Enzymatic Copolymerization to Hybrid Glycosaminoglycans: A Novel Strategy for Intramolecular Hybridization of Polysaccharides

Hirofumi Ochiai, Shun-Ichi Fujikawa, Masashi Ohmae, and Shiro Kobayashi*,†

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

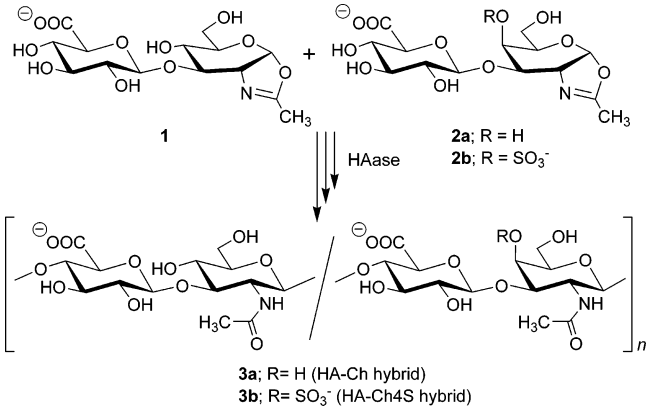
Received January 9, 2007; Revised Manuscript Received April 5, 2007

Hybrid glycosaminoglycans (GAGs) having an intramolecularly hybridized structure of hyaluronan−chondroitin (**3a**) and hyaluronan−chondroitin 4-sulfate (**3b**) have been synthesized via enzymatic copolymerization catalyzed by hyaluronidase (HAase). *N*-Acetylhyalobiuronate (GlcAβ(1→3)GlcNAc)-derived oxazoline (**1**) was copolymerized with *N*-acetylchondrosine (GlcAβ(1→3)GalNAc)-derived oxazoline (**2a**) by HAase catalysis at pH 7.5 and 30 °C, giving rise to copolymer **3a** with M_n 7.4 × 10^3 in a 50% yield. Also, HAase-catalyzed copolymerization of monomer **1** with *N*-acetylchondrosine oxazoline having a sulfate group at C4 on GalNAc (**2b**) was carried out to produce copolymer **3b** with M_n 1.4 × 10^4 in a 60% yield. The copolymer compositions were controllable by varying the comonomer feed ratio. These hybrid GAGs were successfully digested by the catalysis of hyaluronan lyase, clearly exhibiting that the products are not a blend of different homopolymers but an intramolecularly hybridized GAG.

Introduction

Hybrid polysaccharides are novel biomacromolecules composed of different kinds of monosaccharides.[1−4] These are unnatural yet biodegradable polysaccharides with uniform structures synthesized via enzymatic homopolymerization of molecularly designed carbohydrate monomers through nonbiosynthetic pathways.[5] Recently, a novel hybrid glycosaminoglycan[6] (GAG) consisting of chondroitin sulfate and dermatan sulfate (ChS/DS hybrid) was found in vivo and has attracted much attention for its unique bioactivities, particularly in the development of the central nervous system.[7] Such a hybrid GAG is synthesized in vivo through multiple enzymatic actions by specific glycosyltransferases, sulfotransferases, and glucuronate C-5 epimerase.[8] The hybrid has been obtained to date only by extraction from animal tissues, which requires complicated manipulations; therefore, efficient methods for preparing hybrid GAGs have been strongly demanded. Chimeric GAGs have been prepared by transglycosylation utilizing hyaluronidase (EC 3.2.1.35; HAase) catalysis.[9] The method is very interesting; however, the products were obtained as oligosaccharides in very low yields. Very recently, another method to prepare chimeric GAGs employing hyaluronan (HA) synthase and chondroitin (Ch) synthase from *Pasteurella multocida* was reported[10] in which a variety of HA and ChS oligosaccharides were used as acceptors with UDP−sugar donors. This method is also effective, but the strict selectivity of the donor site of the enzyme limits the growing chain structures (β(1→4)-linked *N*-acetylhyalobiuronate by HA synthase and β(1→4)-linked *N*-acetylchondrosine by Ch synthase). Thus, flexible chain designs of the growing polymers are difficult; particularly, those with a sulfate moiety are almost impossible.

