J. Biochem. 127, 695–702 (2000)

# Chimeric Glycosaminoglycan Oligosaccharides Synthesized by Enzymatic Reconstruction and Their Use in Substrate Specificity Determination of *Streptococcus* Hyaluronidase[1]

**Keiichi Takagaki,* Hidekazu Munakata,* Mitsuo Majima,† Ikuko Kakizaki,* and Masahiko Endo*,[2]**

*Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562; and †Kushiro Junior College, Kushiro 085-0814

Received December 13, 1999; accepted February 8, 2000

**A method was developed for the reconstruction of glycosaminoglycan (GAG) oligosaccharides using the transglycosylation reaction of an endo-β-*N*-acetylhexosaminidase, testicular hyaluronidase, under optimal conditions. Repetition of the transglycosylation using suitable combinations of various GAGs as acceptors and donors made it possible to custom-synthesize GAG oligosaccharides. Thus we prepared a library of chimeric GAG oligosaccharides with hybrid structures composed of disaccharide units such as GlcA-GlcNAc (from hyaluronic acid), GlcA-GalNAc (from chondroitin), GlcA-GalNAc4S (from chondroitin 4-sulfate), GlcA-GalNAc6S (from chondroitin 6-sulfate), IdoA-GalNAc (from desulfated dermatan sulfate), and GlcA-GalNAc4,6-diS (from chondroitin sulfate E). The specificity of the hyaluronidase from *Streptococcus dysgalactiae* (hyaluronidase SD) was then investigated using these chimeric GAG oligosaccharides as model substrates. The results indicate that the specificity of hyaluronidase SD is determined by the following restrictions at the nonreducing terminal side of the cleavage site: (i) at least one disaccharide unit (GlcA-GlcNAc) is necessary for the enzymatic action of hyaluronidase SD; (ii) cleavage is inhibited by sulfation of the *N*-acetylgalactosamine; (iii) hyaluronidase SD releases GlcA-GalNAc and IdoA-GalNAc units as well as GlcA-GlcNAc. At the reducing terminal side of the cleavage site, the sulfated residues on the *N*-acetylgalactosamines in the disaccharide units were found to have no influence on the cleavage. Additionally, we found that hyaluronidase SD can specifically and endolytically cleave the internal unsulfated regions of chondroitin sulfate chains. This demonstration indicates that custom-synthesized GAG oligosaccharides will open a new avenue in GAG glycotechnology.**

**Key words: chimeric glycosaminoglycan, enzymatic reconstruction, hyaluronidase from *Streptococcus dysgalactiae*, testicular hyaluronidase, transglycosylation.**

Interest in the synthesis of glycosaminoglycan (GAG) sugar chains has grown along with the clarification of the biological functions of GAGs (*1–4*). Recently, enzymatic synthesis using glycosyltransferase has been performed in addition to the usual chemical synthesis (*5–9*). By the enzymatic methods, it is possible to control the formation of α- and β-anomers and the position of binding sites. However, these methods use nucleotides, which are very expensive, as the sugar donors, and also require glycosyltransferase, which can be obtained by isolation or cloning techniques, but which is not yet available in sufficient quantities for GAG synthesis.

Recently, more attention has been directed toward the reconstruction of carbohydrate chains using glycosidases, which catalyze transglycosylation as a reverse reaction of hydrolysis (*10–12*). The transglycosylation mechanism of testicular hyaluronidase, which is an endo-β-*N*-acetylhexosaminidase, has been investigated with the aim of performing enzymatic synthesis of GAG sugar chains (*13–15*). It was found that disaccharide units (glucuronic acid β 1-3-*N*-acetylglucosamine) are successively released from the nonreducing terminal of a donor hyaluronic acid (HA) and rapidly transferred to the glucuronic acid residue at the nonreducing terminal of an acceptor HA *via* a β 1-4 linkage. Furthermore, the efficiency of the transglycosylation reaction increases when the pH and NaCl concentration are optimized. Therefore, it has become possible to custom-synthesize GAGs by repeating this transglycosylation reaction using suitable combinations of donors and acceptors from different kinds of GAG. We call the products of this

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09358013, 11121203, and 11470029).

[2] To whom correspondence should be addressed. Tel: +81-172-39-5015, Fax: +81-172-39-5016, E-mail: endo-m@cc.hirosaki-u.ac.jp

Abbreviations: hyaluronidase SD, hyaluronidase from *Streptococcus dysgalactiae*; GAG, glycosaminoglycan; HA, hyaluronic acid; Ch, chondroitin; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; DS, dermatan sulfate; ChS-E, chondroitin sulfate E; PA, 2-aminopyridine; GlcA, glucuronic acid; IdoA, iduronic acid; GalNAc, *N*-acetylgalactosamine, GlcNAc, *N*-acetylglucosamine; GalNAc4S, 4-sulfated *N*-acetylgalactosamine; GalNAc6S, 6-sulfated *N*-acetylgalactosamine; GalNAc4,6-diS, 4,6-disulfated *N*-acetylgalactosamine.

enzymatic reconstruction method, which is similar to genetic engineering, "chimeric GAGs."

