Biochemical and Biophysical Research Communications 423 (2012) 344–349

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hyaluronan–chondroitin hybrid oligosaccharides as new life science research tools

Ikuko Kakizaki [a,*], Shinichiro Suto [b], Yota Tatara [a], Toshiya Nakamura [b], Masahiko Endo [a]

[a] Department of Glycotechnology, Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
[b] Department of Glycomedicine, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan

ARTICLE INFO

Article history:
Received 16 May 2012
Available online 31 May 2012

Keywords:
Hyaluronan
Chondroitin
Glycosaminoglycan
Hyaluronidase
Transglycosylation

ABSTRACT

Hyaluronan and chondroitin are glycosaminoglycans well-known as components of pharmaceutical agents and health foods. From these attractive molecules, using transglycosylation reaction of testicular hyaluronidase, we synthesized hybrid neo-oligosaccharides not found in nature. We also found a new site between the chondroitin disaccharide unit and hyaluronan disaccharide unit recognized by a hyaluronan lyase specific to hyaluronan using these hybrid oligosaccharides as substrates. We hope that these hybrid oligosaccharides will help to elucidate the involvement of hyaluronan, chondroitin, and chondroitin sulfates in the mechanisms of cell functions and diseases, based on the structures of these glycosaminoglycans.

1. Introduction

Glycosaminoglycans are linear polysaccharides composed of repeating disaccharide units of a uronic acid linked to a hexosamine and are classified into hyaluronan, chondroitin sulfates, and so on, based on the structure of the disaccharide units [1,2]. Hyaluronan is a non-sulfated polysaccharide composed of repeating disaccharide units of GlcUAβ1-3GlcNAc and occurs ubiquitously as one of the major components of extracellular matrices of tissues [3]. Hyaluronan is involved in various cell functions and diseases including cancer [4–7]. Chondroitin is also a non-sulfated polysaccharide, composed of repeating disaccharide units of GlcUAβ1-3GalNAc. Generally, chondroitin sequences occur as non-sulfated blocks in a chondroitin sulfate chain. Chondroitin is mainly produced through the chemical desulfation of chondroitin sulfates because glycosaminoglycan chains composed of homogeneous chondroitin occur in only limited sources in nature [8,9], therefore, there is not enough information about the functions of chondroitin. Glycosaminoglycans, with the exception of hyaluronan, occur as sugar components of proteoglycan, one of the glycoconjugates. It has been elucidated that specific oligosaccharide sequences carry specific information [2,10–13], however, there is little information about oligosaccharide sequences with confirmed correlation to function.

We have developed a method of reconstructing glycosaminoglycans [14–18] using transglycosylation [19–21] as a reverse reaction of the hydrolysis of testicular hyaluronidase (EC 3.2.1.35), an endoglycosidase [22]. With this method, it is now possible to systematically synthesize glycosaminoglycans as designed and we have constructed a library of hybrid oligosaccharides composed of various combinations of chondroitin sulfates [22,23].

The characteristic features of our system using hyaluronidase are as follows. (1) The proposed mechanism of hyaluronidase-catalyzed transglycosylation is that first, the *N*-acetylhexosaminide linkage at the non-reducing terminal of a donor glycosaminoglycan is hydrolyzed by hyaluronidase. Then, the released disaccharide unit glucronosyl-hexosamine (GlcUAβ1-3HexNAc) is rapidly transferred to the non-reducing terminal of an acceptor oligosaccharide by the same enzyme [14,18,24]. This is the elongation of glycosaminoglycan by transglycosylation of hyaluronidase. (2) The structure of synthesized glycosaminoglycan, i.e., the linkage structure of the aglycone side and anomeric configuration is the same as the native structure of glycosaminoglycan, reflecting the strict specificity of hyaluronidase. (3) Using systematic combinations of donor polysaccharides and acceptor oligosaccharides, hybrid oligosaccharides having the desired structure are custom-made.

Here, we have succeeded in the synthesis of hybrid oligosaccharides using variously organized combinations of two non-sulfated glycosaminoglycans: hyaluronan and chondroitin. These hyaluronan-chondroitin hybrid oligosaccharides are neo-oligosaccharides that have not been found in nature and will be invaluable research tools. We also investigated the new site recognized by a hyaluronan

Abbreviations: GlcUA, glucronic acid; HexNAc, *N*-acetylhexosamine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GlcNAcβ1-4GlcUA, β1,4-*N*-acetylglucosaminide bond; GalNAcβ1-4GlcUA, β1,4-*N*-acetylgalactosaminide bond; PA, 2-pyridylamine; TRU, turbidity reducing unit, BTH, bovine testicular hyaluronidase; GlcN, glucosamine; GalN, galactosamine.

* Corresponding author. Fax: +81 172 39 5016.
E-mail address: kaki@cc.hirosaki-u.ac.jp (I. Kakizaki).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.127

lyase (from *Streptomyces hyalurolyticus*) using these neo-oligosaccharides as substrates.

