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Inhibitory effect of chondroitin sulfate oligosaccharides on bovine testicular hyaluronidase

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ABSTRACT

Hyaluronan and chondroitin sulfates are prominent components of the extracellular matrices of animal tissues; however, their functions in relation to their oligosaccharide structures have not yet been fully elucidated. The oligosaccharides of hyaluronan and chondroitin sulfate were prepared and used to investigate their effects on the hydrolysis and transglycosylation reactions of bovine testicular hyaluronidase when hyaluronan was used as a substrate. Hydrolysis and transglycosylation activities were assessed in independent reaction systems by analyzing the products by HPLC. The hydrolysis and transglycosylation reactions of bovine testicular hyaluronidase were dose-dependently inhibited by chondroitin sulfate oligosaccharides, but not by hyaluronan or chondroitin oligosaccharides. A kinetic analysis of the hydrolysis reaction using hyaluronan octasaccharide revealed that the inhibition mode by chondroitin sulfate oligosaccharides was competitive.

1. Introduction

Hyaluronan (HA) is a linear and high molecular weight polysaccharide that is composed of the repeating disaccharide unit, GlcUAβ1–3GlcNAc, and occurs as sugar chains in free form without covalently binding to a protein core (Fraser, Laurent, & Laurent, 1997). Chondroitin sulfates (ChSs) are also linear polysaccharides that are composed of the repeating disaccharide unit, GlcUAβ1–3GalNAc, and are randomly sulfated. ChSs occur as the glycosaminoglycan (GAG) sugar chains of proteoglycans (PGs) covalently bound to a protein core through a linkage structure (Kjellen & Lindahl, 1991; Rodén, 1980). HA and PGs are ubiquitous as prominent components of the extracellular matrices of animal tissues and are involved in various cell functions including tissue organization, homeostasis, and cell signaling. Previous studies reported that their specific biological activities were based on the specific oligosaccharide structures within a long sugar chain (Achur et al., 2008; Habuchi et al., 1992; Kjellen & Lindahl, 1991; Lindahl, Backstrom, Thunberg, & Leder, 1980; Malavaki, Mizumoto, Karamanos, & Sugahara, 2008). However, the functions of HA and ChSs in relation to their structures and chain lengths have not yet been clarified in detail. Thus, one aim of the present study was to examine the functions of the oligosaccharides of HA and ChSs.

HA-degrading enzymes, referred to as hyaluronidases, have been broadly classified into animal hydrolases and bacterial lyases. Bovine testicular hyaluronidase (BTH) is a hydrolase and is an endo-β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.35) that acts on HA and ChSs at the β1, 4-*N*-acetylhexosaminide bonds (Meyer, 1971; Meyer, Hoffman, & Linker, 1960; Meyer & Rapport, 1950, 1952). BTH simultaneously exhibits both hydrolytic and transglycosylation activities on the initial substrate and reaction products from either reaction (Highsmith, Garvin, & Chipman, 1975; Hoffman, Meyer, & Linker, 1956; Saitoh et al., 1995; Weissmann, 1955). Although the optimal conditions for hydrolysis and transglycosylation by BTH differ; pH 4.0–5.0 in the presence of NaCl versus pH 7.0 in the absence of NaCl (Gorham, Olavesen, & Dodgson, 1975; Saitoh et al., 1995), hydrolysis can occur under the optimal conditions for transglycosylation and vice versa; therefore, the reaction

Abbreviations: HA, hyaluronan; ChS, chondroitin sulfate; GAG, glycosaminoglycan; PG, proteoglycan; BTH, bovine testicular hyaluronidase; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; Ch, chondroitin; Hep, heparin; ChSE, chondroitin sulfate E; ChSD, chondroitin sulfate D; PA, 2-pyridylamine; GlcUA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; K_m, Michaelis constant; V_{max}, maximal enzyme velocity; IC_{50}, 50% inhibitory concentration; SPR, surface plasmon resonance.

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mechanism by which BTH hydrolyzes is complex (Cramer, Bailey, Bailey, & Miller, 1994; Deschrevel, Tranchepain, & Vincent, 2008; Highsmith et al., 1975; Hofinger, Bernhardt, & Buschauer, 2007; Kakizaki, Ibori, Kojima, Yamaguchi, & Endo, 2010; Nakatani, 2002; Takagaki et al., 1994). We previously investigated the HA hydrolysis mechanism by BTH using highly purified HA oligosaccharides with defined structures with or without modifications at the reducing and/or non-reducing termini as substrates. The minimum chain length of HA for hydrolysis by BTH is octasaccharide because hexasaccharide is a poor substrate that is only partially cleaved (Kakizaki et al., 2010). The initial recognition site in each oligosaccharide structure by which BTH hydrolyzes and the potential mechanism underlying HA transglycosylation by BTH were revealed. We utilized the transglycosylation reaction of BTH to engineer the GAG sugar chains of PG (Iwafune et al., 2002; Kakizaki, Suto, Tatara, Nakamura, & Endo, 2012; Takagaki, Ishido, Kakizaki, Iwafune, & Endo, 2002a; Takagaki et al., 2002b; Takagaki, Munakata, Kakizaki, Majima, & Endo, 2000a; Takagaki, Munakata, Majima, Kakizaki, & Endo, 2000b).

The regulators of animal hyaluronidases including BTH are invaluable not only for examining the complex mechanism responsible for their enzymatic reaction, but also for both glycotechnological applications and future medical applications for diseases in which HA is involved. Although a large number of studies have focused on identifying the inhibitors of hyaluronidases, the activators of BTH have not received the same attention. Only a modified donor GAG as the salt of a divalent cation could activate the transglycosylation of BTH (Kakizaki et al., 2011). Mono- or divalent metal cations, plant-derived inhibitors, and sulfated oligosaccharides, as well as inhibitors in mammalian serum, such as the inter-α-trypsin inhibitor, are potential inhibitors of hyaluronidase activity (Girish, Kemparaju, Nagaraju, & Vishwanath, 2009; Mio, Carrette, Maibach, & Stern, 2000; Mio & Stern, 2002; Salmen et al., 2005; Toida, Ogita, Suzuki, Toyoda, & Imanari, 1999; Vercruysse, Ziebell, & Prestwich, 1999). Most studies that have examined the inhibition of hydrolysis have reported inhibitory effects on animal hyaluronidases, whereas, the effects on transglycosylation are not well understood. Another aim of the present study was to identify an inhibitor or activator of BTH.

In the present study, we investigated the effects of the oligosaccharides of HA and ChSs with known structures on the hydrolysis and transglycosylation activities of BTH by HPLC analysis of the enzymatic reaction products. The tetra- or larger oligosaccharides of chondroitin 4-sulfate (Ch4S) and chondroitin 6-sulfate (Ch6S) inhibited both the hydrolysis and transglycosylation activities of BTH in a dose-dependent manner.

2. Materials and methods

2.1. Materials

BTH (type I-S) was purchased from Sigma-Aldrich (St. Louis, MO). HA (from *Streptococcus zooepidemicus*; average M_r of 80,000) was purchased from Food Chemifa (Tokyo, Japan). Ch4S (from whale cartilage; average M_r of 34,000), Ch6S (from shark cartilage; average M_r of 64,000), chondroitin (Ch, chemically desulfated Ch6S; average M_r of 10,000), and chondroitin ABC lyase (from *Proteus vulgaris*) were obtained from Seikagaku Biobusiness (Tokyo, Japan). The resin of Bio-Gel P-4 (extra fine) was from Bio-Rad (Hercules, CA). Other reagents were of analytical grade and obtained from commercial sources.