Scheme 1. Hyaluronidase-Catalyzed Copolymerizations to HA−Ch (**3a**) and HA−Ch4S (**3b**) Hybrids



Recently, we have demonstrated the successful synthesis of GAG polymers by HAase-catalyzed polymerizations.[11] In these polymerizations, a single kind of enzyme catalyzed multiple homopolymerizations of different sorts of sugar oxazoline monomers, giving rise to diverse GAGs such as synthetic HA,[11a] Ch,[11b] and their derivatives[11b−d] with well-defined structures via ring-opening polyaddition. These important findings motivated us to synthesize hybrid GAGs incorporating different repeating sugar units within a molecule via a single-step copolymerization catalyzed by HAase. In this paper, we report a novel and facile method for the synthesis of hybrid GAGs, particularly HA−Ch (**3a**) and HA−Ch-4-sulfate (Ch4S) hybrids (**3b**) by HAase-catalyzed copolymerizations of different sets of sugar oxazoline monomers (**1**, **2a**, and **2b**) (Scheme 1).[12] The monomers were designed according to our concept of a transition-state analogue substrate (TSAS) monomer.[5] The present reaction is the first example of copolymerization, in which the monomers producing the polymers with different main-chain structures have been enzymatically copolymerized. Furthermore, the hybrid with the sulfate group was efficiently produced by the present method,

* Author to whom correspondence should be addressed. Phone/Fax: +81-75-724-7688. E-mail: kobayash@kit.ac.jp.

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

10.1021/bm0700340 CCC: $37.00
Published on Web 05/10/2007

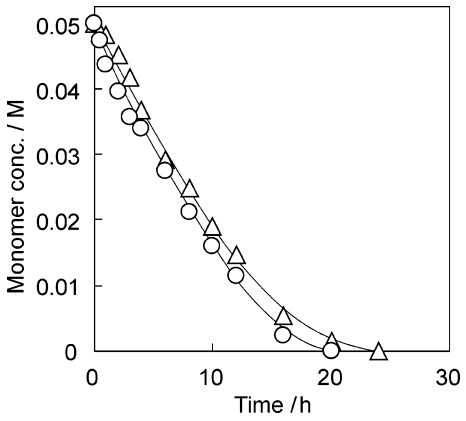


Figure 1. Reaction time courses of **1** (○) and **2a** (△) at equimolar feed ratios (total 0.10 M) with OTH at pH 7.5.

