

Precise Structure Control of Enzymatically Synthesized Polyphenols

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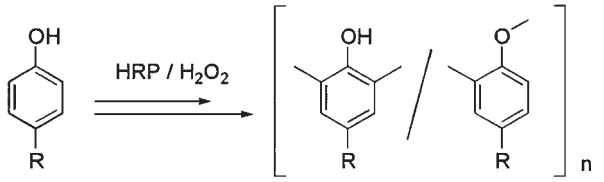
The structural control and biodegradability of enzymatically synthesized polyphenols have been investigated. The peroxidase-catalyzed oxidative polymerization of various 4-substituted phenols was performed in aqueous organic solvents, in which the ratio of phenylene and oxyphenylene units of the polymer product could be precisely controlled by the nature of the solvents and the monomers. The unit ratio strongly depended on their hydrophobic parameters. The control of the structure was also examined by changing the feed ratio of the enzymatic copolymerization of the phenol derivatives. The biodegradability was evaluated by the BOD method. Poly(4-*t*-butylphenol) showed relatively good biodegradability, whereas little degradation of the polyphenol from unsubstituted phenol was observed.

During the last decade, an enzyme-catalyzed polymerization ("enzymatic polymerization") has been developed as a new methodology of polymer synthesis.[1] By utilizing characteristic enzyme catalysis, the synthesis of useful and high-performance polymeric materials has successfully been achieved. Many of these polymers are often difficult to synthesize by conventional methods. Oxidoreductases proved to be efficient catalysts for an oxidative polymerization of phenol and aniline derivatives, yielding polyaromatics. Among them, peroxidases such as horseradish peroxidase (HRP) and soybean peroxidase induced the polymerization of a variety of phenols to produce a new class of polyphenols in good yields.[2] This process is expected to be an environmentally benign route to polyphenols, since conventional phenolic resins (novolak and resol resins) are produced using toxic formaldehyde.

In the oxidative polymerization of phenol derivatives, the structure of the resulting polyphenols is often very complicated since these phenols are multifunctional monomers. Only some 2,6-disubstituted phenols are oxidatively polymerized to give poly(1,4-oxyphenylene)s exclusively.[3] In the case of 4-substituted phenols, the polymers have structures normally composed of a mixture of phenylene and oxyphenylene units, which are formed by the C–C and C–O coupling of phenols, respectively (Scheme 1).[4] Recently, we have preliminarily achieved the precise control of the coupling selectivity (ratio of phenylene and oxyphenylene units) by selection of the monomer substituent and the nature of solvent.[5] In this study, the effects of the hydrophobic parameters on the polymer structure have been systematically investigated. Furthermore, the biodegradability of the polyphenols was preliminarily evaluated by a biochemical oxygen demand (BOD) measurement.



Scheme 1.

Experimental

Materials. HRP was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. An activated sludge was obtained from the Chemicals Evaluation and Research Institute, Japan. Other reagents and solvents were commercially available and were used as received.

Enzymatic Polymerization. A typical run was as follows (entry 10 in Table 1). HRP (2.0 mg, 440 unit) and 4-*t*-butylphenol (0.75 g, 5.0 mmol) 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 over 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 (87% yield).

^{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)).

Determination of Polymer Structure by Titration.[6] The polyphenol (0.10 g) was dissolved in pyridine containing 2.5% acetic anhydride (5.0 mL). The solution was kept at 95–100 °C for 1 h with gentle stirring. After cooling to room temperature, water (0.50 mL) was added to the reaction mixture, and the mixture was then again heated at 95–100 °C for 10 min. The solution was titrated with 0.2 M potassium hydroxide in ethanol in the presence of phenolphthalein as an indicator.