2.2. Preparation of GAG oligosaccharides

The tetrasaccharides or larger oligosaccharides of HA, Ch, Ch4S, and Ch6S with an *N*-acetylhexosamine at the reducing terminus and GlcUA at the non-reducing terminus were prepared by the partial digestion of GAG chains with BTH and purified according to a previously reported method (Kakizaki et al., 2010), with a slight modification for the time of incubation with BTH, which was 3 h for HA and Ch and 24 h for Ch4S and Ch6S. The unsaturated disaccharide compositions of GAGs following the chondroitin ABC lyase digestion were analyzed (Sugahara, Okumura, & Yamashina, 1989; Yoshida, Miyauchi, Kikuchi, Tawada, & Tokuyasu, 1989). The compositions of starting materials were as follows; ΔdiOS:ΔdiHS:ΔdiS = 2.8:74.1:23.1 in Ch4S, 1.8:18.1:80.1 in Ch6S, and 94.2:0:5.8 in Ch. The compositions of the oligosaccharides prepared were as follows; ΔdiOS:ΔdiHS:ΔdiS = 3.8:79.0:17.2 in the Ch4S tetrasaccharide, 2.4:78.9:18.7 in the Ch4S hexasaccharide, 7.0:66.0:27.0 in the Ch4S octasaccharide, 2.0:63.0:35.0 in the Ch4S decasaccharide, 3.9:16.1:80.1 in the Ch6S tetrasaccharide, 1.0:19.4:79.6 in the Ch6S hexasaccharide, 2.0:13.0:85.0 in the Ch6S octasaccharide, 0:13.0:87.0 in the Ch6S decasaccharide, 90.4:0:9.6 in the Ch tetrasaccharide, and 95.2:0:4.8 in the Ch hexasaccharide. Disaccharides, *N*-acetylhyalobiuronic acid (HA disaccharide), and *N*-acetylchondrosin (Ch disaccharide), were prepared by acid hydrolysis of barium salt followed by *N*-acetylation of HA and Ch4S, respectively (Davidson & Meyer, 1954; Gmeiner, Luderitz, & Westphal, 1969; Levene, 1941), and purified by gel filtration chromatography using the Bio-Gel P-4 column (1.8 cm × 122 cm) with 0.5 M pyridine acetate buffer (pH 6.5) as eluent at a flow rate of 0.15 ml/min. The structure and purity of oligosaccharides was assessed by HPLC and/or TLC before or after the digestion with a structurally specific enzyme, and ion-spray mass spectrometry. Oligosaccharides with 100% purity of chain length assessed by HPLC were analyzed by ion-spray mass spectrometry. The mass spectra were used to determine the composition of oligosaccharides with various numbers of sulfate groups (Table 1). The uronic acid content of the fractions was determined by the carbazole sulfuric acid method (Bitter & Muir, 1962). The HA hexasaccharide was pyridylaminated at the reducing termini (Hase, Ibuki, & Ikenaka, 1984; Kon, Takagaki, Kawasaki, Nakamura, & Endo, 1991)

Table 1
The composition of oligosaccharides with various numbers of sulfate groups determined from mass ion-spray spectra.

Oligosaccharides	Composition (%)
Ch4S-4[a]	4–2S[b] (100)
Ch4S-6	6–3S (93), 6–1S (7)
Ch4S-8	8–4S (41), 8–2S (59)
Ch4S-10	10–5S (10), 10–4S (38), 10–3S (52)
Ch6S-4	4–2S (100)
Ch6S-6	6–3S (100)
Ch6S-8	8–4S (88), 8–2S (12)
Ch6S-10	10–4S (23), 10–1S (77)

[a] Oligosaccharides were as follows: Ch4S-4, the Ch4S tetrasaccharide; Ch4S-6, the Ch4S hexasaccharide; Ch4S-8, the Ch4S octasaccharide; Ch4S-10; the Ch4S decasaccharide; Ch6S-4, the Ch6S tetrasaccharide; Ch6S-6, the Ch6S hexasaccharide, Ch6S-8, the Ch6S octasaccharide; Ch6S-10; the Ch6S decasaccharide.
[b] Composition is expressed by chain length (hyphen) the number of sulfate groups. For example, 4-2S is tetrasaccharide with 2 sulfate groups.

2.3. Hydrolysis reaction by BTH

The hydrolysis reaction by BTH was performed on the HA (M_r = 80,000) or HA octasaccharide as a substrate in 50 μl of 0.1 M sodium acetate buffer (pH 5.0), containing 150 mM NaCl, with or without various amounts of the test oligosaccharides as possible inhibitors or activators (di-, tetra-, or hexasaccharides of HA or Ch, tetra-, hexa-, octa-, or decasaccharides of Ch4S, Ch6S) under the conditions (amounts of the substrate and enzyme, incubation time) of the linear part of the enzyme reaction. In the case of HA (M_r = 80,000) as a substrate, the reaction mixture containing

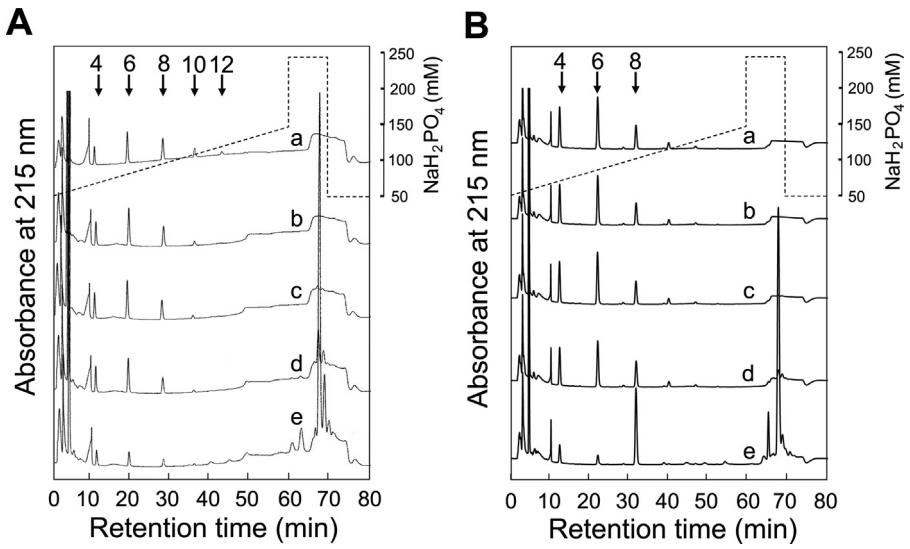


Fig. 1. *HPLC analysis of HA hydrolysis products by BTH in the presence of the Ch4S tetrasaccharide.* The HA (M_r = 80,000) (A) or HA octasaccharide (B) as a substrate was incubated with BTH in the absence (a) or presence ((b) 2; (c) 20; (d) 200; (e) 2000 μM) of the Ch4S tetrasaccharide at 37 °C, as described in Section 2.3. The reaction products were analyzed by HPLC on a YMC-Pack Polyamine II column. The column was eluted with a linear gradient of 50–148 mM NaH_2PO_4 at a flow rate of 1.0 ml/min over a period of 60 min. Oligosaccharides were detected by UV absorbance at 215 nm. Arrows indicate the elution positions of standard HA oligosaccharides with known chain lengths. The dashed line is a NaH_2PO_4 gradient line.

