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Carriers for enzymatic attachment of glycosaminoglycan chains to peptide☆

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Abstract

In the previous study, we have found that the endo-β-xylosidase from *Patinopecten* had the attachment activities of glycosaminoglycan (GAG) chains to peptide. As artificial carrier substrates for this reaction, synthesis of various GAG chains having the linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl, between GAG chain and core protein of proteoglycan was investigated. Hyaluronic acid (HA), chondroitin (Ch), chondroitin 4-sulfate (Ch4S), chondroitin 6-sulfate (Ch6S), and desulfated dermatan sulfate (desulfated DS) as donors and the 4-metylumbelliferone (MU)-labeled hexasaccharide having the linkage region tetrasaccharide at its reducing terminals (MU-hexasaccharide) as an acceptor were subjected to a transglycosylation reaction of testicular hyaluronidase. The products were analyzed by high-performance liquid chromatography and enzyme digestion, and the results indicated that HA, Ch, Ch4S, Ch6S, and desulfated DS chains elongated by the addition of disaccharide units to the nonreducing terminal of MU-hexasaccharide. It was possible to custom-synthesize various GAG chains having the linkage region tetrasaccharide as carrier substrates for enzymatic attachment of GAG chains to peptide.

Keywords: Glycosaminoglycan; Linkage region; Testicular hyaluronidase; Transglycosylation reaction

Biotechnology using genetic engineering has made great progress in recent years. However, many reports have described that those proteins synthesized by gene recombination have few biophysical activities because of the incompletion or the lack carbohydrate chains [1,2]. For this reason, there is a need to reconstruct the carbohydrate chain artificially using "glycotechnology".

Proteoglycans (PGs) are complex glycoconjugates that are composed of core proteins and glycosaminoglycan (GAG) chains. The GAG chains are covalently bound to the core protein via a common core tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl as the linkage region [3,4]. Recently, using the transglycosylation reaction of endo-β-xylosidase, which hydrolyzes the xylosyl serine linkage between a core protein and GAG chains [5], it was succeeded in attachment of the GAG chains to peptide [6]. The natural GAG chains used as carrier substrates have the core tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl, and this linkage region was necessary for the transglycosylation reaction of endo-β-xylosidase.

Also, the transglycosylation mechanism of testicular hyaluronidase, which is an endo-β-*N*-acetylhexosaminidase, has been investigated with the aim of performing enzymatic synthesis of GAG [7–9]. Using this enzymatic reconstruction system, it was possible to custom-synthesize artificial GAG with sequences of nonsulfated and monosulfated disaccharide units, GlcAβ1-3GlcNAc, GlcAβ1-3GalNAc, IdoAα1-3GalNAc, GlcAβ1-3GalNAc4S, and GlcAβ1-3GalNAc6S. In this study, we refer to the enzymatic synthesis of GAG oligosaccharides having the linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl as the carrier substrates for the transglycosylation reaction of endo-β-xylosidase.

☆ *Abbreviations*: DS, dermatan sulfate; Ch6S, chondroitin 6-sulfate; Ch4S, chondroitin 4-sulfate; Ch, chondroitin; HA, hyaluronic acid; PG, proteoglycan; GAG, glycosaminoglycan; GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GalNAc6S, 6-sulfated *N*-acetylgalactosamine; GalNAc4S, 4-sulfated *N*-acetylgalactosamine; PA, 2-aminopyridine; MU, 4-metylumbelliferone; HPLC, high-performance liquid chromatography.

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Materials and methods

Chemicals. Chondroitin 4-sulfate (Ch4S, from whale cartilage), chondroitin 6-sulfate (Ch6S, from shark cartilage), dermatan sulfate (DS, from pig skin), hyaluronic acid (HA, from human umbilical cord), chondroitinase AC-II (from *Arthrobacter aurescens*), and bacterial hyaluronidase (*Streptomyces hyalurolyticus*) were purchased from Seikagaku (Tokyo, Japan). Standard disaccharides, GlcAβ1-3GalNAc4S (Di-4S) and GlcAβ1-3GalNAc6S (Di-6S) were obtained from Funakoshi (Tokyo, Japan), and *N*-acetylchondrosine, GlcAβ1-3GalNAc (Di-0S), was obtained as previously reported [10]. Desulfated DS and chondroitin (Ch) were prepared by desulfation of DS and Ch6S in dimethyl sulfoxide containing 10% methanol according to the procedure of Nagasawa et al. [11]. 4-Metylumbelliferyl glycosaminoglycan (MU-GAG), which has the core tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl, was prepared from the cultured medium of human skin fibroblasts by the method previously reported [12]. Bovine testicular hyaluronidase (Type 1-S) was obtained from Sigma Chemical (St. Louis, MO) and further purified according to the method of Borders and Raftery [13]. Sephadex G-15 was purchased from Pharmacia Biotech (Uppsala, Sweden). Bio-Gel P-4 (400 mesh) was obtained form Bio-Rad (Richmond, CA). 2-Aminopyridine (PA) was purchased from Wako Pure Chemical (Osaka, Japan) and recrystallized from hexane. All other chemicals were obtained from commercial sources.