2. Materials and methods

2.1. Materials

Hyaluronan (from *Streptococcus zooepidemicus*; average molecular weight, 80,000) was purchased from Food Chemifa Co. Ltd. (Tokyo, Japan). Chondroitin (from shark cartilage; average molecular weight, 19,000) and hyaluronan lyase (from *S. hyalurolyticus*) were purchased from Seikagaku Biobusiness Co. (Tokyo, Japan). Bovine testicular hyaluronidase (BTH, type 1-S) was from Sigma–Aldrich (St. Louis, MO.). CNBr-activated Sepharose 4 Fast Flow was from GE Healthcare, Japan (Tokyo, Japan). Other reagents were of analytical grade and obtained from commercial sources.

2.2. Preparation of pyridylaminated oligosaccharides

Oligosaccharides (hexasaccharide, octasaccharide, and decasaccharide) of hyaluronan and chondroitin were prepared by partial digestion with BTH as follows. Two hundred milligrams of hyaluronan or chondroitin was incubated with 4 mg of BTH in 10 ml of 0.1 M sodium acetate buffer, pH 4.0, containing 150 mM NaCl at 37 °C for 24 h and the reaction was stopped by boiling for 10–15 min. The mixture was then clarified by centrifugation, concentrated to about 5 ml and desalted on a Sephadex G-25 column equilibrated with distilled water. Fractions determined to be positive for uronic acid using the carbazole sulfate method [25] were pooled and concentrated to 5 ml. The resulting mixture of oligosaccharides was fractionated by Bio-gel P-10 (Bio-rad, Richmond, CA). The sample was applied to a Biogel P-10 column (dimensions, 2.2 cm × 140 cm), which was equilibrated with 0.5 M pyridine acetate buffer, pH 6.5. The oligosaccharides were eluted at a flow rate of 16.2 ml/h and 5-ml fractions were collected. The fractions determined to be positive for uronic acid were pooled according to the elution profiles, desalted, concentrated and subjected to ion spray mass spectrometry to identify the peaks as hexasaccharides, octasaccharide, and decasaccharide.

Resulting oligosaccharides of hyaluronan or chondroitin were fluorolabeled at the reducing terminal with 2-pyridylamine (PA) by a modified version of the method of Hase et al. [26] as described in our previous report [27]. The oligosaccharides-PA of hyaluronan or chondroitin were used as acceptors in the first step of the transglycosylation of BTH.

2.3. Transglycosylation reaction using immobilized BTH

For transglycosylation, a reaction column stuffed with immobilized resin with BTH was prepared using CNBr-activated Sepharose

Table 1
Substrate specificity of hyaluronan lyase toward hyaluronan-chondroitin hybrid oligosaccharides. Oligosaccharide-PA was incubated with 2.5 TRU of hyaluronan lyase (from *Streptomyces hyalurolyticus*) for 2 h at 60 °C. Then 2.5 TRU of hyaluronan lyase was added and the reaction mixture was further incubated for 16 h at 37 °C. Reaction products were analyzed by HPLC on a YMC Pack Polyamine II column. Arrows indicate recognition sites of hyaluronan lyase predicted from chain lengths of reaction products. Oligosaccharides in bold indicate starting material (substrate).

Substrate oligosaccharide	Starting material after incubation (%)[b]	Oligosaccharide-PA detected after reactions (%)[d]
Decasaccharide-PA		
HHHHH-PA[a]	0	Δ^4HHH-PA[e] (98.2), Δ^4HHHH-PA (1.8)
CHHHH-PA	1.2	**CHHHH-PA** (3.4), Δ^4HHH-PA (96.6)
CCHHH-PA	92.8	**CCHHH-PA** (100)
HCHHH-PA	57.2[c]	**HCHHH-PA** (52.2), Δ^4HHH-PA (47.8)
HHCCC-PA	100.3	**HHCCC-PA** (100)
HCCCC-PA	96.9	**HCCCC-PA** (100)
CHCCC-PA	99.7	**CHCCC-PA** (100)
CCCCC-PA	95.9	**CCCCC-P**A (100)
Dodecasaccharide-PA		
HHHHHH-PA	0	Δ^4HHH-PA (78.8), Δ^4HHHH-PA (21.0), Δ^4HHHHH-PA (0.2)
CHHHHH-PA	2.9	**CHHHHH**-PA (2.7), Δ^4HHH-PA (90.3), Δ^4HHHH-PA (7.0)
CCHHHH-PA	0	Δ^4HHH-PA (92.2), Δ^4HHHH-PA (7.8)
CCCHHH-PA	51.3[c]	**CCCHHH-PA** (46.9), Δ^4HHH-PA (53.1)
HCCHHH-PA	59.4[c]	**HCCHHH-PA** (59.4), Δ^4HHH-PA (40.6)
HHCHHH-PA	2.2	**HHCHHH-PA** (1.6), Δ^4HHH-PA (89.5), Δ^4HCHHH-PA (8.9)
CCHCCC-PA	61.6[c]	**CCHCCC-PA** (58.4), Δ^4HCCC-PA (41.6)
CHHCCC-PA	7.7	**CHHCCC-PA** (7.0), Δ^4HCCC-PA (89.1), Δ^4HHCCC-PA (3.9)
HHHCCC-PA	10.1	**HHHCCC-PA** (10.2), Δ^4HCCC-PA (88.9), Δ^4HHCCC-PA (0.9)
HHCCCC-PA	106.4	**HHCCCC-PA** (100)
HCCCCC-PA	138.8	**HCCCCC-PA** (100)
CCCCCC-PA	114.7	**CCCCCC-PA** (100)