The use of sugar chain degrading-enzymes as glycotechnological tools for structural analysis has advanced and enzymes such as chondroitinase ABC, AC, and B have long been used in the GAG field (*16–18*). However, new enzymatic glycotechnological tools are needed. We have focused our attention on the hyaluronidase of *Streptococcus dysgalactiae*, hyaluronidase SD (*19, 20*), because it catalyzes the eliminative cleavage of the β 1-4 glycosidic linkage between *N*-acetyl-D-glucosamine and D-glucuronic acid residues in HA, yielding disaccharides with Δ4-hexuronate at the nonreducing terminal. However, the precise details of the interaction between this enzyme and chondroitin sulfate (ChS) are not well known because of the variety and complexity of ChS structures compared with HA. In the present study, we synthesized various chimeric GAG oligosaccharides and used them as model substrates to investigate the substrate specificity of hyaluronidase SD toward ChS. This is the first paper to report the use of chimeric GAG oligosaccharides.

## MATERIALS AND METHODS

*Chemicals*—Hyaluronidase from *Sreptococcus dysgalactiae* was obtained from Seikagaku Kogyo (Tokyo). Bovine testicular hyaluronidase (type 1-S) and β-glucuronidase (from *Escherichia coli*) were obtained from Sigma Chemical (St. Louis, MO); the bovine testicular hyaluronidase was further purified according to the method of Borders and Raftery (*21*). It was free of β-glucuronidase and β-*N*-acetylhexosaminidase activities, as measured by the method of Barrett (*22*). Chondroitin 6-sulfate (Ch6S, from shark cartilage), chondroitin 4-sulfate (Ch4S, from whale cartilage), dermatan sulfate (DS, from pig skin), chondroitin sulfate E (ChS-E, from squid cartilage), and chondroitinase ABC (from *Proteus vulgaris*) were purchased from Seikagaku Kogyo. HA was prepared from human umbilical cord by the method of Danishefsky and Bella (*23*), and further purified by AG1-X2 chromatography and Sephacryl S-200HR gel-filtration chromatography as described previously (*24*). Sephadex G-15 and Sephacryl S-200HR were purchased from Pharmacia Biotech. (Uppsala, Sweden). Bio-Gel P-4 (400 mesh) and AG 1-X2 (200–400 mesh) were obtained from Bio-Rad (Richmond, CA). 2-Aminopyridine (PA) was purchased from Wako Pure Chemical (Osaka) and recrystallized from hexane. All other chemicals were obtained from commercial sources.

*Preparation of Desulfated GAGs*—Chondroitin (Ch) was prepared from Ch6S by a modification (*25*) of the method of Kantor and Schubert (*26*). Desulfated DS was prepared by desulfation of DS in dimethyl sulfoxide containing 10% methanol according to the procedure of Nagasawa *et al.* (*27*).

*Preparation of Oligosaccharides*—Saturated GAG oligosaccharides were prepared by partial digestion of HA, Ch, Ch6S, Ch4S, and ChS-E with testicular hyaluronidase using the procedure described in a previous report (*28*). Unsaturated disaccharide to decasaccharide derived from Ch and unsaturated disaccharide to hexasaccharide derived from Ch4S and Ch6S were prepared by digesting Ch, Ch4S, and Ch6S with chondroitinase ABC (*29*). Then, each oligosaccharide was purified by gel filtration on a Bio-Gel P-4 column (1.8 × 112 cm). The oligosaccharides were structurally characterized by chemical analysis, enzymatic analysis, ion-spray mass analysis and HPLC according to the methods reported previously (*28, 30*) as required.

*Preparation of PA-Oligosaccharides*—Fluorescence (PA) labeling of the reducing terminal of each oligosaccharide was carried out as described previously (*28, 31*), based on the method of Hase *et al.* (*32*). Each pyridylaminated oligosaccharide was used as a substrate of hyaluronidase SD, as an acceptor for the transglycosylation reaction, and as a standard marker for HPLC.

*Preparation of Chimeric Oligosaccharides*—Chimeric oligosaccharides were synthesized using the transglycosylation reaction of testicular hyaluronidase according to the previous reports (*14, 15*). Briefly, 50 μg of one GAG (HA, Ch, Ch6S, Ch4S, or desulfated DS) as the donor, 20 nmol of a PA-oligosaccharide as the acceptor, and 5 units of testicular hyaluronidase dissolved in 50 μl of 0.15 M Tris-HCl buffer, pH 7.0, were incubated at 37°C for 1 h. The reaction was terminated by immersion in a boiling water bath at 100°C for 3 min. The resulting product was purified by HPLC, and its structure was confirmed by a method described previously (*13, 14*). Therefore, using systematic combinations of the donor and acceptor molecules, we prepared many oligosaccharides with different types of GAG, and refer to them as chimeric oligosaccharides.

*Digestion with Hyaluronidase SD*—Each sample (0.2 nmol) was incubated in 0.1 M sodium acetate buffer, pH 6.5, containing 0.01 M $CaCl_2$ with hyaluronidase SD (0.02 units) at 37°C for 1 h (*19*). Then, the susceptibility of each PA-oligosaccharide to digestion by hyaluronidase SD was determined by HPLC.

