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NOTE

Synthesis of polyarbutin by oxidative polymerization using PEGylated hematin as a biomimetic catalyst

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INTRODUCTION

Enzymatic polymerization has been extensively developed over the past two decades.[1–7] In particular, horseradish peroxidase (HRP) has been used for enzymatic oxidative polymerization of phenolic compounds. Dordick and co-workers[8,9] reported HRP-catalyzed polymerization of arbutin, a glucose-bearing phenol derivative. Water-soluble polyarbutins with molecular weight (M_n) in the range of 1600–3200 were synthesized. Acidic deglycosylation resulted in polyhydroquinone, which is a redox-active polymer useful for sensing materials. Nakano *et al.*[10] also reported HRP-catalyzed polymerization of arbutin and its derivatives, such as glucosyl, galactosyl and mannosyl arbutins. The resulting polymers exhibited high adsorbability to lectins. Many of these polymers, however, are of low molecular weight, restricting their use as thin films and other forms of practical interest.

Peroxidases are hemoenzymes having ferric complexes of protoporphyrin IX bound to them by a coordinate bond to the imidazole ring of a histidine residue.[11] Hematin, a hydroxylated protoporphyrin IX complex of ferric ion (Fe^{3+}), has been examined as a biomimetic catalyst substituting HRP.[12–15] Hematin, however, has a low solubility in both organic and aqueous solvents. To improve the solubility, Kumar and co-workers[16,17] prepared polyethylene glycol-containing hematin (PEGylated hematin) using *N,N′*-carbonyldiimidazole/1,8-diazabicyclo[5.4.0]undec-7-ene as a condensing agent. PEGylated hematin was shown to catalyze the radical polymerization of sodium styrene sulfonate and the oxidative polymerization of aniline. However, neither the characterization of PEGylated hematin nor the details of the polymerization were reported.

In the present study, we prepared PEGylated hematin by the Mitsunobu reaction using hematin and polyethylene glycol (PEG) monomethyl ether and examined it as a catalyst for polymerization of arbutin in a buffer solution of pH 7.0 (Scheme 1). PEGylated hematin was characterized by ^{1}H nuclear magnetic resonance (NMR), and the polymerization process was examined by gel permeation chromatography (GPC). The polyarbutin obtained was characterized by ^{1}H NMR and infrared spectroscopy. We compared PEGylated hematin, hematin and HRP as a catalyst for arbutin polymerization.

EXPERIMENTAL PROCEDURE

Materials

Arbutin and HRP (150–200 U per mg solid) were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan. Hematin and PEG (DP=45) were obtained from Sigma-Aldrich Japan K.K., Tokyo, Japan. Hydrogen peroxide (30 wt%), diethyl azodicarboxylate (40% in toluene) and triphenylphosphine were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All chemicals and solvents were of reagent grade and were used as received.

Measurements

^{1}H NMR spectra were recorded on a JEOL Lambda 500 MHz Fourier transform NMR spectrometer (JEOL) at the Chemical Analysis Center, Chiba University. UV–vis spectra were obtained with a Hitachi U-3010 UV-Vis spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The average molecular weight of the polymer was determined by gel permeation chromatography using a Shodex OHpak SB-804 HQ column (Showa Denko KK, Miniato, Japan) with aqueous 0.5 M Na_2HPO_4/acetonitrile (90/10) as a mobile phase at a flow rate of 0.8 ml min^{-1} at 40 °C.

Preparation of PEGylated hematin

Diethyl azodicarboxylate (240 μl, 0.60 mmol) was added to PEG (0.5 g, 0.25 mmol), triphenylphosphine (160 mg, 0.60 mmol) and hematin (190 mg, 0.30 mmol) suspended in *N,N*-dimethylformamide (DMF; 10 ml) in a dropwise manner at 0°C under a nitrogen atmosphere. After stirring for 24 h at room temperature, unreacted hematin (~50 mg) was removed by filtration, and the filtrate was poured into diethyl ether. The precipitates were collected, dissolved in acetone and reprecipitated in diethyl ether (430 mg).

