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Enzymatic Oxidative Polymerization of *p*-*t*-Butylphenol and Characterization of the Product Polymer

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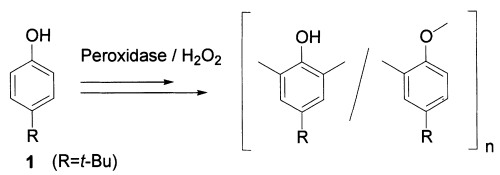
For structural control and analysis of an enzymatically synthesized polyphenol, peroxidase-catalyzed polymerization of *p*-*t*-butylphenol in a mixture of polar organic solvent and phosphate buffer has been examined in detail. The resulting products were subjected to preparative HPLC. Two dimers and one trimer were isolated and their structures were determined by ^{1}H and ^{13}C NMR. ESI-TOF mass analysis showed the formation of two kinds of dimer and of at least four kinds of trimer. These data clearly showed that the polymer was composed of a mixture of phenylene and oxyphenylene units. The ratio of phenylene and oxyphenylene units in the product could be controlled by changing the solvent composition. The phenylene unit linearly increased as a function of the water content in the mixed solvent.

Enzymatic synthesis of polyphenols is considered as a promising alternative to preparation of conventional phenolic resins (novolak and resol resins), owing to no use of toxic formaldehyde, mild reaction conditions (in neutral solvents at room temperature) and facile procedures.[1] Peroxidases were reported to catalyze an oxidative polymerization of various phenol derivatives to produce a new class of useful and functional polyphenols efficiently.[2] Chemoselective polymerization of phenols bearing an unsaturated group took place via the peroxidase catalysis, yielding reactive polyphenols.[3]

Structures of the polyphenols are often very complicated; in the case of *p*-substituted phenols, we described the polymer as mainly consisting of phenylene and oxyphenylene units;[4] however, other groups reported exclusive formation of the polymer having an *ortho–ortho* linkage.[5] Very recently, we briefly reported the control of the coupling selectivity (ratio of phenylene and oxyphenylene units) in the peroxidase-catalyzed polymerization of *p*-substituted phenols by selection of the monomer substituent and nature of solvent.[6] The hydrophobic parameters of the monomer substituent and organic solvent strongly affected the polymer structure.

In an oxidative coupling of *p*-substituted phenols, three reaction positions are conceivable, whereas unsubstituted and *m*-substituted phenols have four sites. Formation of Pummerer's ketone is involved as a side-reaction in the oxidative coupling of *p*-substituted phenols with small substituents.[7] In this study, therefore, we used *p*-*t*-butylphenol (**1**) as a model monomer for structural control and analysis of an enzymatically synthesized polyphenol (Scheme 1). In order to obtain more data on the structure of the polyphenols, some oligomers were isolated and their structures were determined by NMR. The solvent composition was systematically investigated for precise control of the polymer structure.



Scheme 1.

Results and Discussion

Isolation and Identification of Oligophenols. In this study, horseradish and soybean peroxidases (HRP and SBP, respectively) were used as catalysts. The HRP-catalyzed oxidative polymerization of **1** was first performed using hydrogen peroxide as oxidizing agent in an equivolume mixture of methanol and phosphate buffer (pH 7) at room temperature under air. Hydrogen peroxide (5%) was added dropwise to the reaction mixture for 2 h. Powdery polymeric precipitates were quickly formed by the addition of hydrogen peroxide. Then, after 1 h, the polymer was isolated by filtration (yield 83%). HPLC analysis of the reaction mixture showed that 90% of **1** was consumed. The molecular weight of the polymer was measured by size exclusion chromatography (SEC). The num-

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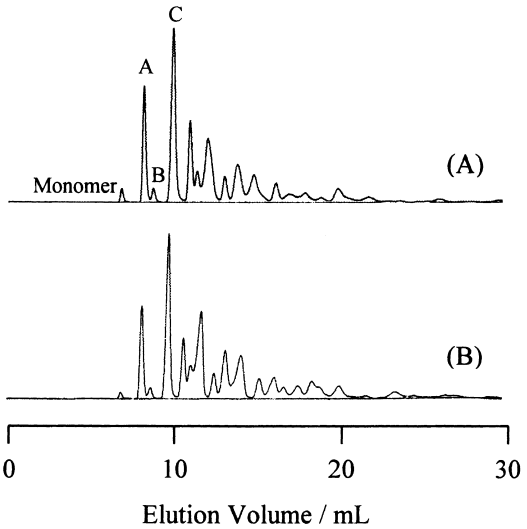

Fig. 1. HPLC traces of the product obtained from **1** in an equivolume mixture of methanol and phosphate buffer using (A) HRP and (B) SBP catalyst.

ber-average molecular weight (M_n) was 570 and its index was 1.9.

