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One-pot synthesis of glycosyl poly(arbutin) by enzymatic glycosylation followed by polymerization with peroxidase

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Abstract

Polyaromatic compounds that carry several side residues of D-glucose, D-galactose, and D-mannose were synthesized from hydroquinone β-glucoside (arbutin) through two successive enzymatic reactions: glycosylation of the glucose residue in arbutin, followed by oxidative polymerization of the hydroquinone moieties. In the first step, *Bacillus macerans* cyclodextrin glucanotransferase, *B. circulans* β-galactosidase, and jack bean α-mannosidase were used to obtain glycosylation products in yields of 56.0, 29.5, and 25.2%, respectively, where the main products were a series of α-1,4-linked glucosyl/maltooligosyl arbutin, β-1,4-linked galactosyl arbutin, and α-1,6-linked mannosyl arbutin, respectively. In the second step, horseradish peroxidase was used and the enzyme efficiently polymerized glycosyl arbutin(s) together with the remaining arbutin using hydrogen peroxide as an oxidant. The water-soluble products, glycosyl poly(arbutin)s, showed approximate molecular weight distribution of 0.5–25 kDa which was estimated by gel permeation chromatography calibrated with maltooligosaccharides and pullulan markers. There were no differences in the phenolic main chain structures of glycosyl poly(arbutin)s and poly(arbutin), where the binding of neighboring hydroquinone moieties occurred at *ortho*-positions. Glycosyl poly(arbutin)s containing α-linked mannose and β-linked galactose residues exhibited increased absorbability to immobilized concanavalin A and castor bean lectin (RCA_{120}), respectively.

Keywords: Arbutin; Glycosylation; Polymerization; Peroxidase; Lectin

1. Introduction

Peroxidase (EC 1.11.1.7) catalyzes the oxidative polymerization of various aromatic compounds using hydrogen peroxide (H_2O_2) as an electron acceptor (oxidant) [1–7]. The enzyme has been applied to remove unfavorable aromatic compounds from wastewater by converting them into insoluble precipitates [5]. The enzyme has also been attracting interest as a catalyst for the synthesis of polyaromatic compounds [1–4,6,7] because of the novel structures of products, mild reaction conditions, and avoidance of poisonous organic solvents, compared to conventional polymerization processes. Recently, Wang et al. reported the polymerization of 4-hydroxyphenyl β-D-glucoside (hydroquinone β-glucoside, arbutin, Arb) by horseradish peroxidase (HRP), where the binding of adjacent hydroquinone moieties occurred at *ortho*-positions [6]. However, the authors subsequently removed the glucose (Glc) residues of the product, poly(Arb), by acid hydrolysis to obtain poly(hydroquinone), a redox-active polymer with possible applications for batteries, sensors, and antioxidants. As far as we know, however, no attention has been paid to poly(Arb), a water-soluble polymer retaining Glc residues, which may cause biological responses when the glycosyl structures are appropriately modified.

This report deals with the synthesis of the glycosylated derivatives of poly(Arb)s, which have several additional D-Glc, D-galactose (Gal), and D-mannose (Man) residues on poly(Arb). Introducing these glycosyl residues influences their binding affinity to lectins. The synthetic methodology involves two successive enzymatic reactions (Fig. 1): glycosylation of the Glc residues of Arb with glycosylating en-

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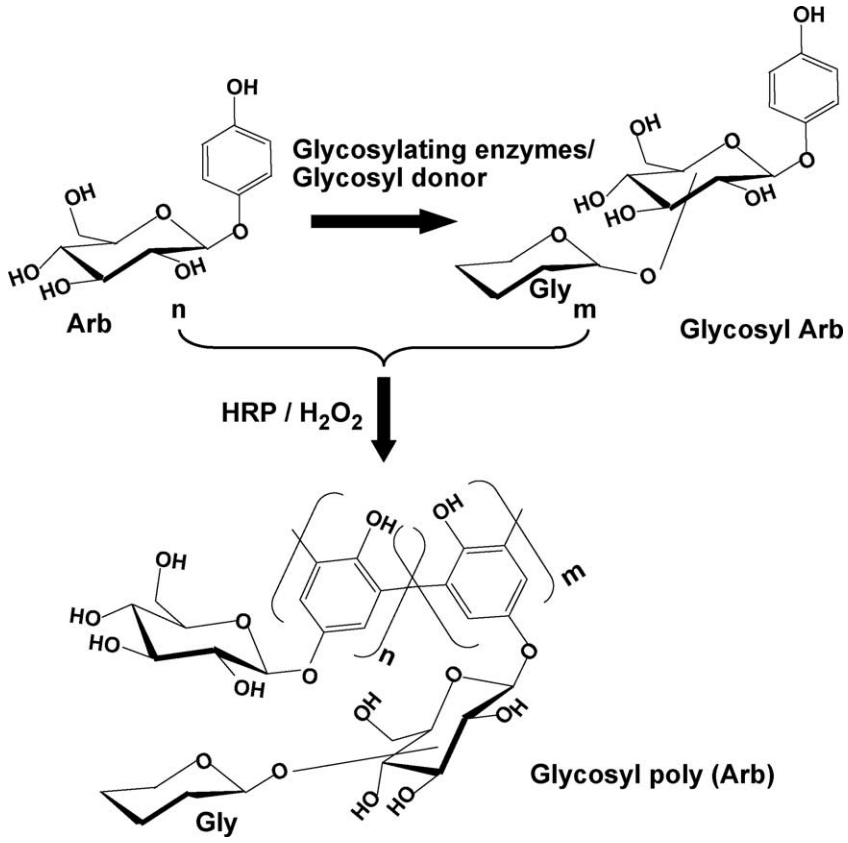

