CUSTOM SYNTHESIS OF HYALURONAN/CHONDROITIN SULFATE HYBRID OLIGOSACCHARIDES: FOR FUTURE MEDICAL APPLICATIONS

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Abstract Hyaluronan and chondroitin sulfate sugar chains play important roles as major components of extracellular matrix. They are attractive molecules for biochemical research and are also used in pharmaceutical agents. However, details of the relationships between their oligosaccharide structures and functions are not known because of a lack of availability of model oligosaccharides with known structures. In order to, in the future, investigate their functions based on structure, we synthesized hybrid oligosaccharides by the *in vitro* transglycosylation reaction of hyaluronidase using various combinations of acceptors and donors. A total of 18 kinds of pyridylaminated hyaluronan/chondroitin sulfate hybrid oligosaccharides (from octasaccahride to dodecasaccharide), which have not been found in nature, were synthesized.

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 Key words: hyaluronan; chondroitin sulfate; hyaluronidase; transglycosylation; oligosaccharide

Introduction

Hyaluronan (HA) is a linear polysaccharide composed of repeating disaccharide units of GlcUAβ1-3GlcNAc that occurs ubiquitously as one of the major components of extracellular matrices of tissues and is involved in many biological processes and disease processes, including tissue organization, wound healing, tumor invasion and cancer metastasis, through its interactions with other extracellular matrix components[1-3]. It is important to note that activities of HA depend on the molecular weight, for instance, the functions of HA oligosaccharides are completely different from high molecular weight HA. Chondroitin sulfates (ChSs) are also linear polysaccharides, composed of repeating disaccharide units of GlcUAβ1-3GalNAc with sulfate groups and occur as sugar components of proteoglycan, one of the glycoconjugates[4]. The fine structure of native chondroitin sulfate has considerable variation since the patterns of sulfation or epimerization are variable even on the same sugar chain. Chondroitin sulfates have domain structures consisting of specific oligosaccharide sequences demonstrating specific biological functions. However, there are very few domain structures with confirmed correlation between the structure and the function[5-7]. Therefore, we established an enzymatic reconstruction method for custom-synthesizing new hybrid glycosaminoglycans in order to clarify biological functions of glycosaminoglycans[8]. Our method using bovine testicular hyaluronidase (BTH) is capable of synthesizing hybrid oligosaccharides from different kinds of chondroitin sulfates[9-11]. In this study, we synthesized hybrid oligosaccharides from hyaluronan and chondroitin sulfate species using an immobilized enzyme.

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Materials and method

Materials. HA（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）, chondroitin 4-sulfate（Ch4S, from whale cartilage; average molecular weight, 34,000）and chondroitin 6-sulfate（Ch6S, from shark cartilage; average molecular weight, 64,000）were purchased from Seikagaku Biobusiness（Tokyo, Japan）. BTH（type 1-S）was from Sigma-Aldrich（St. Louis, MO, U.S.A.）, CNBr-activated Sepharose 4 Fast Flow was from GE Healthcare, Japan（Tokyo, Japan）. Other reagents were of analytical grade and obtained from commercial sources.

Preparation of pyridylaminated hexasaccharides. Hexasaccharides of HA, Ch, Ch4S, and Ch6S were prepared by partial digestion of HA, Ch, Ch4S, and Ch6S chains with BTH and purified according to a previous report[12]. The hexasaccharides were fluorolabeled at the reducing terminus with 2-pyridylamine（PA）by a modified version of the method of Hase *et al.*[13] as described in our previous report[14]. The hexasaccharide-PA was used as an acceptor in the transglycosylation reaction of BTH.

Transglycosylation reaction of BTH. Various combinations of 1.5 mg（by weight）of GAG hexasaccharide-PA as an acceptor and 45 mg（by weight）of GAGs as donors were incubated in immobilized BTH columns（dimensions, 4.8 cm x 2 cm）in 0.1 M Tris-HCl buffer, pH 7.0 at optimal temperature for optimal time depending on the kind of donor: 37℃/1 h for HA and Ch, 4℃/24 h for Ch4S, and 4℃/72 h for Ch6S. 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. The products were purified based on chain length by HPLC on a TSKgel Amide-80 column and their structure confirmed by ion spray mass spectrometry.

HPLC analysis. HPLC was performed using a Hitachi ELITE LaChrom system equipped with a fluorescence detector（model L-2485; Hitachi）. A TSKgel Amide-80 column（dimensions, 4.6 x 250 mm, Tosoh Co.,Tokyo,Japan）was used for the analysis and 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, adjusted to pH 7.3 with triethylamine. 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 to 100% of solution B over 60 min. The eluted material was detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