HPLC analysis of the product was performed using an inverse-phase silica-gel column with methanol/water (98:2 vol%) eluent. On the HPLC chart of the reaction mixture, many peaks were observed (Fig. 1(A)). The main fractions A–C were separated by using preparative HPLC column and their structures were analyzed by NMR spectroscopy. Two dimers **2**, **3** and one trimer **4** were identified by ^{1}H and ^{13}C NMR spectroscopies (Fig. 2). In the SBP-catalyzed polymerization of **1** under similar reaction conditions, the peak pattern of the product was very similar to that obtained for HRP (Fig. 1(B)). These data suggest little structural difference between the polymers obtained by using HRP and SBP catalysts.

ESI-TOF Mass Analysis of Poly(1). In order to confirm the polymer structure consisting of a mixture of phenylene and oxyphenylene units, a liquid chromatography/electrospray ionization-time of flight mass (LC/ESI-TOF MS) measurement was carried out. There were many peaks observed in a total ion chromatogram of poly(**1**) obtained by HRP catalyst in an equivolume mixture of methanol and the buffer (Fig. 3(A)), although the peak separation was not good. The peaks' pattern was somewhat different from that by UV detector (Fig. 1), probably owing to the difference of the detection methods. In the detection of the mass of a dimer plus a sodium ion (m/z = 321.1), two sharp peaks were detected (Fig. 3(B)), supporting the formation of two dimers **2** and **3**. If the oxidative coupling of **1** proceeds via the formation of C–C and C–O bonds, five trimers are conceivable (Chart 1). In case of the molecular mass of a trimer (m/z = 469.2), four or five peaks were detected (Fig. 3(C)), which may be due to these trimers. As for a tetramer (m/z = 617. 3) and a pentamer (m/z = 765.4), many peaks were observed in their spectra (Figs. 3(D) and 3(E), respectively). These data support the conclusion that the polymer structure was of a mixture of phenylene and oxyphenylene units.

Solvent Engineering for Control of Polymer Structure.

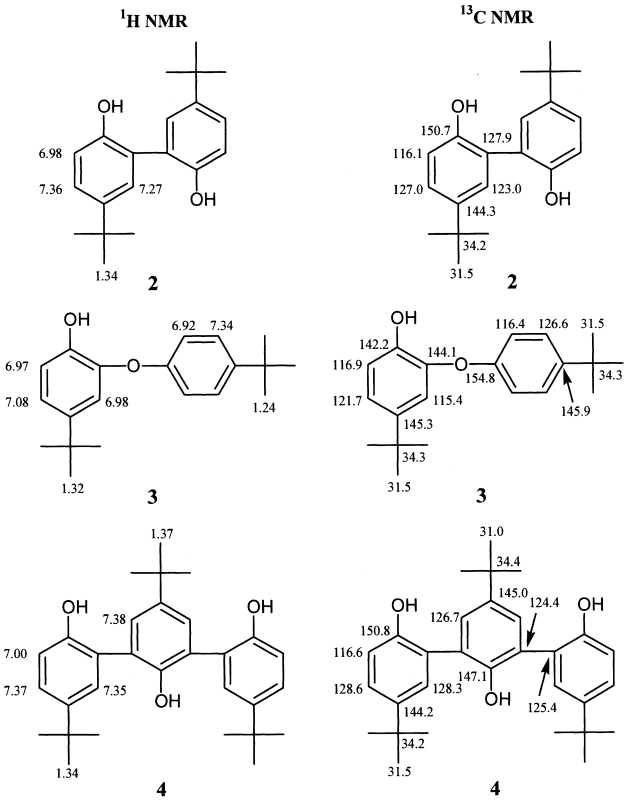

Fig. 2. Structure of isolated dimers (**2** and **3**) and trimer (**4**) and their assignment of ^{1}H and ^{13}C NMR peaks.