Fig. 1. Scheme for the enzymatic synthesis of glycosyl poly(Arb), $n = 4–7$, $m = 3–6$.

zymes, and the co-polymerization of Arb and several resulting glycosyl Arb with HRP. These syntheses were carried out in a one-pot system, and therefore, troublesome isolation of the glycosylated products was avoidable and Arb was sufficiently utilized for the polymer synthesis.

2. Materials and methods

2.1. Materials

Arb, lactose (Lac), D-mannose (Man), and methyl α-mannoside (Me-Man) were purchased from Nakalai Tesque Co. (Tokyo). α-Cyclodextrin (α-CD) and maltooligosaccharides were supplied by the Bio Research Corporation of Yokohama (Yokohama). Concanavalin A (ConA)–sepharose and castor bean lectin (RCA_{120})–agarose were commercial products of Seikagaku Kogyo Co. (Tokyo). Pullulan for molecular weight markers were purchased from Showa Denko Co. (Tokyo). The other chemicals used were of the highest grade commercially available.

2.2. Enzymes

HRP and jack bean α-mannosidase (α-Mase, EC 3.2.1.25) were purchased from Toyobo Co. (Kyoto) and Sigma Chemicals Co. (St. Louis, MO, USA), respectively. Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from *Bacillus macerans* and β-galactosidase (β-Gase, EC 3.2.1.23) from *B. circulans* were supplied by Amano Enzyme Inc. (Nagoya) and Daiwa Kasei Co. (Shiga), respectively.

2.3. HPLC

High-performance liquid chromatography (HPLC) was performed under two conditions using a Shimadzu LC-10AD (Shimadzu, Kyoto) equipped with a Shimadzu RID-10A refractive index detector (RI) and a SPD-10AV UV–vis detector (UV): (1) condition (A) for sugar analysis; column, Polyspher CH-Pb (Showa Denko, Tokyo); temperature, 80 °C; solvent, H_2O; flow rate, 0.4 ml/min. (2) Condition (B) for gel permeation chromatography (GPC); column, Shodex OHpak SB-802.5HQ (fractionation range for proteins, less than 50 kDa; exclusion limit of pullulan, 10 kDa; Showa Denko Co.); temperature, 40 °C; solvent, 25 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl; flow rate, 0.7 ml/min. The composition of glycosylation mixtures was measured under condition (B) using distilled water as a solvent.

2.4. Spectrometry

2.4.1. Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectra were recorded at 75 MHz (^{13}C) or at 300 MHz (^{1}H) with a JEOL

AL-300 FT NMR spectrometer (Tokyo). Glycosyl Arb(s) were dissolved in D_2O, and acetone (δ 31.4 ppm) was added as a shift reference. Deglycosylated polymers for ^{1}H and ^{13}C NMR analyses were prepared as described in Section 2.9 and dissolved in dimethyl sulfoxide (DMSO)-d6.

2.4.2. Mass spectrometry

Matrix-assisted laser desorption ionization (MALDI)–time-of-flight mass spectrometry (TOF-MS) was performed using Voyager^{TM} (PerSeptive Biosystems, Framingham, MA, USA). α-Cyano-4-hydroxy-cinnamic acid was used as a matrix.

2.4.3. Infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) was measured with a Shimadzu FTIR-8300 spectrometer. Deglycosylated polymers in KBr pellets were used for the measurement.

2.5. Enzymatic glycosylation of Arb

2.5.1. α-Glycosylation with CGTase

A reaction mixture (2.0 ml) containing 250 mg Arb, 250 mg α-CD, 25 mM acetate buffer (pH 6.0), and *B. macerans* CGTase [8,9] (25 U/ml) was incubated at 40 °C for 24 h. After incubation, the mixture was boiled for 10 min to stop the reaction, filled up to 5.0 ml with water, filtered, and the resulting solution was used for HRP-catalyzed polymerization as described in Section 2.7 or for product isolation as described in Section 2.6.