General procedure of polymerization

The catalyst (PEGylated hematin, hematin or HRP) was added to arbutin (30 mg, 0.1 mmol) dissolved in a phosphate buffer solution (pH 7.0, 300 μl). An aqueous H_2O_2 solution (6 wt%) was then added dropwise. The mixture was incubated at 30 °C with gentle stirring. The polymerization process was

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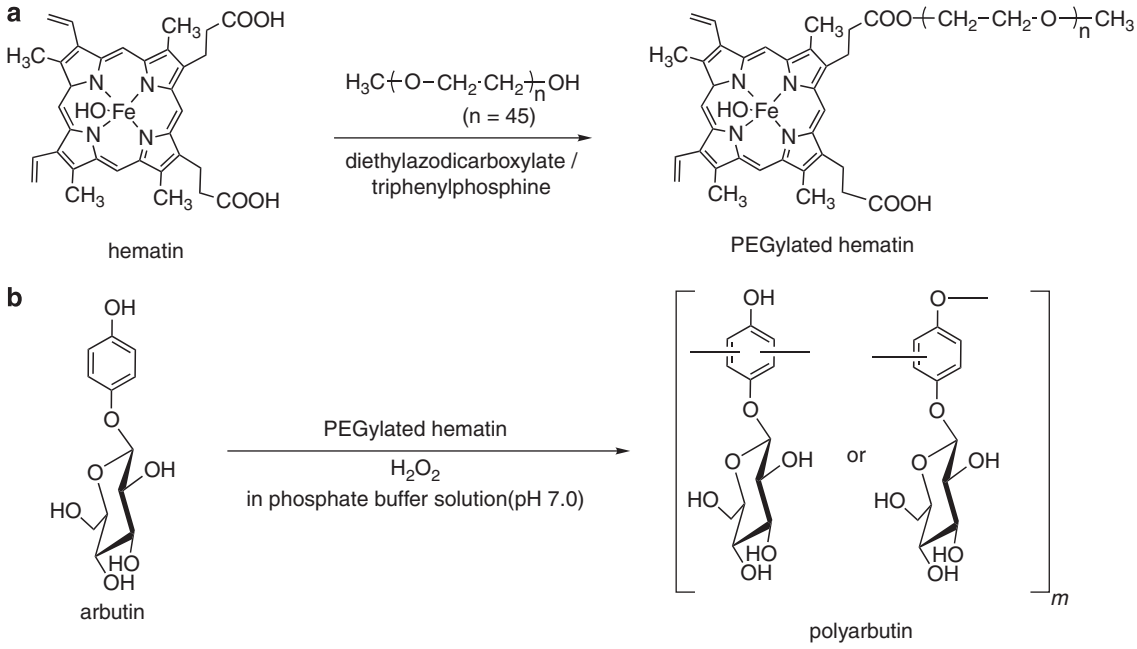

Scheme 1 (**a**) Preparation of PEGylated hematin and (**b**) polymerization of arbutin catalyzed by PEGylated hematin.

followed by GPC using UV detection of the hydroquinone absorption at 285 nm. The polyarbutin obtained was purified by precipitation in cold methanol and dried *in vacuo*. No polymer was obtained in the absence of the catalyst or H_2O_2. M_n and M_w were determined from the GPC traces obtained by UV detection together with molecular weight calibration using RI detection and pulluran standards.

RESULTS AND DISCUSSION

Synthesis and characterization of PEGylated hematin

The product obtained by the Mitsunobu reaction, that is, crude PEGylated hematin, was soluble in water, as well as in organic solvents such as toluene, acetone, chloroform and tetrahydrofuran. Figure 1 shows the ^{1}H NMR spectrum of hematin and that of the crude PEGylated hematin in DMF-d_7. Hematin and PEG protons normally appear at 5.2–6.6 and at 3.0–4.0 p.p.m., respectively. The carboxylic protons of hematin around 10 p.p.m. virtually disappeared after PEGylation, and new methylene protons appeared at 4.1 p.p.m., indicating successful introduction of PEG moieties into hematin by esterification. Determination of the degree of PEG substitution on the hematin moiety, however, was difficult owing to the broadness of the peaks and the presence of water in the solvent. (We performed ^{1}H NMR measurements of PEGylated hematin in D_2O and in $CDCl_3$ and observed only the signals due to PEG chains; virtually no signals due to the hematin moieties were observed, suggesting that PEGylated hematin has a limited solubility in these solvents.) When Matrix Assisted Laser Desorption Ionization (MALDI-TOF-MS) measurement was performed on the crude PEGylated hematin, we observed large peaks around *m/e* 2150, each separated by ~44, corresponding to unreacted PEG; this suggested its DP to be ~48 as opposed to nominal DP=45 (see Supplementary Figure S1). When we looked at the spectrum closely, we observed peaks around *m/e* 2800, which are attributable to mono-PEGylated hematin. We also note the presence of di-PEGylated hematin around *m/e* 4850. The ratio of di-PEGylated hematin:mono-PEGylated hematin:PEG was roughly 5:15:80, indicating the presence of a large amount of unreacted PEG in the PEGylated hematin.