Biodegradation Test. The inherent biodegradability was evaluated by the OECD method 302C (Modified MITI Test (II)).[7] A sample (9.0 mg), activated sludge (30 mg), and basal culture medium (300 mL) were combined in a bottle, and the oxygen con-

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Table 1. Polymerization of 4-Substituted Phenols in an Equivolume Mixture of Organic Solvent and Buffer[a)]

Entry	Monomer	Organic solvent	log *P*	Conv.[b)]/%	Polymer			
					Yield/%	M_n[c)]	M_w/M_n[c)]	Ph/Ox[d)]
1	4-CHP	Ethylene glycol	−1.36	89	85	470	2.2	94/6
2	4-CHP	1,4-Dioxane	−0.42	97	86	600	2.0	63/37
3	4-CHP	2-Propanol	0.05	99	93	1000	1.5	58/42
4	4-TBP	Ethylene glycol	−1.36	92	38	390	2.0	85/15
5	4-TBP	*N*,*N*-Dimethylformamide	−1.01	95	70	760	1.8	68/32
6	4-TBP	Methanol	−0.77	90	83	570	1.9	72/28
7	4-TBP	1,3-Dioxane	−0.42	84	79	1300	1.6	61/39
8	4-TBP	1,4-Dioxane	−0.42	95	98	930	1.8	64/36
9	4-TBP	Acetone	−0.24	93	67	1500	1.6	56/44
10	4-TBP	2-Propanol	0.05	92	87	1400	1.5	56/44
11	4-TBP	1-Propanol	0.25	78	64	1600	1.5	47/53
12	4-TBP	*t*-Butyl alcohol	0.35	96	93	1600	1.5	51/49
13	4-IPP	Ethylene glycol	−1.36	99	77	400	2.3	74/26
14	4-IPP	Methanol	−0.77	99	90	910	1.5	68/32
15	4-IPP	1,4-Dioxane	−0.42	99	84	1400	1.7	55/45
16	4-IPP	2-Propanol	0.05	98	83	1600	1.6	53/47
17	4-IPP	1-Propanol	0.25	97	89	1500	1.7	44/56
18	4-EP	Ethylene glycol	−1.36	98	85	480	1.9	48/52
19	4-EP	Methanol	−0.77	99	76	590	1.8	38/62
20	4-EP	2-Propanol	0.05	97	94	610	1.6	21/79
21	4-EP	1-Propanol	0.25	100	93	650	1.7	15/85
22	4-MP	1,4-Dioxane	−0.42	100	68	610	1.9	42/58
23	4-MP	2-Propanol	0.05	100	95	610	1.7	24/76

a) Polymerization of 4-substituted phenols (5.0 mmol) using HRP as 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.

sumption was measured continuously at 25 °C for 81 days by means of an Ohkura OM2001A biochemical oxygen demand (BOD) apparatus. The biodegradability was estimated by the percent of the amount of oxygen consumed, corrected for a blank test to the theoretical amount of oxygen required for complete oxidation of the sample.

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 as the 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 with methanol/17 mM phosphoric acid as the eluent at a flow rate of 1.8 mL min^{-1}. ^{1}H NMR spectra were recorded on a JEOL JNM-LA 600 spectrometer. The amount of total organic carbon (TOC) in the supernant of the BOD test was measured by a Shimadzu TOC-5000A apparatus.

Results and Discussion

Structure Control. Our previous studies on the HRP-catalyzed polymerization of phenol in an aqueous methanol showed that the polymer structure depended on the methanol content of the mixed solvent,[8] suggesting that the polyphenol structure can be controlled by the solvent properties. Recently, we reported that the hydrophobicity of the solvents and monomers greatly affected the coupling selectivity (regioselectivity) in the polymerization of 4-substituted phenols in a mixture of water-miscible organic solvents and buffer.[5] In this study, relationships between some parameters of the organic solvents and monomer substituents and the structure of the resulting polyphenols have been examined in detail.

The HRP-catalyzed oxidative polymerization of 4-substituted phenols was performed in an equivolume mixture of a polar organic solvent and phosphate buffer (pH 7) at room temperature under air. Hydrogen peroxide (oxidizing agent) was added dropwise to the reaction mixture for 2 h.[4c] With the addition of hydrogen peroxide, powdery polymeric precipitates were quickly formed. After 1 h, the polymer was separated by centrifugation. The molecular weight of the polymer was measured by SEC. The unit ratio of phenylene/oxyphenylene (Ph/Ox) was determined by titration of the phenolic hydroxy group in the polymer.[6]

In this study, five 4-substituted phenols, 4-cyclohexylphenol, 4-*t*-butylphenol, 4-isopropylphenol, 4-ethoxyphenol, and 4-methoxyphenol (4-CHP, 4-TBP, 4-IPP, 4-EP, and 4-MP, respectively), were used as monomers. The polymerization results are summarized in Table 1. In most cases, the polymer was obtained in good yields. The polymers in the range of 15 to 94% phenylene unit content were formed. The molecular weight was around 1×10^3 and dependent on the monomer substituents and organic solvents.

