syringe filter (Micron Separations Inc., Westboro, MA). Concentrations of the three dimers, phenol, and peroxide in the filtrate were determined with HPLC and UV/VIS methods described below. The precipitates left on the filter were washed with 2 × 1 mL of distilled deionized water and dissolved in 1 mL of methanol by injecting the methanol through the precipitate three times. The methanol solution was analyzed to determine the content of dimers in the precipitates.

Oxidations of the three dimers (**I**, **II**, and **III**) were investigated separately in the same manner. The initial dimer concentrations were about 0.1 mM, while the molar ratio of hydrogen peroxide to dimer was controlled at 1. Depending on the dimer investigated, the peroxidase concentration in the reactor had a range from 0.08 to 19.2 nM and PEG doses ranged from 3 to 150 mg/L.

Identification of the products left in the aqueous solution was carried out after a 2-h reaction. After centrifugation for 40 min (2410*g*), the supernatant was extracted with ethyl acetate and concentrated by evaporation. The products in the extract were analyzed with thin-layer chromatography (TLC) and separated by column chromatography. The molecular masses of the compounds were determined by mass spectrometry (MS). Their structures were determined by GC/MS and nuclear magnetic resonance (NMR) spectroscopy.

UV/VIS Analysis. The concentration of HRP stock solution was determined by the absorbance at 404 nm with a 8451A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) based on an extinction coefficient of 102 000 M^{-1} cm^{-1} (*18*). Peroxidase activity in reaction solutions was assayed with a phenol/4-aminoantipyrine (AAP) chromogen system (*19*). The assay solution contained 10 mM phenol, 2.4 mM AAP, 0.2 mM hydrogen peroxide, and 50 mM phosphate buffer (pH 7.0) and peroxidase. With an extinction coefficient based on hydrogen peroxide of 6000 M^{-1} cm^{-1}, the rate of absorbance change monitored at 510 nm was converted to peroxidase activity: one unit (U) of activity was defined as the number of micromoles of peroxide utilized per minute at pH 7.0 and 25 °C. One nM HRP concentration based on heme absorbance was equivalent to 25 U/L as measured with the phenol/AAP method. This AAP–phenol chromogen system was also used to determine the hydrogen peroxide concentration in an end-point method with the concentrations of other reagents being high enough that the color formation was dependent only on the concentration of hydrogen peroxide (*6*). The peroxide concentration was determined from the absorbance of assay solution at 510 nm and the extinction coefficient of 6000 M^{-1} cm^{-1}. In both of the foregoing colorimetric assays, color in the sample aliquot was not a problem due to dilution and to the assay wavelength of 510 nM.

HPLC Analysis. A high-performance liquid chromatography (HPLC) system (Millipore, Milford, MA), equipped with a Radial-PAK Resolve C18 (10 μm) reverse phase cartridge, was used for the routine determination of phenol and the dimers. The mobile phase (1.5 mL/min) was a mixture of solvent A (water with 1% glacial acetic acid) and solvent B (methanol) and had a gradient change in the composition: 75% A (25% B) to 5% A (95% B) in 5 min and then 5% A (95% B) for 5 min. The UV detector was set at 272 nm, the maximum for phenol absorbance.

TLC and Column Chromatography. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (GHLF, 20 x 20 cm, layer thickness 0.25 mm, Analtech Inc., Newark, DE) with mobile phases of (a) *n*-hexane/acetone (70/30 v/v), (b) acetic acid/ethyl acetate/*n*-hexane (4/36/60 v/v), and (c) toluene/chloroform/acetone (40/25/35 v/v). The separated compounds were detected by exposure to UV at 254 nm after drying. By using the same solvents, the product compounds were separated by column chromatography on silica gel (230–400 mesh, Merck & Co., Rahway, NJ, 22 mm × 45 cm) into small fractions. The content of the fractions was checked by TLC, and pure fractions were kept for further analysis.