Preparation of the GAG-protein linkage region hexasaccharide derived from MU-GAG. The MU-hexasaccharide having the core tetrasaccharide corresponding to the linkage region of PG, GlcAβ1-3GalNAc(6S)β1-4-GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-4MU, was prepared from MU-GAG by digestion with testicular hyaluronidase using the procedure described in a previous report [14]. From the hydrolyzed materials, the MU-hexasaccharides were purified by a Bio-Gel P-4 column (1.6 × 110 cm) using 0.5 M pyridinium acetate buffer, pH 6.5, as the eluent. Eluates were monitored for fluorescence of MU derivatives at excitation and emission wavelengths of 325 and 380 nm, respectively. Fraction I was separately pooled as indicated in Fig. 1, and further purified by high-performance liquid chromatography (HPLC) with a Polyamine-II column (4.6 × 250 mm; YMC, Tokyo, Japan). Similarly, MU-oligosaccharides (from octasaccharide to hexadecasaccharide) were prepared.

Pyridylamination of oligosaccharides. Fluorescence labeling with PA of the reducing terminal sugar of saturated disaccharides was performed as described previously [15], based on the method of Hase et al. [19].

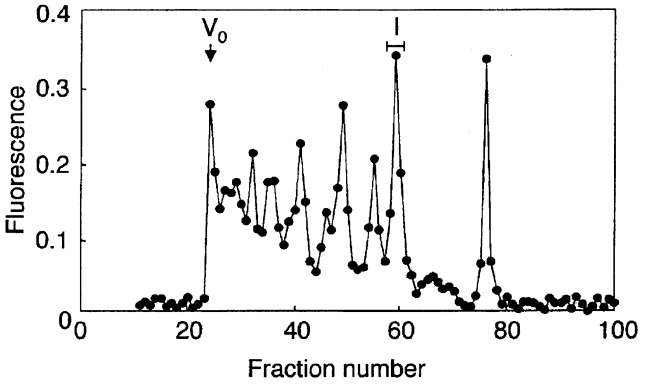

Fig. 1. Gel filtration column chromatography of MU-oligosaccharides on Bio-Gel P-4. The MU-GAG was digested with testicular hyaluronidase. The digests were fractionated on a Bio-Gel P-4 column (1.6 × 110 cm) using 0.5 M pyridinium acetate buffer, pH 6.5, as the eluent at a flow rate of 12.0 ml/h. Fractions (3.0 ml) were monitored for fluorescence of MU derivatives at excitation and emission wavelengths of 325 and 380 nm, respectively.

HPLC. A high-performance liquid chromatograph (Hitachi L-6200, Hitachi) connected to a fluorescence detector (Hitachi F-1050) was used. Fractionation of MU-oligosaccharides derived from MU-GAG was carried out with a Polyamine-II column (4.6 × 250 mm; YMC, Tokyo, Japan) using a linear gradient of NaH_2PO_4 from 16 to 500 mM over a 30-min period at a flow rate of 1.0 ml/min at 30 °C. Eluates containing MU-oligosaccharides were monitored at excitation and emission wavelengths of 325 and 380 nm, respectively. Analysis of PA-disaccharides was carried out with the same column using the same linear gradient of NaH_2PO_4 at a flow rate of 1.0 ml/min at 45 °C. Eluates containing PA-disaccharides were monitored at excitation and emission wavelengths of 320 and 400 nm, respectively.