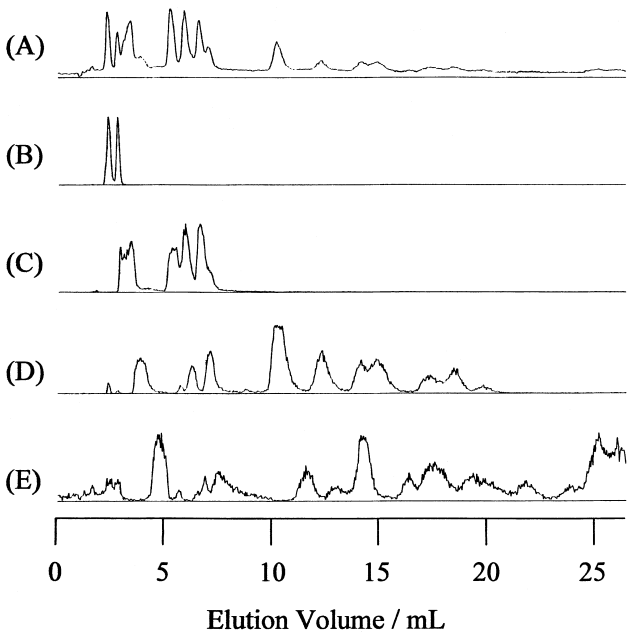

Fig. 3. LC/ESI-TOF mass chromatograms of poly(**1**) with detector of (A) total ion, (B) m/z 321.1, (C) m/z 469.2, (D) m/z 617.3, and (E) m/z 765.4.

In the HPR-catalyzed polymerization of phenol in a mixture of methanol and buffer, the polymer structure could be controlled by changing the methanol content of the mixed solvent.[2b,2c] In the HRP-catalyzed oxidative polymerization of *p*-substituted

Table 1. Peroxidase-Catalyzed Polymerization of **1** in a Mixture of 2-Propanol and Phosphate Buffer[a)]

Entry	Catalyst	Content of 2-propanol/%	Conv.[b)]/%	Polymer			
				Yield/%	Mn[c)]	Mw/Mn[c)]	Ph/Ox[d)]
1	HRP	30	97	95	800	1.7	71/29
2	HRP	40	94	92	1000	1.7	60/40
3	HRP	50	92	87	1400	1.5	56/44
4	HRP	60	63	51	1400	1.5	50/50
5	HRP	70	33	9	1500	1.6	45/55
6	SBP	30	100	91	920	1.6	68/32
7	SBP	40	98	89	1200	1.4	60/40
8	SBP	50	98	91	1300	1.5	57/43
9	SBP	60	93	86	1400	1.5	53/47
10	SBP	70	68	44	1500	1.6	45/55

a) Polymerization of **1** (0.75 g, 5.0 mmol) using HRP (2.0 mg, 440 units) or SBP (8.0 mg, 420 units) catalyst in a mixture of 2-propanol and pH 7 phosphate buffer (25 mL) at room temperature for 3 h under air. b) Determined by HPLC. c) Determined by SEC using DMF as eluent with polystyrene standards. d) Determined by titration.

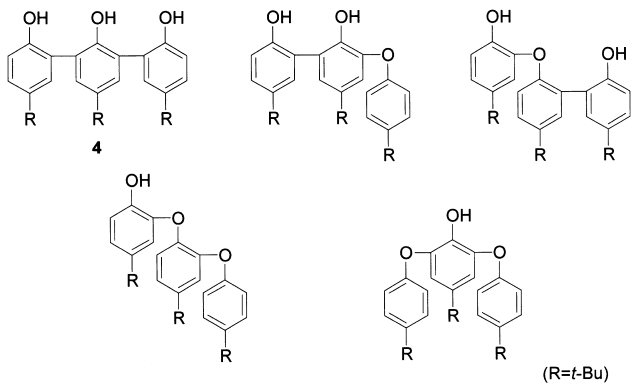

Chart 1.

phenols, the coupling selectivity could be controlled by changing the hydrophobic parameters of the solvent and monomer substituent, yielding soluble polyphenols with a different ratio of phenylene/oxyphenylene units.[6] Here, such solvent effects on the structural control in the peroxidase-catalyzed oxidative polymerization of **1** in an aqueous organic solvent were investigated in detail.