2.5.2. β-Galactosylation with β-Gase

A reaction mixture (0.5 ml) containing 250 mg Arb, 250 mg Lac, 25 mM acetate buffer (pH 6.0), and *B. circulans* β-Gase [10] (10 U/ml) was incubated at 40 °C for 4 h. After the incubation, the mixture was treated similarly to that described in Section 2.5.1.

2.5.3. α-Mannosylation with α-Mase

A reaction mixture (about 0.3 ml) containing 250 mg Arb, 1500 mg Man, 0.4 ml of 25 mM acetate buffer (pH 6.0), and jack bean α-Mase [11] (0.1 ml, 1.75 U) was incubated at 55 °C for 48 h. After the reaction, the mixture was treated similarly to that described in Section 2.5.1.

2.6. Isolation of glycosyl Arb(s)

2.6.1. α-Glycosylation product

The glycosylation mixture appropriately diluted with water (about 5.0 ml) was loaded onto a column of Bio-Gel P-2 (2.5 cm × 93 cm, Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 5.0% (v/v) ethanol. The elution was performed at 20 ml/h, and 5-ml fractions were collected. Saccharides in the fractions were monitored using the phenol–sulfuric acid method, ultraviolet adsorption at 260 nm, and thin-layer chromatography. The fractions were separated into three parts that contained each of products B1–B3 in Fig. 2B. Products B2 and B3 were subjected to re-chromatography on Bio-Gel P-2. The preparations, thus, obtained were evaporated and lyophilized. Product B1 (isolated yield, 76 mg) and product B2 (32 mg) appeared as single peaks in HPLC analysis under condition (B). The preparation of product B3 (17 mg) contained small amounts of other saccharides (purity judged by peak area, 86%).

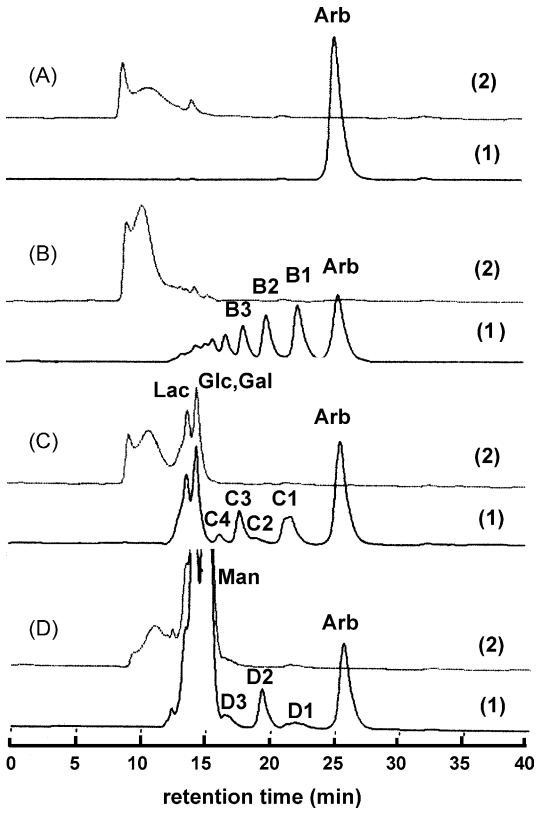

Fig. 2. HPLC chromatogram of glycosyl Arb(s) and their polymerization. Glycosylation and polymerization were carried out as described in Sections 2.5 and 2.7, respectively. The reaction mixtures were analyzed by HPLC under condition (B) described in Section 2.3. A-(1), Arb; A-(2), polymerization of A-(1); B-(1), α-glycosylation of Arb by the CGTase; B-(2), polymerization of B-(1); C-(1), β-galactosylation of Arb by the β-Gase; C-(2), polymerization of C-(1); D-(1), α-mannosylation of Arb by the α-Mase; D-(2), polymerization of D-(1). Peaks B1–B3, C1–C4, and D1–D3 were of the glycosylation products.

2.6.2. β-Galactosylation product

Abundant products C1 and C3 in Fig. 2C were purified almost similarly to that described in Section 2.6.1 except that Bio-Gel P-2 chromatography was repeated twice for each. Product C1 was accompanying with a shoulder in HPLC analysis under condition (B) (isolated yield, 37 mg; purity, 90%). Product C3 (15 mg) eluted as a single peak of unsymmetrical shape.

2.6.3. α-Mannosylation product

Abundant product D2 in Fig. 2D was purified almost similarly to that described in Section 2.6.1. Product D2 (isolated

yield, 21 mg) eluted as a single peak in HPLC under condition (B).

2.7. Polymerization with HRP

The glycosylation mixtures obtained as described in Section 2.5 were diluted appropriately with water (3.0 ml, 50 mg as Arb/ml, 0.551 mmol). To the solutions were added HRP (250 μg, 1.67 μg per mg of Arb) and then 6% (2.36 M) H_2O_2 solution drop by drop (20 μl each at 5-min intervals) to become 1.5 mol equivalent to the glycosides (about 0.83 mmol). The mixtures were incubated at 20 °C for 4 h with gentle stirring.