*HPLC of PA-Oligosaccharides*—HPLC of PA-oligosaccharides was carried out on a PALPAK Type S column (4.0 × 250 mm, Takara Shuzo, Kyoto). Solution A was 3% acetic acid, adjusted to pH 7.0 with triethylamine and acetonitrile (20:80 v/v). Solution B was the same agents at a ratio of 50:50. The column was equilibrated with solution A, and the ratio of solution B to solution A was increased linearly from 0% to 100% over 60 min after sample injection; the flow rate was fixed at 1.0 ml/min and the column temperature was 30°C. A Hitachi L-6200 equipped with a fluorescence detector (Model F-1150, Hitachi, Tokyo) was used. Fluorescence of PA was detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

*Ion-Spray Mass Spectrometry*—Mass spectra were obtained on an API-100 LC/MS system (PE Siex, Thornhill, Ontario, Canada) equipped with an atomospheric-pressure ionization source as described previously (*28*). Each sample was dissolved in 0.5 mM ammonium acetate–acetonitrile (50:50) and injected at 2 μl/min with a micro-HPLC syringe pump (Pump 22, Harvard Apparatus, MA). In the negative mode, scanning was done from $m/z$ 200 to 1,200 during the 1-min scan (six cycles).

*Other Methods*—Reduction of oligosaccharides was accomplished with 1.0 M sodium borohydride in 0.05 M NaOH at 45°C for 24 h as described previously (*33*). β-Glucuronidase digestion was performed by the method of Himeno *et al.* (*34*).

## RESULTS

*GAG Oligosaccharides as Substrates for Hyaluronidase SD*—GAG oligosaccharides (Tables I and II) were prepared

TABLE I. **Sensitivity of oligosaccharides to hydrolysis by hyaluronidase SD.**

| Number | Structures | Digestibility with hyaluronidase SD |
|---|---|---|
| 1 | GlcA-GalNAc-PA | - |
| 2 | GlcA-GalNAc-GlcA-GalNAc-PA | - |
| 3 | GlcA-GalNAc↓-GlcA-GalNAc-GlcA-GalNAc-PA | + |
| 4 | GlcA-GalNAc↓-GlcA-GalNAc↓-GlcA-GalNAc-GlcA-GalNAc-PA | + |
| 5 | GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-PA | - |
| 6 | GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | - |
| 7 | GlcA-GalNAc↓-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-PA | + |
| 8 | GlcA-GalNAc↓-GlcA-GalNAc↓-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-PA | + |
| 9 | GlcA-GalNAc↓-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | + |
| 10 | GlcA-GalNAc↓-GlcA-GalNAc↓-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | + |
| 11 | GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-GlcA-GalNAc↓-GlcA-GalNAc-GlcA-GalNAc-PA | + |
| 12 | GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc↓-GlcA-GalNAc-GlcA-GalNAc-PA | + |
| 13 | GlcA-GalNAc(4S)-GlcA-GalNAc↓-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | + |

The arrows indicate the glycosidic linkages cleaved by hyaluronidase SD

TABLE II. **Hydrolysis of oligosaccharides by hyaluronidase SD.**

| Number | Structures | Hydrolysis (%)[a] |
|---|---|---|
| 14 | GlcA-GalNAc\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 100 |
| 15 | GlcA-GalNAc(4S)\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 0 |
| 16 | GlcA-GalNAc(6S)\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 0 |
| 17 | GalNAc\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 0 |
| 18 | GlcA-GlcNAc\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 100 |
| 19 | IdoA-GalNAc\|GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(6S)-PA | 62 |
| 20 | GlcA-GalNAc\|GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-GlcA-GalNAc(4S)-PA | 100 |
| 21 | GlcA-GalNAc\|GlcA-GalNAc(4, 6S)-GlcA-GalNAc(4, 6S)-GlcA-GalNAc(6S)-PA | 100 |

[a]Hydrolysis (%) was measured by HPLC of PA-labeled products. [b]The vertical broken line indicates the sites the of cleavage by hyaluronidase SD.

and used as model substrates for hyaluronidase SD. After partial hydrolysis of Ch, Ch4S, and Ch6S, seven oligosaccharides (Nos. 1–6 and 16) were purified on a Bio-Gel P-4 column and then selectively labeled with a PA. Other chimeric oligosaccharides were reconstructed using the transglycosylation reaction of testicular hyaluronidase according

to the methods described in previous reports (*14*, *15*). Oligosaccharides Nos. 7–10 were chimeric oligosaccharides made of one or two disaccharide units that we transferred from Ch to the nonreducing terminal or PA-Ch4S-hexasaccharide or PA-Ch6S-hexasaccharide. Similarly, chimeric oligosaccharides Nos. 11 and 12 were reconstructed by transferring to PA-Ch-hexasaccharide two disaccharide units derived from Ch4S and Ch6S, respectively. Chimeric oligosaccharide No. 13 was reconstructed by transferring successively Ch and then Ch4S disaccharide units to PA-Ch6S-hexasaccharide. Chimeric oligosaccharides Nos. 15, 18, and 19 were reconstructed by transferring to PA-Ch6S-hexasaccharide one disaccharide unit derived from Ch4S, HA, and desulfated DS, respectively. Chimeric oligosaccharide No. 17 was obtained by β-glucuronidase digestion of chimeric oligosaccharide No. 9. Chimeric oligosaccharide No. 21 was reconstructed by transferring a disaccharide unit derived from Ch to the PA-hexasaccharide derived from ChS-E (Takagaki, K., Majima, M., and Endo, M., submitted for publication).