MS and NMR. The molecular mass of the separated products was determined with a Finnigan-4000 mass spectrometer (Finnigan MAT, San Jose, CA) by electron impact ionization (EI) at 70 or 20 eV. The structures of some products were confirmed with proton nuclear magnetic resonance (NMR) spectroscopy. The spectra were obtained on AC-200 or AC-300 instruments (Bruker Spectrospin Ltd., San Jose, CA) with acetone-d_6 as the solvent. Homonuclear spin–spin decoupling was performed in some cases to determine ortho and/or meta protons on the aromatic rings.

GC/MS. A gas chromatograpy/mass spectrometry (GC/MS) instrument (Hewlett-Packard, Palo Alto, CA) was used to determine the structures of the dimers based on retention times of standard compounds and mass spectra analysis by the instrument. An HP-1 fused silica capillary column (0.2 mm × 25 m) coated with 5% phenyl methyl silicon underwent a gradient temperature change; 50 °C for 1 min, 50–150 °C at 10 °C/min, 150–250 °C at 20 °C/min, and then 250 °C for 13 min. The carrier gas (helium) was set at 25 cm/s.

Results

Phenol polymerization was initiated by adding H_2O_2 into a stirred solution of phenol, PEG, and HRP. After peroxide was added, dark-brown precipitates were formed quickly. Two control solutions, one containing all reagents except H_2O_2 and another containing all reagents except HRP, demonstrated no phenol conversion in the 2-h reaction period.

Kinetics of Phenol Conversion and Peroxidase Inactivation. Figure 1 gives the typical time courses of phenol conversion, peroxide consumption, and peroxidase inactivation. Peroxidase-catalyzed phenol oxidation starts with the oxidation of enzyme (E) by peroxide, forming a

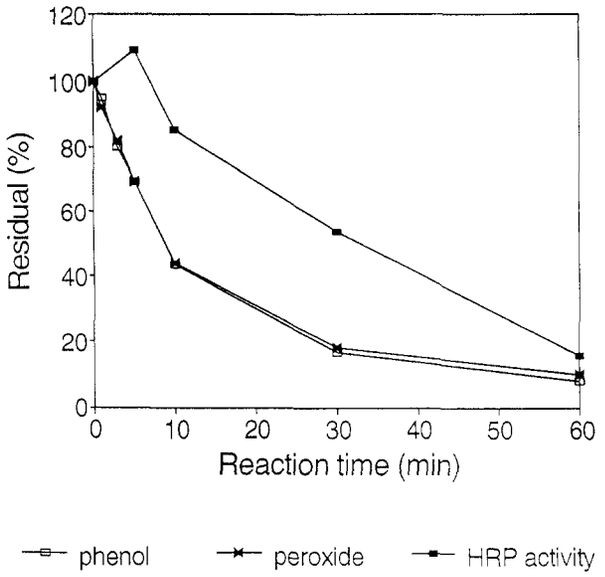


Figure 1. Time courses of phenol conversion ($[Ph]_0$ = 2 mM), H_2O_2 consumption ($[H_2O_2]_0$ = 2 mM), and inactivation of HRP ($[E]_0$ = 2.4 nM) in the presence of PEG (30 mg/L).