2.8. Separation of glycosyl poly(Arb)s

After polymerization, the mixtures were put onto a column (1.0 cm × 14 cm) of ODS-AQ (Organo Co., Tokyo) equilibrated with water. After washing with water, the polymers were eluted with 25% ethanol, collected, evaporated, and dialyzed exhaustively against distilled water in dialysis tubes (with a molecular weight cut-off of 12 kDa, Japan Medical Science Co., Osaka). After exhaustive dialysis, the samples were lyophilized and stocked at −20 °C.

2.9. Acid hydrolysis of poly(Arb) and glycosyl poly(Arb)s

The reaction mixtures (0.4 ml) containing the polymers (absorbance at 260 nm, A_{260}, 460–490) and 2.0 M trifluoroacetic acid (TFA) were boiled for 2 h. The black precipitates were washed with water several times using centrifugation. The supernatant solutions were combined, evaporated, and lyophilized. The samples were dissolved in water (0.4 ml), filtered, and subjected to the HPLC analysis performed under condition (A) to determine the sugar components. The precipitates, deglycosylated polymers, were dried well under reduced pressure and used for IR and NMR analyses as described in Section 2.4.

2.10. Adsorption of glycosyl poly(Arb)s to immobilized lectin

Columns (1.0 cm × 1.5 cm) of ConA–sepharose and RCA_{120}–agarose were equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. Poly(Arb) and glycosyl poly(Arb)s (about 75 μg) dissolved in the buffer (10–20 μl) were loaded onto the columns at 15 °C. After washing the gels with the buffer (5.0 ml), sequential elution was carried out with either 2 mM and then 20 mM Me-Man in the buffer (for ConA) or 2 mM and then 10 mM Lac (for RCA_{120}). The polymers were measured with A_{260}.

3. Results and discussion

3.1. Enzymatic glycosylation of Arb

3.1.1. α-Glycosylation by B. macerans CGTase

The Glc residue in Arb was effectively transglycosylated by *B. macerans* CGTase [8] as reported by Sugimoto et al. [9]. As shown in Fig. 2B-(1), CGTase gave several transfer products (peaks B1–B3, etc.). The total yield of transfer products was 56.0% from the reduction in Arb. Products B1–B3, which were separated by gel filtration, gave molecular ion peaks ($[M + Na]^+$) at *m*/*z* 457, 619, and 782 in MALDI–TOF-MS, indicating that they were glucosyl, glucobiosyl, and glucotriosyl Arb(s) (Glc-Arb–Glc_3-Arb), respectively. The following ratios were quantified according to the UV adsorption intensities on the HPLC analysis: Arb (44.0%), Glc-Arb (24.4%), Glc_2-Arb (13.8%), Glc_3-Arb (7.9%), and larger transfer products (9.9%). Glc-Arb, the simplest and the most abundant product, was confirmed to be 4-*O*-α-glucosyl Arb [9] with ^{13}C NMR (Table 1), and the more elongated products were reasonably estimated to be α-1,4-linked maltooligosyl Arb(s) according to the well-known action of CGTases [8,9].

3.1.2. β-Galactosylation by B. circulans β-Gase

Four products (C1–C4) were detected in the transgalactosylation of Arb as shown in Fig. 2C-(1). A total transfer yield of 29.5% was estimated based on the reduction in Arb. Abundant products C1 and C3 were suggested to be galactosyl Arb (Gal-Arb) and further glycosylated Arb with two Gal residues (galactobiosyl Arb or di-galactosyl Arb collectively abbreviated as Gal_2-Arb) from the molecular ion peaks at *m*/*z* 457 and 619 detected by TOF-MS, respectively. ^{13}C NMR

Table 1
^{13}C NMR data for Arb and major glycosyl Arb(s)

	Glycoside			
	Arb	B1 (Glc-Arb)	C1 (Gal-Arb)	D2 (Man-Arb)
C-aromatic				
1	152.4	152.5	152.8	152.5
2, 6	119.6	119.6	119.7	119.6
3, 5	117.4	117.4	117.5	117.4
4	151.6	151.6	151.5	151.5
C-Glc residue at reducing end				
1′	102.6	102.4	102.4	102.4
2′	74.2	74.0	73.9	74.1
3′	76.8	77.2	76.1	77.0
4′	70.6	77.9	79.4	70.7
5′	77.2	75.9	76.6	75.3
6′	61.8	61.7	61.2	67.8
C-glycosyl residue at non-reducing end				
1″		100.9	104.1	100.7
2″		72.9	72.2	71.1
3″		74.0	73.8	71.8
4″		70.5	69.8	66.8
5″		73.9	75.4	73.9
6″		61.7	62.2	62.0

