Hyaluronidase-Catalyzed Copolymerization for the Single-Step Synthesis of Functionalized Hyaluronan Derivatives

Hirofumi Ochiai, Masashi Ohmae, Tomonori Mori,† and Shiro Kobayashi*,‡

Department of Materials Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan

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Hyaluronidase-catalyzed copolymerization was carried out with monomer combinations of 2-methyl (**1a**)/2-vinyl (**1b**), 2-methyl (**1a**)/2-ethyl (**1c**), 2-methyl (**1a**)/2-*n*-propyl (**1d**), and 2-vinyl (**1b**)/2-ethyl (**1c**) oxazoline derivatives of hyalobiuronate [GlcAβ(1→3)GlcN]. All copolymerization reactions proceeded successfully in a regio and stereoselective manner, giving rise to hyaluronan derivatives bearing different *N*-acyl groups at the C2 position of the glucosamine unit in the polymer chain. The composition of the *N*-acyl groups was controlled by varying the comonomer feed ratio. The copolymerization mechanism was also discussed.

Introduction

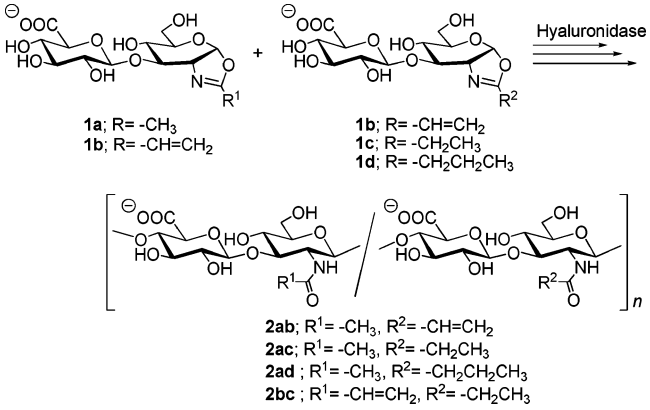
Hyaluronan (HA) is a naturally occurring bioactive polysaccharide comprising β(1→4)-linked β-D-glucuronyl-(1→3)-*N*-acetyl-D-glucosamine [GlcAβ(1→3)GlcNAc] disaccharide units.[1] HA is a non-sulfated glycosaminoglycan (GAG)[2] existing in extracellular matrixes.[3] It attracts increasing attention because of its inherent biological activities and physicochemical properties.[4−9] Such multifunctions of HA are frequently utilized in the medical and pharmaceutical fields.[10−17] Many HA derivatives have been successfully synthesized by the modification of natural HA with the aim of obtaining multipurpose biomaterials.[18] However, deacetylation of HA (hydrolysis of the acetamido group to the amino group) causes its degradation, leading to the reduction of the molecular mass because of the basic reaction conditions,[19] and this problem is still unsolved.

Previously, we have demonstrated the synthesis of HA and its derivatives with perfectly controlled structure via hyaluronidase (HAase)-catalyzed polymerization. Several oxazoline monomers were successfully homo-polymerized by enzyme catalysis, giving rise to the corresponding polysaccharide.[20−22] The present article reports the synthesis of HA derivatives bearing various amido functional groups (**2**) via enzymatic copolymerization of 2-substituted oxazoline monomers from hyalobiuronate (**1**) (Scheme 1). The copolymerization will lead to various unnatural HA derivatives, which may show new properties. Furthermore, the results clearly show the fact that the cross propagation of these monomers is feasible by using hyaluronidase as catalyst; therefore, this enzymatic copolymerization is probably one of the key technologies for constructing important GAGs.

Experimental Section

Measurements. NMR spectra were recorded on a Bruker DPX-400 spectrometer. For solutions in D_2O, acetone served as reference δ 2.22 (^{1}H) and δ 30.89 (^{13}C). High-performance liquid chromatography (HPLC) was performed by using a Tosoh LC-8020 system equipped with refractive index (RI) and UV detectors under the following conditions: Shodex Asahipak NH2P-50 4E column (4.6 × 250 mm) and phosphate buffer (10 mM, pH 7.0)−MeCN mixed solution (30:70, v/v) eluent at a flow rate of 0.5 mL/min at 30 °C. Size-exclusion chromatography (SEC) was carried out on a Tosoh GPC-8020 system equipped with an RI detector under the following conditions: Shodex OHpak SB-803HQ column (8.0 × 300 mm; exclusion limit, 100 000, determined by using pullulan standards) and 0.1 M aqueous $NaNO_3$ eluent at a flow rate of 0.5 mL/min at 40 °C. The calibration curves were obtained by using hyaluronan (M_n = 800, 2000, 4000; M_v = 50 000, 100 000) as standards. MALDI-TOF/MS analysis of the product was performed with a Jeol JMS−ELITE spectrometer by using 2,5-dihydroxybenzoic acid as a matrix on a Nafion-coated plate[23] under negative ion mode. Circular dichroism spectra were recorded in a H_2O solution at 20 °C on a CD spectrometer (J-600, Jasco) using an optical cell of 0.1 cm path length.