[a] H, –GlcUAβ1-3GlcNAc–; C, –GlcUAβ1-3GalNAc–; PA, 2-pyridylamine (fluorescence).
[b] The percentages shown are the relative peak areas of the substrate oligosaccharides after incubation with peak areas before incubation defined as 100%.
[c] HCHHH-PA, CCCHHH-PA, HCCHHH-PA, and CCHCCC-PA were digested completely by longer incubation with hyaluronan lyase or with a larger amount of hyaluronan lyase.
[d] The percentages shown are the relative peak areas of individual oligosaccharides when the total peak areas of all of the oligosaccharides detected after reaction is defined as 100%.
[e] Δ^4HHH-PA, HHH-PA having unsaturated GlcUA at the non-reducing terminus.

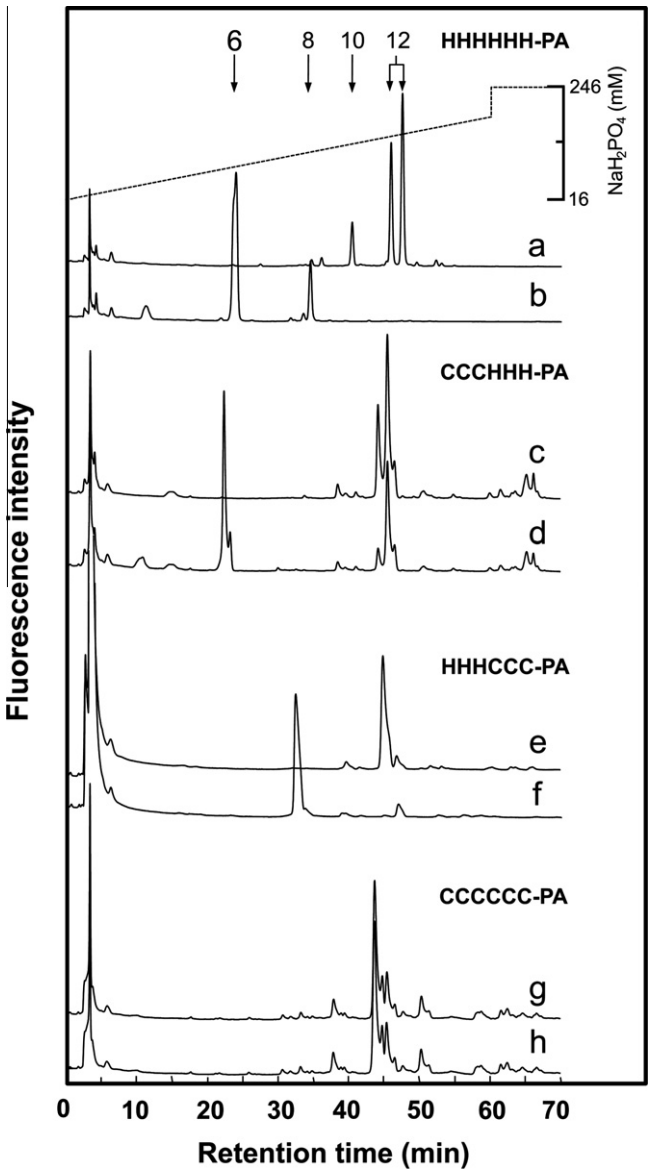

Fig. 1. HPLC of the oligosaccharides produced by hyaluronan lyase. Hyaluronan dodecasaccharide-PA (a, HHHHHH-PA), hyaluronan-chondroitin hybrid dodecasaccharide-PAs (c, CCCHHH-PA; e, HHHCCC-PA), or chondroitin dodecasaccharide-PA (g, CCCCCC-PA) were incubated with 2.5 TRU of hyaluronan lyase (from *Streptomyces hyalurolyticus*) for 2 h at 60 °C. Then 2.5 TRU of hyaluronan lyase was added and the reaction mixture was further incubated for 16 h at 37 °C. Starting oligosaccharides and reaction products were analyzed by HPLC on a YMC Pack Polyamine II column. (a, c, e, and g), before incubation; (b, d, f, and h), after incubation. Arrows indicate the elution positions of standard pyridylaminated hyaluronan oligosaccharides numbered by sugar length. H, –GlcUAβ1-3GlcNAc–; C, –GlcUAβ1-3GalNAc–; PA, 2-pyridylamine (fluorescence).

4 Fast Flow according to the instructions supplied. For the first step of transglycosylation, 3 mg of the acceptor oligosaccharide-PA and 90 mg of donor polysaccharide (hyaluronan or chondroitin) were incubated in the BTH-immobilized column (dimensions, 4.8 cm × 4.0 cm; bed vol., 72 ml) in 0.1 M Tris–HCl buffer, pH 7.0, at 37°C for 1 h. The conditions (pH 7.0, without NaCl) for the transglycosylation of BTH are different from those (pH 4.0, with 150 mM NaCl) for hydrolysis. Reaction products were eluted using distilled water and fractions having fluorescence of PA (ex. 320 nm, and em. 400 nm) were collected and boiled, then concentrated to 3 ml. The products were purified based on chain length by HPLC on a TSKgel Amide-80 column and their structure confirmed from ion spray MS analysis and HPLC analysis after digestion with specific enzyme for specific sequences.