Chemical shifts were given in ppm.

analysis indicated that product C1 contained two glycosides, 4-*O*-β-galactosyl Arb and 3-*O*-β-galactosyl Arb. The major glycoside was identified as 4-*O*-β-galactosyl Arb principally because of the carbon signal assignable to 4′ (79.4 ppm), which shifted to the downfield region (8.8 ppm) compared with the corresponding signal of Arb at 70.6 ppm (Table 1). Smaller peaks were assigned to the minor glycoside, 3-*O*-β-galactosyl Arb. This gave a peak at 85.5 ppm, which was assignable to the shifted signal of 3′ carbon (data not shown). Byproduct C2 with unidentified linkage(s) seemed to be another galactosyl Arb from its elution position in HPLC. In summary, the composition of the products was: Arb (70.5%), Gal-Arb (product C1, 15.0%; C2, 0.9%), Gal_2-Arb (product C3, 12.3%), and product C4, (1.3%).

3.1.3. α-Mannosylation by jack bean α-Mase

Jack bean α-Mase [11] mannosylated 25.2% of Arb in the condensation (reverse hydrolysis) reaction of Man and Arb. Main product D2 (Fig. 2D-(1)) was identified to be 6-*O*-α-mannosyl Arb (Man-Arb) from TOF-MS ($[M + Na]^+$ at *m*/*z* 457) and ^{13}C NMR analyses (Table 1), where the carbon signal assignable to 6′ (67.8 ppm) had a downfield shift value of 6.0 ppm from the corresponding 6′ signal of Arb (61.8 ppm). Byproducts D1 and D3 were considered to be another mannosyl Arb and further glycosylated Arb with two Man residues (mannobiosyl Arb or di-mannosyl Arb collectively abbreviated as Man_2-Arb), respectively, from the elution positions on HPLC. The reaction mixture consisted of Arb (74.8%), Man-Arb (product D1, 5.0%; D2, 17.0%), and Man_2-Arb (product D3, 3.2%).

3.2. Polymerization of Arb and glycosyl Arb(s) with HRP

The HRP-catalyzed polymerization was performed in a one-pot system after enzymatic glycosylation of Arb. After the addition of HRP to the glycosylation mixtures, the oxidant (H_2O_2) solution was added drop-wise to prevent the inactivation of HRP. A red–brown color developed with the progress of the reactions. Fig. 2 shows the GPC chromatogram for the reaction mixtures. The reaction occurred efficiently: Arb and glycosyl Arb(s) disappeared completely to form polymerized products (retention time, 9–14 min). The polymerization was also specific for phenolic glycosides: although the mixtures contained Arb and glycosyl Arb(s) as well as substrates and hydrolysis products, etc., the phenolic glycosides were selectively polymerized and the usual saccharides such as Glc, Gal, Lac, and Man remained unchanged.

In this report, poly(Arb) derivatives with additional side residues of Glc, Gal, and Man are abbreviated as Glc_n-poly(Arb), Gal_n-poly(Arb), and Man_n-poly(Arb), respectively, and these polymers are collectively referred as glycosyl poly(Arb)s.

3.3. Properties of glycosyl poly(Arb)s

3.3.1. Purification

Poly(Arb) and glycosyl poly(Arb)s were retained in dialysis tubes (12 kDa cut-off), and smaller saccharides were diffused out. The polymers were also adsorbed to the ODS resin. After washing with water, the polymers were eluted with ethanol solutions. Alternatively, they are separable as precipitates in the presence of ethanol (50–66%, v/v). In this study, the polymers were purified by ODS chromatography followed by dialysis as described in Section 2.8.

3.3.2. Glcosyl side chain residues

The sugar compositions of glycosyl poly(Arb)s and poly(Arb) were determined after hydrolysis with TFA (Table 2). The results prove that glycosylated Arb(s) (monomers) are incorporated into the polymers; Glc_n-poly(Arb) showed a 2.5-times higher Glc/A_{260} ratio, which represented the frequency of Glc residue to the phenolic backbone, than the other polymers, indicating the former contained additional Glc residues. It was also confirmed that Gal and Man residues exist in the respective glycosyl poly(Arb)s. The percentages of Gal/Glc (38.9%) and Man/Glc (31.3%) were somewhat larger than the respective glycosylation yields (29.5% and 25.2%) because of the existence of the glycosyl Arb(s) with multiple residues such as Gal_2-Arb and Man_2-Arb.