Physicochemical parameters of the organic solvents, log *P*, dielectric constants, and dipole moments were employed to examine the relationships between the solvent nature and the polymer structure. Log *P* is one of the most popular hydrophobic parameters. *P* is a partition coefficient for a given solvent

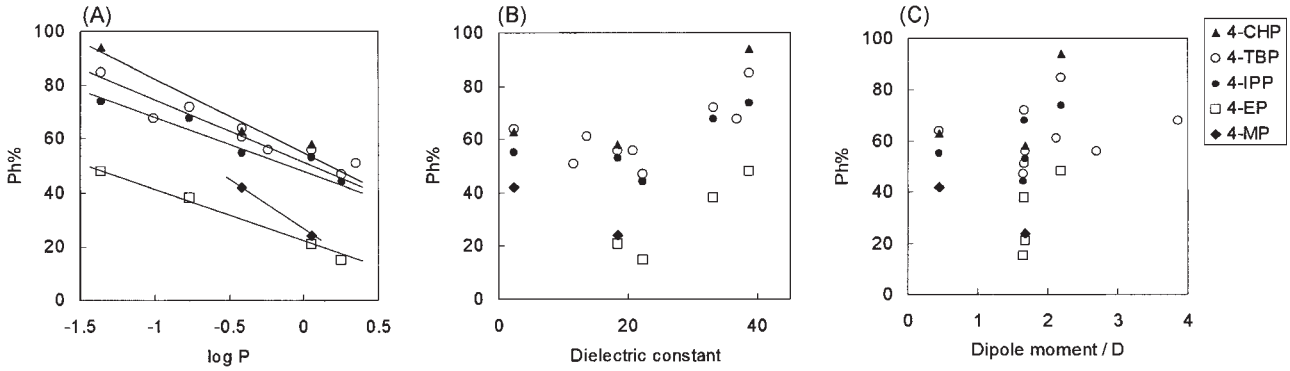

Fig. 1. Relationships between the nature of organic solvents and the phenylene unit of polymers in the polymerization of 4-substituted phenols in an equivolume mixture of organic solvent and phosphate buffer. Parameters of organic solvent: (A) log *P*, (B) dielectric constant, (C) dipole moment. Values of log *P* of the solvents are shown in Table 1. Dielectric constant: ethylene glycol, 38.66; *N*,*N*-dimethylformamide, 36.71; methanol, 33.1; 1,3-dioxane, 13.57; 1,4-dioxane, 2.24; acetone, 20.7; 2-propanol, 18.3; 1-propanol, 22.2; *t*-butyl alcohol, 11.4. Dipole moment: ethylene glycol, 2.2; *N*,*N*-dimethylformamide, 3.86; methanol, 1.66; 1,3-dioxane, 2.13; 1,4-dioxane, 0.45; acetone, 2.69; 2-propanol, 1.68; 1-propanol, 1.66; *t*-butyl alcohol, 1.66.