*Action of Hyaluronidase SD on Ch Oligosaccharides*—The action of hyaluronidase SD on Ch oligosaccharides, which have no sulfate residue, was investigated. PA-Ch-oligosaccharides (Nos. 1–4) were incubated with hyaluronidase SD, and the reaction products were analyzed by HPLC using a size fractionation column. PA-Ch-disaccharide and PA-Ch-tetrasaccharide were not degraded (Fig. 1, A and B). When PA-Ch-hexasaccharide and PA-Ch-octasaccharide were each used as substrates, they were degraded and a new peak (I) appeared close to the PA-Ch-tetrasaccharide standard (Fig. 1, C and D). After separation and purification of peak I, the molecular mass was determined by ion-spray mass spectrometry. A molecular ion at $m/z$, 835 $[M-H]^-$ and a doubly-charged ion at $m/z$, 417 $[M-2H]^{2-}$ were observed (Fig. 2). Therefore, peak I was PA-Ch-tetrasaccharide with Δ4-hexouronate at its nonreducing terminal (ΔGlcA-GalNAc-GlcA-GalNAc-PA). These results indicate that the minimum size requirement for this enzyme is PA-hexasaccharide, and that its product is unsaturated PA-tetrasaccharide when the substrate is a PA-Ch-oligosaccharide.

To investigate the cleavage pattern of hyaluronidase SD on Ch-oligosaccharides, aliquots of a Ch-dodecasaccharide were used in two separate experiments. The first aliquot of Ch-dodecasaccharide was labeled with the fluorescent reagent, PA, and then incubated with hyaluronidase SD. PA-labeled products in the incubation mixture were analyzed

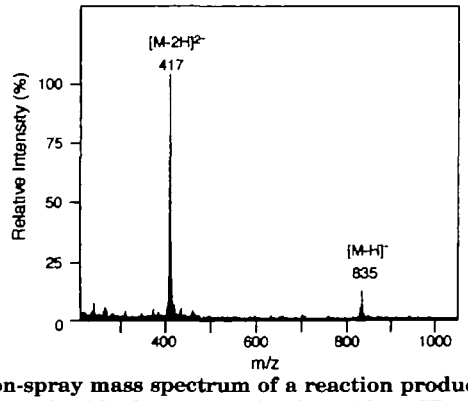


Fig. 2. **Ion-spray mass spectrum of a reaction product of PA-Ch-hexasaccharide digested by hyaluronidase SD.** A reaction product (peak 1 in Fig. 1C) was recovered and analyzed by ion-spray mass spectrometry. The conditions for mass spectrometry are described in "MATERIALS AND METHODS."

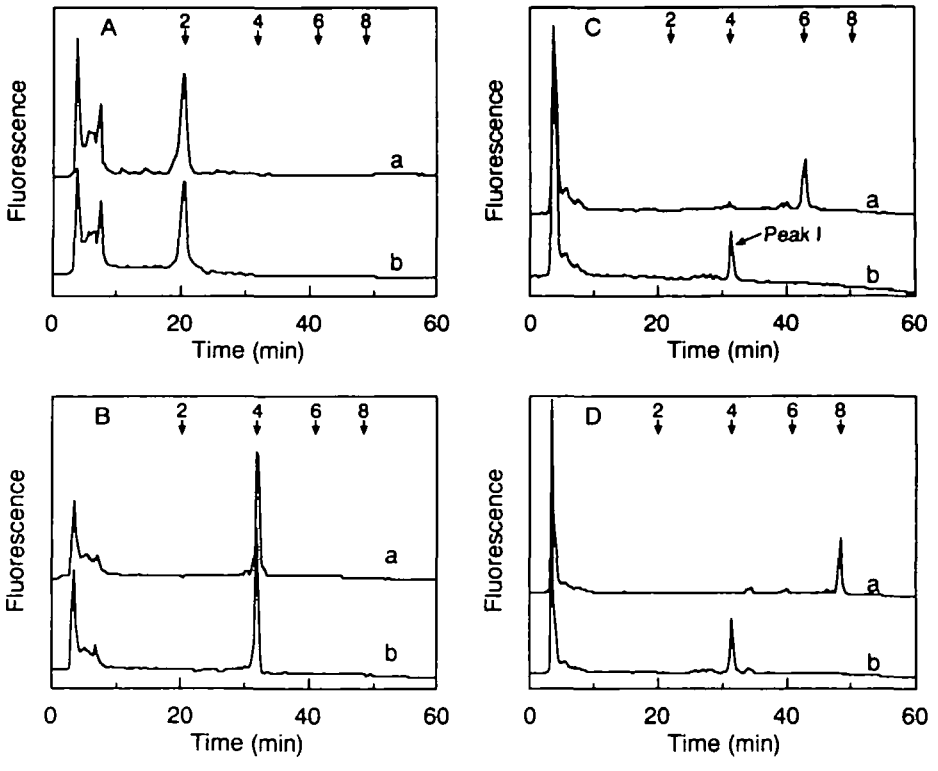


Fig. 1. **HPLC of the products generated from PA-Ch-disaccharide (A), -tetrasaccharide (B), -hexasaccharide (C), and -octasaccharide (D) by hyaluronidase SD.** PA-Ch-oligosaccharides were incubated without (a) or with (b) hyaluronidase SD and then analyzed by HPLC. The chromatographic conditions are described in "MATERIALS AND METHODS." Arrows numbered 2–8 indicate the elution positions of standards: PA-unsaturated Ch-disaccharide to octasaccharide were prepared by chondroitinase ABC digestion of Ch.

by HPLC. Time-sequence changes in the hyaluronidase SD digests are shown in Fig. 3A. As dodecasaccharide decreased, decasaccharide showed an increase and then a fall, followed by an increase of octasaccharide. Next, hexasaccharide showed an increase and then a fall, and the final product was found to be tetrasaccharide. This suggests that Ch-dodecasaccharide lost successive disaccharide units from its nonreducing terminal.