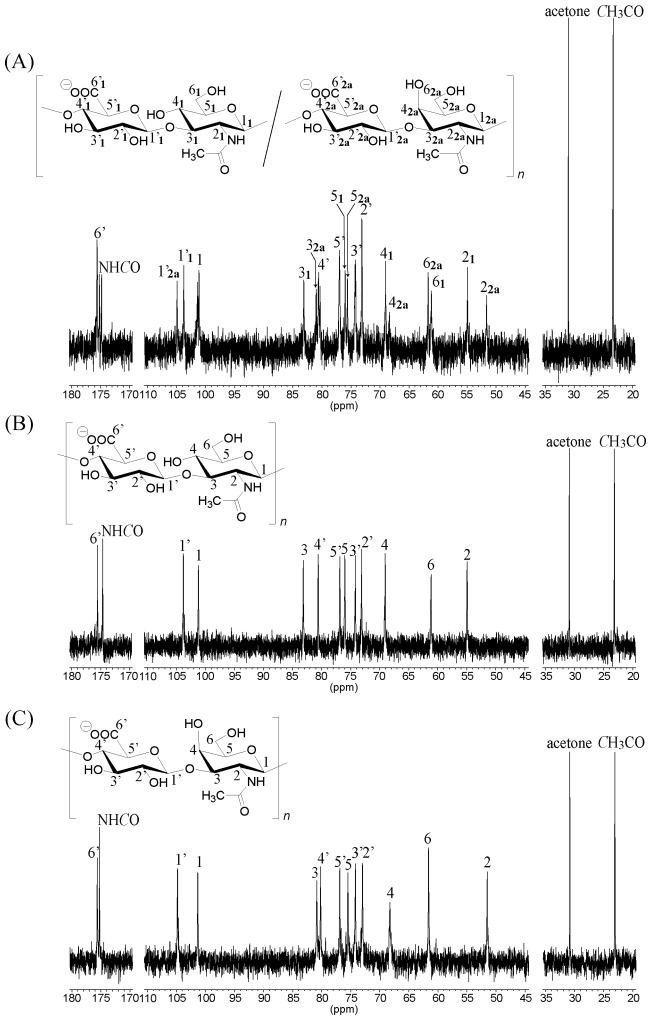


Figure 2. ^{13}C NMR spectra of (A) copolymer **3a**, (B) natural HA, and (C) natural Ch.

which is difficult to generate via a biosynthetic synthase system. Thus, this study is the first step leading to the tailor-made synthesis of hybrid GAGs with ordered sequences, which will be a potent tool in medical and pharmaceutical uses.

Experimental Section

Measurements. NMR spectra were recorded on a Bruker DPX-400 spectrometer. For solutions in D_2O, acetone served as a reference at δ 2.22 (for ^1H) and 30.89 (for ^{13}C). High-performance liquid

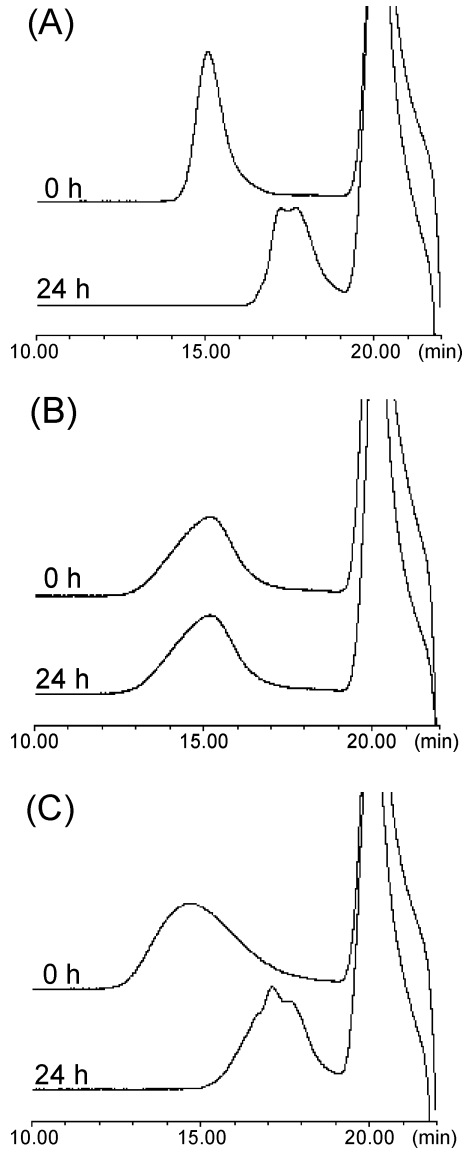


Figure 3. SEC profiles of (A) HA, (B) Ch, and (C) **3a** in *S. hyalurolyticus* hyaluronidase digestion at 0 and 24 h.

chromatography (HPLC) was performed using a Tosoh LC8020 system equipped with refractive index (RI) and UV detectors under the following conditions: Shodex Asahipak NH2P-50 4E column (4.6 mm × 250 mm) and phosphate buffer (10 mM, pH 7.0)−MeCN mixed solution (30:70, v/v) as the 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 a RI detector under the following conditions: Shodex OHpak SB-803HQ column (8.0 mm × 300 mm) and 0.1 M aqueous $NaNO_3$ as the 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 the standard. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis was performed with a Jeol JMS-ELITE spectrometer by using 2,5-dihydroxybenzoic acid as a matrix on a Nafion-coated plate[13] under negative ion mode.

Materials. Monomers **1**,[11a] **2a**,[11b] and **2b**[11d] were prepared according to the procedures reported previously. Ovine testicular HAase (OTH; lot no. 9303B, 560 units/mg, and lot no. 122k1378, 3720 units/mg) was purchased from ICN Biochemicals, Inc. The hyaluronan lyase, hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.1), was obtained from Seikagaku Co. (Tokyo, Japan). All enzymes were used without further purification.