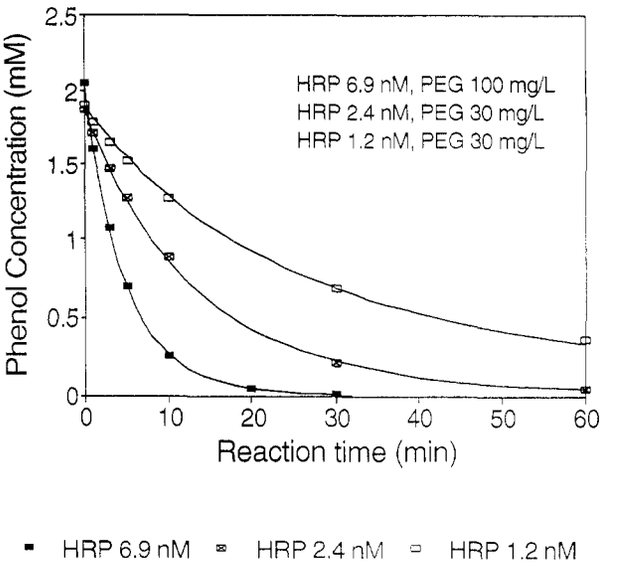


Figure 2. Phenol conversion at different HRP doses and $[PEG]/[E]_0$ ratios. The lines are the best fits for eqs 7 and 9 to the experimental points.

"compound I" (E_I). Compound I is reduced by phenol in a one-electron step to "compound II" (E_{II}), which cycles back to the resting state by accepting another electron from phenol. The two phenol molecules are released as phenoxy radicals (Ph•), which may combine to form a dimeric product (Ph:Ph). Equations 1–4 show the steps of reaction involved (*18*).

$$E + H_2O_2 \xrightarrow{k_1} E_I \quad (1)$$

$$E_I + Ph \xrightarrow{k_2} E_{II} + Ph^\bullet \quad (2)$$

$$E_{II} + Ph \xrightarrow{k_3} E + Ph^\bullet \quad (3)$$

$$Ph^\bullet + Ph^\bullet \xrightarrow{k_4} Ph{:}Ph \quad (4)$$

A two-substrate reaction model has been proposed for phenol conversion (*18*)

$$r_{Ph} = -\frac{d[Ph]}{dt} = \frac{2k_1[E_a][Ph]}{\frac{[Ph]}{[H_2O_2]} + \frac{k_1}{k_2} + \frac{k_1}{k_3}} \quad (5)$$

Where $[E_a]$ refers to the active enzyme concentration. The phenoxy radical concentration may be expressed as (*20*)

$$[Ph^\bullet] = \sqrt{\frac{r_{Ph}}{k_4}} \quad (6)$$

An initial equimolar ratio of H_2O_2 to phenol has been found to give the best phenol removal (*10*) and was used throughout these experiments. Figure 1 shows that at this particular initial ratio, hydrogen peroxide concentration dropped at the same rate as phenol concentration did. The instananeous molar concentration ratio of phenol to peroxide, $[Ph]/[H_2O_2]$, remained constant during the reaction. Therefore, the phenol removal rate, r_{Ph}, as indicated by eq 5 can be simplified into a first-order reaction with respect to phenol concentration as shown in eq 7, in which [Ph] and $[E_a]$ represent the concentrations of phenol and active enzyme, respectively. The rate constant k_{Ph} is an overall kinetic parameter consisting of k_1, k_2, k_3, and $[Ph]/[H_2O_2]$, which was a constant during the reaction.

$$r_{Ph} = -\frac{d[Ph]}{dt} = 2k_{Ph}[E_a][Ph] \quad (7)$$

Figure 1 also shows that the peroxidase activity declined with reaction time. One of the proposed mechanisms (*16, 21, 22*) attributes the inactivation to the attack of phenoxy radicals to the enzyme active center (*21*). On the basis of this assumption, the peroxidase inactivation rate, $-d[E_a]/dt$, may be expressed as a second-order reaction model and may be correlated with the phenol conversion rate after the radical concentration being represented by eq 6. Where k_d ($mM^{-1/2}\,min^{-1/2}$) is an overall inactivation rate constant including a second-order-specific deactivation rate, k_d' and k_4 (see eq 4):

$$-\frac{d[E_a]}{dt} = k_d'[E_a][Ph^\bullet] = \frac{k_d'}{(k_4)^{1/2}}[E_a](r_{Ph})^{1/2} = k_d[E_a](r_{Ph})^{1/2} \quad (8)$$