The ratio of phenylene and oxyphenylene units (Ph/Ox) was determined by conventional titration methods;[8] the polymer was reacted with an excess of acetic anhydride in pyridine and the acetylated amount was determined by the titration. Figure 4 shows FT-IR spectra of the polymer before and after the acetylation at 95–100 °C for 1 h. A characteristic broad peak due to phenolic O–H bond was observed at 3400 cm^{-1} in poly(**1**) (Fig. 4(A)). After the acetylation, this peak completely disappeared (Fig. 4(B)), indicating that the acetylation proceeded quantitatively under the present reaction conditions; thus, the residual phenolic group in poly(**1**) can be correctly determined by the titration. By ^{1}H NMR analysis of the acetylated poly(**1**), the quantitative acetylation of the phenolic group was confirmed (data not shown).

In a mixed solvent of 2-propanol and phosphate buffer (pH 7), the effects of the mixed ratio have been systematically examined (Table 1). When the 2-propanol content was less than 20%, the monomer was partly insoluble in the medium. In the

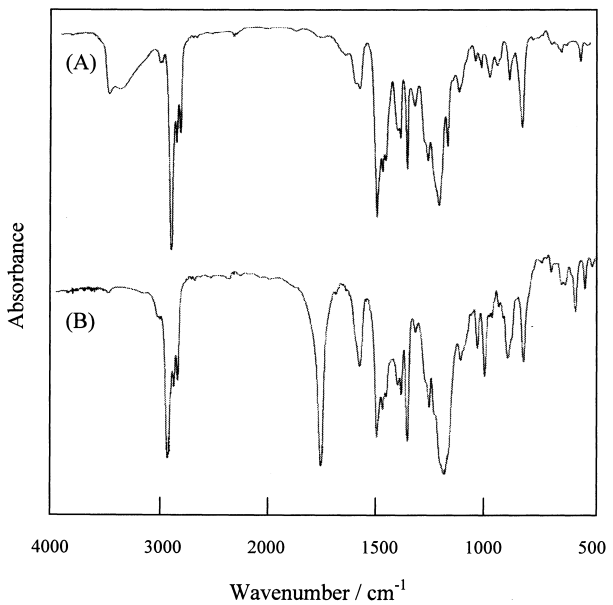

Fig. 4. FT-IR spectra of poly(**1**) (entry 3 in Table 1) (A) before acetylation and (B) after acetylation.

HRP-catalyzed polymerization of **1**, the polymer was formed in high yields when the 2-propanol content was in the range from 30 to 50% and the molecular weight increased with increasing the 2-propanol content (entries 1–3). When the 2-propanol content was higher than 60%, the monomer conversion and polymer yield became smaller (entries 4 and 5). This is probably due to the HRP denaturation in the solvent with the high content of 2-propanol.[9] In using SBP as catalyst (entries 6–10), a similar reaction pattern was observed except for the higher conversion and yield in the 2-propanol content of 60 or 70% (entries 9 and 10). This may be because the activity loss of SBP in such a solvent is smaller than that of HRP.

The unit ratio of the polymer was determined by the titration. For both enzymes, the phenylene content linearly decreased as a function of the 2-propanol content (Fig. 5); correlation coefficients for HRP and SBP catalysts were 0.984 and 0.986, respectively. These data suggest that the precise control

Table 2. Peroxidase-Catalyzed Polymerization of **1** in an Equivolume Mixture of Organic Solvent and Phosphate Buffer[a)]