0.2 μg/μl of HA and 0.2 μg/μl of BTH was incubated at 37 °C for 1 h. In the case of the HA octasaccharide as a substrate, the reaction mixture containing 0.65 μg/μl of the HA octasaccharide and 0.15 μg/μl of BTH was incubated at 37 °C for 3 h. The reaction was stopped by boiling for 5 min, and the reaction mixture was centrifuged for 5 min at 8000 × *g* at 4 °C and filtered. The filtrate was analyzed by HPLC. Hydrolysis activity (%) by BTH was assessed according to the production of HA tetra- and hexasaccharides, which were the final products. Hydrolysis activity in the absence of the test oligosaccharides was defined as 100%. This was assessed from the amounts of the peak areas of HA tetra- and hexasaccharides on HPLC. Incidentally, the HA tetra- and hexasaccharides produced could be distinguished from the test HA oligosaccharides by subtracting the peak areas of the pre-reaction from those of the post-reaction because HA tetra- and hexasaccharides were either not, or barely hydrolyzed by BTH, respectively (Kakizaki et al., 2010). Inhibitory activity (%) was calculated by subtracting hydrolysis activity in the presence of the test oligosaccharides from that in their absence.

In the kinetic experiments using the HA octasaccharide as a substrate, the mole amount of the HA tetrasaccharide produced during the reaction was determined from the standard curve of the HPLC peak areas using the standard HA tetrasaccharide with a known mole amount. The Michaelis constant (K_m) and maximal enzyme velocity (V_{max}) values were calculated by Lineweaver–Burke plots. K_m was expressed in mM and V_{max} was expressed in pmol/min.

2.4. Transglycosylation reaction by BTH

The transglycosylation reaction by BTH was performed using 13.0 ng/μl of pyridylaminated HA hexasaccharide (PA–HA hexasaccharide) as an acceptor and 0.2 μg/μl of HA (M_r = 80,000) as a donor with 0.08 μg/μl of BTH in 0.1 M Tris–HCl buffer, pH 7.0 (NaCl free) at 37 °C for 1 h in the absence or presence of various amounts of the test oligosaccharides (di-, tetra-, or hexasaccharides of HA or Ch, tetra-, hexa-, octa-, or decasaccharides of Ch4S, Ch6S). The reaction was stopped by boiling for 5 min, and the reaction mixture was centrifuged for 5 min at 8000 × *g* at 4 °C and filtered. The filtrate was analyzed by HPLC. The transglycosylation activity of BTH (%) was estimated by the total peak areas of the PA oligosaccharides (the PA–HA octasaccharide to the PA–HA eicosasaccharide) after the reaction. The inhibitory activities (%) of the test oligosaccharides were calculated by subtracting transglycosylation activity in the presence of the test oligosaccharides from transglycosylation activity in their absence.

2.5. HPLC analysis

HPLC was performed using a Hitachi ELITE LaChrom system equipped with a UV detector (model L-2420; Hitachi, Tokyo, Japan) and/or fluorescence detector (model L-2485; Hitachi). A YMC-Pack Polyamine II column (4.6 × 250 mm; YMC Co., Tokyo, Japan) was used to analyze the reaction products of BTH. Two solutions were prepared: solution A was 50 mM NaH_2PO_4, and solution B was 246 mM NaH_2PO_4. Reaction products were injected onto the column equilibrated with solution A. Oligosaccharides were eluted with a linear gradient of 0–60% of solution B (corresponding to 50–148 mM of NaH_2PO_4) at a flow rate of 1.0 ml/min over a period of 60 min. Oligosaccharides were monitored by UV absorbance at 215 nm and/or the fluorescence of PA (ex. 320 nm and em. 400 nm).

2.6. Ion-spray mass analysis

Mass spectra of GAG oligosaccharides were obtained on a PE-Sciex API-100 single-quadrupole mass spectrometer (Thornhill, Ontario, Canada), equipped with an atmospheric pressure ionization source, as described previously (Takagaki et al., 1992). The mass spectrometer was operated in the negative ion mode. Samples dissolved in 50% of isopropanol were ionized by ESI and continuously infused into the ESI chamber at a flow rate of 5 μl/min. Oligosaccharide compositions were determined by the mass spectra.

3. Results

3.1. Inhibitory effects of ChS oligosaccharides on HA hydrolysis by BTH

The effects of HA and ChS oligosaccharides on HA hydrolysis by BTH were investigated. The hydrolysis mechanism of BTH using higher molecular weight HA as a substrate is known to

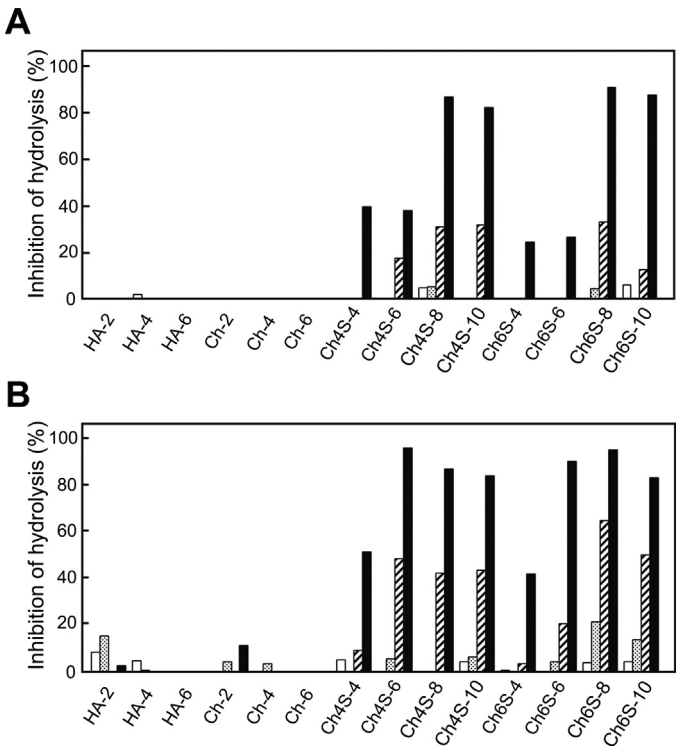


Fig. 2. *Effects of GAG oligosaccharides on HA hydrolysis by BTH.* The HA (M_r = 80,000) (A) or HA octasaccharide (B) was incubated with BTH in the absence or presence (2, 20, 200, and 2000 μM) of test GAG oligosaccharides at 37 °C, as described in Section 2.3. The reaction products were analyzed by HPLC on a YMC-Pack Polyamine II column, as shown in Fig. 1. The hydrolysis activity of BTH was assessed by the total peak areas of the HA tetra- and hexasaccharides because the digestion with BTH mainly yielded a mixture of tetra- and hexasaccharides as the final products. The inhibitory effects of GAG oligosaccharides were determined on the basis of hydrolysis activity. Complete inhibition in the reaction was defined as 100%. HA-2, the HA disaccharide; HA-4, the HA tetrasaccharide; HA-6, the HA hexasaccharide; Ch-2, the Ch disaccharide; Ch-4, the Ch tetrasaccharide; Ch-6, the Ch hexasaccharide; Ch4S-4, the Ch4S tetrasaccharide; Ch4S-6, the Ch4S hexasaccharide; Ch4S-8, the Ch4S octasaccharide; Ch4S-10; the Ch4S decasaccharide; Ch6S-4, the Ch6S tetrasaccharide; Ch6S-6, the Ch6S hexasaccharide, Ch6S-8, the Ch6S octasaccharide; Ch6S-10; the Ch6S decasaccharide. White bar, 2 μM; dotted bar, 20 μM; diagonally lined bar, 200 μM; solid bar, 2000 μM.