Combining eqs 7 and 8 gives the peroxidase inactivation rate

$$-\frac{d[E_a]}{dt} = k_d\sqrt{2k_{Ph}[E_a]^3[Ph]} \quad (9)$$

The two kinetic parameters in eqs 7 and 9, (k_{Ph} and k_d) were estimated by using a least-squares fitting method which included a fourth-order Runge–Kutta numerical integration program and a nonlinear parameter search program. Figure 2 displays the time courses of three experiments under different enzyme and PEG dosages. The lines in Figure 2 are the best fits for eqs 7 and 9 to the experimental data with the estimated kinetic parameters (k_{Ph} = 0.0105 $nM^{-1}\,min^{-1}$). The enzyme inactivation coefficient, k_d, was found to have a logarithmic relationship with the initial molar ratio of poly(ethylene glycol) to

Table 1. Identification of Soluble Products in Aqueous Solution from Phenol Polymerization

compound	name	TLC R_f[a] D1	D2	D3	GC rt[b] (min)	MM[c]
A1	unknown	0.70	0.79	0.78		
A2 (V)	*o*-phenoxyphenol	0.46	0.65	0.70	14.18	186.1
A3 (III)	*p*-phenoxyphenol	0.38	0.54	0.65	15.78	186.1
A4	unknown	0.24	0.48	0.61		
A5 (II)	*o,o'*-biphenol	0.27	0.48	0.57	15.26	186.1
A6 (VI)	trimer[d]	0.21	0.44	0.60	17.42	277.9
A7 (IV)	*o,p'*-biphenol	0.18	0.44	0.53	16.69	186.1
A8 (I)	*p,p'*-biphenol	0.14	0.41	0.52	17.78	186.1
A9	trace	0.18	0.41	0.58	23.18	
A10	trace	0.07	0.35	0.45		

[a] TLC retardation factors (R_f) were obtained from the developers: D1 (*n*-hexane/acetone, 70/30), D2 (acetic acid/ethyl acetate/*n*-hexane, 4/36/60), D3 (toluene/chloroform/acetone, 40/25/35). [b] GC retention time (rt) and mass spectra of five stable dimeric products were compared with standard compounds and matched with the instrument's database. [c] Molecular mass (MM) of six compounds was obtained in direct MS spectrometry. [d] The trimer's molecular structure, 4-(phenoxyphenoxy)phenol (**VI**), was assigned from its NMR spectrum: 6.8 (d, 2H, J = 1.94); 6.82–6.87 (m, 3H); 6.93 (d, 4H, J = 8.72); 7.49 (d, 4H, J = 8.70); 8.88 (s, 1H).

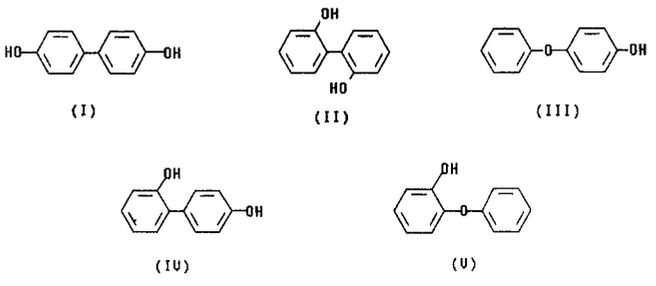
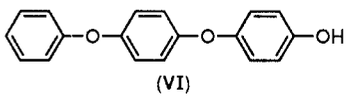


Figure 3. Five stable dimeric products in phenol coupling: *p,p'*-biphenol (**I**), *o,o'*-biphenol (**II**), *p*-phenoxyphenol (**III**), *o,p'*-biphenol (**IV**), and *o*-phenoxyphenol (**V**).

peroxidase, $[PEG]/[E]_0$, in the range from 2500 to 13000 (eq 10). This means the protection of the enzyme by PEG (*16*) asymptotically approached a maximum with increase in PEG/enzyme ratio.