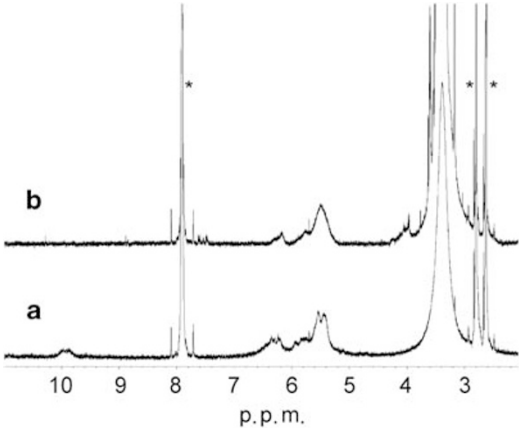

Figure 1 ^{1}H NMR spectra of (**a**) hematin and (**b**) PEGylated hematin in DMF-d_7. Peaks due to partially deuterated DMF are indicated by the asterisk. Large peaks at ~3.5 p.p.m. are due to PEG and water.

The amount of the hematin moiety, relative to PEG, was also examined using the molar extinction coefficient (70 000 l mol^{-1} cm^{-1}) obtained from the calibration curve of the hematin sample in DMF, and we found the ratio of hematin to PEG to be roughly 15:85, a value consistent with the MALDI-TOF-MS results. For the intended use of PEGylated hematin as a biomimetic catalyst, the presence of unreacted PEG will not affect its efficiency or evaluation thereof. Unlike hematin, crude PEGylated hematin was soluble in water at pH 7 and showed strong absorption, with λ_{max} at 390 nm attributable to the $\pi-\pi^*$ transition of the porphyrin ring (Soret band).

Polymerization of arbutin

The polymerization of arbutin by PEGylated hematin was carried out at pH 7.0 to avoid the hydrolysis of arbutin. (When the reaction was performed in deionized water (pH ~6.0), the resulting polyarbutin was hydrolyzed and the release of glucose residue was detected by ^{1}H

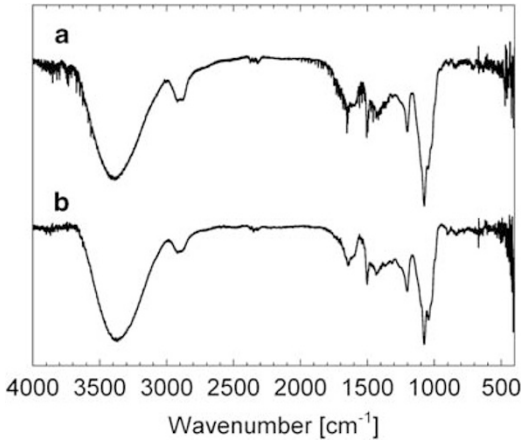

Figure 2 Infrared spectra of polyarbutins prepared by using (**a**) PEGylated hematin and (**b**) horseradish peroxidase as a catalyst.

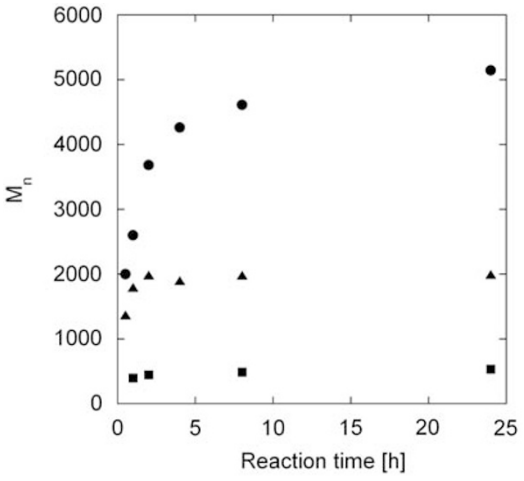

Figure 3 Polymerization of arbutin catalyzed by (●) PEGylated hematin (30 mg), (■) hematin (3 mg) and (▲) horseradish peroxidase (30 μg). Polymerization was carried out at 30 °C for 24 h with a H_2O_2 concentration of 1.7 wt%. The amounts of hematin moieties (in terms of moles) were comparable, considering the content of hematin in PEGylated hematin. As for HRP, see text.

Table 1 Dependence of PEGylated hematin-catalyzed polymerization of arbutin on reaction time and on H_2O_2 concentration[a]

Entry	*Reaction time (h)*	*H_2O_2 (wt%)*	M_n[b]	M_w[b]	M_w/M_n[b]	*Yield (%)*
1	0.5	1.7	2000	5600	2.8	ND
2	2	1.7	3700	8300	2.2	ND
3	8	1.7	4600	9800	2.1	ND
4	24	1.7	5100	11 000	2.1	80
5	24	1.0	3700	7400	2.0	65
6	24	3.3	900	1900	2.1	47

Abbreviations: ND, not determined; PEGylated hematin, polyethylene glycol-containing hematin.
[a]Polymerization was carried out using arbutin (30 mg, 0.1 mmol) catalyzed by PEGylated hematin (30 mg, 1.7 μmol based on hematin content) in a phosphate-buffered solution (pH 7.0, 300 μl) at 30 °C.
[b]Determined by GPC (see text).