The average structures of the polymers are shown in Fig. 3. Since HRP-catalyzed polymerization occurred efficiently, the frequency of the side residues could be estimated from the ratios of glycosyl Arb(s) to free Arb. The glycosylation yields in the reactions of CGTase (56.0%), β-Gase (29.5%), and α-Mase (25.2%) implied that the polymerized products possessed approximately 5–6, 3, and 2–3 of the introduced glycosyl residues in every 10 Arb backbone units,

Table 2
Sugar composition of acid hydrolyzates of poly(Arb) and glycosyl poly(Arb)s

Compound	A_{260}*	Composition (mg/ml)			Glc/A_{260}* (%)	Gal, Man/Glc
		Glc	Gal	Man		
Poly(Arb)	484	10.5	0	0	2.17	0
Glc_n-poly(Arb)	476	28.3	0	0	5.95	0
Gal_n-poly(Arb)	492	10.2	3.97	0	2.07	0.389
Man_n-poly(Arb)	462	9.65	0	3.02	2.09	0.313

The concentrations of the polymers were adjusted to be almost the same (460–490) in terms of A_{260}. Hydrolysis was carried out as described in Section 2.9. The sugar composition of the hydrolyzate was determined by HPLC performed under the condition (A).

* A_{260} values before hydrolysis.

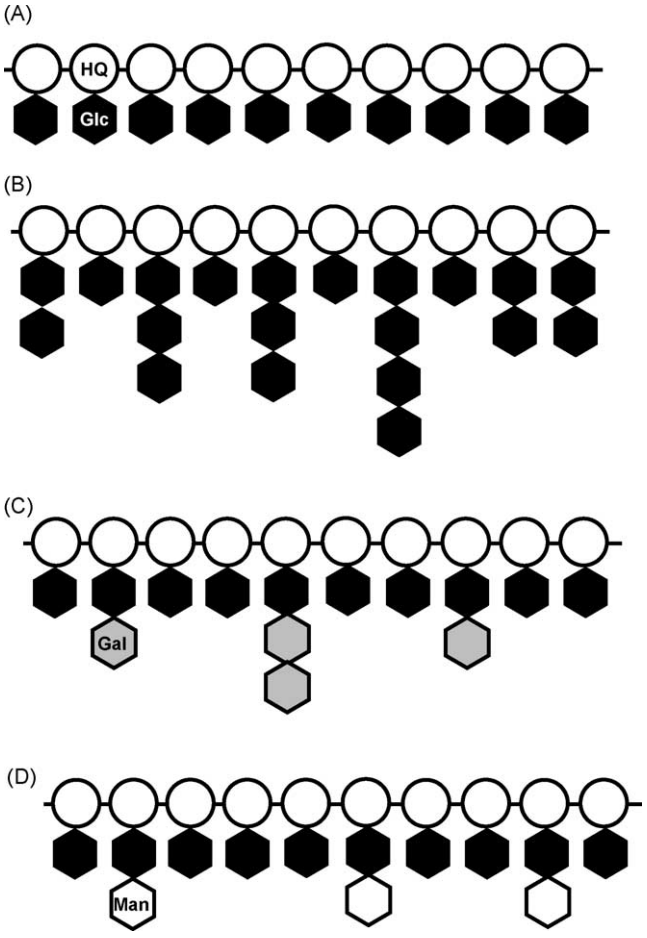

Fig. 3. Illustration of the average structures of glycosyl poly(Arb)s. (A) Poly(Arb); (B) Glc_n-poly(Arb); (C) Gal_n-poly(Arb); (D) Man_n-poly(Arb). White circle, hydroquinone moiety; closed hexagon, Glc residue; shaded hexagon, Gal residue; white hexagon, Man residue.

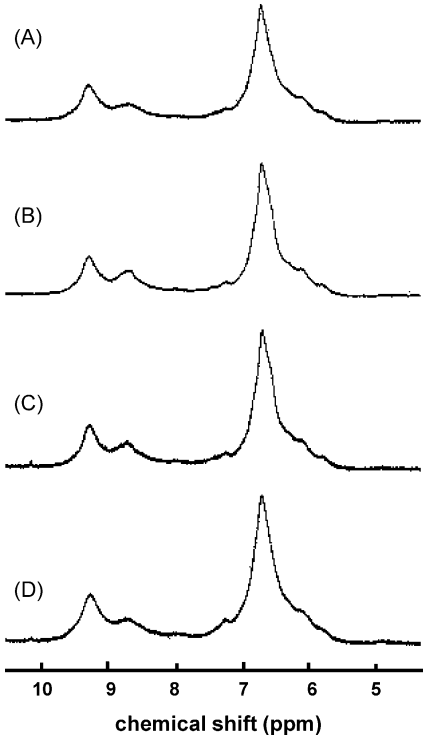

Fig. 4. ^{1}H NMR spectra of deglycosylated polymers. (A) Poly(Arb); (B) Glc_n-poly(Arb); (C) Gal_n-poly(Arb); (D) Man_n-poly(Arb).

respectively. Similarly, it was roughly estimated that Glc_n-poly(Arb) contained Glc-Arb–Glc_3-Arb at a ratio of 3:2:1, and that Gal_n-poly(Arb) contained Gal-Arb and Gal_2-Arb at a ratio of 2:1. In Man_n-poly(Arb), Man-Arb was the predominant constituent. We assume that the glycosyl residues are randomly distributed in the polymerized products since HRP does not appear to strictly recognize Arb or glycosyl Arb(s) as substrates, which is evidenced by the fact that Glc-Arb, Glc_2-Arb, Glc_3-Arb, Gal-Arb, and Man-Arb showed almost the same decreasing rates as Arb during polymerization (data not shown).