Results and discussion

One-step transglycosylation reaction in a BTH-immobilized column was performed as shown in Figure 1. When HA hexasaccharide-PA（HHH-PA）as an acceptor and Ch4S polysaccharide as a donor were used in the reaction, new oligosaccharides having GlcUAβ1-3GalNAc4S units at the non-reducing terminal; 4HHH-PA（octasaccharide）, 44HHH-PA（decasaccharide）, and 444HHH-PA（dodecasaccharide）and larger hybrid oligosaccharides were synthesized as transglycosylated products.（Here, we named the disaccharide units as "H", -4GlcUAβ1-3GlcNAc- and "4", -4GlcUAβ1-3GalNAc4S-, respectively.）The transglycosylated products are shown in the chromatogram in Figure 1. In each reaction, 1.9-2.8 mg of octa- to decasaccharides from H units and 4 units were obtained（Table 1）. We performed multiple reactions and pooled the resulting hybrid oligosaccharides in order to obtain an adequate amount of
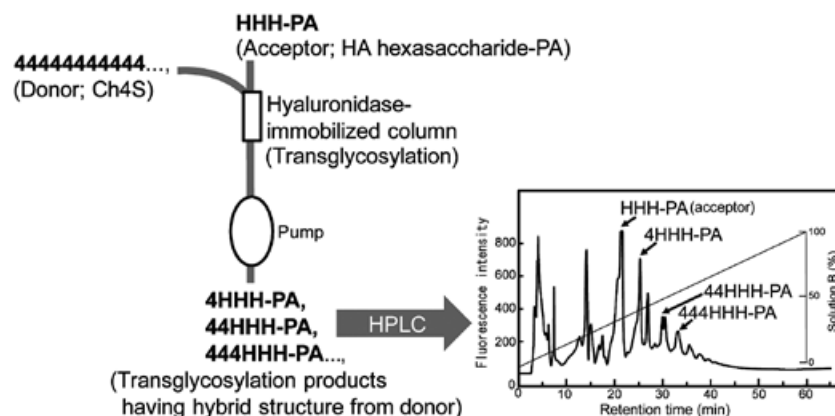
Fig. 1. Transglycosylation reaction in a BTH-immobilized column.
Hybrid oligosaccharides having disaccharide units from donor polysaccharide were synthesized by the combinations of acceptors and the donors using transglycosylation of BTH. An example using HA hexasaccharide-PA （HHH-PA） as an acceptor and Ch4S polysaccharide as a donor is shown. Transglycosylated products （4HHH-PA, 44HHH-PA, and 444HHH-PA） were analyzed by HPLC on a TSKgel Amide-80 column. H, -4GlcUAβ1-3GlcNAc- and 4, -4GlcUAβ1-3GalNAc4S-.

Table 1. Synthesized hybrid oligosaccharides.
The list shows products （octasaccharide to dodecasaccharide） by transglycosylation reactions of BTH using various combinations of acceptors and donors. Amounts of oligosaccharides （mg） in each reaction are provided in the parentheses.

Acceptor \ Donor	HA	Ch	Ch4S	Ch6S
HHH[a]-PA[b]	HHHH-PA （0.52[c]）	0HHH-PA （1.12）	4HHH-PA （1.90）	6HHH-PA （1.49）
	HHHHH-PA （0.38）	00HHH-PA （1.26）	44HHH-PA （2.84）	66HHH-PA （2.10）
	HHHHHH-PA （0.39）	000HHH-PA （0.62）	444HHH-PA （1.95）	666HHH-PA （2.10）
000-PA	H000-PA （0.62）	0000-PA （1.09）	4000-PA （1.21）	6000-PA （1.66）
	HH000-PA （0.52）	00000-PA （0.60）	44000-PA （1.14）	66000-PA （0.42）
	HHH000-PA （0.53）	000000-PA （0.43）	444000-PA （1.26）	66000-PA （0.25）
444-PA	H444-PA （0.33）	0444-PA （0.80）	4444-PA （1.58）	6444-PA （0.41）
	HH444-PA （0.16）	00444-PA （1.08）	44444-PA （1.60）	66444-PA （0.87）
	HHH444-PA （0.38）	000444-PA （0.90）	444444-PA （1.40）	666444-PA （0.73）
666-PA	H666-PA （0.31）	0666-PA （0.95）	4666-PA （2.81）	6666-PA （0.69）
	HH666-PA （0.29）	00666-PA （0.52）	44666-PA （2.86）	66666-PA （0.63）
	HHH666-PA （0.34）	000666-PA （0.37）	444666-PA （2.35）	66666-PA （1.01）

[a]Here disaccharide unit of hyaluronan, chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate were showed as follows. H, -4GlcUAβ1-3GlcNAc-; 0, -4GlcUAβ1-3GalNAc-; 4, -4GlcUAβ1-3GalNAc4S-; 6, -4GlcUAβ1-3GalNAc6S-. [b]PA, 2-pyridylamine. [c]The amount of each oligosaccharide was calculated from uronic acid content measured by carbazole sulfate method.

oligosaccharides for biological experiments. Using this immobilized enzyme system, the problems of low yields and contamination of enzymes are resolved. In the same way, using various combinations of acceptors and donors, we synthesized various other hybrid oligosaccharides （Table 1）. Here, we named the disaccharide units as "6", -4GlcUAβ1-3GalNAc6S- and "0", -4GlcUAβ1-3GalNAc-. Among these, HA-ChS （HA-Ch） hybrid oligosaccharides （hybrid chondroitin [sulfate] oligosaccharides containing H units） are neo-oligosaccharides that have not

been found in nature and that will be useful new research tools.

HA oligosaccharides induce cell proliferation, angiogenesis, wound recovery, by modulating signal transductions through the regulation of phospholylation of proteins or plasma membrane translocation[15, 16]. Specific oligosaccharide sequences from chondroitin sulfates also control tissue regeneration by modulating signal transductions[17, 18] or have potential clinical applications including carriers for transport targeting of drugs[19, 20]. Our new hybrid oligosaccharides with defined structures will also be invaluable for exploration of biological functions of HA and ChS, and may possibly contribute to the studies on transplant and regenerative medicine.

Footnotes

[1]The abbreviations used are: HA, hyaluronan; ChS, chondroitin sulfate; Ch, chondroitin; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; BTH, bovine testicular hyaluronidase; GlcUA, glucronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GalNAc-4-sulfate, GalNAc4S; GalNAc-6-sulfate, GalNAc6S; PA, 2-pyridylamine.

Acknowledgements

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