Consumption of Comonomers in Enzymatic Copolymerization. A typical procedure for monitoring comonomer consumption is given

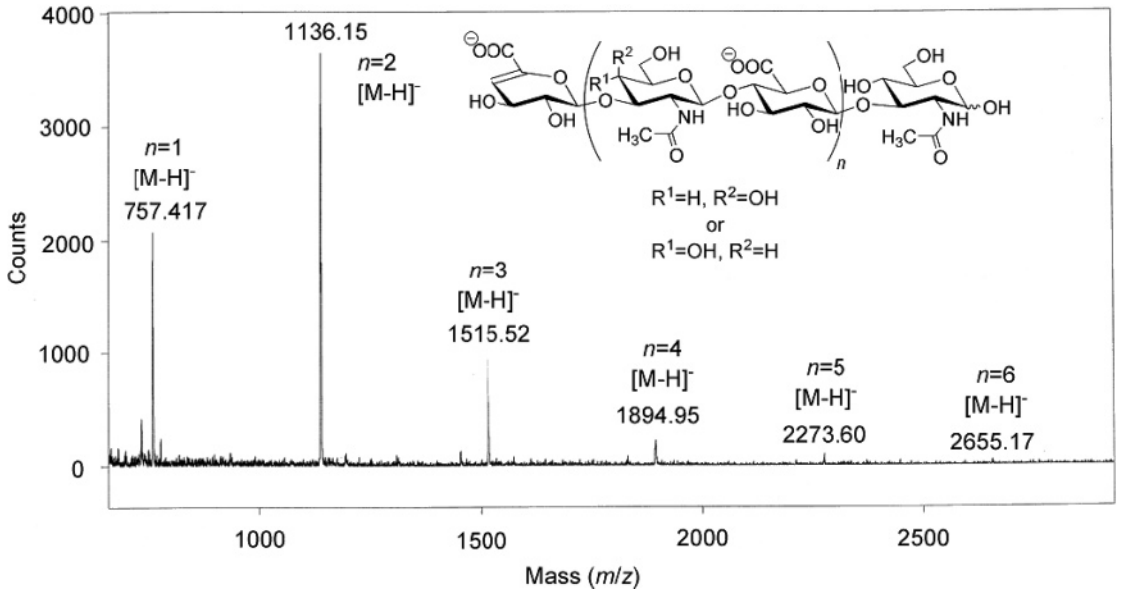


Figure 4. MALDI-TOF mass spectrum of the oligosaccharides having a 4,5-unsaturated terminal unit obtained by *S. hyalurolyticus* hyaluronidase digestion of **3a**.

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

	copolymerization[a]			copolymer (**3a**)				
	comonomer, feed ratio			composition[b]				
entry	**1**	**2a**	time/h	**1**	**2a**	yield[c]/%	M_n[d] $\times 10^{-3}$	M_w[d] $\times 10^{-3}$
1	1	0	3.5	1	0	55	15	25
2	0.75	0.25	4.0	0.75	0.25	53	8.0	15
3	0.50	0.50	4.0	0.51	0.49	50	7.4	14
4	0.25	0.75	4.0	0.26	0.74	48	5.8	11
5	0	1	2.0	0	1	45	5.0	7.5

[a] In 50 mM phosphate buffer at pH 7.5; total monomer concentration, 0.10 M; enzyme, OTH (3720 units/mg),[15] 10 wt % for total monomers; reaction at 30 °C. [b] Determined by ^{1}H NMR analysis. [c] Isolated yields after purification [(weight of the isolated copolymer/weight of the feed comonomers) × 100]. [d] Determined by SEC calibrated with hyaluronan standards.

as follows: Compounds **1** (2.0 mg, 5.0 μmol) and **2a** (2.0 mg, 5.0 μmol) were mixed and dissolved in a phosphate buffer (50 mM, pH 7.5, 100 μL). Then the mixture was incubated with OTH (0.4 mg) at 30 °C. During the reaction, a small portion (5.0 μL) of the mixture was sampled at desired intervals, and 1 M aqueous NaOH (1.0 μL) was added to terminate the reaction. The mixture was lyophilized, dissolved in D_2O, and analyzed by ^{1}H NMR. Concentrations of **1** and **2a** were calculated from the integration values of the signals from the H-2 proton of **1**, H-4 proton of **2a**, and the methyl protons. Concentrations of **1** and **2b** were calculated from the integration values of the signals from the H-1 protons of **1** and **2b**, H-2 proton of **1**, and the methyl protons.