The second aliquot was reduced with sodium borohydride in advance, and the reduced Ch-dodecasaccharide, with *N*-actylgalactosaminitol at the reducing terminal, was used as the substrate. After incubation with hyaluronidase SD, the newly exposed reducing terminal of the digestion product was labeled with PA, and the fluorescence-labeled product was monitored by HPLC using a size fractionation column. Only disaccharide was detected as a PA-labeled digestion product at the beginning of the incubation (Fig. 3B). These data confirm that hyaluronidase SD exhibits an exo-mode cleavage pattern, successively releasing disaccharide units from the nonreducing terminal of Ch-oligosaccharides.

*Action Studies of Hyaluronidase SD Using ChS Oligosaccharides and Chimeric Oligosaccharides*—The action of hyaluronidase SD on ChS oligosaccharides composed of sulfated repeating disaccharide units (glucuronic acid β1-3-*N*-acetylgalactosamine; GlcA-GalNAc) was investigated. First, homo type-ChS hexasaccharides derived from Ch, Ch4S, and Ch6S were each incubated with hyaluronidase SD. This enzyme acted on Ch-hexasaccharide (Fig. 1C), but not on Ch4S-hexasaccharide or Ch6S-hexasaccharide (data not shown). Therefore, hyaluronidase SD does not act on ChS regardless of whether the sulfate is at the C4 or C6 position of the *N*-acetylgalactosamine residue in the disaccharide units.

Next, to investigate the effects of intrachain variation in the sulfate position on hyaluronidase SD action, three kinds of chimeric ChS oligosaccharides (Nos. 8, 11, and 13) were each incubated with hyaluronidase SD and the reaction products were analyzed by HPLC. When PA-Ch4S-

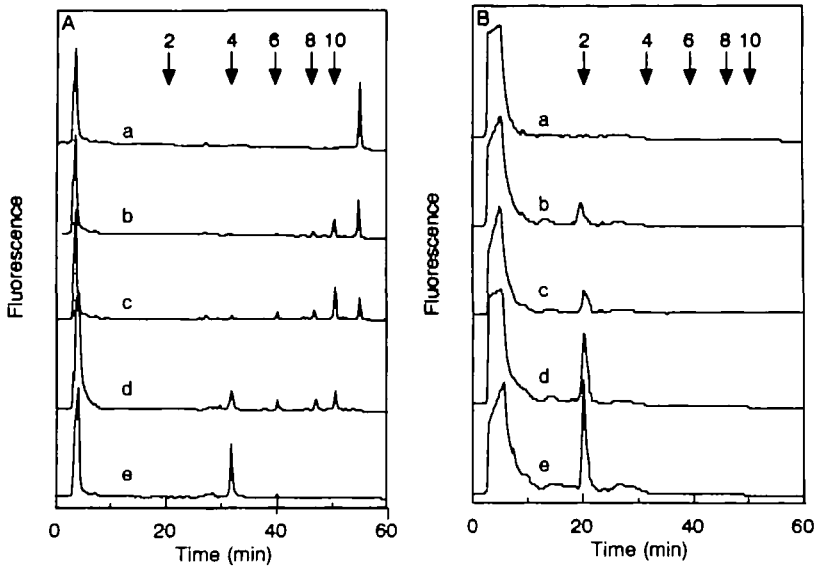


Fig. 3. **HPLC of oligosaccharide intermediates generated at different stages of digestion of Ch-dodecasaccharide by hyaluronidase SD.** PA-Ch-dodecasaccharide (A) and reduced Ch-dodecasaccharide (B) were incubated with hyaluronidase SD for 0 min (a), 5 min (b), 15 min (c), 30 min (d), and 60 min (e). The products of the reduced Ch-dodecasaccharide (B) with newly exposed reducing terminals, were labeled with PA and then the digestion products were analyzed by HPLC (as described in the legend to Fig. 1). Arrows numbered 2–10 indicate the elution positions of the standards: PA-unsaturated Ch disaccharide to decasaccharide.