Scheme 1. Enzymatic Copolymerization to Copolymers (**2**)



Materials. All monomers **1a**−**d** were prepared as described.[20b] Ovine testicular HAase (OTH, ICN Biochemicals, Inc., Lot No. 9303B, 560 units/mg) was used without further purification.

Consumption of Comonomers 1a and 1b in Enzymatic Copolymerization. A typical procedure of monitoring comonomer consumption is as follows: Compounds **1a** (10.0 mg, 24.9 μmol) and **1b** (10.3 mg, 24.9 μmol) were mixed and dissolved in a carbonate-buffered D_2O solution (50 mM, pD 7.5, 498 μL). Then the mixture was incubated with OTH (2.0 mg) at 30 °C. Concentrations of **1a** and **1b** were calculated from the integration values of the signals from the H-3

* Corresponding author. Tel/Fax: +81-75-724-7688. E-mail: kobayash@kit.ac.jp.

† Present address: RIKEN, The Institute of Physical and Chemical Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Tel: +81-48-467-9431. E-mail: t-mori@riken.jp.

‡ Present address: R&D Center for Bio-based Materials, Kyoto Institute of Technology, Kyoto 606-8585, Japan.

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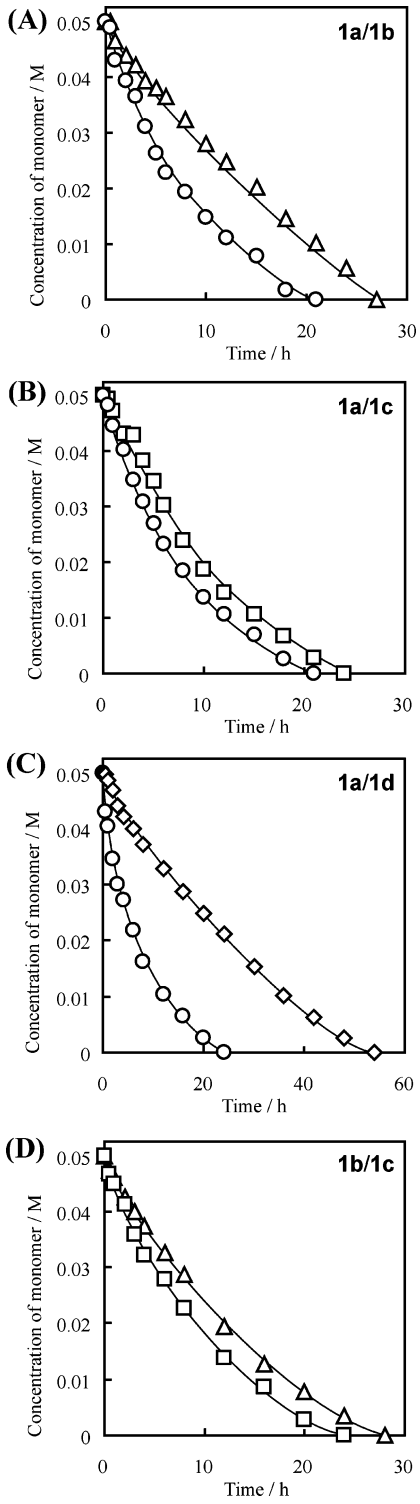

Figure 1. Monomer consumptions in the enzymatic copolymerization of (A) **1a** (○)/**1b** (△); (B) **1a** (○)/**1c** (□); (C) **1a** (○)/**1d** (◇); and (D) **1b** (△)/**1c** (□) with an equimolar feed (total 0.10 M) with OTH at pD 7.5 in D_2O.

protons and the methyl protons by ^{1}H NMR spectroscopy. After 5, 10, and 27 h, a small part of the reaction mixture was sampled and heated at 90 °C for 5 min to inactivate the enzyme. Each mixture was purified by HPLC through a Shodex OHpak SB-803HQ column using 0.1 M aqueous $NaNO_3$ as the eluent followed by dialysis against distilled water using a Spectra/Por CE dialysis membrane (molecular weight cut off: 1000) to afford **2ab**.