Analysis of MU-oligosaccharides was carried out with a PALPAK Type S column (4.6 × 250 mm; Takara Shuzo, Kyoto, Japan) under the following conditions. Solution A containing 3% acetic acid, adjusted to pH 7.0 with triethylamine, and acetonitrile at a ratio of 20:80, and solution B containing the same agents at a ratio of 50:50, were prepared. The column was equilibrated with solution A, and the ratio of solution B to solution A was increased linearly to 100% over a 60-min period at a flow rate of 1.0 ml/min at 30 °C. The eluates were monitored at excitation and emission wavelengths of 325 and 380 nm, respectively.

Conditions of transglycosylation reaction by testicular hyaluronidase. A typical transglycosylation reaction was carried out as follows. Five μg of GAGs as donors, 2 nmol of MU-hexasaccharides as acceptors, and 1.0 NFU of testicular hyaluronidase in 0.1 M Tris–HCl buffer, pH 7.0, were incubated at 37 °C for 60 min. The reaction was terminated by immersion in a boiling water bath at 100 °C for 3 min.

Enzymatic digestions. Samples were digested with chondroitinase AC-II (0.1 M sodium acetate buffer, pH 6.0) [17], and bacterial hyaluronidase (0.1 M sodium acetate buffer, pH 6.0) [18] for 1 h at 37 °C and 60 °C, respectively.

Mass spectrum measurements. Mass spectra were obtained on an API-III triple-quadrupole mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an atmospheric-pressure ionization source, as described previously [14]. The samples were dissolved in 0.5 mM ammonium acetate–acetonitrile (50:50) and injected at 2 μl/min with a micro-HPLC syringe pump (pump 22, Harvard Apparatus, MA). In negative mode, scanning was done from *m/z* 200 to 1400 during the 1-min scan (six cycles).

Results and discussion

Isolation of the GAG–protein linkage region hexasaccharide derived from MU-GAG

The GAG bear MU at its reducing terminals, MU-GAG, was digested with testicular hyaluronidase, and the digest was subjected to fractionation by gel filtration using a Bio-Gel P-4 column. The MU-oligosaccharides were monitored for fluorescence (Fig. 1). In this study, the fraction I expected to contain MU-hexasaccharides, on the basis of previous chromatographic data [14] for Ch6S, was pooled and further purified by HPLC on a Polyamine-II column. The fraction II indicated with a bar in Fig. 2 was pooled and subjected to structural analyses as described below.

Structural analysis of MU-hexasaccharide

The molecular weight of this MU-oligosaccharide (Fr. II) derived from MU-GAG was determined by ion spray

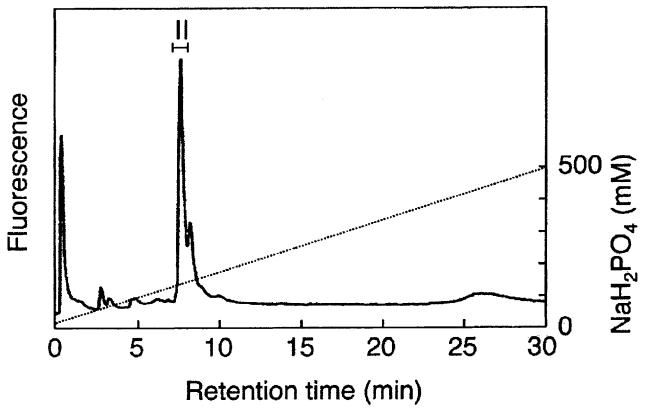

Fig. 2. Subfractionation of fraction I by HPLC on a Polyamine-II column chromatography. Fraction I in Fig. 1 was purified by a Polyamine-II column (4.6×250 mm) using a linear gradient of NaH_2PO_4 from 16 to 500 mM over a 30-period at a flow rate of 1.0 ml/min. The eluate was monitored for fluorescence of MU derivatives at excitation and emission wavelengths of 325 and 380 nm, respectively.

mass analysis in the negative ion mode. As shown in Fig. 3, negative charged ions $[M\text{-}3H]^{3-}$, $[M\text{-}2H]^{2-}$, and $[M\text{-}H]^{-}$ at m/z 421.5, 632.8, and 1266.1, respectively, were revealed. The molecular weight of MU-oligosaccharide was computed to be 1267.4 ± 0.3 based on the presence of these ions. Takagaki et al. [12] showed that the MU-GAG had the MU-labeled linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl-MU; and, the molecular weight of MU-oligosaccharide was the same as that of the MU-hexasaccharide with one sulfate residue, one disaccharide unit, GlcAβ1-3GalNAc, and core tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl, corresponding to the linkage region of PG.