The second step of transglycosylation was performed in the BTH-immobilized column (dimensions, 1.5 cm × 2.0 cm; bed vol., 3.6 ml) using a product (hybrid oligosaccharide-PA) of the first transglycosylation as an acceptor (180 μg), and polysaccharide (hyaluronan or chondroitin) as a donor (4.5 mg) in 0.1 M Tris–HCl buffer, pH 7.0, at 37°C for 15 min. The processes (elution and purification of oligosaccharides) were carried out in a similar manner to those after the first transglycosylation. HCHHH-PA, HHCHHH-PA, CHCCC-PA, CCHCCC-PA, HCCHHH-PA, and CHHCCC-PA were the products of the second step of transglycosylation using products of the first step (CHHH-PA, HCCC-PA, CCHHH-PA, and HHCCC-PA) as acceptors. Here, we named the disaccharide units as H, –GlcUAβ1-3GlcNAc– and C, –GlcUAβ1-3GalNAc–, respectively.

Resulting hybrid oligosaccharide-PAs by transglycosylation were purified by HPLC on a TSKgel Amide-80 column. Purified oligosaccharide-PA was analyzed by HPLC on a YMC-Pack polyamine II column as is or after digestion with structural specific enzymes. Also, they were hydrolyzed with 4 N HCl at 100 °C for 6 h and the resulting monosaccharides were pyridylaminated, then, the compositions of glucosamine-PA (GlcN-PA) and galactosamine-PA (GalN-PA) of hybrid oligosaccharide-PAs were analyzed by high performance anion exchange chromatography on a Carbopac MA1 column (4 × 250 mm, Dionex, Sunnyvale, CA) equipped with a fluorescence detector (model F-1150, Hitachi, Tokyo, Japan) and a pulsed amperometric detection (Dionex, Sunnyvale, CA). Elution was performed using 500 mM NaOH at 0.4 ml/min at 40 °C. GlcN and GalN (Dionex) were pyridylaminated and used as monosaccharide-PA standards. Fluorescence of PA was detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

2.4. Reaction of hyaluronan lyase

Each oligosaccharide-PA (1 μg) was incubated with 2.5 TRU of hyaluronan lyase (from *S. hyalurolyticus*) in 50 mM sodium acetate buffer, pH 6.0, containing 150 mM NaCl, at 60 °C for 2 h. The reaction mixture was further incubated with an additional 2.5 TRU of hyaluronan lyase at 37 °C for 16 h. For time course and dose response experiments, various incubation times and various amounts of enzymes were used. The reaction was stopped by boiling for 5 min, and the reaction mixture was clarified by centrifugation for 5 min at 8000*g* at 4 °C and filtrated. The filtrate was then analyzed by HPLC using a polyamine II column. Potential recognition sites by hyaluronan lyase (Table 1) were estimated from the composition of oligosaccharides-PA in HPLC by monitoring fluorescence to detect unsaturated oligosaccharides having PA at the reducing terminal, generated after enzymatic reaction. For example, when hyaluronan dodecasaccharide-PA (HHHHHH-PA) is used as a substrate (e.g. Fig. 1b), it is known that detected hexasaccharide-PA and octasaccharide-PA have an unsaturated glucuronic acid at the non-reducing terminal due to the general features of this enzyme and the method of detection. Therefore, potential recognition sites can be estimated from chromatograms of the reaction products. Decasaccharide-PA and dodecasaccharide-PA were used as substrate of the hyaluronan lyase because preliminary experiments showed that hyaluronan decasaccharide-PA is the minimum substrate for the hyaluronan lyase (data not shown). On the other hand, octasaccharide, either saturated or unsaturated, without PA, is the minimum substrate for the hyaluronan lyase [28].

For kinetic analysis, various amounts of HHHHHH-PA or CCCHHH-PA (0, 0.1, 1, 2, 5, 10, 15, 20, 40, 60, 80, 100, 150, 200 μg) were used as substrates and incubated with 4 TRU of hyaluronan lyase for 16 h, and 1/10 of reaction mixture was analyzed