be extremely complex, while the octasaccharide is the minimum chain length of HA for hydrolysis by BTH; therefore, experiments were performed using the HA octasaccharide as a substrate in addition to experiments using HA (M_r = 80,000) as a substrate. Representative chromatograms on which the inhibitory effects of the oligosaccharide were observed are shown in Fig. 1A and B. The Ch4S tetrasaccharide as the test oligosaccharide was eluted after 65 min with multiple peaks only being observed at the higher concentrations; therefore, these peaks did not overlap with the HA oligosaccharides as hydrolysis products. The other ChS oligosaccharides used as test oligosaccharides were also eluted after 65 min (data not shown). When HA oligosaccharides were used, their peaks (test oligosaccharide control) were subtracted from the peaks of HA oligosaccharides in the reaction mixture after the reaction in order to define the HA oligosaccharides as reaction products. Without a test oligosaccharide, the digestion of HA with BTH mainly yielded a mixture of HA tetra- and hexasaccharides as the final products (Fig. 1A-a and 1B-a). When oligosaccharides inhibited or activated BTH, the peak areas of the HA tetra- and hexasaccharides were correspondingly decreased or increased. No oligosaccharide activated HA hydrolysis by BTH. The inhibitory effects of oligosaccharides were assessed based on the production of HA tetra- and hexasaccharides (Fig. 2). Nonsulfated oligosaccharides, i.e. the di-, tetra- and hexasaccharides of HA and Ch, did not have any inhibitory

Table 2
Inhibitory effects of the ChS oligosaccharides on HA hydrolysis by BTH. The HA octasaccharide as a substrate was incubated with BTH in the presence (2, 20, 50, 100, 200, 250, 500, 1000, 2000, and 4000 μM) of the tetra-, hexa-, octa-, or decasaccharides of Ch4S or Ch6S at 37 °C, as described in Section 2.3. The reaction products were analyzed by HPLC. The hydrolysis activity of BTH was assessed by the total peak areas of the HA tetra- and hexasaccharides produced (final products), as shown in Fig.3 and IC_{50} values were calculated.

Oligosaccharides	IC_{50} (μM)
Ch4S-4	1.91×10^3
Ch4S-6	0.21×10^3
Ch4S-8	0.27×10^3
Ch4S-10	0.23×10^3
Ch6S-4	2.55×10^3
Ch6S-6	0.55×10^3
Ch6S-8	0.08×10^3
Ch6S-10	0.21×10^3

Ch4S-4, the Ch4S tetrasaccharide; Ch4S-6, the Ch4S hexasaccharide; Ch4S-8, the Ch4S octasaccharide; Ch4S-10; the Ch4S decasaccharide; Ch6S-4, the Ch6S tetrasaccharide; Ch6S-6, the Ch6S hexasaccharide, Ch6S-8, the Ch6S octasaccharide; Ch6S-10; the Ch6S decasaccharide.

effects on HA hydrolysis by BTH with either of the HA (M_r = 80,000) or HA octasaccharides as substrates. In contrast, sulfated oligosaccharides, i.e. the tetra-, hexa, octa, and decasaccharides of Ch4S and Ch6S, had inhibitory effects on HA hydrolysis by BTH in both substrate cases. The inhibitory effects of ChSs appear to be higher in larger oligosaccharides. Hydrolysis activity was almost completely inhibited at 2000 μM of octasaccharides of ChS in the case of HA (M_r = 80,000) as a substrate and at 2000 μM of hexasaccharides of ChS in the case of HA octasaccharide as a substrate.

In order to examine dose-dependent inhibition with ChS oligosaccharides in more detail and determine 50% inhibitory concentration (IC_{50}) values, an inhibition experiment was performed with more dose points with the HA octasaccharide as the substrate. Dose-dependent changes in the peak areas of the HA tetra- and hexasaccharides were similar to their total peak area (Fig. 3). IC_{50} values were subsequently determined based on the total peak areas of the tetra- and hexasaccharides, as shown in Fig. 3C (Table 2). The highest to lowest inhibitory effects observed were broadly the Ch6S octa- > Ch4S hexa-, octa-, deca-, Ch6S deca- > Ch6S hexa- > Ch4S tetra- > Ch6S tetrasaccharide. The IC_{50} values of hexasaccharides or larger oligosaccharides were significantly smaller than those of tetrasaccharides (Table 2). When HA (M_r = 80,000) was used as the substrate, the reaction was difficult to analyze because of the complex reaction mechanism; however, IC_{50} values in this study were calculated from the experiment shown in Fig. 2A in which the dose points used were lower. IC_{50} values were 2.93×10^3, 7.61×10^3, 0.44×10^3, 0.56×10^3, 5.37×10^3, 4.99×10^3, 0.39×10^3, and 0.63×10^3 μM for the Ch4S tetra-, Ch4S hexa-, Ch4S octa-, Ch4S deca-, Ch6S tetra-, Ch6S hexasa-, Ch6S octa-, and Ch6S decasaccharide, respectively.

Lineweaver-Burk plots were generated to determine the mode of inhibition (Fig. 4) when tetra- and hexasaccharides of ChS were used as inhibitors. No significant difference was observed in V_{max} determined at 500 μM of ChS oligosaccharides and in the absence of ChS oligosaccharides, suggesting that these inhibitory effects were competitive.

3.2. *Inhibitory effects of oligosaccharides on HA transglycosylation by BTH*

Hyaluronidase is known to catalyze the transglycosylation reaction as a reverse reaction of hydrolysis. The effects of the test oligosaccharides on HA transglycosylation by BTH were investigated in the reaction system that used pyridylaminated HA hexasaccharide (PA–HA hexasaccharide) as an acceptor and HA