To investigate the disaccharide unit at the nonreducing terminal site, MU-hexasaccharide was digested with chondroitinase AC-II, and then labeled with the fluorescent reagent PA. PA-labeled saturated disaccharides were analyzed by HPLC. Chondroitinase AC-II,

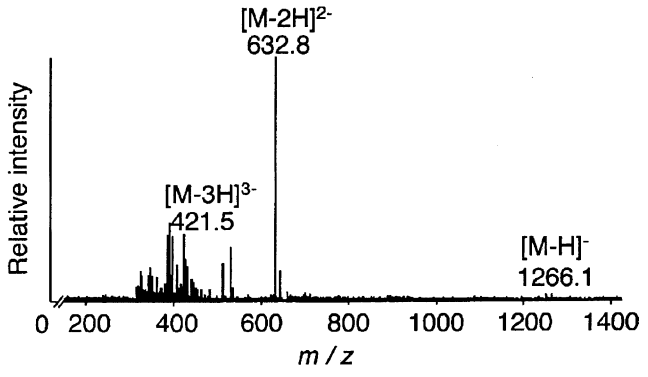

Fig. 3. Ion spray mass spectra of fraction II. Negative-mode mass spectra of the MU-oligosaccharide (fraction II) purified by a Polyamine-II column was analyzed. The spectra was acquired after injection of the samples dissolved in a mobile phase of 0.5 mM ammonium acetate-acetonitrile (50:50).

Table 1
Disaccharide analysis at the nonreducing terminal of MU-hexasaccharide

Composition	PA-Di-0S	PA-Di-4S	PA-Di-6S
Molar proportion (%)[a]	7.3	0	92.7

[a] Percent recovery were calculated based on the peak area in HPLC and were expressed in molar proportions of the disaccharides produced by digestions.

which is a bacterial eliminase, should degrade MU-hexasaccharide to a saturated disaccharide unit derived from the nonreducing terminal site. The results were shown in Table 1. The saturated disaccharide unit generated upon digestion with chondroitinase AC-II was PA–Di-6S, suggesting that the structure of the disaccharide unit at the nonreducing terminal of MU-hexasaccharide was GlcAβ1-3GalNAc(6S). Therefore, its structure was identified to be GlcAβ1-3GalNAc(6S)β1-4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-MU.

Enzymatic synthesis of HA chains having the linkage region

We used the transglycosylation activity of testicular hyaluronidase, endo-β-*N*-acetylhexosaminidase, for synthesis of HA chain having the linkage region as a model of the artificial PG. HA as a donor and MU-hexasaccharide as an acceptor were incubated with testicular hyaluronidase under the optimal conditions (0.1 M Tris–HCl buffer, pH 7.0, in the absence of NaCl at 37 °C for 60 min). The elongation of the HA chain was examined by tracing the fluorescence of the MU-hexasaccharides by HPLC (Fig. 3). Disaccharide units from HA were transferred to the nonreducing terminal of MU-hexasaccharide, and MU-oligosaccharides with various chain lengths were observed. To examine the newly synthesized MU-oligosaccharide chains, the MU-dodecasaccharide fraction (the bar in Fig. 4B) was recovered (Fig. 4C) and incubated with bacterial hyaluronidase. After incubation with the enzyme, the retention time of the MU-dodecasaccharide was shifted closest to that of MU-octasaccharide (Fig. 4D). Therefore, it was revealed that the MU-dodecasaccharide elongated by the addition of disaccharide units, GlcAβ1-3GlcNAc, derived from HA to the nonreducing terminal of MU-hexasaccharide having the linkage region (Fig. 5).