Entry	Catalyst	Organic solvent	log *P*	Conv.[b)]/%	Polymer			
					Yield/%	Mn[c)]	Mw/Mn[c)]	Ph/Ox[d)]
1[e)]	HRP	Ethylene Glycol	−1.36	92	38	390	2.0	85/15
2	HRP	*N*,*N*-Dimethylformamide	−1.01	95	70	760	1.8	68/32
3[e)]	HRP	Methanol	−0.77	90	83	570	1.9	72/28
4[e)]	HRP	1,4-Dioxane	−0.42	95	98	930	1.8	64/36
5	HRP	Acetone	−0.24	93	67	1500	1.6	56/44
6[e)]	HRP	2-Propanol	0.05	92	87	1400	1.5	56/44
7[e)]	HRP	1-Propanol	0.25	78	64	1600	1.5	47/53
8	HRP	*t*-Butyl Alcohol	0.35	96	93	1600	1.5	51/49
9	SBP	Methanol	−0.77	98	96	670	1.6	73/27
10	SBP	1,4-Dioxane	−0.42	100	96	1100	1.8	64/36
11	SBP	Acetone	−0.24	94	88	1500	1.7	56/44
12	SBP	2-Propanol	0.05	98	91	1300	1.5	57/43

a) Polymerization of **1** (0.75 g, 5.0 mmol) using HRP (2.0 mg, 440 units) or SBP (8.0 mg, 420 units) catalyst in an equivolume mixture of organic solvent and pH 7 phosphate buffer (25 mL) at room temperature for 3 h under air. b) Determined by HPLC. c) Determined by SEC using DMF as eluent with polystyrene standards. d) Determined by titration. e) Data from Ref. 6.

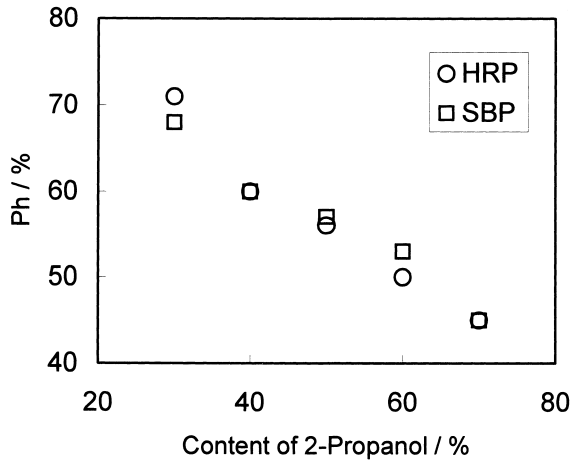

Fig. 5. Relationships between 2-propanol content and polymer structure in the peroxidase-catalyzed polymerization of **1** in a mixture of 2-propanol and phosphate buffer.

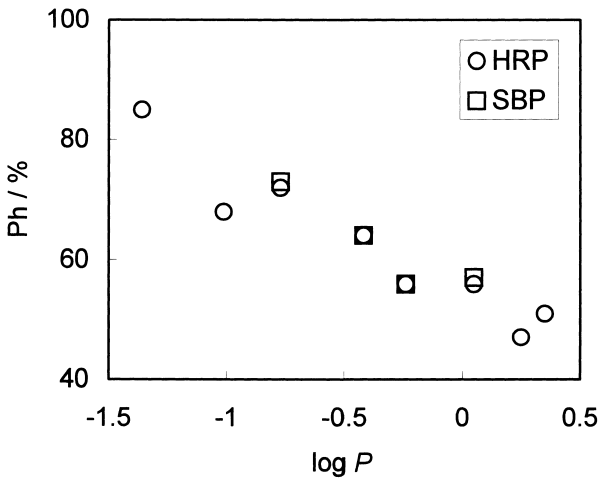

Fig. 6. Relationships between log *P* of organic solvents and polymer structure in the peroxidase-catalyzed polymerization of **1** in an equivolume mixture of organic solvent and phosphate buffer.

of the polyphenol structure can be achieved by changing the mixing ratio. A similar tendency was observed in the HRP-catalyzed polymerization of unsubstituted phenol in aqueous methanol.[2b,2c]

Table 2 shows polymerization results in equivolume mixtures of various polar organic solvents and the phosphate buffer. Here, log *P* was used as a measure of hydrophilicity of the solvent.[6,10] In most cases, the polymer was obtained in good yields. When the log *P* value of the cosolvent was less than −0.4, the molecular weight of the polymer was relatively low (< 1000). The peroxidase origin affected the polymerization behaviors only slightly; the polymerization results obtained by using HRP catalyst were very similar to those by SBP.