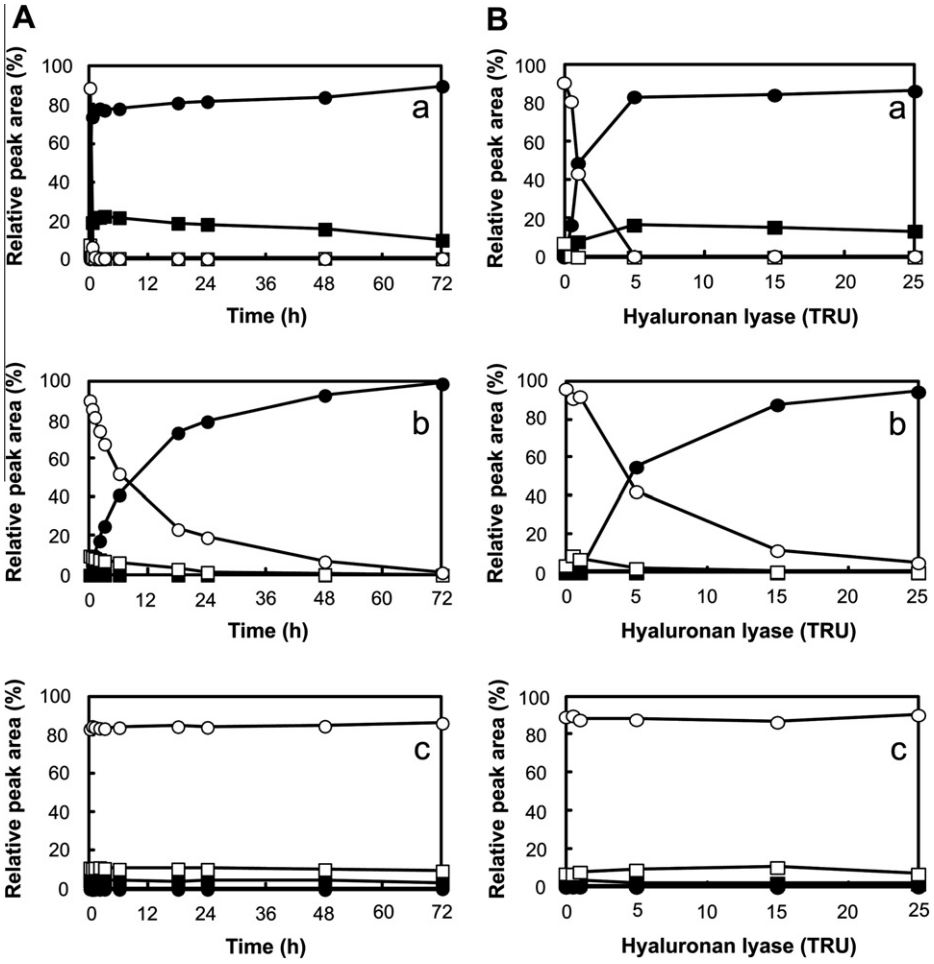

Fig. 2. Characterization of the enzymatic activity of hyaluronan lyase acting on the linkage of hyaluronan-chondroitin hybrid oligosaccharides. (A) Dodecasaccharide-PAs were incubated with 2.5 TRU of hyaluronan lyase (from *Streptomyces hyalurolyticus*) for various periods (0, 0.5, 1, 2, 3, 6, 16, 24, 48, and 72 h) at 60 °C. (B) Dodecasaccharide-PAs were incubated with various amounts of hyaluronan lyase (from *Streptomyces hyalurolyticus*) (0, 0.5, 1, 5, 15, and 25 TRU) for 16 h at 60 °C. Reaction products of both (A) and (B) were analyzed by HPLC on a YMC Pack Polyamine II column. The percentages shown are the relative peak areas of the oligosaccharide-PAs with the total peak area of all reaction products defined as 100%. (a) HHHHHH-PA, (b) CCCHHH-PA, and (c) CCCCCC-PA. Symbols: filled circles, hexasaccharide; filled squares, octasaccharide; open squares, decasaccharide; open circles, dodecasaccharide. H, –GlcUAβ1-3GlcNAc–; C, –GlcUAβ1-3GalNAc–; PA, 2-pyridylamine.

by HPLC. The mole number was calculated from the peak area (fluorescence intensity) based on the peak areas of standard hyaluronan hexasaccharide-PA (HHH-PA) with known mole numbers (100, 200, 400, and 800 pmol). The standard curve was linear intersecting at the zero point.

2.5. HPLC analyses

HPLC was performed using a Hitachi ELITE LaChrom system equipped with a fluorescence detector (model L-2485; Hitachi) and a UV detector (model L-2420; Hitachi).

A TSKgel Amide-80 column (4.6 × 250 mm, Tosoh Co., Tokyo, Japan) was used for the purification of oligosaccharide-PA. Two solutions were prepared: solution A was a 20:80 (v/v) mixture of 3% acetic acid and acetonitrile, adjusted to pH 7.3 with triethylamine, while solution B was a 50:50 (v/v) mixture of 3% acetic acid and acetonitrile. The flow rate was set at 1.0 ml/min. Samples were injected onto the column equilibrated with solution A and eluted with a linear gradient of 0–100% of solution B over 60 min. The eluted material was detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

A polyamine II column (4.6 × 250 mm; YMC Co., Kyoto, Japan) was used for the analysis of the purified oligosaccharides and reaction products of hyaluronan lyase. Two solutions were prepared: solution A was 16 mM NaH_2PO_4, and solution B was 246 mM NaH_2PO_4. Samples were injected onto the column equilibrated with solution A. The oligosaccharides were eluted with a linear gradient of 0–75% of solution B at a flow rate of 1.0 ml/min over a period of 60 min. Oligosaccharides were monitored by fluorescence detection (ex. 320 nm, em. 400 nm) and by UV absorbance at 232 nm.

2.6. Ion spray mass spectrometry

Oligosaccharide-PAs were characterized by ion spray mass spectrometry as described in a previous paper [29] using an API-100 single quadrupole instrument equipped with an ESI source (PE-Sciex, Toronto, Canada).