The transfer of various GAGs used as donors to the GAG–protein linkage region hexasaccharide

To elongate the various GAG chains, Ch, Ch4S, Ch6S, and desulfated DS as donors and MU-hexasaccharide as an acceptor were incubated with testicular hyaluronidase. The reaction products were analyzed by HPLC. It was found that disaccharide units released from various GAGs were transferred to the nonreducing

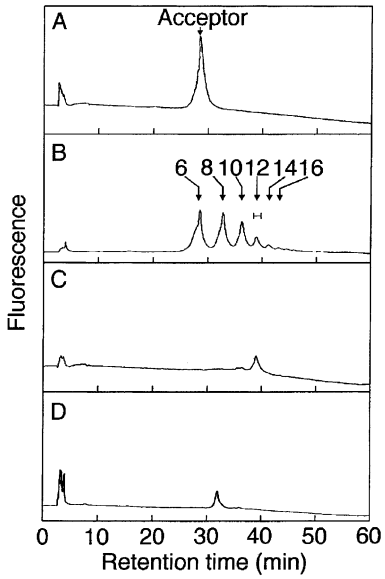

Fig. 4. HPLC chromatograms of MU-oligosaccharides. MU-hexasaccharide as an acceptor and HA as a donor were incubated without (A) and with (B) testicular hyaluronidase. The reaction products were subjected to HPLC, and then MU-dodecasaccharide fraction (bar in B) was recovered and purified (C). An aliquot of the purified sample was digested bacterial hyaluronidase (D), and then subjected to HPLC. Arrows indicated the elution positions of MU-oligosaccharide standards.

Table 2
The transglycosylation reaction of various glycosaminoglycans used as donors

Donors	Chain length of reaction products					
	6[a]	8	10	12	14	16
HA (%)[b]	39.8	31.3	20.1	6.9	1.4	0.5
Ch (%)[b]	71.7	10.8	6.3	5.5	3.4	2.9
Ch4S (%)[b]	97.2	1.5	0.9	0.4		
Ch6S (%)[b]	98.5	1.5				
Desulfated DS (%)[b]	86.9	7.4	4.5	1.2		

[a] *Note.* The GAG–protein linkage region hexasaccharide was used as an acceptor.
[b] Values indicate percentage of total products.

In the present study, artificial GAG chains were synthesized using the transglycosylation reaction of testicular hyaluronidase, which was a reverse reaction of hydrolysis [7–9]. MU-hexasaccharide having the linkage region tetrasaccharide was prepared in advance, and used as an acceptor in the transglycosylation reaction. As the results, the various disaccharide units derived from HA, Ch, Ch4S, Ch6S, or desulfated DS were transferred to the nonreducing terminals of the MU-hexasaccharide. Therefore, it was possible to custom-synthesize natural GAGs or unnatural GAG as HA

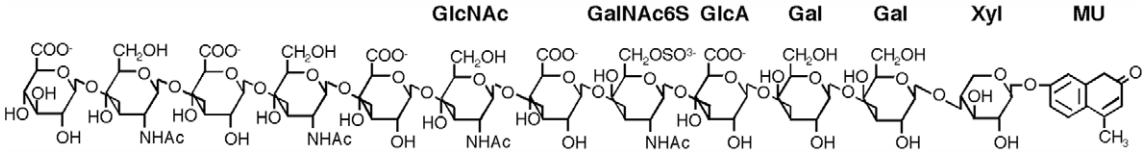

Fig. 5. HA sugar chain having the core tetrasaccharide.

terminal of MU-hexasaccharide (Table 2). Therefore, it was possible to elongate the various disaccharide units, GlcAβ1-3GalNAc, GlcAβ 1-3GalNAc(4S), GlcAβ1-3GalNAc(6S) or IdoA1-3GalNAc, to MU-hexasaccharide having the linkage region.

Recently, Ishido and co-workers [6] succeeded in the attachment of the GAG chains to peptide by using the GAG chain transfer reaction of endo-β-xylosidase as a glycotechnological tool for artificial synthesis of PG. It was shown that three kinds of GAG chains (chondroitin sulfate, DS, and HS) could be transferred to the peptide. However, HA was not transferred by this reaction at all. GAG chains of PG were attached to the serine residue of the core protein through the linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xyl [11]. However, it was known that HA was a GAG without a linkage region and did not bind to the core protein [4]. Then, HA could not be the substrate of endo-β-xylosidase because it did not have the xylosyl serine linkage. Therefore, HA was not transferred to peptide by the GAG chain transfer reaction of endo-β-xylosidase.

having the linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl, at their reducing sites. These artificial GAG chains are expected to be used as the carrier substrates for the GAG chain transfer reaction of endo-β-xylosidase. Furthermore, by using the HA chain having the linkage region as a donor, it is possible to synthesize a novel PG having HA chains.

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