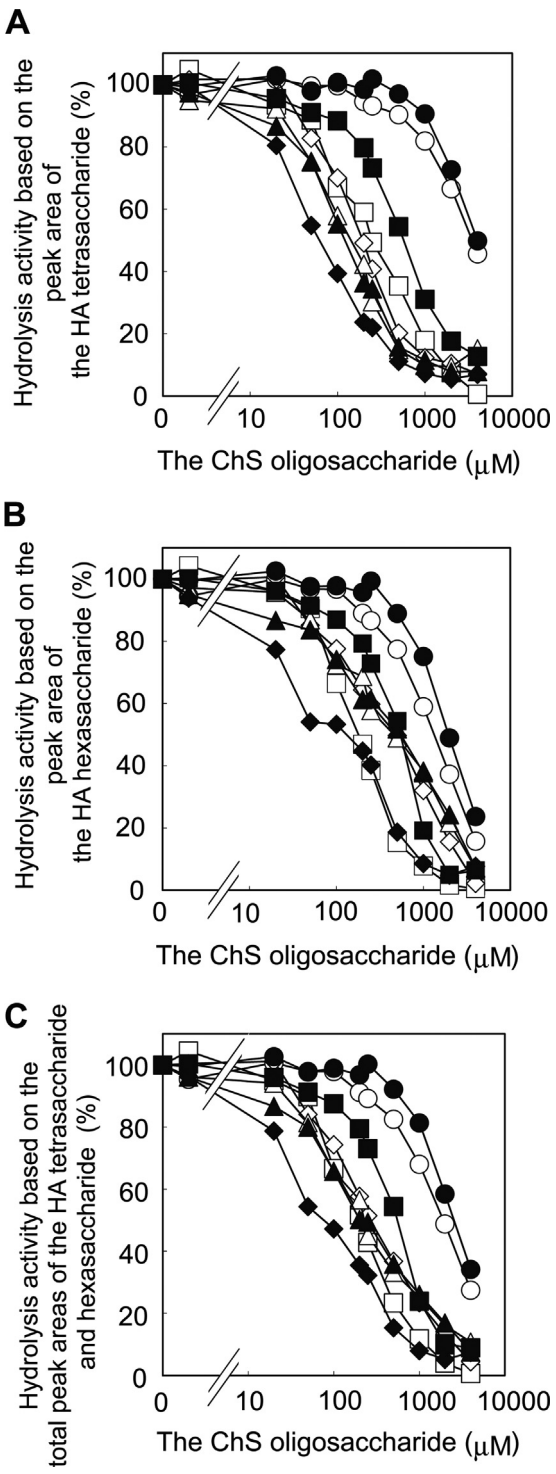


Fig. 3. Concentration-dependent inhibition of HA hydrolysis by BTH by ChS oligosaccharides using the HA octasaccharide as a substrate. The HA octasaccharide as a substrate was incubated with BTH in the absence or presence (2, 20, 50, 100, 200, 250, 500, 1000, 2000, and 4000 μM) of the tetra-, hexa-, octa-, or decasaccharides of Ch4S or Ch6S at 37 °C, as described in Section 2.3. The reaction products were analyzed by HPLC on a YMC-Pack Polyamine II column. The hydrolysis activity of BTH was assessed by the peak area of the HA tetrasaccharide (A), by that of the HA hexasaccharides (B), or by the total peak areas of the HA tetrasaccharide and HA hexasaccharide (C). The peak areas in the control reaction without the ChS oligosaccharide were defined as 100%. IC_{50} values were calculated from these plots and shown in Table 1. Open circle, the Ch4S tetrasaccharide; open square, the Ch4S hexaccharide; open diamond, the Ch4S octasaccharide; open triangle, the Ch4S decasaccharide; closed circle, the Ch6S tetrasaccharide; closed square, the Ch6S hexasaccharide; closed diamond, the Ch6S octasaccharide; closed triangle, the Ch6S decasaccharide.

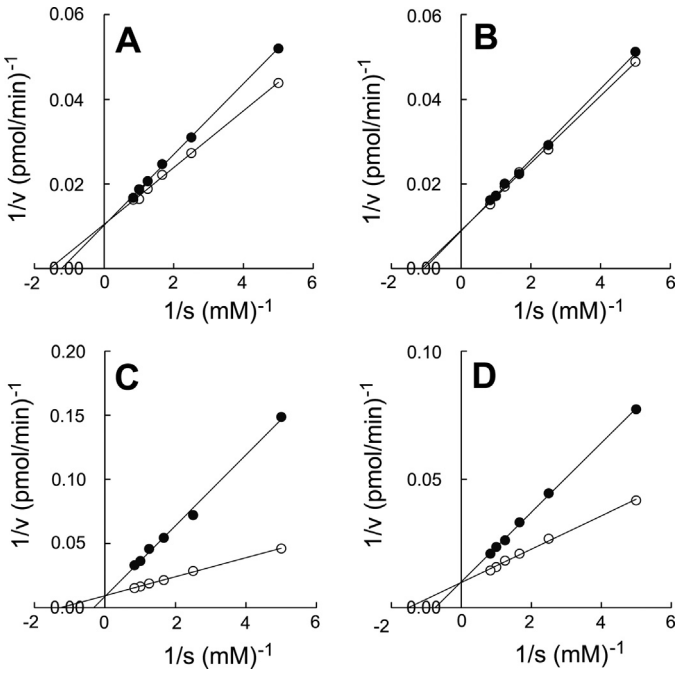


Fig. 4. *Kinetic analysis of BTH hydrolysis inhibition by ChS oligosaccharides.* Lineweaver–Burke plots were drawn based on the hydrolysis activity of BTH on the HA octasaccharide (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM) as a substrate. The hydrolysis activity of BTH was assessed by the peak area of the HA tetrasaccharide on HPLC. Open circle, in the absence of the ChS oligosaccharide; closed circle, in the presence of 500 μM of the ChS oligosaccharide ((A) the Ch4S tetrasaccharide; (B) the Ch6S tetrasaccharide; (C) the Ch4S hexasaccharide; (D) the Ch6S hexasaccharide). S represents the initial substrate HA concentration (mM). *V* is the velocity (pmol/min) of the reaction defined as pmol of the HA tetrasaccharide generated per min during the reaction.

(M_r = 80,000) as a donor. Representative chromatograms of the transglycosylation reaction products are shown in Fig. 5A and C. Without the test oligosaccharides (Fig. 5A-a and C-a), the peaks of the PA–HA octasaccharide and larger PA oligosaccharides as transglycosylated products (elongated PA–HA oligosaccharides) were detected in addition to the peak of the acceptor PA–HA hexasaccharide. Chromatograms on which the inhibitory/activatory effects of the test oligosaccharides are observed had fewer/more of the peaks of the larger PA oligosaccharides with smaller/larger peak areas. The effects of the test oligosaccharides were assessed based on the total peak areas of the elongated PA oligosaccharides (from the PA octasaccharide to the PA eicosasaccharide) in relation to the control without the test oligosaccharides (Fig. 6). In contrast to the hydrolysis reaction, decrease in the transglycosylated products was observed even with the tetra- and hexasaccharides of HA or Ch at higher concentrations. Also, ChS tetrasaccharides showed inhibition as high as larger oligosaccharides. IC_{50} values were calculated from this experiment as a reference (Table 3). The range of the IC_{50} values in the transglycosylation reaction was similar to that in the hydrolysis reaction. The IC_{50} values of ChS tetrasaccharides compared to those of larger oligosaccharides.