NMR.) The reaction mixture remained homogeneous during the polymerization. The ^{1}H NMR spectrum of the obtained polyarbutin in D_2O showed broad peaks at 3.0–4.0 p.p.m. (6H), at 4.8−5.0 p.p.m. (1H) and at 6.5–7.5 p.p.m. (2H) attributed to the sugar, anomeric and aromatic protons, respectively (see Supplementary Figure S2). The infrared spectrum gives a broad peak at 3400 cm^{-1} due to the O–H stretching vibrations of the phenolic and hydroxyl groups of arbutin. Rather sharp peaks occur at 1200 and 1075 cm^{-1} and can be ascribed to the asymmetric stretching vibrations of the C–O–C and C–O–H groups (Figure 2a). The spectra pattern was very similar to that of polyarbutin obtained by HRP (Figure 2b).

The effects of reaction time and H_2O_2 concentration on the molecular weight of the obtained polyarbutin are shown in Table 1. The M_n increased from 2000 to 5100 with reaction time (entries 1–4), while polydispersity remained at ∼2. When H_2O_2 concentration was lowered from 1.7 to 1.0 wt% (entries 4, 5), the molecular weight obtained after 24 h of polymerization decreased. When a larger amount of H_2O_2 (3.3 wt%) was used (entry 6), a significant amount of the monomer remained in the reaction mixture, and only polymers of lower molecular weights were obtained (see Supplementary Figure S3). Apparently, excess H_2O_2 resulted in an inactive form of PEGylated hematin.[12]

The catalytic activities of hematin, HRP and PEGylated hematin, in terms of the molecular weights of the obtained polyarbutin, are shown in Figure 3. The amount of each catalyst used is given in the caption. With hematin as a catalyst, most of the arbutin remained unpolymerized; only a small amount of oligomers with M_n=∼500 was obtained, as hematin is a heterogeneous catalyst in water at pH 7. On the other hand, HRP-catalyzed polymerization proceeded, though the reaction stopped after ∼2 h. The M_n of the polyarbutin obtained was ∼2000. The conditions were optimized by considering those of previous reports.[8–10] The best result was obtained by using 30 μg, giving M_n=∼2000, which is in agreement with the M_n reported in Wang *et al.*[8,9] and Nakano *et al.*[10] When a larger amount of HRP (3 mg) was used, M_n decreased to ∼1000, in accord with a report by Guerra *et al.*,[18] who showed that an increase in the ratio of HRP to lignin monomer led to a decrease in molecular weight. The phenomenon is not yet fully understood. In contrast, PEGylated hematin-catalyzed polymerization continued much longer to yield an M_n of ∼5100 at 24 h. (When a smaller amount of PEGylated hematin, for example, 10 mg, was used, M_n also increased to ∼5000, even though it required a longer reaction time (3 days).) After precipitation, the yield of polyarbutin was ∼80%.

In HRP-catalyzed oxidative polymerization of phenolic compounds, free radicals are suggested to be generated on monomers and polymers.[19,20] The resulting free radicals couple with one another, and further polymerization proceeds. In the HRP-catalyzed polymerization, the conversion of arbutin was almost quantitative after ∼0.5 h, as with the PEGylated hematin-catalyzed polymerization (see Supplementary Figure S4). M_n rapidly increased, but stopped increasing in a short period of time, suggesting that HRP recognizes only monomeric and low-molecular weight oligomeric arbutin. This is probably due to the steric hindrance between HRP and oligomeric arbutin of higher molecular weights. When PEGylated hematin was used as a catalyst, higher-molecular-weight polyarbutin (M_n=∼5100) was obtained as shown above. Apparently, PEGylated hematin is active toward oligomeric arbutin of larger molecular weights, as PEGylated hematin is a smaller molecule compared with HRP.

CONCLUSION

Water-soluble PEGylated hematin was synthesized by the Mitsunobu reaction. PEGylated hematin showed high catalytic activity for arbutin polymerization, and the M_n of polyarbutin reached ∼5100,

indicating PEGylated hematin to be a better catalyst than HRP. Although a large amount of free PEG is present in the PEGylated hematin, it apparently does not affect achieving a high-molecular-weight polyarbutin that, to our knowledge, has not been obtained previously.

Further studies, including preparation of polyarbutin thin films and specific adsorption to lectins, are now in progress and will be reported in a future publication.

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Supplementary Information accompanies the paper on Polymer Journal website (http://www.nature.com/pj)