Enzymatic Copolymerization of 1a/1b, 1a/1c, 1a/1d, and 1b/1c. A mixed solution of **1a** (5.0 mg, 12.5 μmol) and **1b** (5.1 mg, 12.3 μmol) in a carbonate buffer (50 mM, pH 7.5, 248 μL) was incubated with OTH (1.0 mg) at 30 °C. The consumption of **1a** and **1b** was monitored by HPLC analysis. After 48 h, the resulting suspension was heated at 90 °C for 5 min to inactivate the enzyme. A small portion of the mixture was analyzed by SEC measurements (yield 69%, M_n 6900, and M_w 18000). The reaction mixture was purified by HPLC through a Shodex OHpak SB-803HQ column using 0.1 M aqueous $NaNO_3$ as the eluent. The combined fractions were desalted by dialysis against distilled water using a Spectra/Por CE dialysis membrane (molecular weight cut off: 1000) to give **2ab** (4.3 mg, 43%): ^{1}H NMR (400 MHz, D_2O, acetone) δ 6.30−6.15 (m, 1H, CH_AH$_B$=CH$_C$, CH$_A$H$_B$=CH_C), 5.75 (d, 0.50H, J = 8.43 Hz, CH$_A$$H_B$ = CH$_C$), 4.59 (m, 0.50H, H-1**b**), 4.53 (m, 0.50H, H-1**a**), 4.46 (d, 1H, J = 7.03 Hz, H-1′×2), 3.98−3.68 (m, 6H, H-2 × 2, H-3 × 2, H-6 × 4, H-4′×2, H-5′×2), 3.60−3.44 (m, 3H, H-4 × 2, H-5 × 2, H-3′×2), 3.33 (m, 1H, H-2′×2), 2.00 (s, 1.50H, CH_3CO); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.54, 174.81 (C-6′×2), 169.84 (NH*C*O), 130.77 (*C*H$_2$=CH), 128.47 (CH$_2$=*C*H), 103.69 (C-1′), 100.79 (C-1**b**), 100.12 (C-1**a**), 83.23 (C-3), 80.48 (C-4′**a**), 79.95 (C-4′**b**), 76.78 (C-5′), 76.01 (C-5), 74.12 (C-3′), 73.09 (C-2′), 69.08 (C-4), 61.13 (C-6), 54.96 (C-2), 23.10 (*C*H$_3$CO).

A solution of the mixture of **1a** (5.0 mg, 12.5 μmol) and **1c** (5.2 mg, 12.5 μmol) in a carbonate buffer (50 mM, pH 7.5, 250 μL) was incubated with OTH (1.0 mg) at 30 °C. The consumption of **1a** and **1c** was monitored by HPLC analysis. After 48 h, the resulting suspension was heated at 90 °C for 5 min to inactivate the enzyme. A small amount of the mixture was analyzed by SEC measurements (yield 59%, M_n 6300, and M_w 17300). The mixture was purified by HPLC on a SEC column (Shodex OHpak SB-803HQ, 0.1 M aqueous $NaNO_3$). The combined fractions were desalted by dialysis against distilled water using a Spectra/Por CE dialysis membrane (molecular weight cut off: 1000) to provide **2ac** (4.3 mg, 49%): ^{1}H NMR (400 MHz, D_2O, acetone) δ 4.61−4.55 (m, 1H, H-1 ×2), 4.47 (d, 1H, J = 6.53 Hz, H-1′×2), 3.92−3.70 (m, 7H, H-2 ×2, H-3 × 2, H-6 ×4, H-2′ ×2, H-4′ ×2, H-5′ ×2), 3.58−3.45 (m, 3H, H-4 ×2, H-5 ×2, H-3′ ×2), 3.39−3.31 (m, 1H, H-2′ ×2), 2.29 (m, 1H, CH$_3$CH_2), 2.01 (s, 1.5H, CH_3CO), 1.10 (t, 1.5H, J = 7.53 Hz, CH_3CH$_2$).