Monitoring the reaction products by UV absorbance at 215 nm in parallel with monitoring the fluorescence of PA revealed the production of non-pyridylaminated HA oligosaccharides in a manner that was dependent on the addition of HA oligosaccharides as test oligosaccharides (Fig. 5D). This was also observed when Ch oligosaccharides but not ChS oligosaccharides were added as the test oligosaccharides. As shown in Fig. 5B, when ChS oligosaccharides were added in this transglycosylation reaction system, decrease rather than increase in the peaks of non-pyridylaminated HA oligosaccharides was observed. At 2000 μM

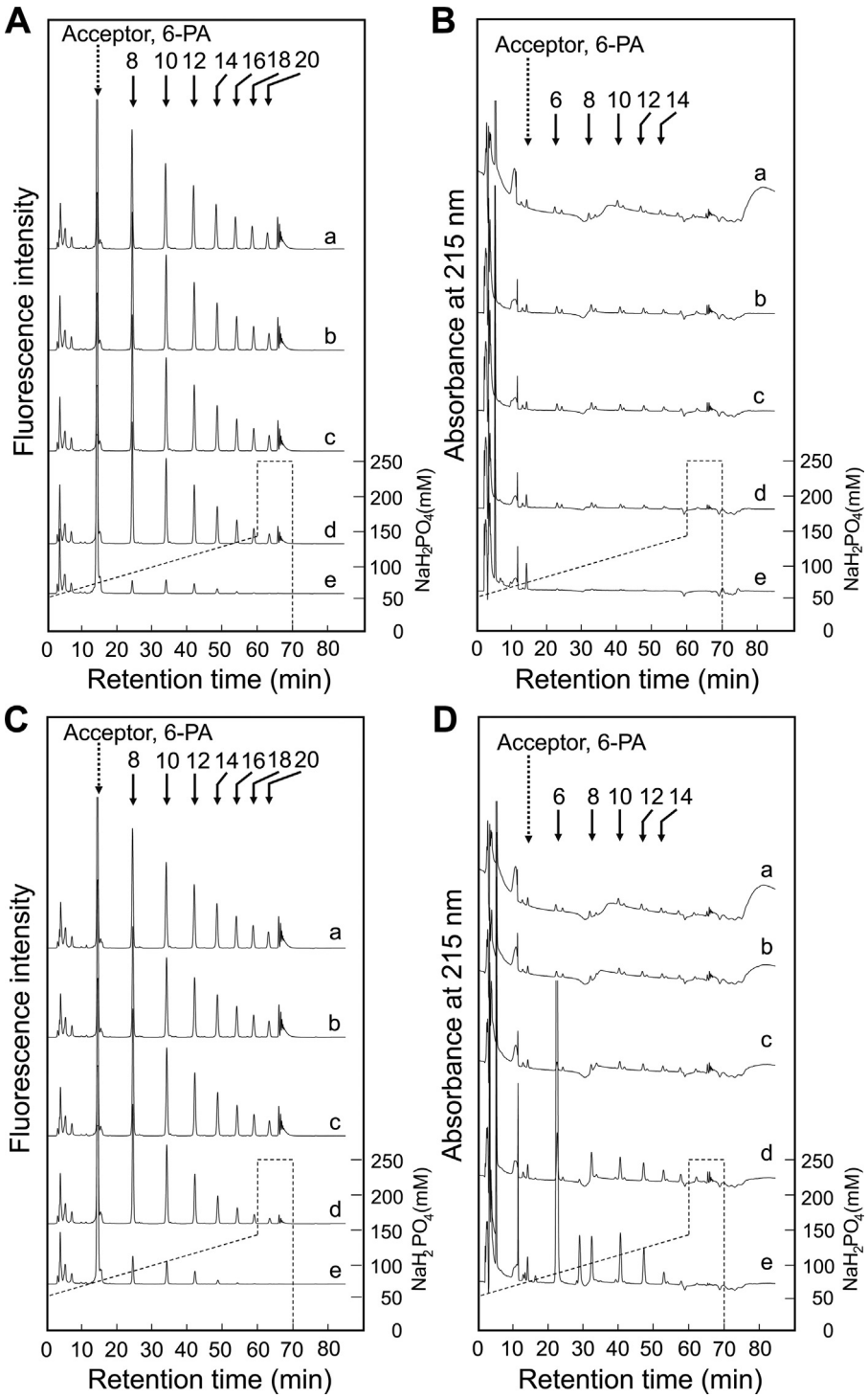


Fig. 5. *HPLC analysis of HA transglycosylation products by BTH.* The PA–HA hexasaccharide as an acceptor and HA (M_r = 80,000) as a donor were incubated with BTH in the absence (a) or presence ((b) 2; (c) 20; (d) 200; (e) 2000 μM) of the Ch4S hexasaccharide (A and B) or HA hexasaccharide (C and D) at 37 °C, as described in Section 2.4. The reaction products were analyzed by HPLC. Oligosaccharides were detected by both the fluorescence of PA (ex. 320 nm, and em. 400 nm) (A and C) and UV absorbance at 215 nm (B and D). Dashed arrows indicate the elution positions of the PA–HA hexasaccharide as an acceptor. Solid arrows indicate the elution positions of standard PA–HA oligosaccharides (A and C) or non-labelled HA oligosaccharides (B and D) with known chain lengths. Dashed lines are the NaH_2PO_4 gradient lines.

of ChS oligosaccharides, they were detected in only trace amounts. The inhibition of PA–HA hexasaccharide elongation by HA hexasaccharide (Fig. 5A) was correlated with the production of non-pyridylaminated oligosaccharides by HA hexasaccharides (Fig. 5B). In order to examine whether these non-pyridylaminated HA oligosaccharides increased by addition of HA hexasaccharide are from only hydrolysis products of donor HA, or also from transglycosylated products between HA oligosaccharides, transglycosylation reaction of BTH was performed under the conditions (a–e) shown in the table embedded in Fig. 7. Regardless of the presence or absence of the acceptor (PA–HA hexasaccharide), in the conditions where the HA hexasaccharide was added as the test oligosaccharide (Fig. 7D-c and D-d), there were larger amounts of non-pyridylaminated HA oligosaccharides produced than those

Table 3
Inhibitory effects of GAG oligosaccharides on transglycosylation by BTH. The transglycosylation reaction of BTH was performed using the PA–HA hexasaccharide as an acceptor and long chain HA as a donor in the presence (2, 20, 200, and 2000 μM) of the oligosaccharides of HA, Ch4S, or Ch6S at 37 °C, as described in Section 2.4. The reaction products were analyzed by HPLC in the same manner as shown in Fig. 5A. The transglycosylation activity of BTH was assessed by the total peak areas of oligosaccharides from the octasaccharide to docosasaccharide, and IC_{50} values were calculated.

Oligosaccharides	IC_{50} (μM)
HA-2	ND
HA-4	2.50×10^3
HA-6	0.57×10^3
Ch-2	ND
Ch-4	1.68×10^3
Ch-6	1.04×10^3
Ch4S-4	0.20×10^3
Ch4S-6	0.51×10^3
Ch4S-8	0.13×10^3
Ch4S-10	0.20×10^3
Ch6S-4	0.46×10^3
Ch6S-6	0.28×10^3
Ch6S-8	0.09×10^3
Ch6S-10	0.53×10^3

ND, not determined; HA-2, the HA disaccharide; HA-4, the HA tetrasaccharide; HA-6, the HA hexasaccharide; Ch-2, the Ch disaccharide; Ch-4, the Ch tetrasaccharide; Ch-6, the Ch hexasaccharide; Ch4S-4, the Ch4S tetrasaccharide; Ch4S-6, the Ch4S hexasaccharide; Ch4S-8, the Ch4S octasaccharide; Ch4S-10; the Ch4S decasaccharide; Ch6S-4, the Ch6S tetrasaccharide; Ch6S-6, the Ch6S hexasaccharide, Ch6S-8, the Ch6S octasaccharide; Ch6S-10; the Ch6S decasaccharide.