3. Results

Neo-oligosaccharides, in variously organized combinations of two non-sulfated disaccharide units from hyaluronan and chondroitin, were synthesized using the transglycosylation reaction of hyaluronidase. These oligosaccharides have fluorescence of 2-pyridylamine at the reducing terminal to facilitate identification of the oligosaccharide in HPLC. Using these oligosaccharides as substrates we examined the substrate specificity of hyaluronan lyase from *S. hyalurolyticus* (EC 4.2.2.1), which has been known to act on

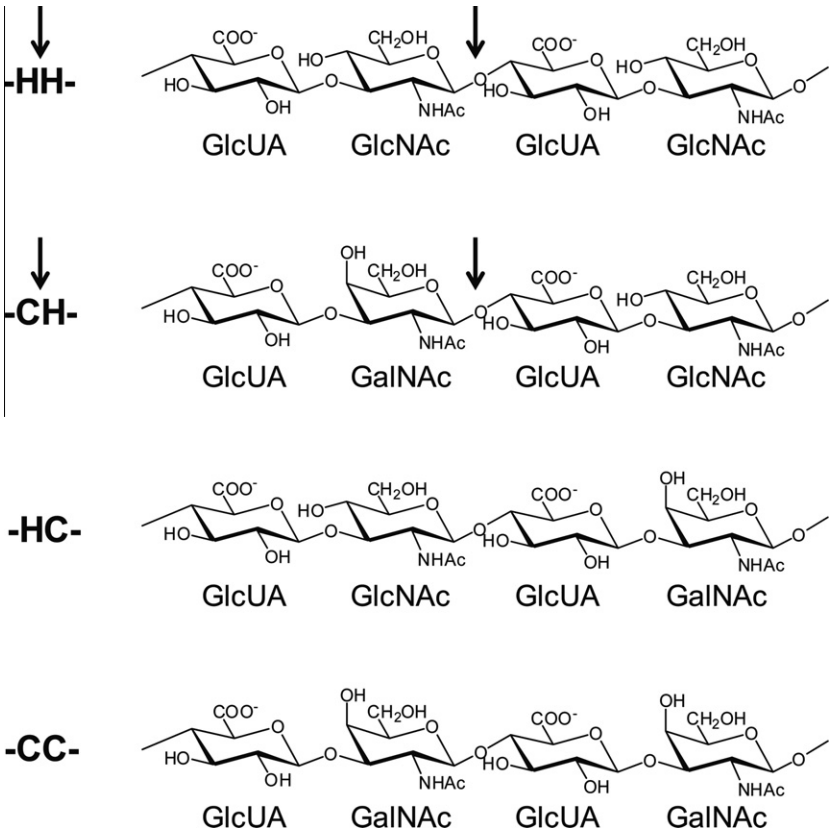

Fig. 3. Chemical structure of the recognition sites by hyaluronan lyase. Arrows indicate the sites on which hyaluronan lyase (from *Streptomyces hyalurolyticus*) acts. H, –GlcUAβ1-3GlcNAc–; C, –GlcUAβ1-3GalNAc–.

hyaluronan and not on chondroitin [28]. Hyaluronan and chondroitin are extremely similar in structure: they have no sulfate group and the linkage structure between uronic acid and hexosamine is the same sterically, the only difference is that the hexosamine is GlcNAc in hyaluronan while it is GalNAc in chondroitin. The only structural difference between GlcNAc and GalNAc is in the steric conformation of a hydroxyl group at the C-4 position of the hexosamine. Therefore, we thought it would be interesting to examine whether this hyaluronan lyase acts on the hybrid oligosaccharides with disaccharide units from hyaluronan and chondroitin, that is, GlcUAβ1-3GlcNAc and GlcUAβ1-3GalNAc. Oligosaccharides listed in Table 1 are the minimum necessary patterns to analyze the site recognized by this hyaluronan lyase. These substrate oligosaccharides were digested with hyaluronan lyase under optimal conditions for exhaustive digestion toward authentic substrate (hyaluronan polysaccharide) and reaction products were analyzed by normal phase HPLC using a YMC Pack Polyamine II column. Representative chromatograms are shown in Fig. 1. Hyaluronan dodecasaccharide-PA (HHHHHH-PA) was completely digested and a 78.8% of unsaturated hexasaccharides-PA (Δ^4HHH-PA) and 21.0% of unsaturated octasaccharide-PA (Δ^4HHHH-PA) were generated (Fig. 1a and b and Table 1). Chondroitin dodecasaccharide-PA (CCCCCC-PA) was not digested (Fig. 1g and h and Table 1). HHHCCC-PA, one of the hyaluronan-chondroitin hybrid dodecasaccharide-PAs, was almost completely digested and 88.9% of unsaturated octasaccharide-PA (Δ^4HCCC-PA) was generated but unsaturated hexasaccharide-PA (Δ^4CCC-PA) was not generated (Fig. 1e and f and Table 1), showing that this hyaluronan lyase does not act on the glycosyl bond (GlcNAcβ1-4GlcUA) between an H-unit and a C-unit when the H-unit proceeds the C-unit. However, other hyaluronan-chondroitin hybrid dodecasaccharide-PA (CCCHHH-PA) was digested with half of the initial amount and only unsaturated hexasaccharide-PA (Δ^4HHH-PA) was generated (Fig. 1c and d and Table 1). All substrate oligosaccharides used in this experiment and the results of digestion are shown in Table 1. From the deduced structure of reaction products, the possible site of recognition by hyaluronan lyase on oligosaccharides was determined. It was confirmed that hyaluronan lyase recognizes and acts on the hyaluronan linkage as thought previously: hyaluronan lyase acts on the internal β1,4-*N*-acetylglucosaminide bonds (GlcNAcβ1-4GlcUA) of the linkage between the two hyaluronan disaccharide units. In addition, we found for the first time, this enzyme acted on the internal β1,4-*N*-acetylgalactosaminide bonds (GalNAcβ1-4GlcUA) of the linkage between the chondroitin disaccharide unit and the hyaluronan disaccharide unit, depending on the sequences, after a longer incubation or with a larger amount of enzyme (Fig. 2). That is, hyaluronan lyase acts on the glycosyl bond (GalNAcβ1-4GlcUA) in the hybrid oligosaccharide when it consists of a C unit followed by an H unit but not on the glycosyl bond (GlcNAcβ1-4GlcUA) when they are an H unit followed by a C unit (Fig. 3).