3.3.3. Phenolic backbone structure

Poly(Arb) and glycosyl poly(Arb)s were hydrolyzed with TFA to remove glycosyl residues for the elucidation of the polymer backbone structure. The deglycosylated polymers derived from glycosyl poly(Arb) gave almost the same ^{13}C NMR spectra as that from poly(Arb) [6]: broad and splitting carbon peaks were observed at δ 16–124 ppm assignable to C-2, -3, -5, and -6, and at δ 143–154 ppm assignable to C-1 and -4 of hydroquinone moiety. In ^{1}H NMR spectra (Fig. 4), broad signals ascribable to aromatic proton and phenolic hydroxyl proton were observed at around δ 6.7 ppm and δ 8.7–9.3 ppm, respectively, which were coincidental with the previous data of poly(hydroquinone) [6,7] obtained from HRP-catalyzed synthesis. Splitting of the latter peak may be due to two phenol groups in hydroquinone moiety. The signal area of the aromatic proton was almost twice larger than that of the phenolic hydroxyl proton, which may be attributable to inaccurate integration of the spreading signals. There were also no significant differences in the IR spectra of the deglycosylated polymers. Following typical adsorptions suggested the polymers were constructed of similar phenolic main chains; at 3350 cm^{-1} due to the O–H linkage, at 1601 and 1507 cm^{-1} due to the C=C linkage in aromatic ring, and at 1198 cm^{-1} due to the C (aromatic)-OH linkage.

From these results, we concluded that glycosyl poly(Arb)s had the same hydroquinone backbone structure as poly(Arb) [6,7], which was reported to have linkages at the *ortho*-positions among adjacent hydroquinone moieties (Fig. 1).

3.3.4. Molecular weight

Fig. 5 shows the GPC chromatogram of the polymers in HPLC analysis. Poly(Arb) and three glycosyl poly(Arb)s eluted in an almost same retention time range of 9–14 min, which corresponded to an approximate molecular weight distribution of 0.5–25 kDa, when maltooligosaccharides and pullulan were used as marker substances. The peak top positions of the glycosylated polymers were also similar and their molecular weight value was estimated to be ap-

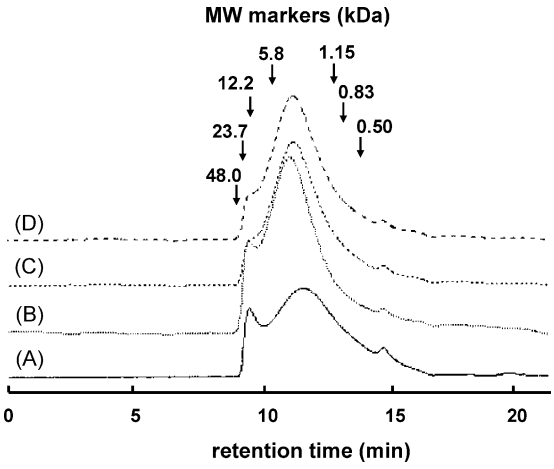

Fig. 5. HPLC analysis of the polymers. The polymers were separated by ODS chromatography followed by dialysis and analyzed by HPLC equipped with a GPC column and RI detector under condition (B) as described in Section 2.3. (A) Poly(Arb); (B) Glc_n-poly(Arb); (C) Gal_n-poly(Arb); (D) Man_n-poly(Arb). The elution positions of maltotoriose (0.50 kDa), maltopentaose (0.83 kDa), maltoheptaose (1.15 kDa), and the pullulan markers (5.80, 12.2, 23.7, and 48.0 kDa) are indicated by arrows.