A mixture of compounds **1a** (4.6 mg, 11.5 μmol) and **1d** (4.9 mg, 11.4 μmol) in a carbonate buffer (50 mM, pH 7.5, 229 μL) were incubated with OTH (1.0 mg) at 30 °C. The consumption of **1a** and **1d** was monitored by HPLC analysis. After 48 and 60 h, an aliquot of the reaction mixture was sampled and heated at 90 °C for 5 min to inactivate the enzyme. A small amount of the mixture was analyzed by SEC measurements (yield 49%, M_n 4600, and M_w 11400 at 48 h; yield 54%, M_n 4300, and M_w 10400 at 60 h). Each mixture was purified by HPLC through a Shodex OHpak SB-803HQ column using 0.1 M aqueous $NaNO_3$ as the eluent followed by dialysis against distilled water using a Spectra/Por CE dialysis membrane (molecular weight cut off: 1000) to afford **2ad** (27% at 48 h, 32% at 60 h): ^{1}H NMR (400 MHz, D_2O, acetone) δ 4.55 (d, 1H, J = 6.53 Hz, H-1 ×2), 4.45 (d, 1H, J = 6.53 Hz, H-1′ ×2), 3.92−3.71 (m, 7H, H-2 ×2, H-3 ×2, H-6 ×4, H-2′ ×2, H-4′ ×2, H-5′ ×2), 3.59−3.45 (m, 3H, H-4 ×2, H-5 ×2, H-3′ ×2), 3.34 (t, 1H, H-2′ ×2), 2.20 (m, 0.28H, CH$_3$CH$_2$CH_2), 2.01 (s, 2.58H, CH_3CO), 1.61 (m, 0.28H, CH$_3$CH_2CH$_2$), 0.93 (m, 0.42H, CH_3-CH$_2$CH$_2$).

Compounds **1b** (2.1 mg, 5.1 μmol) and **1c** (2.1 mg, 5.1 μmol) were mixed in a carbonate buffer (50 mM, pH 7.5, 102 μL) and then incubated with OTH (0.4 mg) at 30 °C. The consumption of **1b** and **1c** was monitored by HPLC analysis. After 48 h, the enzyme was thermally inactivated at 90 °C for 5 min. HPLC purification of the mixture was carried out with a Shodex OHpak SB-803HQ column using 0.1 M aqueous $NaNO_3$ as the eluent followed by dialysis against distilled water using a Spectra/Por CE dialysis membrane (molecular weight cut off: 1000) to provide **2bc** (1.7 mg, 40%): ^{1}H NMR (400 MHz, D_2O, acetone) δ 6.24−6.22 (m, 0.98H, CH_AH$_B$=CH$_C$, CH$_A$H$_B$=CH_C), 5.78 (d, 0.49H, J = 8.54 Hz, CH$_A$$H_B$=CH$_C$), 4.58 (m, 1H, H-1 ×2), 4.42 (m, 1H, H-1′ ×2), 3.93−3.67 (m, 7H, H-2 ×2, H-3 ×2, H-6 ×4, H-2′ ×2, H-4′ ×2, H-5′ ×2), 3.55−3.49 (m, 3H, H-4 ×2, H-5 ×2, H-3′

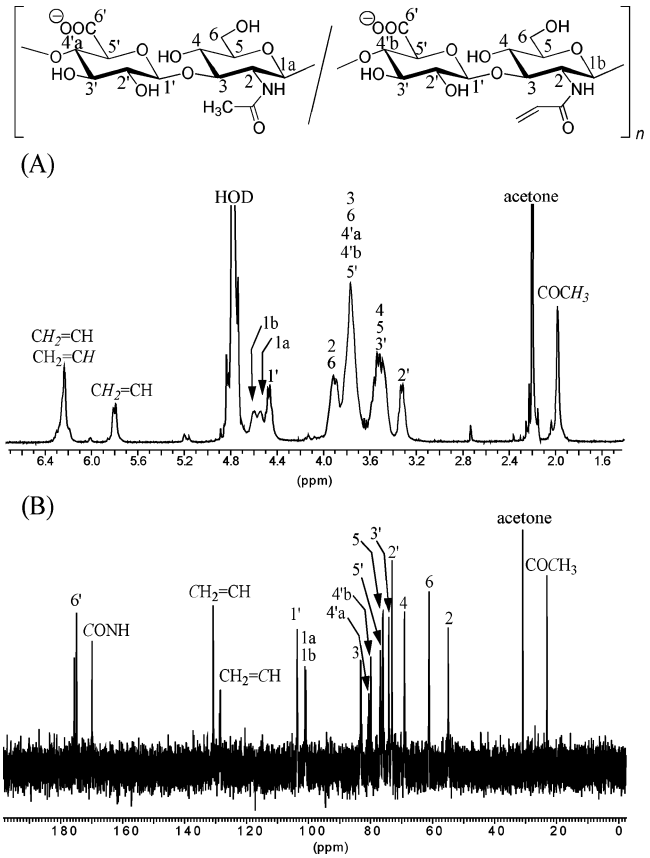

Figure 2. (A) ^{1}H and (B) ^{13}C NMR spectra of **2ab**.