Enzymatic Copolymerization of 1/2a and 1/2b. A mixture of **1** (10.0 mg, 24.9 μmol) and **2a** (10.0 mg, 24.9 μmol) in a phosphate buffer (50 mM, pH 7.5, 498 μL) was incubated with OTH (2.0 mg) at 30 °C. Consumptions of **1** and **2a** were monitored by HPLC measurements with a Shodex Asahipak NH2P-50 4E column (4.6 mm × 250 mm) eluting with phosphate buffer (10 mM, pH 7.0)−MeCN mixed solution (30:70, v/v; flow rate, 0.5 mL/min; 30 °C). After complete consumption of comonomers, the mixture was heated at 90 °C for 5 min to inactivate the enzyme. 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 **3a** (10.0 mg, 50%). ^{1}H NMR (400 MHz, D_2O, acetone): δ 4.59−4.43 (m, 2H, H-1, H-1′), 4.10 (s, 0.49H, H-4$_{2a}$), 4.02−3.41 (m, 8.51H, H-2, H-3, H-4$_1$, H-5, H-6a, H-6b, H-3′, H-4′, H-5′), 3.34 (m, 1H, H-2′), 2.00 (s, 3H, CH_3CO). ^{13}C NMR (100 MHz, D_2O, acetone): δ 175.61, 175.22, 174.82, 174.78 (C-6′, NH*C*O), 104.92 (C-1′$_{2a}$), 103.74 (C-1′$_1$), 101.45 (C-1$_{2a}$), 101.15 (C-1$_1$), 83.10 (C-3$_1$), 80.97 (C-3′$_{2a}$), 80.55 (C-4′$_1$), 80.32 (C-4′$_{2a}$), 76.95 (C-5′$_1$, C-5′$_{2a}$), 75.98 (C-5$_1$), 75.58 (C-5$_{2a}$), 74.28, 74.18 (C-3′$_1$, C-3′$_{2a}$), 73.09 (C-2′$_1$, C-2′$_{2a}$), 69.03 (C-4$_1$), 68.31 (C-4$_{2a}$), 61.71 (C-6$_{2a}$), 61.15 (C-6$_1$), 54.91 (C-2$_1$), 51.61 (C-2$_{2a}$), 23.11 (*C*H_3CO).

A solution of the mixture of **1** (2.0 mg, 5.0 μmol) and **2b** (2.5 mg, 5.0 μmol) in a phosphate buffer (50 mM, pH 7.5, 100 μL) was incubated with OTH (0.45 mg) at 30 °C. After the complete consumption of the comonomers, the mixture was heated at 90 °C for 5 min to inactivate the enzyme. The product 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 provide **3b** (2.7 mg, 60%). ^{1}H NMR (400 MHz, D_2O, acetone): δ 4.71 (m, 0.49H, H-4$_{2b}$), 4.66−4.51 (m, 2H, H-1, H-1′), 4.02−3.41 (m, 8.51H, H-2, H-3, H-4$_1$, H-5, H-6a, H-6b, H-3′, H-4′, H-5′), 3.34 (m, 1H, H-2′), 2.00 (s, 3H, CH_3CO). ^{13}C NMR (100 MHz, D_2O, acetone): δ 175.44, 173.41 (C-6′, NHCO), 104.37 (C-1′$_{2b}$), 103.56 (C-1′$_1$), 101.57, 101.37 (C-1$_1$, C-1$_{2b}$), 82.89 (C-3$_1$), 80.88 (C-4′$_{2b}$), 80.67 (C-4′$_1$), 76.96 (C-4$_{2b}$, C-5′$_1$, C-5′$_{2b}$), 76.31 (C-3$_{2b}$), 75.92 (C-5$_1$), 75.62 (C-5$_{2b}$), 74.16 (C-3′$_1$, C-3′$_{2b}$), 72.76 (C-2′$_1$, C-2′$_{2b}$), 68.88 (C-4$_1$), 61.59 (C-6$_{2b}$), 61.10 (C-6$_1$), 54.89 (C-2$_1$), 52.15 (C-2$_{2b}$), 23.04 (*C*H_3CO).