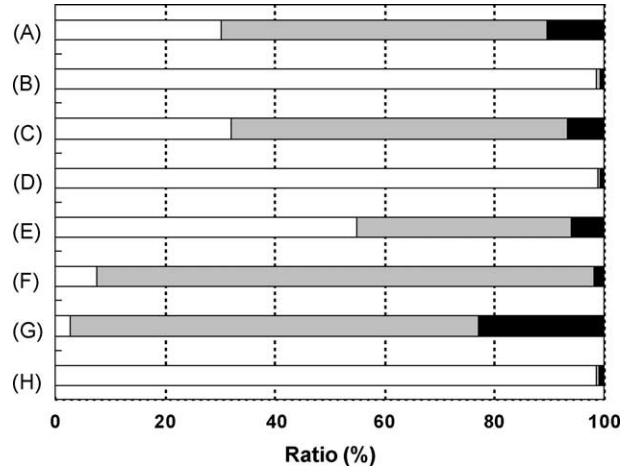

Fig. 6. Adsorption of the polymers to immobilized Con A and immobilized RCA_{120} gels. The adsorptions of the polymers to the immobilized lectins were evaluated as described in Section 2.10. (A and B) Poly(Arb); (C and D) Glc_n-poly(Arb); (E and F) Gal_n-poly(Arb); (G and H) Man_n-poly(Arb). (A, C, E, and G) Con A–sepharose; (B, D, F, and H) RCA_{120}–agarose. White bar, eluate with buffer (unabsorbed polymers); shaded bar, eluate with 2 mM Me-Man for Con A and 2 mM Lac for RCA_{120}; closed bar, eluate with 20 mM Me-Man for Con A and 10 mM Lac for RCA_{120}.

proximately 3.2 kDa, which corresponded to a polymerization degree of about 12. Shoulder and small peak were observed in a higher molecular weight region of around 12–24 kDa.

The molecular size of poly(hydroquinone), which was derived from poly(Arb), was reported to be 1.6–3.2 kDa, which was estimated by GPC calibrated with polyethylene glycol markers [6]. The apparent molecular weight elucidated for the most abundant glycosyl polymer species (3.2 kDa), therefore, was considered to be reasonable, when the presence of sugar residues in the polymers and the difference in the marker substances used were taken into account. The polymers, however, did not diffuse through a dialysis membrane with a cut-off size of 12 kDa as described in Section 3.3.1. This may be attributable to the particular molecular shape or aggregate formation of the polymers. Detailed molecular sizes and structural properties especially in aqueous solutions, therefore, remains a topic to be studied.

3.3.5. *UV adsorption spectra*

Glycosyl Arb(s) showed the same UV-spectrum as Arb, and an adsorption maximum was observed at around 280 nm. The spectra of poly(Arb) and glycosyl poly(Arb)s were also the same and showed a broad, gentle adsorption peak at around 300 nm (data not shown). The solution of the polymers was reddish brown, although no obvious adsorption maximums were observed in the visible region.

3.4. *Adsorption to immobilized lectins*

Lectins are proteins that react reversibly with specific sugar residues. Concanavalin A (Con A) from the jack bean (*Canavalia ensiformis*) binds molecules that contain α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues [12]. RCA_{120} from the castor bean (*Recinus communis*) specifically recognizes β-D-galactopyranosyl residue [13]. This study evaluates the interactions of the polymers with these two lectins in terms of absorbability to immobilized lectins (Fig. 6).

Most Man_n-poly(Arb) was adsorbed to the immobilized Con A gel under the experimental conditions and eluted with Me-Man, whereas about 50–70% of poly(Arb) and other glycosyl poly(Arb)s showed no absorbability. Man_n-poly(Arb) separated into two fractions, one eluted with 2.0 mM and the other with 20 mM Me-Man. The density and/or length of the glycosyl residues seemed to cause these different behaviors. Gal_n-poly(Arb) was adsorbed almost entirely to immobilized RCA_{120} and eluted with the Lac solution, whereas the other polymers showed no interactions. These behaviors coincided with the binding specificity of RCA_{120} lectin [13].

4. Conclusions

The methodology presented here provides a one-pot synthesis of polymers constructed from the hydroquinone backbone and Glc side residue, some of which carry additional glycosyl residues. This synthetic strategy involves two enzymatic reactions. First, the Glc residue of Arb is glycosylated by appropriate enzymes. The introduced linkages depend on the regio- and stereo-selectivity of the glycosylating enzymes used. In addition to the CGTase, β-Gase,

and α-Mase used in this report, other glycosydases and glycosyltransferases may be similarly applicable to give more variations for the side chains. Glycosyl Arb and the remaining Arb were subjected to the second polymerization step. The oxidative polymerization by HRP proceeded efficiently, and thus the frequency of the introduced side residues can be controlled by the glycosylation yields in the first step. Furthermore, when a certain glycosyl Arb is isolated and used as a monomer, a polymer with a uniformed side chain structure is expected to be synthesized similarly.

The resulting glycosyl poly(Arb)s are regarded as kinds of so-called artificial glycoconjugate polymer or glycopolymer that mimic biological glycoconjugate molecules [14]. Artificial glycopolymers constructed from the main chains of polypeptides, polysaccharides or vinyl polymers with glycosyl side chains have shown wide applicability in cellbiology because of their affinity with certain cells or lectin-like proteins [14]. Furthermore, the potential application of some glycopolymers are considered in the medical field, for example, for use as materials for tissue cell culture, as inhibitors for binding of viruses or bacteria to cells, or as neutralization agents for bacterial toxins. From the viewpoint of application, therefore, glycosyl pol(Arb)s with binding affinity to lectins may have similar potentialusefulness.

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