×2), 3.32 (m, 1H, H-2′ ×2), 2.30 (m, 1. H, CH_3CH_2), 1.10 (t, 1.5H, J = 7.28 Hz, CH_3CH_2).

Results and Discussion

Copolymerization of monomer **1a** with **1b** with OTH catalysis was carried out at a monomer feed ratio of 0.50/0.50. Figure 1A shows the monomer consumption of **1a** and **1b**; **1a** was consumed a little more rapidly than **1b**. On completion of the reaction, the enzyme was thermally inactivated, and the resulting mixture was analyzed by SEC. The SEC chart of the product exhibited a single peak, suggesting that the copolymer is the sole product. Figure 2 shows the ^{1}H (A) and ^{13}C (B) NMR spectra of the purified product. The broad signals at δ 4.59 and 4.53 in A were assigned to the anomeric proton derived from *N*-acryloyl-D-glucosamine and *N*-acetyl-D-glucosamine units, respectively. The signals at δ 6.30−6.15, 5.75, and 2.00 indicate the existence of both *N*-acryloyl and *N*-acetyl groups. The ^{13}C NMR spectrum of the product (Figure 2B) is similar in all respects to the combined spectra of the two homopolymers. These results support the fact that the product (**2ab**) has the copolymer structure having β(1→4)-linked GlcAβ(1→3)GlcNAc and GlcAβ(1→3)GlcNHCOCH=CH_2 repeating units.

MALDI-TOF mass spectroscopy was used for the structural analysis of the copolymer. Figure 3 indicates the spectrum of product **2ab**, obtained by the equimolar reaction of the monomers.[24] Four groups of peaks were observed (1−4). These correspond to tetra (1), hexa (2), octa (3), and decasaccharides (4), which involve **1a** and **1b** units in various proportions. Table 1 gives the data for complete assignments of the peaks. All peaks were assigned as deprotonated ions ([M−H]$^-$). In group 1, the ratio of monomers **1a** and **1b** ranges from 2/0 to 0/2. The peak with maximum intensity at *m*/*z* 787.23 accords with the

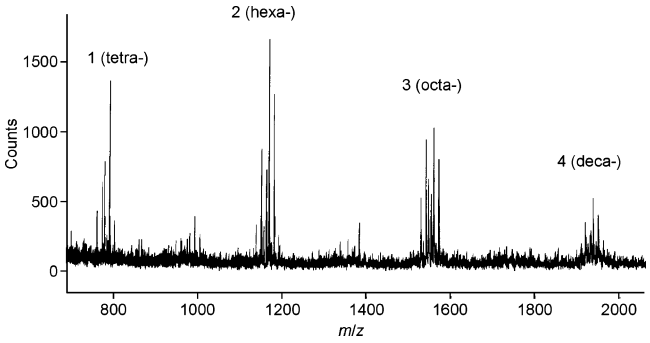

Figure 3. MALDI-TOF mass spectrum of copolymer **2ab** (oligomer portions) obtained from the equimolar reaction of **1a** and **1b**.

Table 1. Analytical Data for the MALDI-TOF Mass Spectrum of Copolymer **2ab** (Oligomer Portions) Obtained from the Equimolar Reaction of **1a** and **1b**

group no.	saccharides	*m*/*z*[a]	peak intensity	**1a**/**1b** unit ratio[b]	relative content in the group/%
1	tetra	775.25	790	2/0	32
		757.25 (−18)	434		
		787.23*	1369	1/1	52
		769.24 (−18)	642		
		799.31	366	0/2	16
		781.30 (−18)	260		
2	hexa	1154.41	329	3/0	12
		1136.32 (−18)	342		
		1166.34*	1675	2/1	44
		1148.33 (−18)	886		
		1178.34	1280	1/2	35
		1160.34 (−18)	736		
		1190.27	266	0/3	9
		1172.50 (−18)	213		
3	octa	1545.33	675	3/1	26
		1527.40 (−18)	539		
		1557.33*	1042	2/2	44
		1539.35 (−18)	957		
		1569.37	817	1/3	30
		1551.33 (−18)	565		
4	deca	1936.39*	536	3/2	56
		1918.46 (−18)	362		
		1948.31	413	2/3	44
		1931.30 (−18)	301		