$$k_d = 3.255([PEG]/[E]_0)^{-0.429} \quad (10)$$

Soluble Products in Aqueous Phase. Ten soluble products were detected and separated by using TLC and column chromatography as listed in Table 1. Two of them (A9 and A10) were of trace amounts. High unpaired electron densities have been detected at oxygen, ortho and para atoms of a monophenoxy radical (*23*, *24*), and hence, the coupling of two radicals may produce five stable products as shown in Figure 3 (*25*). All five stable dimers were separated and identified by GC/MS. Compound A6 (mp 206–206.5 °C) formed yellow brown needles and had the molecular mass of a trimer (277.9) determined by MS. Its structure is assigned as 4-(4-phenoxyphenoxy)phenol (**VI**) based on its ^1H NMR spectrum (Table 1).



(VI)

Dimer Formation Rate and Distribution. If the monophenoxy radicals coupled with each other to generate dimers, the total formation rate of all dimers, r_{dim}, is half of the phenol conversion rate:

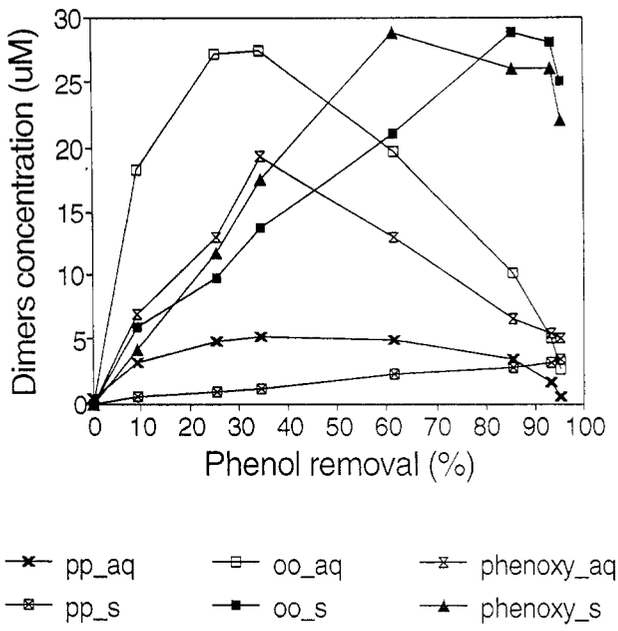


Figure 4. Partitioning of *p,p'*-biphenol (pp), *o,o'*-biphenol (oo), and *p*-phenoxyphenol (phenoxy) between aqueous (aq) and precipitate (s) phases with phenol removal; $[Ph]_0$ = 2 mM, $[H_2O_2]_0$ = 2 mM, $[E]_0$ = 2.4 nM, PEG = 30 mg/L.

$$r_{dim} = \frac{r_{Ph}}{2} = k_{Ph}[E_a][Ph] \quad (11)$$

Some of the dimers were precipitated out of water with other insoluble products during the reaction. The contents of the three dimers, *o,o'*-biphenol, *p,p'*-biphenol, and *p*-phenoxyphenol, were measured and expressed as "concentrations" as if the dimers had been dissolved in the original solution aliquot. Figure 4 gives the partitioning of the three dimers between aqueous and solid phases with phenol conversion. In the aqueous phase, the three dimers reached their concentration peaks at 35% phenol conversion.

After the phenol conversion exceeded 95%, the soluble *o,o'*-biphenol, *p*-phenoxyphenol, and *p,p'*-biphenol dropped to 3, 5, and 0.6 μM, respectively. In contrast, the contents of the three dimers in the precipitates increased continuously.

The dimers in the final precipitates after 2-h reaction accounted for 6.7% of the total dry mass of the precipitates. About 40% of the precipitate mass was insoluble in methanol, but could be dissolved in tetrahydrofuran or dioxane. Analysis continues on the precipitates.