Kinetic analyses were also performed to determine the Michaelis–Menten constants K_m values and V_{max} values for the representative substrates (HHHHHH-PA and CCCHHH-PA). Lineweaver–Burk plots were drawn based on the amounts of hexasaccharide-PA (Δ^4HHH-PA), as a common product for both substrates (Supplemental Fig. 1), although octasaccharide-PA (Δ^4HHHH-PA) was also generated when HHHHHH-PA was used as a substrate. K_m was 59.13 for HHHHHH-PA and 395.1 μM for CCCHHH-PA and V_{max} was 0.567 μM/min for HHHHHH-PA and 0.691 μM/min for CCCHHH-PA. Incidentally, when Lineweaver–Burk plots were drawn based on the amounts of Δ^4HHHH-PA as another product for HHHHHH-PA, K_m was 59.87 μM and V_{max} was 0.285 μM/min. It was suggested that hyaluronan lyase preferentially recognizes

and acts on the linkage of GlcNAcβ1-4GlcUA in -HH- than GalNAcβ1-4GlcUA in –CH–.

4. Discussion

As shown here, hyaluronan-chondroitin hybrid oligosaccharides, and by extension, also other hybrid oligosaccharides covering variations in the numbers and positions of sulfated groups, might be developed as tools to examine the functions of hyaluronan, chondroitin, and chondroitin sulfates. The possible practical uses of hybrid oligosaccharides are unlimited, ranging from their addition to existing bioassay systems to applications in studies for the discovery of new drugs regulating specific reactions in the body. Millions of organic compounds have been found in nature or synthesized artificially, and exciting studies on the activities of these compounds are being performed. However, there are many cases of these compounds persisting in the living body. Our hybrid oligosaccharides are not found in nature but they are guaranteed to be degraded by enzymes in the body. Mammalian hyaluronan-degrading enzymes also act on chondroitin sulfates and chondroitin, suggesting very similar mechanisms of degradation among these glycosaminoglycans [30,31]. Therefore, once valuable applications are found, these hybrid oligosaccharides will enable breakthroughs in the study of mechanisms of cell functions and diseases, and further the development of medical applications. Recently, we have been able to synthesize hybrid oligosaccharides in large quantities with no contamination by enzyme, so that they can be applied to life science studies on, for example, cancer, inflammation, or mechanisms of infection. Exactly how these hybrid oligosaccharides will be applied will depend in large part on the ideas of the readers of this paper as well as progress in our research.

Acknowledgments

This work was supported by Grants-in-Aid (Nos. 19570119 and 23570157) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Fund for Cooperation for Innovative Technology and Advanced Research in an Evolution Area (City Area). We would also like to thank Sunstar Inc. (Osaka, Japan) for their generous grant to perform research on proteoglycans.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.127.