[a] The value with an asterisk shows the peak at the highest intensity in the group. [b] The ratio was calculated from the combination of the molecular masses of **1a** and **1b** units.

molecular mass of the tetrasaccharides involving the equimolar amount (1/1) of **1a** and **1b** units. Hexasaccharides with a ratio of **1a**/**1b** ranging from 3/0 to 0/3 were observed in group 2. The peak at *m*/*z* 1166.34 with the maximum intensity is derived from the hexasaccharide containing two **1a** units and one **1b** unit. The peak intensities derived from hexasaccharides of homounits, **1a** and **1b**, were low. Group 3 has the top at *m*/*z* 1557.33, giving a ratio of **1a**/**1b** equal to 2/2. No peaks due to homo-octasaccharide units (4/0 or 0/4) of **1a** or **1b** were observed. In group 4, the peaks derived from **1a**/**1b** in 3/2 and 2/3 ratios were found, and the peak at *m*/*z* 1936.39 corresponds to the decasaccharides with **1a** and **1b** in a 3/2 ratio in maximum intensity. These results give direct evidence that monomers **1a** and **1b** were actually copolymerized via an almost random fashion, involving a tendency of monomer **1a** to show a slightly higher reactivity as observed in Figure 1. Notably, the formation

Table 2. Enzymatic Copolymerization of **1a** and **1b**

	copolymerization[a,b]			copolymer (**2ab**)				
	comonomer, feed ratio			composition[c]				
entry	1a	1b	time/h	1a	1b	yield[d]/%	M_n[e]	M_w[e]
1[a]	0.50	0.50	5	0.60	0.40	18	9500	18200
2[a]	0.50	0.50	10	0.56	0.44	29	8600	17400
3[a]	0.50	0.50	27[f]	0.52	0.48	43	6700	12100
4[b]	0.25	0.75	48[f]	0.25	0.75	41	9500	16400
5[b]	0.50	0.50	48[f]	0.50	0.50	43	9700	19000
6[b]	0.75	0.25	48[f]	0.76	0.24	44	9700	19400
7[b]	0.91	0.09	48[f]	0.91	0.09	50	10200	19400

[a] In entries 1−3, copolymerization data were taken from the experiments shown in Figure 1A, in a carbonate buffer at pD 7.5 at 50 mM in D_2O; total monomer concentration, 0.10 M; enzyme, OTH (560 unit/mg), 10 wt % for the total monomers; reaction at 30 °C. [b] In entries 4−7, copolymerizations were performed in a manner similar to that described above, except for using H_2O instead of D_2O at pH 7.5. [c] Determined by ^{1}H NMR, measuring the integration of the methyl proton of the *N*-acetyl group and the vinyl protons of the *N*-acryloyl group. [d] Isolated yields after purification (weight of the isolated copolymer/weight of the feed comonomers × 100). [e] Determined by SEC calibrated with hyaluronan standards. [f] Indicating the time for complete monomer consumption.

Table 3. Enzymatic Copolymerization of **1a**/**1c**, **1a**/**1d**, and **1b**/**1c**

	copolymerization[a]			copolymer					
	comonomer, feed ratio				composition[c]				
entry	**I**	**II**	time[b]/h	structure	I	II	yield[d]/%	M_n[e]	M_w[e]
1	**1a** (0.50)	**1c** (0.50)	48	**2ac**	0.50	0.50	49	10200	18700
2	**1a** (0.50)	**1d** (0.50)	48[f]	**2ad**	0.86	0.14	27	8300	18100
3	**1a** (0.50)	**1d** (0.50)	60	**2ad**	0.78	0.22	32	8100	17400
4	**1b** (0.50)	**1c** (0.50)	48	**2bc**	0.51	0.49	40	9200	17100