Figure 6 shows relationships between log *P* of the organic cosolvent and the phenylene unit of poly(**1**). A relatively good first-order correlation was observed and the phenylene unit decreased as log *P* increased. Data of Figs. 5 and 6 clearly showed that the phenylene unit decreased as the solvent hydrophobicity increased. The structure of the polymer obtained by using HRP catalyst was almost the same as that obtained by SBP.

Conclusion

For structural analysis of enzymatically synthesized poly(**1**), two dimers and one trimer were isolated from the resulting polymer by using preparative HPLC and their structure was determined by NMR analysis. LC/ESI-TOF MS analyses suggest the formation of at least four kinds of trimers. These data clearly indicate that the enzymatically synthesized polyphenol consisted of phenylene and oxyphenylene units. The solvent composition affected the control of the polyphenol structure. In the polymerization of **1** in a mixture of 2-propanol and phosphate buffer, the phenylene unit content linearly decreased as a function of 2-propanol content. A relatively good first-order correlation between log *P* of organic solvent and phenylene

unit was observed in a mixed solvent of polar organic solvent and phosphate buffer.

Experimental

Materials. HRP and SBP were purchased from Wako Pure Chemical Industries, Ltd. and Sigma Chemical Co., respectively, and were used without further purification. Other reagents and solvents were commercially available and were used as received.

Enzymatic Polymerization. A typical run was as follows (entry 3 in Table 1). *p-t*-Butylphenol (0.75 g, 5.0 mmol) and HRP (2.0 mg, 440 unit) were dissolved in an equivolume mixture of 2-propanol and 0.1 M phosphate buffer (pH 7) (25 mL). To this solution, 3.2 mL of 5% hydrogen peroxide (5.3 mmol) was added dropwise for 2 h. The mixture was stirred at room temperature under air. After 1 h, the precipitated materials were collected by centrifugation and washed with a mixture of methanol and water (1:1 vol) repeatedly, followed by drying in vacuo to give 0.66 g of the polymer (yield 87%). ^{1}H NMR (DMSO-d_6) δ 0.8–1.5 (m, CH_3), 6.5–7.5 (m, Ar). IR (KBr) 3400 (ν O-H), 2963, 2906, 2869 (ν C-H), 1586, 1508 (ν C=C of Ar), 1217 (ν C(Ar)–O–C(Ar) and C(Ar)–OH), 1120 cm^{-1} (ν C(Ar)–O–C(Ar)).

Titration. Poly(**1**) (0.1 g) was dissolved in pyridine containing 2.5% acetic anhydride (5 mL). The solution was kept at 95–100 °C for 1 h under gentle stirring. After cooling to room temperature, water (0.5 mL) was added to the reaction mixture, then the mixture was again heated at 95–100 °C for 10 min. The solution was titrated with 0.2 M (1 M = 1 mol dm^{-3}) potassium hydroxide in ethanol in the presence of phenolphthalein as indicator.

Measurements. For SEC and HPLC measurements, a Tosoh SC8020 apparatus was used. SEC analysis was carried out by using a refractive index (RI) detector at 60 °C under the following conditions: two TSKgel α-M columns and DMF containing 0.09 M LiCl eluent at a flow rate of 1.0 mL min^{-1}. The calibration curves were obtained using polystyrene standards. HPLC analysis was performed using a UV monitor (278 nm) at 40 °C under the following conditions: two YMC-Pack ODS AM-312 columns and methanol/17 mM phosphoric acid eluent at a flow rate of 1.8 mL min^{-1} or a TSKgel ODS-80Ts column and methanol/water (98:2 vol%) eluent at a flow rate of 0.5 mL min^{-1}. NMR spectra was recorded on JEOL JNM-LA 600 and Bruker DPX400 spectrometers. LC/ESI-TOF MS analyses were carried out using a PE Biosystems Mariner ESI/TOF equipped with a Hewlett Packard HP 1100 under the following LC conditions: an HP-ODS Hypersil column and methanol/water (90:10 vol%) eluent at a flow rate of 1 mL/min. FT-IR measurements were carried out with a Perkin-Elmer Paragon 1000 spectrometer.

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