Further Oxidation of Dimers. The further oxidation of the three dimers (**I**, **II**, and **III**) that have the typical chemical bonds (para–para, ortho–ortho, and oxygen–para) was investigated separately. Other two dimers (**IV** and **V**) were not studied because of the lack of chemical supply. After the oxidation was initiated by adding H_2O_2 to a solution of dimer, PEG, and HRP, the reaction products precipitated out of water; brownish precipitates from *p,p*- and *o,o'*-biphenol whereas white solids were formed from *p*-phenoxyphenol. The time courses of *p,p'*-biphenol conversion at various peroxidase doses are shown in Figure 5, and the other two dimers had similar behavior.

Oxidation of dimers was obviously catalyzed by HRP since a higher enzyme dose gave a faster reaction as depicted by Figure 5. Enzyme activity was measured during the reactions and found to be very close to its initial

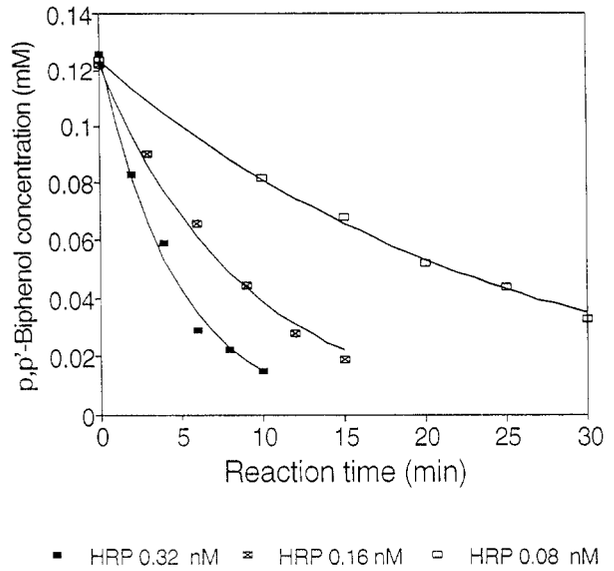


Figure 5. Oxidation of *p,p'*-biphenol catalyzed by HRP; $[H_2O_2]_0$ = 0.12 mM, PEG = 3 mg/L. The lines are the best fit of eq 12 to the experimental data under three HRP initial concentrations.

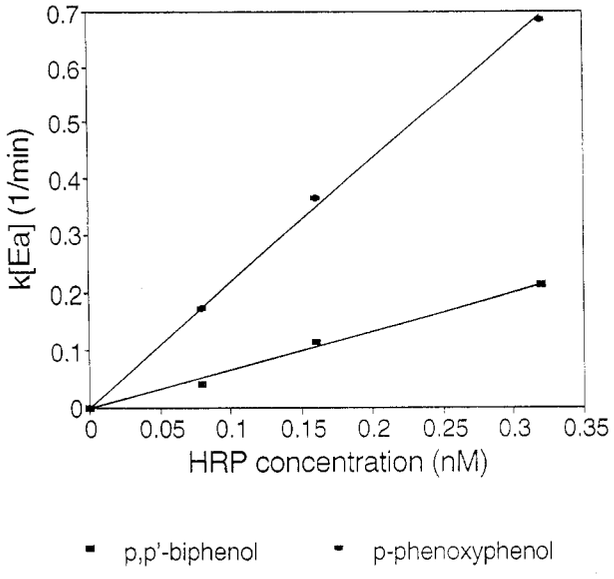


Figure 6. Estimation of specific reaction rates of *p,p'*-biphenol and *p*-phenoxyphenol. The rate constants are calculated from the slopes of the lines.