The copolymer composition in **3a** was determined from ^{1}H NMR signals by the integration value of the H-4 proton of the Ch unit (δ 4.10) and the H-1 protons of the HA and Ch units (δ 4.59−4.43). The copolymer composition in **3b** was calculated from the integration value of ^{1}H NMR signals assigned to the H-4 proton of the Ch4S unit (δ 4.71) and the H-1 protons of the HA and Ch units (δ 4.66−4.51).

Enzymatic Digestion of HA, Ch, 3a, and 3b. A typical procedure for enzymatic digestion of HA, Ch, **3a**, and **3b** is given as follows: Hybrid GAG **3a** (M_n 4.7 × 10^3, 1.0 mg) in an acetate buffer (20 mM,

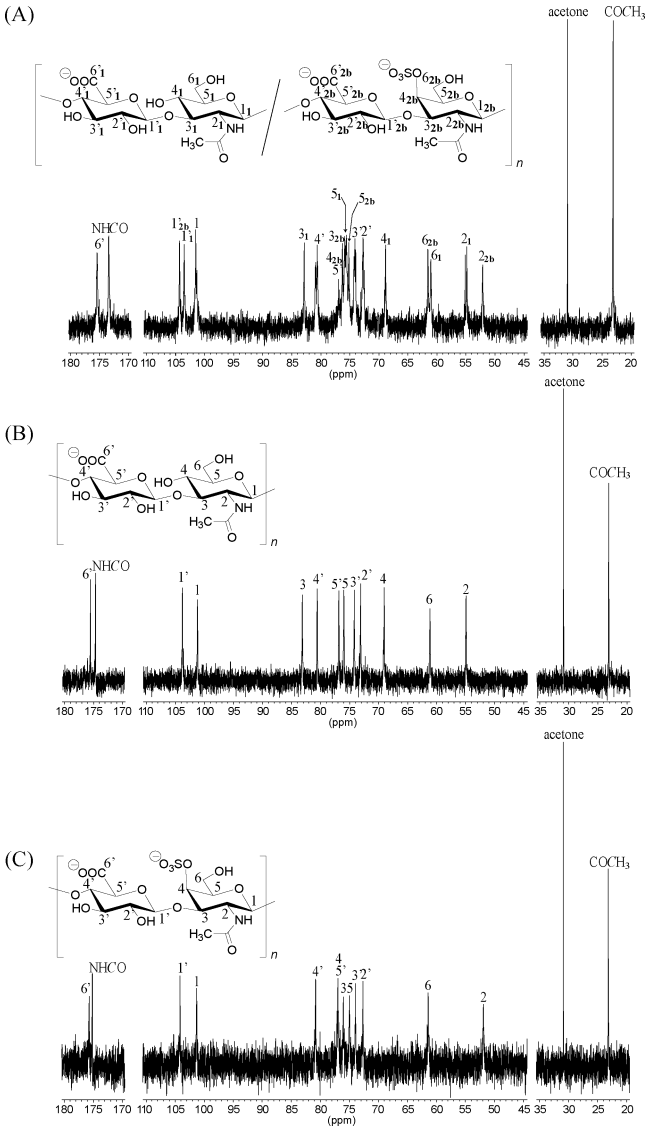


Figure 5. ^{13}C NMR spectra of (A) copolymer **3b** obtained by copolymerization at equimolar feed ratios, (B) natural HA, and (C) synthetic Ch4S.

pH 6.0, 400 μL) was incubated with *S. hyalurolyticus* hyaluronidase (5 TRU) at 60 °C. After 24 h, an aliquot of the reaction mixture was sampled and heated at 90 °C for 5 min to inactivate the enzyme, then analyzed by SEC measurements.