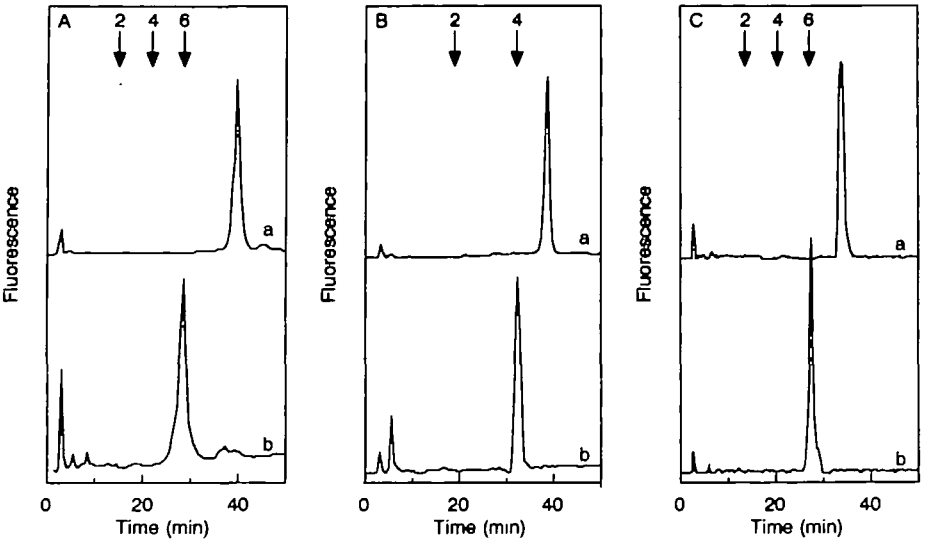


Fig. 4. **HPLC of the products generated from chimeric oligosaccharides.** Chimeric oligosaccharides Nos. 8 (A), 11 (B), and 13 (C) were incubated without (a) or with (b) hyaluronidase SD and then analyzed by HPLC as described in the legend to Fig. 1. Arrows numbered 2–6 indicate the elution positions of the standards, PA-unsaturated disaccharide to hexasaccharide derived from Ch4S (A), Ch (B), and Ch6S (C), which were prepared by chondroitinase ABC digestion of Ch4S, Ch and Ch6S.

decasaccharide with two unsulfated disaccharide units (GlcA-GalNAc) at the nonreducing terminal sites (No. 8) was the substrate, the retention time of the fluorescent product was equivalent to that of unsaturated PA-Ch4S-hexasaccharide (Fig. 4A). Thus, hyaluronidase SD exolytically removes the unsulfated moieties from the nonreducing terminal site of this oligosaccharide.

Next, when PA-Ch-decasaccharide with two 4-sulfated disaccharide units (GlcA-GalNAc4S) at the nonreducing terminal sites (No. 11) was the substrate, the retention time of the PA-decasaccharide shifted to that of the unsaturated PA-Ch-tetrasaccharide (Fig. 4B). Thus, hyaluronidase SD jumps over the 4-sulfated moieties at the nonreducing terminal site and acts on the unsulfated moieties of this oligosaccharide. The sensitivities of other chimeric oligosaccharides towards hyaluronidase SD are summarized in Table I. These results suggest that hyaluronidase SD is able to cut GAG chains endolytically in the unsulfated regions. To confirm this finding, a PA-ChS-decasaccharide with an unsulfated disaccharide unit (GlcA-GalNAc) at the internal position (No. 13) was synthesized and then incubated with hyaluronidase SD. The HPLC retention time of the fluorescent product was equivalent to that of the unsaturated PA-Ch6S-hexasaccharide (Fig. 4C). Therefore we conclude that hyaluronidase SD can cleave ChS chains endolytically at internal unsulfated regions.

Next, the effect of the structure adjacent to the cleavage site was investigated using eight oligosaccharides as model substrates (Table II). Each oligosaccharide was incubated with hyaluronidase SD and the reaction product was analyzed by HPLC. The sensitivities of the oligosaccharides towards hyaluronidase SD are summarized in Table II. Hyaluronidase SD cleaved the unsulfated *N*-acetylgalactosaminide and *N*-acetylglucosaminide linkages of the tested oligosaccharides, but not the oligosaccharides (Nos. 15 and 16) that contained the 4-sulfated and 6-sulfated *N*-acetylgalactosamine, respectively, at the nonreducing terminal side adjacent to the cleavage site. However, among the tested oligosaccharides, hyaluronidase SD did not cleave the unsulfated *N*-acetygalactosaminide linkage only in oligosaccharide No. 17, in which the *N*-acetylgalactosamine is positioned at the nonreducing terminal. Oligosaccharide No. 19, which has an iduronic acid at the nonreducing terminal, unlike a glucuronic acid such as in oligosaccharide No. 9, was degraded slowly compared with oligosaccharide No. 9 under the same conditions, and about 40% remained at the end of the incubation (Fig. 5). However, when oligosaccharide No. 19 was incubated using a 2-fold higher concentration of the enzyme, it was completely digested. Additionally, the oligosaccharides with 4-sulfated, 6-sulfated, and 4,6-disulfated *N*-acetylgalactosamine residues in disaccharide units at the reducing terminal side of the cleavage site (Nos. 7, 9, and 21) were also degraded by the enzyme. After fractionation of the enzyme by preparative PAGE, all enzymatic activities were observed in the same position (data not shown).

Judging from these results, the specifity of hyaluronidase SD is determined by the following restrictions at the nonreducing terminal side of the cleavage site: (i) at least one disaccharide unit (GlcA-GlcNAc) is necessary for the enzymatic action of hyaluronidase SD; (ii) the cleavage is inhibited by sulfation of the *N*-acetylgalactosamine; and (iii) hyaluronidase SD can release GlcA-GalNAc and IdoA-GalNAc disaccharide units as well as GlcA-GlcNAc from the nonreducing terminal of oligosaccharides. In contrast, sulfated residues on the *N*-acetylgalactosamine of the disaccharide units at the reducing terminal side of the cleavage site have no influence on cleavage.

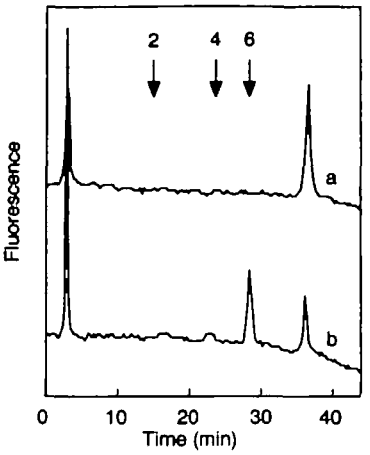


Fig. 5. **HPLC of the products generated from chimeric oligosaccharide.** Chimeric oligosaccharide No. 19 was incubated without (a) or with (b) hyaluronidase SD and then analyzed by HPLC analysis as described in the legend to Fig. 1. Arrows numbered 2–6 indicate the elution positions of the standards, PA-unsaturated Ch6S disaccharide to hexasaccharide (No. 19).