activity ($[E_a] = [E]_0$). As with phenol conversion, the oxidation of the three dimers followed the first-order reaction model with respect to their aqueous concentrations as described by eqs 12–14, in which [pp], [oo], and

$$r_{pp} = -\frac{d[pp]}{dt} = k_{pp}[E_a][pp] \quad (12)$$

$$r_{oo} = -\frac{d[oo]}{dt} = k_{oo}[E_a][oo] \quad (13)$$

$$r_{phenoxy} = -\frac{d[phenoxy]}{dt} = k_{phenoxy}[E_a][phenoxy] \quad (14)$$

[phenoxy] are the concentrations of *p,p'*-biphenol, *o,o'*-biphenol, and *p*-phenoxyphenol, respectively, and the subscripts, pp, oo, and phenoxy, represent the corresponding dimers. The lines in Figure 5 are the best fits of eq 12 to *p,p'*-biphenol concentrations. Based on the enzyme doses used, the specific reaction rates of the three dimers (k_{pp}, k_{oo}, and $k_{phenoxy}$) were estimated from the slopes of straight lines as shown in Figure 6 for *p,p'*-biphenol and *p*-phenoxyphenol and are listed in Table 2 along with the constant for phenol.

Table 2. Specific Reaction Rates of Phenol and Dimers

	p-phenoxyphenol	*p,p'*-biphenol	phenol	*o,o'*-biphenol
k (nM^{-1} min^{-1})[a]	2.172	0.671	0.0105	0.00453

[a] The constants are based on initial HRP concentration (nM), which was determined from the heme absorbance at 404 nM (*18*).

Discussion

Since few dimers were left in the aqueous solution at the end of reaction, and only about 7% of dry mass in the final precipitates was attributed to the three dimers (**I**, **II**, and **III**), most of the phenol molecules were polymerized to higher oligomers which had greater hydrophobicity as indicated by their longer retention time in the reverse-phase HPLC column. Comparing the specific reaction rates of the dimers and phenol (Table 2) indicates that some dimers like *p*-phenoxyphenol are better substrates of peroxidase than phenol. These dimers were converted very fast even at very low concentrations. For example, at 9% of phenol conversion, a comparison of conversion rates of *p*-phenoxyphenol and phenol shows that both of them were converted at comparable rates even though phenol concentration was 270 times higher than *p*-phenoxyphenol concentration (eq 15).

$$\frac{r_{phenoxy}}{r_{Ph}} = \frac{k_{phenoxy}[phenoxy]}{2k_{Ph}[Ph]} = \frac{2.172 \times 0.007}{2 \times 0.0105 \times 1.88} = 0.385 \quad (15)$$

Theoretically, an 1:2 molar ratio of peroxide to phenol can oxidize all the phenol molecules to monophenoxy radicals as the reaction stoichiometry (steps 1–4) indicates. A 1:1 molar ratio, however, was required in the experiments for greater than 95% phenol conversion. A theoretical molar ratio could only oxidize about 50% of phenol. A possible explanation is that some dimers like *p*-phenoxyphenol and *p,p'*-biphenol are better substrates of peroxidase and compete with phenol for peroxide.

Figure 4 shows that *o,o'*-biphenol had relatively high accumulation in both the aqueous and solid phases. Whether or not it was a prevailing dimerization product from the phenoxy radical coupling is analyzed as follows. At the early stage of the reaction with high phenol concentration and low product concentration, monophenoxy radicals mainly couple with each other to generate dimers and couple with other oligomeric radicals in negligible amount. The total dimer formation rate, r_{dim}, could be estimated with eq 11. An approximate state to the initial reaction condition was when the phenol conversion was about 9% (residual phenol concentrtion 1.88 mM) and the total dimer concentration in solution was less than 0.028 mM as shown in Figure 4. Comparison of the further conversion rate of each dimer (r_{pp}, r_{oo}, and $r_{phenoxy}$) with the total dimer formation rate (r_{dim}) at this instantaneous reaction time had the following values:

$$\frac{r_{pp}}{r_{dim}} = \frac{k_{pp}E_a[pp]}{k_{Ph}E_a[Ph]} = 0.1 \quad (16)$$

$$\frac{r_{oo}}{r_{dim}} = \frac{k_{oo}E_a[oo]}{k_{Ph}E_a[Ph]} = 0.004 \quad (17)$$

$$\frac{r_{phenoxy}}{r_{dim}} = \frac{k_{phenoxy}E_a[phenoxy]}{k_{Ph}E_a[Ph]} = 0.77 \quad (18)$$