[a] In a carbonate buffer at pH 7.5 at 50 mM in H_2O; total monomer concentration, 0.10 M; enzyme, OTH (560 unit/mg), 10 wt % for the total monomers; reaction at 30 °C. [b] Indicating the time for the complete consumption of both monomers except for entry 2. [c] Determined by ^{1}H NMR measurements. [d] Isolated yields after purification ((weight of the isolated copolymer/weight of the feed comonomers) × 100). [e] Determined by SEC calibrated with hyaluronan standards. [f] Indicating the time for the complete consumption of **1a**; **1d** remained partly unreacted.

of more than four successive homounits of comonomer **1a** or **1b** was not detected in the copolymer molecule. In the MALDI-TOF mass spectroscopic analysis, peak intensity does not always quantitatively reflect the amount of the peak's compound. However, relative intensity for compounds with similar mass and structure may afford significant information regarding the relative amount of the compound produced in the same group. Therefore, from peak intensity values, the relative content (%) in the four groups was calculated and given in Table 1 for reference.

Table 2 shows the results of the OTH-catalyzed copolymerization of **1a** and **1b** to produce copolymer **2ab** bearing *N*-acetyl and *N*-acryloyl groups in varied proportions on the HA chain. In entries 1−3, the copolymerization was carried out as shown in the experiment in Figure 1A, with sampling the reaction mixture after 5, 10, and 27 h. In accord with the observation in Figure 1A, the composition of the isolated copolymer **2ab** was rich in **1a** units when the reaction time was shorter; the content of the **1a** unit decreased from 0.60 to 0.56 and eventually to 0.52 after 27 h when both monomers were completely consumed. In entries 4−7, the copolymerization was performed by varying the feed ratio of **1a** and **1b** until both monomers completely disappeared (48 h),[25] giving rise to copolymer **2ab** in isolated yields between 41 and 50%. The copolymer compositions were very close to or identical to that of the feed ratio of **1a** and **1b** in these four runs. In all runs (entries 1−7), the SEC chart of **2ab** showed a single peak, supporting the fact that **2ab** is not a mixture of homopolymers but a copolymer derived from **1a** and **1b**. The M_n value of the product reached 10 200 (approximately 50 saccharides).

Enzymatic copolymerization of three combinations in **1a**/**1c**, **1a**/**1d**, and **1b**/**1c** was carried out with the feed ratio of 0.50/0.50 in all runs under reaction conditions that were similar to those in Figure 1A. The consumption rates of comonomers are shown in Figure 1B, C, and D. In both copolymerizations of **1a**/**1c** (Figure 1B) and **1b**/**1c** (Figure 1D), the comonomers exhibited a close copolymerization reactivity, whereas in the case of **1a**/**1d** (Figure 1C), comonomer **1d** showed much less reactivity.

Table 3 indicates these copolymerization results. The copolymerization of **1a** with **1c** afforded copolymer **2ac** with M_n 10 200 (approximately 50 saccharides) in 49% yield (entry 1). The copolymer composition, determined by ^{1}H NMR, was identical to the feed ratio in this case. In contrast, the content of **1d** in copolymer **2ad** was relatively low (entries 2 and 3); it was 0.14 after 48 h when **1a** was completely consumed and increased to 0.22 after 60 h when both monomers were completely consumed. In entry 4, copolymer **2bc** was obtained by the copolymerization of **1b** with **1c** in 40% yield (M_n 9200, approximately 44 saccharides), and its composition was very close to the feed ratio. All of the results are qualitatively in accord with the observations in Figure 1. SEC charts of products **2ac**, **2ad**, and **2bc** revealed a single peak, suggesting that copolymers are the sole products. The structures of these copolymers were definitely determined by ^{1}H and ^{13}C NMR as HA derivatives with different amido-pendent groups in the glucosamine unit. It should be pointed out that each monomer has different reactivity in the enzyme catalysis as estimated from the different complete consumption times in copolymerization (Figure 1). Therefore, a higher reactive monomer will be easier to be incorporated into a copolymer structure rather than a lower one, suggesting the formation of a copolymer with an intramolecular gradient structure. Indeed, in the cases of monomer combinations of **1a**/**1b** and **1a**/**1d**, the composition of **1a** unit in each copolymer decreased with the increase in reaction time (entries 1−3 in Table 2, and entries 2 and 3 in Table 3); in both copolymerizations, **1a** was consumed faster than the others, suggesting that **1a** was more reactive.