## DISCUSSION

The development of new GAG-degrading enzymes to be used as glycotechnological tools is very important. To examine various characteristics of such enzymes, including their substrate specificities, many kinds of GAG oligosaccharides are needed to be used as model substrates. Recently, attempts have been made to synthesize oligosaccharides corresponding to GAG-protein linkage regions using chemical (*35*), enzymatic (*10*) and cell technologies (*36*). On the other hand, we have developed a method for reconstructing oligosaccharides using testicular hyaluronidase to customize the oligosaccharides in the GAG-chain region. The transglycosylation mechanism of testicular hyaluronidase, which is an endo-β-*N*-acetylhexosaminidase, was investigated previously with the aim of performing enzymatic synthesis of GAG sugar chains (*13–15*). It was found that GAG sugar chains are sequentially elongated along with disaccharide units. It is also known that testicular hyaluronidase acts on GAGs such as ChS, as well as on HA. Therefore, using various GAGs as the acceptor and donor molecules, we attempted to synthesize natural and artificial GAG oligosaccharides, and produced various chimeric oligosaccharides with two or three kinds of disaccharide units, such as GlcA-GlcNAc (from HA), GlcA-GalNAc (from Ch), GlcA-GalNAc4S (from Ch4S), GlcA-GalNAc6S (from Ch6S), IdoA-GalNAc (from desulfated DS), and GlcA-GalNAc4,6-diS (from ChS-E), which we used as model substrates for the investigation of hyaluronidase SD. This is the first report of the successful utilization of "Custom-synthesized" GAG oligosaccharides using a transglycosylation of testicular hyaluronidase. A system for the enzymatic reconstraction of chimeric GAG oligosaccharides could open a new avenue in GAG research.

Hyaluronidases from bacterial sources, such as *Streptomyces* and *Streptococcus*, have been used to measure HA (*37*). The former enzyme acts only on HA (*38*), but the latter acts not only on HA but also on Ch (*19*, *20*). However, the precise details of the interacion between this enzyme and Ch and ChS are not well known. Therefore we investigated the properties of the hyaluronidase from *Streptococcus dysgalactia*, which belongs to group C and is now commercially available, with a view to using it as a glycotechnological tool. We found that hyaluronidase SD acts on Ch but not on Ch4S and Ch6S. Jandik *et al.* (*39*) classified bacterial GAG-degrading enzymes according to cleavage patterns from exolytic to endolytic activity. Under their classification scheme, hyaluronidase SD exhibited an exo-mode action pattern, because it successively releases disaccharide units from the nonreducing terminals of Ch oligosaccharides. The effect of GAG sulfation on the action of hyaluronidase SD was examined using chimeric ChS oligosaccharides with unsulfated moieties inside the chain as substrates. We found that the hyaluronidase SD jumps over the sulfated moieties at the nonreducing terminals and endolytically cleaves the internal unsulfated moieties in the ChS. Recently, the hyaluronidase from *Streptococcus agalactiae*, which belongs to group B rather than group C, has been shown to cleave ChS specifically (*40*), but it is not commercially available at present. In this study we have shown that the hyaluronidase from *Streptococcus dysgalactiae* has a specificity that will be useful in the structural analysis of ChS.

The use of restriction enzymes acting on DNA has accelerated the progress of genetic engineering. On the other hand, restriction enzymes for GAG chains have received little attention. Successive reports have indicated that GAG chains have certain domains that are concerned with their specific biological functions (*41*, *42*). To isolate functional domains from a GAG chain, GAG-degrading endo-type enzymes, used as restriction enzymes, have become very important tools. Thus, the exploitation of the limited specific cleavage of ChS by hyaluronidase SD, to release the sulfated domains of ChS, is expected to assist in the progress of glycoengineering.