Results and Discussion

Copolymerization of 1 with 2a. Copolymerization of **1** with **2a** was carried out at 30 °C with OTH (560 units/mg) catalysis at a comonomer feed ratio of 0.50:0.50. The copolymerization proceeded homogeneously during the reaction. Comonomers **1** and **2a** were consumed effectively with comparative rates (Figure 1).

After complete consumption of the comonomers, the enzyme was thermally inactivated, and the mixture was analyzed by SEC. Only a single peak was observed on the chromatogram, suggesting the formation of copolymer **3a** without homopolymers. The ^{13}C NMR spectrum of the product (Figure 2A) is very close to the combined spectrum of HA (Figure 2B) and Ch (Figure 2C) homopolymers. These results support that the product is copolymer **3a** having β(1→4)-linked GlcAβ(1→3)-GlcNAc and GlcAβ(1→3)GalNAc repeating units.

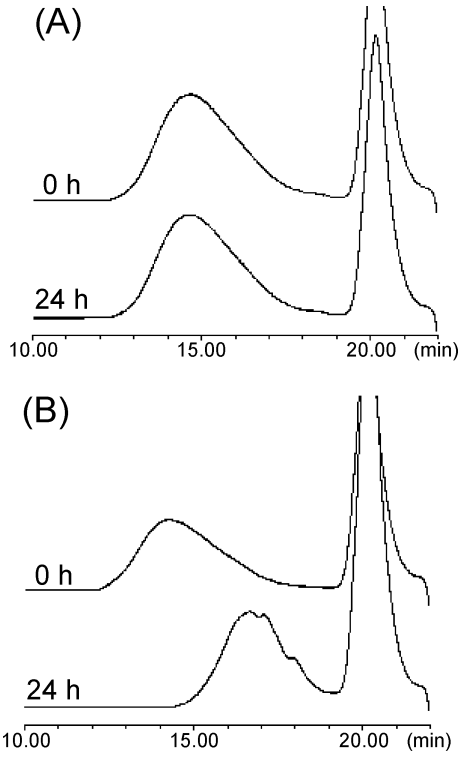


Figure 6. SEC profiles of (A) Ch4S and (B) **3b** in *S. hyalurolyticus* hyaluronidase digestion at 0 and 24 h.

The structure of **3a** was further confirmed by enzymatic digestion with hyaluronidase from *S. hyalurolyticus* (EC 4.2.2.1).[14] This enzyme is a lyase catalyzing endolytic cleavage of the β-(1→4)-*N*-acetylglucosaminide linkage by β-elimination exclusively in HA, which does not cleave the other GAG bonds. Synthetic HA and Ch and **3a** were treated with the lyase for 24 h and then analyzed by SEC (Figure 3). HA was digested by the enzyme effectively, mainly yielding a mixture of unsaturated hexa- and tetrasaccharides (Figure 3A). Ch was not degraded by the enzyme at all (Figure 3B). Copolymer **3a** was successfully digested as shown in Figure 3C. MALDI-TOF/MS analysis of this digested mixture showed the formation of oligosaccharides having an unsaturated terminal unit up to tetradecasaccharide (Figure 4); *m*/*z* 757.417 ([M − H]$^-$, $n = 1$, tetrasaccharide), 1136.15 ([M − H]$^-$, $n = 2$, hexasaccharide), 1515.52 ([M − H]$^-$, $n = 3$, octasaccharide), 1894.95 ([M − H]$^-$, $n = 4$, decasaccharide), 2273.60 ([M − H]$^-$, $n = 5$, dodecasaccharide), 2655.17 ([M − H]$^-$ ($n = 6$, tetradecasaccharide). These results indicate clearly that **3a** is composed of both GlcAβ(1→3)GlcNAc and GlcAβ(1→3)GalNAc units; a bimodal distribution would be found on the chromatogram if a mixture of HA and Ch was obtained.