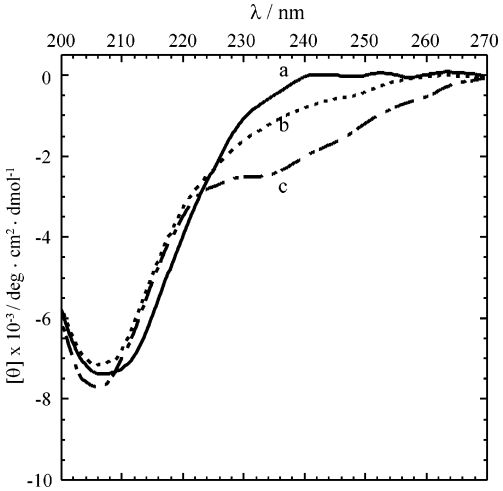

Figure 4. Circular dichroism of (a) synthetic HA (M_n = 14000), (b) **2ab** (M_n = 5000), and (c) HA derivative with an *N*-acryloyl group in all glucosamine units (M_n = 5100). Concentration of the sample was 1.00 mM.

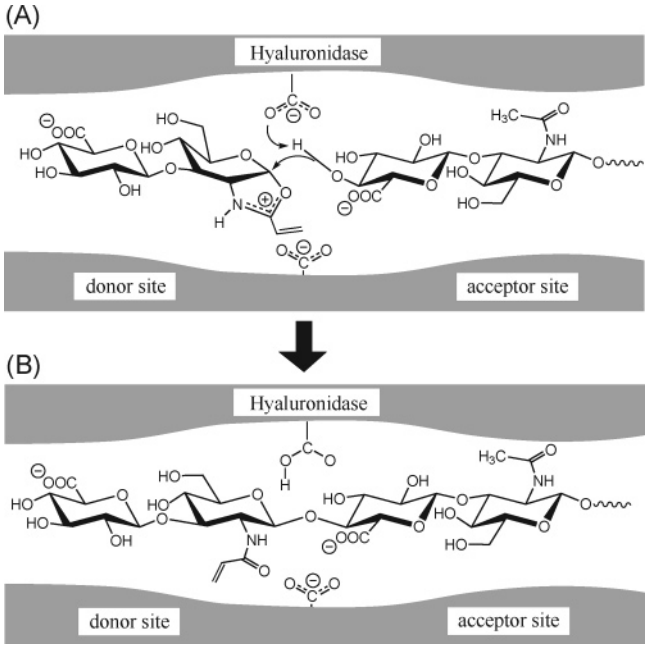

Figure 5. A postulated transition state of the copolymerization of **1a** and **1b** to copolymer **2ab** showing the cross-propagation.

Circular dichroism for copolymer **2ab** as well as the natural type HA (Figure 4a; a homopolymer produced by the polymerization of **1a**) and HA derivative with the *N*-acryloyl group in all glucosamine units (Figure 4c; a homopolymer produced by the polymerization of **1b**)[20b] are shown in Figure 4. In Figure 4b and c, the dichroism was observed at 205 nm because of the amido group and at the 220−270 nm region because of the conjugated amido group. For natural type HA (Figure 4a), the dichroism was observed at 207 nm because of the amido group, which is very similar to that of the natural HA reported previously.[26]

Copolymerization between two monomers involves four elementary reactions. Figure 5 illustrates a postulated transition state for the cross-propagation step in the copolymerization of monomer **1a** with **1b**, which is to be compared with that of homopolymerization.[20] The C1 carbon of the protonated oxazolinium species of **1b** in the donor site is nucleophilically attacked from the β-side by 4′-OH group of the monomer **1a** unit placed at the propagating-chain end in the acceptor site to open the oxazolinium ring (Figure 5A), giving rise to the $\beta(1\rightarrow4)$ glycosidic bond formation (Figure 5B). The other cross-propagation is the reverse way, the glycosidation of the oxazolinium species from **1a** being attacked by the 4′-OH group of the **1b** unit placed at the propagating-chain end. Additionally, two homopropagations of **1a** and **1b** take place.

Conclusions

Enzymatic copolymerization was successfully induced by HAase catalysis, which produces HA derivatives bearing different *N*-acyl groups in the polymer chain in various proportions. The present method permits a tailored production of various HA derivatives for medical and pharmaceutical uses, if necessary. Some of these HA derivatives contain a reactive vinyl group capable of cross-linking via radical process, which will lead to macromonomers, telechelics, and graft copolymers in future work. They have the potential to serve as new HA-related biomaterials. Furthermore, the present reaction allows the production of various hybrid GAG derivatives with different sugar units. Related works are in progress to prepare such GAG derivatives.

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