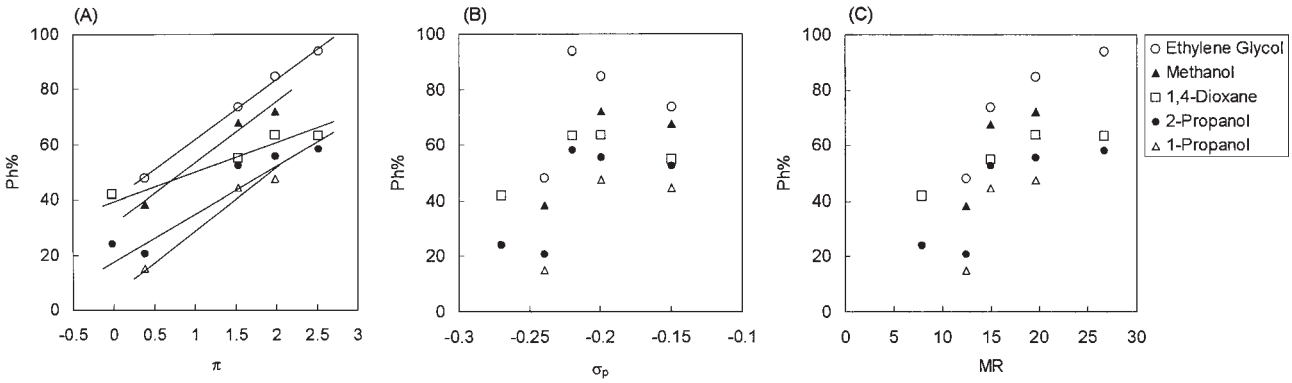

Fig. 2. Relationships between the basic physicochemical parameter of monomer substituents and the phenylene unit of polymers in the polymerization of 4-substituted phenols in an equivolume mixture of organic solvent and phosphate buffer. Monomer substituent: (A) hydrophobic parameter (π), (B) electronic parameter (σ_p), and (C) steric parameter (MR). Hydrophobic parameter (π): cyclohexyl, 2.51; *t*-butyl, 1.98; isopropyl, 1.53; ethoxy, 0.38; methoxy, −0.02. Electronic parameter (σ_p): cyclohexyl, −0.22; *t*-butyl, −0.20; isopropyl, −0.15; ethoxy, −0.24; methoxy, −0.27. Steric parameter (MR): cyclohexyl, 26.69; *t*-butyl, 19.62; isopropyl, 14.98; ethoxy, 12.47; methoxy, 7.87.

between 1-octanol and water.[9] For log *P*, a relatively good first-order correlation was observed and the slope of all the monomers examined was relatively close to each other (Fig. 1(A)). The phenylene unit decreased as the log *P* value increased. In contrast, no correlation was found in the case of other solvent parameters, as illustrated in Figs. 1(B) and 1(C). These data clearly indicate that the polymer structure strongly depends on the hydrophobic parameter of the reaction solvent. Furthermore, the molecular weight increased as a function of the hydrophobic parameter (Table 1). This may be due to the difference in the polymer solubility in the solvent.

In order to elucidate the effects of the monomer substituents on the coupling selectivity (regioselectivity) in the present polymerization, relationships between the hydrophobic parameter (π), electronic parameter (σ_p), and steric parameter (MR) of the substituents and the regioselectivity have been examined.[10] A relatively good linearity was found in the case of the hydrophobic parameter (Fig. 2(A)). On the other hand, there was no clear correlation with the other parameters (Figs. 2(B) and 2(C)). These results indicate that the hydrophobic parameters of the monomer substituents strongly affect the polymer structure.

The data in Figs. 1 and 2 suggest that the regioselectivity of the oxidative polymerization of phenols can be controlled by changing the hydrophobic parameters of the solvent and monomer substituents, leading to the precise synthesis of polyphenols with a defined structure.

Structural Control by Enzymatic Copolymerization of Phenols. The good first-order correlation between the hydrophobic parameter of the monomer substituents and polymer structure encouraged us to attempt to control the polymer structure by the peroxidase-catalyzed copolymerization of two phenol derivatives with different hydrophobicity. We selected 4-TBP and 4-MP as comonomers for structural control of the polyphenols through the enzymatic copolymerization.

The copolymerization was carried out in an equivolume mixture of 2-propanol and phosphate buffer. Table 2 summarizes the copolymerization results. The copolymer composition, determined by ^{1}H NMR, was similar to the feed ratio. The phenylene unit content decreased as a function of the feed ratio of 4-

Table 2. Copolymerization of 4-TBP and 4-MP in an Equivolume Mixture of 2-Propanol and Buffer[a)]

Entry	Feed	Polymer				
	Molar ratio 4-TBP/4-MP	Yield/%	M_n[b)]	M_w/M_n[b)]	Unit ratio 4-TBP/4-MP[c)]	Unit ratio Ph/Ox[d)]
1	100/0	87	1400	1.5	100/—	56/44
2	80/20	87	730	1.8	78/22	43/57
3	60/40	86	580	1.9	58/42	37/63
4	40/60	86	500	1.9	34/66	32/68
5	20/80	87	450	1.8	15/85	23/77
6	0/100	95	610	1.7	—/100	24/76

a) Copolymerization of monomers (5.0 mmol) using HRP as catalyst in an equivolume mixture of 2-propanol and pH 7 phosphate buffer (25 mL) at room temperature for 3 h under air. b) Determined by SEC using DMF as eluent with polystyrene standards. c) Determined by ^{1}H NMR. d) Determined by titration.