The conversion rate of *o,o'*-biphenol was only about 0.4% of the formation rate of all the dimers. This kinetic analysis suggests that the coupling of monophenoxy radicals hardly occurs at ortho–ortho atoms. Otherwise, a significant amount of *o,o'*-biphenol would accumulate in the precipitates. Therefore, *o,o'*-biphenol was a less possible dimerization product, and its accumulation during the reaction was due to its slow conversion to higher oligomers. However, the conversion rate of *p*-phenoxyphenol was about 77% of the total dimer formation rate, which means that most monophenoxy radicals went to oxygen–para combination since this dimer had almost the same accumulation in the precipitates as *o,o'*-biphenol had. Furthermore, at this instantaneous time, the three dimers (**I**, **II**, and **III**) that were investigated quantitatively in this study had a collective formation more than 88% of the total dimers formation. Therefore, other two dimers, *o,p'*-biphenol (**IV**) and *o*-phenoxyphenol (**V**), where not the major dimeric products; their collective formation should be less than 12% of the total dimer formation. The presence of an aromatic ring at the ortho position might actually inhibit the approach of *o,o'*-biphenol to peroxidase's active center and, hence, the formation of dimeric phenoxy radicals. If the products of higher molecular mass are formed through the coupling of various radicals (*25*), the predominant polymerization bonds in the final reaction precipitates may be the oxygen–para connection followed by the para–para connection. Many trimeric products may be formed during the reaction (*9*). The fact that only one trimer, 4-(4-phenoxyphenoxy)phenol (**VI**) with two oxygen–para connections, was detectable in the aqueous solution indicates that it was the major trimeric product in the precipitates.

In phenol conversion, the dimers could be oxidized directly by HRP/H_2O_2 to form dimeric radicals which could then couple with each other to generate tetramers or couple with monophenoxy radicals to form trimers (*25*). The dimers might also become dimeric radicals through radical transfer with monophenoxy radicals and generate trimers and tetramers through the radical coupling (*26*). The comparison of direct dimer oxidation by H_2O_2/enzyme with the total dimer formation discussed above shows that the direct oxidation could be the major dimer conversion mechanism (>88%).

Conclusions

Phenol conversion rate can be expressed as a first-order reaction model with respect to phenol concentration at equal molar concentrations of H_2O_2 and phenol. The presence of poly(ethylene glycol) can reduce the peroxidase inactivation rate, but this 'protection' function asymptotically approached a limit with increasing the dosage ratio of PEG to enzyme. Five stable dimeric products and one trimer were identified in the aqueous solution at high phenol conversion. The time courses of three dimers, *p,p'*-biphenol, *o,o'*-biphenol, and *p*-phenoxyphenol, demonstrated that they were substrates of peroxidase. After more than 95% of phenol was removed, the aqueous concentration of each of the three dimers dropped below 1 mg/L. In the final reaction precipitates, the three dimeric products accounted for about 7% of dry mass. Some dimers like *p*-phenoxyphenol and *p,p'*-biphenol were better substrates of peroxidase. These dimeric intermediates competed with phenol for hydrogen peroxide and consumed extra hydrogen peroxide over the theoretical requirement for oxidizing phenol only. The ortho–ortho coupling of two phenoxy radicals was not a favorable coupling route. The predominant products of higher molecular mass and hydrophobicity in the reaction precipitates are expected to have oxygen–para connections and/or para–para ones. At least 88% of dimers generated from phenol coupling were directly oxidized by H_2O_2/peroxidase. The generation of dimeric radicals through radical transfer may be a minor mechanism of dimer conversion.

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