The copolymerization was performed by varying the feed ratio of **1** and **2a** (Table 1). Copolymer **3a** was obtained in good yields (entries 2−4). The copolymer composition (**1**/**2a**), determined by the ^1H NMR spectrum, was very close to the feed ratio of **1** and **2a**, as speculated from Figure 1. Yields and molecular weights of **3a** increased with the increasing proportion of **1** in the feed, suggesting that **1** is preferable to elongate by HAase compared to **2a**. It should be pointed out that the copolymerization required a longer time than expected for complete consumption of the monomers (entries 2−4) compared with that in homopolymerization (entries 1 and 5). This might be considered as the monomers with different stereochemistry are capable of inhibition against each other during recognition by the donor site of the enzyme, which makes the reaction

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

	copolymerization[a]			copolymer (**3b**)				
	comonomer, feed ratio			composition[c]				
entry	**1**	**2b**	time[b]/h	**1**	**2b**	yield[d]/%	M_n[e] $\times 10^{-4}$	M_w[e] $\times 10^{-4}$
1	1	0	3.5	1	0	55	1.5	2.5
2	0.75	0.25	7.0	0.74	0.26	56	1.4	2.4
3	0.50	0.50	7.0	0.50	0.50	60	1.4	2.5
4	0.25	0.75	8.0	0.25	0.75	63	1.5	2.6
5	0	1	1.5	0	1	68	1.6	2.7

[a] In 50 mM phosphate buffer at pH 7.5; total monomer concentration, 0.10 M; enzyme, OTH (3720 units/mg),[15] 10 wt % for total monomers; reaction at 30 °C. [b] Indicating the time for complete consumption of both monomers. [c] Determined by ^{1}H NMR analysis. [d] Isolated yields after purification. [e] Determined by SEC calibrated with hyaluronan standards.

slower in the rate-determining step of HAase-catalyzed polymerization. However, the details are not well known yet.

Copolymerization of 1 with 2b. Enzymatic copolymerization of **1** with **2b** was carried out with a feed ratio of 0.50:0.50 under similar reaction conditions to those mentioned above, which proceeded homogeneously. The SEC profile of the reaction mixture showed again a single molecular weight distribution, indicating that the copolymerization proceeded successfully to give copolymer **3b** without the formation of homopolymers. ^{13}C NMR analysis showed that **3b** has both repeating unit structures of HA and Ch4S (Figure 5).

Copolymer **3b** was also enzymatically digested by the lyase employed above to verify the structure (Figure 6). Synthetic Ch4S[11d] was not digested by the enzyme at all (Figure 6A). In contrast, **3b** was effectively degraded after 24 h (Figure 6B). These results indicate that **3b** is a copolymer having both GlcAβ(1→3)GlcNAc and GlcAβ(1→3)GalNAc4S units.

Table 2 shows the results of copolymerization of **1** and **2b** with varying comonomer feed ratios. The copolymerization gave copolymer **3b** with M_n over 1.4×10^4 (approximately 62−66 saccharides) in good yields (entries 2−4). The copolymer composition was very close to or identical to the feed ratio. As mentioned above, the copolymerization also required a longer reaction time.

Conclusion

Hybrid GAGs have been successfully prepared for the first time via a single-step copolymerization catalyzed by HAase, which provides the first example of enzymatic copolymerization between monomers giving polymers with a different main-chain structures. Comonomers **1** and **2a** were copolymerized in a regio- and stereoselective manner, giving rise to HA−Ch hybrid **3a** with M_n around several thousand in good yields. HAase-catalyzed copolymerization of **1** with **2b** proceeded also successfully to produce HA−Ch4S hybrid **3b** with M_n over 1.4×10^4 in good yields. Copolymer compositions of these hybrids were controllable by varying the comonomer feed ratio. Furthermore, these hybrids were effectively digested by the catalysis of *S. hyalurolyticus* hyaluronidase. Thus, the present study demonstrated a novel and facile method for the production of various hybrid GAGs. This method will open a new door to easy access to intramolecular polysaccharide hybridization. Such hybrid GAGs may serve as a novel class of tools for investigation of their functions in various scientific fields such as polymer chemistry, biochemistry, organic chemistry, medicine, pharmaceutics and enzymology.

Acknowledgment. The authors thank Dr. T. Miyoshi and Dr. T. Morikawa of DENKA Co. (Tokyo, Japan) for their gift of hyaluronan samples for SEC calibration standards. This study was partially supported by the 21st COE program for a United Approach to New Materials Science at Kyoto University.

Supporting Information Available. ^{1}H NMR spectra for the determination of the comonomer concentration in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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BM0700340