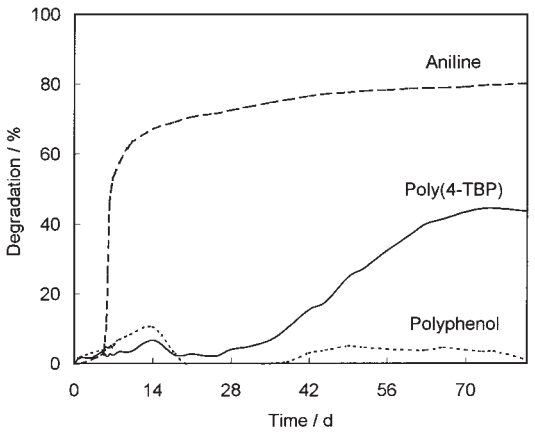

Fig. 3. Biodegradation of poly(4-TBP) (entry 10 in Table 1) and polyphenol in activated sludge.

TBP, indicating that the polyphenol structure strongly depends on the feed ratio of the phenol monomers with different hydrophilicities of the substituents. To our knowledge, this is the first example of structural control of a polyphenol by the copolymerization of phenols.

For reference data, the biodegradable properties of the product will be useful for future applications. Recently, the biodegradation of poly(*p*-phenylphenol) formed by the peroxidase-catalyzed polymerization was reported.[11] Here, the inherent biodegradability[7] of poly(4-TBP) (entry 10 in Table 1) was investigated. The degradation behavior in an activated sludge was evaluated by a BOD measurement (Fig. 3). The degradation of aniline as a positive control substance exceeded 40% and 65% after 7 and 14 days, respectively, indicating that this test is valid. The degradation of poly(4-TBP) took place gradually and reached 45% after 73 days. Afterwards, the degradation proceeded very slowly. These results indicate that poly(4-TBP) shows relatively good biodegradability. For comparison, the biodegradability of the polymer obtained from an unsubstituted phenol ($M_n = 2.0 \times 10^3$, $M_w/M_n = 3.6$)[8] was also tested. The degradation ratio was only 3% after 73 days. These preliminary data suggest that the biodegradability of the enzymatically synthesized polyphenols depends on their structure. The molecular weight of the polyphenols may also affect biodegradability.

After the cultivation, the degradation product of poly(4-TBP) was analyzed. The molecular weight of the precipitated polymer, which was recovered from the culture medium by filtration, was estimated by SEC. M_n and its index of the recovered product were 1000 and 1.5, respectively. Only a slight decrease of the molecular weight was observed, and the pattern of the SEC trace was very similar to that before the cultivation.

TOC is a useful and convenient assay of water-soluble organic substances in an aqueous solution. The water-soluble TOC concentration of the culture filtrate in the biodegradation test of poly(4-TBP) was measured. The TOC value was 1.4 mg/L, which was almost the same as that of the control blank test (the basal culture medium and activated sludge), indicating that water-soluble organic compounds are scarcely formed during the degradation. These results suggest that the degradation proceeds gradually from the terminal of the polymer and the degradation product is completely converted into carbon dioxide and water.[12]

Conclusion

In the HRP-catalyzed oxidative polymerization of 4-substituted phenols in aqueous organic solvents, structural control of the polymer was achieved. The polyphenol structure strongly depended on the hydrophobic parameters of the organic solvents and monomer substituents ($\log P$ and π, respectively). The structure could be controlled by the feed ratio in the HPR-catalyzed copolymerization of phenolic monomers with different hydrophobicities. These results will be useful for the design and precise synthesis of polyphenols.

A BOD analysis of the enzymatically synthesized polyphenols showed that the biodegradability was dependent on the structure. Poly(4-TBP) exhibited relatively good biodegradability, whereas the polyphenol from unsubsitituted phenol was hardly biodegradable. The TOC measurement of the supernant in the BOD test suggests that the biodegradation gradually takes place from the terminal ends of the polymer and converts to carbon dioxide and water completely.