References

[1] L. Rodén, Structure and metabolism of connective tissue proteoglycans, in: W.J. Lannarz (Ed.), The Biochemistry of Glycoproteins and Proteoglycans, Plenum Press, New York, 1980, pp. 267–371.
[2] L. Kjellen, U. Lindahl, Proteoglycans: structures and interactions, Annu. Rev. Biochem. 60 (1991) 443–475.
[3] K. Meyer, J. Palmer, The polysaccharide of the vitreous humor, J. Biol. Chem. 107 (1934) 629–634.
[4] J. McDonald, V.C. Hascall, Hyaluronan minireview series, J. Biol. Chem. 277 (2002) 4575–4579.
[5] M.I. Tammi, A.J. Day, E.A. Turley, Hyaluronan and homeostasis: a balancing act, J. Biol. Chem. 277 (2002) 4581–4584.
[6] B.P. Toole, Hyaluronan promotes the malignant phenotype, Glycobiology 12 (2002) 37R–42R.
[7] E.A. Turley, P.W. Noble, L.Y. Bourguignon, Signaling properties of hyaluronan receptors, J. Biol. Chem. 277 (2002) 4589–4592.
[8] H.Y. Hwang, S.K. Olson, J.D. Esko, H.R. Horvitz, Caenorhabditis elegans early embryogenesis and vulval morphogenesis require chondroitin biosynthesis, Nature 423 (2003) 439–443.
[9] S. Mizuguchi, T. Uyama, H. Kitagawa, K.H. Nomura, K. Dejima, K. Gengyo-Ando, S. Mitani, K. Sugahara, K. Nomura, Chondroitin proteoglycans are involved in cell division of *Caenorhabditis elegans*, Nature 423 (2003) 443–448.
[10] C. Malavaki, S. Mizumoto, N. Karamanos, K. Sugahara, Recent advances in the structural study of functional chondroitin sulfate and dermatan sulfate in health and disease, Connect. Tissue Res. 49 (2008) 133–139.
[11] U. Lindahl, G. Backstrom, L. Thunberg, I.G. Leder, Evidence for a 3-O-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin, Proc. Natl. Acad. Sci. USA 77 (1980) 6551–6555.
[12] R.N. Achur, I. Kakizaki, S. Goel, K. Kojima, S.V. Madhunapantula, A. Goyal, M. Ohta, S. Kumar, K. Takagaki, D.C. Gowda, Structural interactions in chondroitin 4-sulfate mediated adherence of *Plasmodium falciparum* infected erythrocytes in human placenta during pregnancy-associated malaria, Biochemistry 47 (2008) 12635–12643.
[13] H. Habuchi, S. Suzuki, T. Saito, T. Tamura, T. Harada, K. Yoshida, K. Kimata, Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor, Biochem. J. 285 (Pt 3) (1992) 805–813.
[14] H. Saitoh, K. Takagaki, M. Majima, T. Nakamura, A. Matsuki, M. Kasai, H. Narita, M. Endo, Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase, J. Biol. Chem. 270 (1995) 3741–3747.
[15] K. Takagaki, H. Munakata, M. Majima, M. Endo, Enzymatic reconstruction of a hybrid glycosaminoglycan containing 6-sulfated, 4-sulfated, and unsulfated *N*-acetylgalactosamine, Biochem. Biophys. Res. Commun. 258 (1999) 741–744.
[16] K. Takagaki, H. Munakata, I. Kakizaki, M. Majima, M. Endo, Enzymatic reconstruction of dermatan sulfate, Biochem. Biophys. Res. Commun. 270 (2000) 588–593.
[17] K. Takagaki, K. Ishido, I. Kakizaki, M. Iwafune, M. Endo, Carriers for enzymatic attachment of glycosaminoglycan chains to peptide, Biochem. Biophys. Res. Commun. 293 (2002) 220–224.
[18] K. Takagaki, T. Nakamura, J. Izumi, H. Saitoh, M. Endo, K. Kojima, I. Kato, M. Majima, Characterization of hydrolysis and transglycosylation by testicular hyaluronidase using ion-spray mass spectrometry, Biochemistry 33 (1994) 6503–6507.
[19] B. Weissmann, The transglycosylative action of testicular hyaluronidase, J. Biol. Chem. 216 (1955) 783–794.
[20] P. Hoffman, K. Meyer, A. Linker, Transglycosylation during the mixed digestion of hyaluronic acid and chondroitin sulfate by testicular hyaluronidase, J. Biol. Chem. 219 (1956) 653–663.
[21] S. Highsmith, J.H. Garvin Jr., D.M. Chipman, Mechanism of action of bovine testicular hyaluronidase. Mapping of the active site, J. Biol. Chem. 250 (1975) 7473–7480.
[22] M. Endo, S. Hase, K. Yamamoto, K. Takagaki (Eds.), Endoglycosidases-Biochemistry, Biotechnology, Application, KODANSHA and Springer, 2006.
[23] K. Takagaki, K. Ishido, Synthesis of chondroitin sulfate oligosaccharides using enzymatic reconstruction, Trends Glycosci. Glycotechnol. 12 (67) (2000) 295–306.
[24] I. Kakizaki, N. Ibori, K. Kojima, M. Yamaguchi, M. Endo, Mechanism for the hydrolysis of hyaluronan oligosaccharides by bovine testicular hyaluronidase, FEBS J. 277 (2010) 1776–1786.
[25] Z. Dische, A new specific color reaction of hexuronic acids, J. Biol. Chem. 167 (1947) 189–198.
[26] S. Hase, T. Ibuki, T. Ikenaka, Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins, J. Biochem. 95 (1984) 197–203.
[27] A. Kon, K. Takagaki, H. Kawasaki, T. Nakamura, M. Endo, Application of 2-aminopyridine fluorescence labeling to glycosaminoglycans, J. Biochem. 110 (1991) 132–135.
[28] E. Shimada, G. Matsumura, Degradation process of hyaluronic acid by *Streptomyces hyaluronidase*, J. Biochem. 88 (1980) 1015–1023.
[29] K. Takagaki, K. Kojima, M. Majima, T. Nakamura, I. Kato, M. Endo, Ion-spray mass spectrometric analysis of glycosaminoglycan oligosaccharides, Glycoconj. J. 9 (1992) 174–179.
[30] K. Meyer, Hyaluronidases, in: P.D. Boyer, E.G. Krebs (Eds.), The Enzymes, Academic Press, New York, 1971, pp. 307–320.
[31] K. Meyer, M.M. Rapport, The hydrolysis of chondroitin sulfate by testicular hyaluronidase, Arch. Biochem. 27 (1950) 287–293.