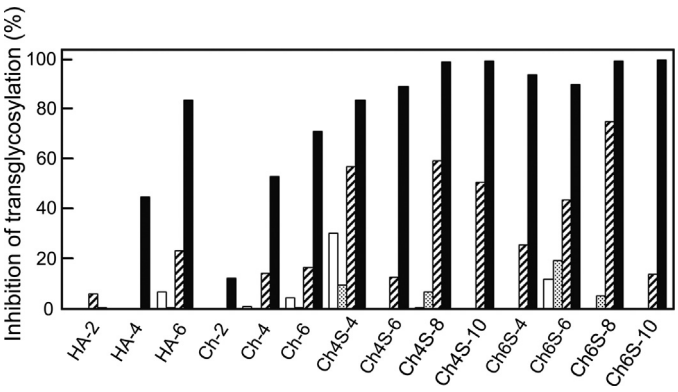


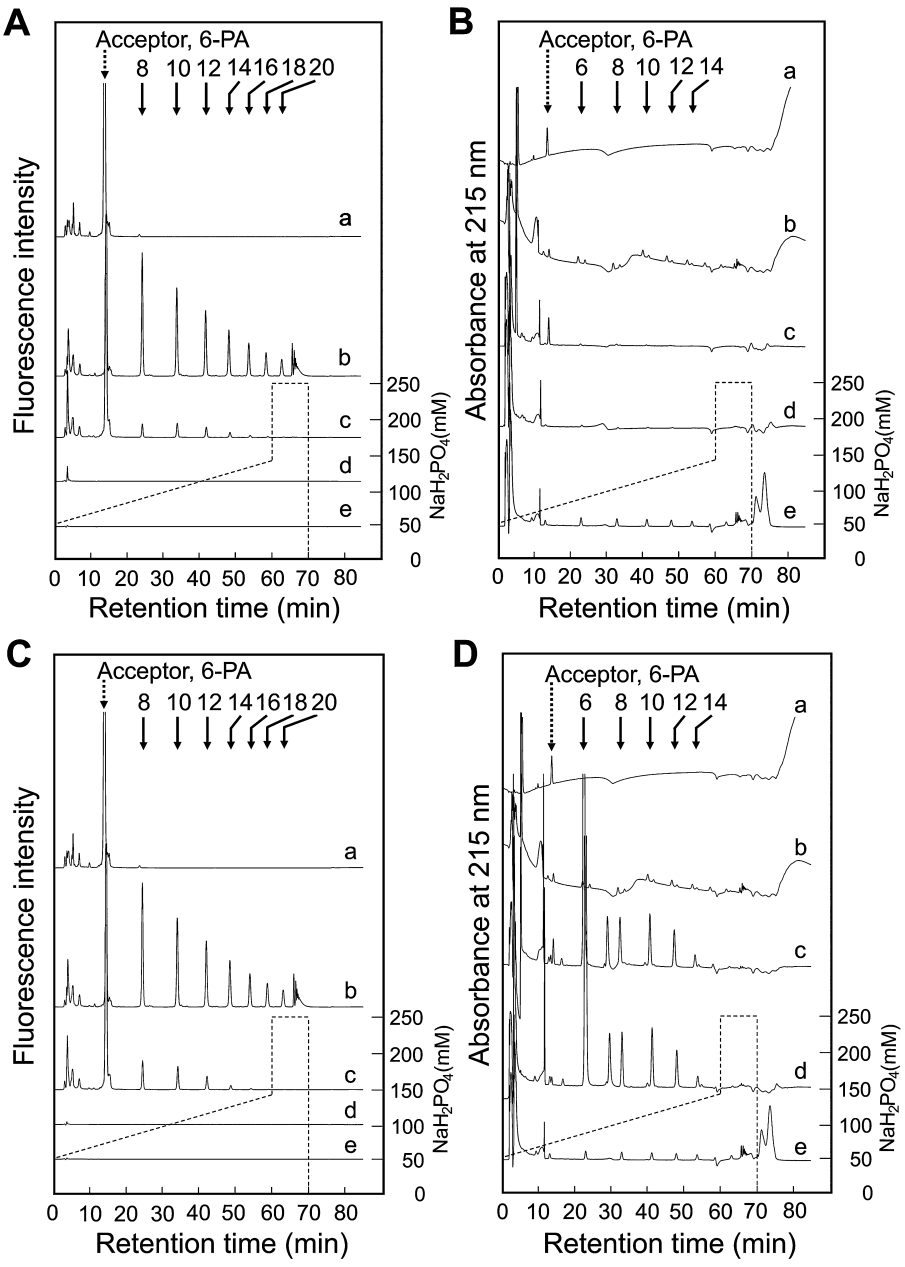
Fig. 6. *Effects of GAG oligosaccharides on transglycosylation to the acceptor PA–HA hexasaccharide from the donor HA by BTH.* The PA–HA hexasaccharide as an acceptor and HA (M_r = 80,000) as a donor were incubated with BTH in the absence or presence (2, 20, 200, and 2000 μM) of the test oligosaccharides at 37 °C, as described in Section 2.4. The reaction products were analyzed by HPLC on a YMC-Pack Polyamine II column, as shown in Fig. 5A. The transglycosylation activity of BTH was assessed by the total peak areas of the elongated PA–HA oligosaccharides (from the PA octasaccharide to the PA eicosasaccharide). The inhibitory effects of GAG oligosaccharides were determined on the basis of transglycosylation activity. Complete inhibition in the reaction was defined as 100%. HA-2, the HA disaccharide; HA-4, the HA tetrasaccharide; HA-6, the HA hexasaccharide; Ch-2, the Ch disaccharide; Ch-4, the Ch tetrasaccharide; Ch-6, the Ch hexasaccharide; Ch4S-4, the Ch4S tetrasaccharide; Ch4S-6, the Ch4S hexasaccharide; Ch4S-8, the Ch4S octasaccharide; Ch4S-10; the Ch4S decasaccharide; Ch6S-4, the Ch6S tetrasaccharide; Ch6S-6, the Ch6S hexasaccharide, Ch6S-8, the Ch6S octasaccharide; Ch6S-10; the Ch6S decasaccharide. White bar, 2 μM; dotted bar, 20 μM; diagonal bar, 200 μM; solid bar, 2000 μM.

when HA hexasaccharide was not added (Fig. 7D-b and D-e). Peaks of non-pyridylaminated HA detected in Fig. 7D-e contain both hydrolysis products of donor HA and their transglycosylated products. In Fig. 7D-d, those peaks were increased compared to Fig. 7D-e. Because HA oligosaccharide did not enhance HA hydrolysis this increase was suggested to be due to transglycosylation between non-pyridylaminated HA oligosaccharides.