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References

1 For reviews on enzymatic polymerization, see: a) S. Kobayashi, S. Shoda, and H. Uyama, *Adv. Polym. Sci.*, **121**, 1 (1995). b) S. Kobayashi, *J. Polym. Sci., Polym. Chem. Ed.*, **37**, 3041 (1999). c) S. Kobayashi, H. Uyama, and M. Ohmae, *Bull. Chem. Soc. Jpn.*, **74**, 613 (2001). d) R. A. Gross, A. Kumar, and B. Kalra, *Chem. Rev.*, **101**, 2097 (2001). e) S. Kobayashi, H. Uyama, and S. Kimura, *Chem. Rev.*, **101**, 3793 (2001). f) S. Matsumura, *Macromol. Biosci.*, **2**, 105 (2002).

2 For recent papers on enzymatic polymerization of phenols, see: a) T. Fukuoka, Y. Tachibana, H. Tonami, H. Yuma, and S. Kobayashi, *Biomacromolecules*, **3**, 768 (2002). b) P. Xu, J. Kumar, L. Samuelson, and A. L. Cholli, *Biomacromolecules*, **3**, 889 (2002). c) L. Mejias, M. H. Reihmann, S. Sepulveda-Boza, and H. Ritter, *Macromol. Biosci.*, **2**, 24 (2002). d) M. H. Reihmann and H. Ritter, *J. Macromol. Sci., Pure Appl. Chem.*, **A39**, 1369 (2002). e) A. Cui, A. Singh, and D. L. Kaplan, *Biomacromolecules*, **3**, 1353 (2002). f) S. K. Sahoo, W. Liu, L. A. Samuelson, J. Kumar, and A. L. Cholli, *Macromolecules*, **35**, 9990 (2002). g) Z. Xia, T. Yoshida, and M. Funaoka, *Biotechnol. Lett.*, **25**, 9 (2003). h) M. Kurisawa, J. E. Chung, Y.-J. Kim, H. Uyama, and S. Kobayashi, *Biomacromolecules*, **4**, 469 (2003). i) Y.-J. Kim, H. Uyama, and S. Kobayashi, *Macromolecules*, **36**, 5058 (2003).

3 a) A. S. Hay, H. S. Blanchard, G. F. Endres, and J. W. Eustance, *J. Am. Chem. Soc.*, **81**, 6335 (1959). b) A. S. Hay, *J. Polym. Sci., Polym. Chem. Ed.*, **36**, 505 (1998).

4 a) H. Kurioka, I. Komatsu, H. Uyama, and S. Kobayashi, *Macromol. Rapid Commun.*, **15**, 507 (1994). b) H. Uyama, H. Kurioka, J. Sugihara, I. Komatsu, and S. Kobayashi, *J. Polym. Sci., Polym. Chem. Ed.*, **35**, 1453 (1997). c) N. Mita, N. Maruichi, H. Tonami, R. Nagahata, S. Tawaki, H. Uyama, and S. Kobayashi, *Bull. Chem. Soc. Jpn.*, **76**, 375 (2003). d) N. Mita, S. Tawaki, H. Uyama, and S. Kobayashi, *Macromol. Biosci.*, **3**, 253 (2003).

5 N. Mita, S. Tawaki, H. Uyama, and S. Kobayashi, *Chem. Lett.*, **2002**, 402.

6 JIS K 0070-1992.

7 OECD Guideline for Testing of Chemicals, 302C (Modified MITI Test (II)): Inherent Biodegradability, adopted in 12 May 1981.

8 a) T. Oguchi, S. Tawaki, H. Uyama, and S. Kobayashi, *Macromol. Rapid Commun.*, **20**, 401 (1999). b) T. Oguchi, S. Tawaki, H. Uyama, and S. Kobayashi, *Bull. Chem. Soc. Jpn.*, **73**, 1389 (2000).

9 C. Laane, S. Boeren, K. Vos, and C. Veeger, *Biotechnol. Bioeng.*, **30**, 81 (1987).

10 a) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964). b) C. Hansch, A. Leo, S. H. Unger, K.-H. Kim, D. Nikaitani, and E. J. Lien, *J. Med. Chem.*, **16**, 1207 (1973).

11 R. Farrell, M. Ayyagari, J. Akkara, and D. Kaplan, *J. Environ. Polym. Degrad.*, **6**, 115 (1998).

12 OECD Guideline for Testing of Chemicals, 301: Ready Biodegradability, adopted in 17 July 1992.