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Hyaluronan–chondroitin hybrid oligosaccharides as new life science research tools

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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.

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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.