4. Discussion

It is conceivable that inhibitory effect of oligosaccharides on the hydrolysis of BTH is determined by sulfation degree, steric constitution (by positions of bulky sulfate groups), and affinity for BTH in relation to their oligosaccharide structures. The results of the present study revealed that the inhibition of ChS hexasaccharides or larger oligosaccharides were greater than that of tetrasaccharides, and this was attributed to oligosaccharides having longer chain lengths and more sulfate groups, thereby increasing electrostatic interactions with enzyme proteins. In the case of using HA octasaccharide as a substrate, similar inhibition activity among Ch4S hexa-, octa- and decasaccharide and Ch6S decasaccharide is suggested to be due to longer oligosaccharides having relatively low sulfation degree with respect to chain length (Table 1). By contrast, the highest inhibition activity of Ch6S octasaccharide is suggested to be due to higher sulfation degree than that of Ch4S octa-, Ch4S deca-, and Ch6S decasaccharides. Sulfated GAGs have been reported to show an inhibitory effect enhanced by chemically full *O*-sulfation on the HA hydrolysis by BTH (Toida et al., 1999). Non-sulfated HA did not inhibit BTH activity, whereas fully *O*-sulfated HA oligosaccharides with an unsaturated GlcUA at the nonreducing terminus did in a manner that was dependent on the chain length (Suzuki, Toyoda, Toida, & Imanari, 2001). Ch4S was previously shown to more effectively inhibit testicular hyaluronidase than Ch6S (Afify, Stern, Guntenhoner, & Stern, 1993). In the present study, the inhibition of Ch4S oligosaccharides on BTH hydrolysis was significantly greater than that of Ch6S oligosaccharides when the sulfation degree in the same chain length is similar. However, in longer chain lengths the sulfation degree makes a greater contribution to inhibition than the sulfation position. During preparation of ChS oligosaccharides a problem is that larger ChS oligosaccharides have considerable heterogeneity in sulfation degree due to the limitation of separation capacity of current chromatography. Therefore, tetra- and hexasaccharide will be invaluable for future applications since the heterogeneity is relatively low. Testicular hyaluronidase has been known to show a preference towards HA over Ch4S as a hydrolysis substrate and that was consistent at pH 5, 37 °C (typical condition), whereas it showed a preference towards Ch4S over HA in selective conditions such as at pH 6, 45 °C (Knudson, Gundlach, Schmid, & Conrad, 1984). Recently, it was demonstrated that Ch4S is a better substrate than HA for testicular hyaluronidase (recombinant SPAM1) at pH 4 or lower pH, whereas HA is a better substrate than Ch4S above pH 4 (Honda, Kaneiwa, Mizumoto, Sugahara, & Yamada, 2012). In either condition in both reports Ch6S was a poorer substrate than HA and Ch4S. Their kinetic data suggested that testicular hyaluronidase has higher affinity for Ch4S and HA than for Ch6S (Honda et al., 2012). These findings may explain why that when both the chain length and sufation degree are similar Ch4S oligosaccharides are better inhibitors than Ch6S oligosaccharides for BTH from the aspect of the affinity with the enzyme. This indicates that the position of the sulfate group on the oligosaccharides may significantly contribute to the affinity of inhibitors with BTH.

The competitive inhibition of HA hydrolysis by BTH from ChS oligosaccharides indicated that ChS oligosaccharides bound to the active center of BTH. Once an oligosaccharide with bulky sulfate groups enters the active center of BTH, it may not separate from the active center easily, and these bulky GAGs may interfere with the entrance of substrate HA into the same centers. A study on interactions between BTH and various GAGs using a surface plasmon resonance (SPR) biosensor by another research group identified the highest to lowest affinities of GAGs with BTH as heparin (Hep) > ChSE > ChSD > Ch4S although it is unclear to what extent GAG hydrolysis by BTH affected the SPR results (Shen, Shimmon, Smith, & Ghosh, 2003). For comparison, order of the case of the hydrolysis by BTH of these GAGs is Ch4S > ChSD > ChSE from the



	a	b	c	d	e
Acceptor (PA-HA hexasaccharide)	+	+	+	-	-
Donor (HA)	-	+	+	+	+
Inhibitor (2000 μM)	-	-	+	+	-

Fig. 7. *Effects of HA hexasaccharides on the production of non-pyridylaminated HA oligosaccharides.* Transglycosylation reaction was performed as described in Section 2.4 in the absence of donor HA and inhibitor (a), in the absence of inhibitor (b), in the presence of acceptor (PA-HA hexasaccharide), donor HA, and inhibitor (c), in the absence of acceptor (PA–HA hexasaccharide) (d), and in the absence of acceptor (PA–HA hexasaccharide) and inhibitor (e). As an inhibitor, 2000 μM of Ch4S hexasaccharide (A and B) or HA hexasaccharide (C and D) was used. Oligosaccharides were detected by both the fluorescence of PA (ex. 320 nm, and em. 400 nm) (A and C) and UV absorbance at 215 nm (B and D). Dashed arrows indicate the elution positions of the PA–HA hexasaccharide as an acceptor. Solid arrows indicate the elution positions of standard PA–HA oligosaccharides (A and C) or non-labeled HA oligosaccharides (B and D) with known chain lengths. Dashed lines are the NaH_2PO_4 gradient lines. Symbols in the embedded table: "+" is in the presence and "−" is in the absence.

highest to lowest and Hep is not a substrate of BTH. However there is currently no information on the affinity of HA and Ch6S for BTH. Based on these findings by SPR, the affinity of GAGs for BTH may be higher if GAGs have many sulfate groups. The mode of the glycosidic bond between uronic acid and hexosamine (α or β) and whether the GAG become substrate or not did not appear to critically contribute to the affinity of GAG with BTH. Also, even the differences of their interacting site on the enzyme protein and inhibition mechanism did not appear to critically contribute to their affinity. Incidentally, Hep was shown to inhibit BTH through a non-competitive mechanism (Girish et al., 2009; Mio & Stern, 2002; Suzuki et al., 2001; Toida et al., 1999). It remains unclear how hyaluronidase activities

are regulated in each tissue after these enzymes are translated into proteins. Hyaluronidases, which are found broadly in HA-rich extracellular matrices, may regulate their enzymatic activities by interacting with tissue-specific hyaluronidase inhibitors through a tissue-specific mechanism to maintain HA homeostasis. Molecules whose structural moiety is a ChS chain, such as the inter-α-trypsin inhibitor, have been identified as hyaluronidas inhibitors (Mio et al., 2000). The ChS chains of other proteoglycans may also be adequate candidates as regulators of hyaluronidases in order to control HA at optimal amounts in tissues via an interaction in the active center of BTH.

The inhibition of HA transglycosylation by ChS can be explained by the inhibition of donor HA hydrolysis by ChS because transglycosylation does not occur until hydrolysis progresses. Indeed, this was supported by the comparison of Fig. 5A and B. However, in contrast to HA hydrolysis, apparent inhibition was even observed by the tetra- and hexasaccharides of HA or Ch in the transglycosylation system using a fluorescent acceptor. In these cases, during the reaction, HA oligosaccharides, as hydrolysis products, increased because HA hydrolysis by BTH was not inhibited by the HA or Ch oligosaccharides. Both HA oligosaccharides generated as hydrolysis products and the test oligosaccharides of HA and Ch could be acceptors for transglycosylation by BTH. Therefore, transglycosylation from the donor to these non-pyridylaminated acceptors was increased. This may represent the mechanism of apparent inhibition on HA transglycosylation used by non-sulfated oligosaccharides from HA or Ch.

In conclusion, the results of the present study demonstrated a new function of ChS oligosaccharides as inhibitors of BTH. Hydrolysis using BTH-immobilized resin (Suto, Kakizaki, Nakamura, & Endo, 2014) recently enabled us to prepare the tetrasaccharides or larger oligosaccharides of HA or ChSs without contamination by BTH. The application of these ChS oligosaccharides in the elucidation of the complex mechanism underlying the BTH reaction and in glycotechnological or medical areas is expected.

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