## REFERENCES


1. Choay, J., Petitou, M., Lormeau, J.C., Sinay, P., Casu, B., and Gatti, G. (1983) Structure–activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti–factor Xa activity. *Biochem. Biophys. Res. Commun.* **116**, 492–499
2. van Boeckel, C.A.A., Beetz, T., Vos, J.N., de Jong, A.J.M., van Aelst, S.F., van den Bosch, R.H., Mertens, J.M.R., and van der Vlugt, F.A. (1985) Synthesis of a pentasaccharide corresponding to the antithrombin III binding fragment of heparin. *J. Carbohydr. Chem.* **4**, 293–321
3. Ichikawa, Y., Monden, R., and Kuzuhara, H. (1986) Synthesis of a heparin pentasaccharide fragment with a high affinity for antithrombin III employing cellobiose as a key starting material. *Tetrahedron Lett.* **27**, 611–614
4. Goto, F. and Ogawa, T. (1993) Recent aspects of glycoconjugate synthesis: a synthetic approach to the linkage region of proteoglycans. *Pure Appl. Chem.* **65**, 793–801
5. Toone, E.J., Simon, E.S., Bednarski, M.D., and Whitesides, G.M. (1989) Enzyme-catalyzed synthesis of carbohydrates. *Tetrahedron* **45**, 5365–5422
6. David, S., Auge, C., and Gautheron, C. (1992) Enzymic methods in preparative carbohydrate chemistry. *Carbohydr. Chem. Biochem.* **49**, 175–237
7. Gijsen, H.J.M., Qiao, L., Fitz, W., and Wong, C.-H. (1996) Recent advances in the chemoenzymic synthesis of carbohydrates and carbohydrate mimetics. *Chem. Rev.* **96**, 443–473
8. Wong, C.-H., Haynie, S.L., and Whitesides, G.M. (1982) Enzyme-catalyzed synthesis of *N*-acetyllactosamine with in situ regeneration of uridine 5′-diphosphate glucose and uridine 5′-diphosphate galactose. *J. Org. Chem.* **47**, 5416–5418
9. Zervosen, A. and Elling, L. (1996) A novel three-enzyme reaction cycle for the synthesis of *N*-acetyllactosamine with *in situ* regeneration of uridine 5′-diphosphate glucose and uridine 5′-diphosphate galactose. *J. Am. Chem. Soc.* **118**, 1836–1840
10. Yasukouchi, T., Fukase, K., Suda, Y., Takagaki, K., Endo, M., and Kusumoto, S. (1997) Enzymatic synthesis of 4-methylumbelliferyl glycosides of trisaccharide, and core tetrasaccharide, Gal(β 1-3)Gal(β 1-4)Xyl and GlcA(β 1-3)Gal(β 1-3)Gal(β 1-4)Xyl, corresponding to the linkage region of proteglycans. *Bull. Chem. Soc. Jpn.* **70**, 2719–2725
11. Yoon, J.-H. and Ajisaka, K. (1996) The synthesis of galactopyranosyl derivates with β-galactosidases of different origins. *Carbohydr. Res.* **292**, 153–163
12. Usui, T., Morimoto, S., Hayakawa, Y., Kawaguchi, M., Murata, T., Matahira, Y., and Nishida, Y. (1996) Regioselectivity of β-D-galactosyl-disaccharide formation using the β-D-galactosidase from *Bacillus circulans*. *Carbohydr. Res.* **285**, 29–39
13. Takagaki, K., Nakamura, T., Izumi, J., Saitoh, H., and Endo, M. (1994) Characterization of hydrolysis and transglycosylation by testicular hyaluronidase using ion-spray mass spectrometry. *Biochemistry* **33**, 6305–6507
14. Saitoh, H., Takagaki, K., Majima, M., Nakamura, T., Matsuki, A., Kasai, M., Narita, H., and Endo, M. (1995) Enzymic reconstruction of glycosaminoglycan oligosaccharide chain using the transglycosylation reaction of bovine testicular hyaluronidase. *J. Biol. Chem.* **270**, 3741–3747
15. Takagaki, K., Munakata, H., Majima, M., and Endo, M. (1999) Enzymatic reconstruction of a hybrid glycosaminoglycan containing 6-sulfated, 4-sulfated and unsulfated *N*-acetylgalactosamine. *Biochem. Biophys. Res. Commun.* **258**, 741–744
16. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* **243**, 1523–1535
17. Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, N., Kawai, Y., and Furuhashi, T. (1968) Formation of three types of disulfated disaccharides from chondroitin sulfates by chondroitinase digestion. *J. Biol. Chem.* **243**, 1543–1550
18. Michelacci, Y.M. and Dietrich, C.P. (1974) Isolation and partial characterization of an induced chondroitinase B from *Flavobacterium heparinum*. *Biochem. Biophys. Res. Commun.* **56**, 973–980
19. Linker, A., Meyer, K., and Hoffman, P. (1956) The production of unsaturated uronides by bacterial hyaluronidases. *J. Biol. Chem.* **219**, 13–25
20. Hamai, A., Morikawa, K., Horie, K., and Tokuyama, K. (1989) Purification and characterization of hyaluronidase from *Streptococcus dysgalactiae*. *Agric. Biol. Chem.* **53**, 2163–2168
21. Borders, C.L., Jr. and Raftery, M.A. (1968) Purification and partial characterization of testicular hyaluronidase. *J. Biol. Chem.* **243**, 3756–3762
22. Barrett, A.J. (1972) Lysosomal enzymes in *Lysosomes* (Dingle, J.T., ed.) pp. 46–135, North-Holland Publishing, Amsterdam
23. Danishefsky, I. and Bella, A., Jr. (1996) The sulfated mucopolysaccharides from human umbilical cord. *J. Biol. Chem.* **241**, 143–146
24. Nakamura, T., Majima, M., Kubo, K., Takagaki, K., Tamura, S., and Endo, M. (1990) Hyaluronidase assay using fluorogenic hyaluronate as a substrate. *Anal. Biochem.* **191**, 21–24
25. Nakamura, T., Takagaki, K., Majima, M., Kimura, S., Kubo, K., and Endo, M. (1990) A new type of exo-β-glucuronidase acting only on non-sulfated glycosaminoglycans. *J. Biol. Chem.* **265**, 5390–5397
26. Kantor, T.G. and Schubert, M. (1957) A method for the desulfa-

tion of chondroitin sulfate. *J. Am. Chem. Soc.* **79**, 152–153
27. Nagasawa, K., Inoue, Y., and Tokuyasu, T. (1979) An improved method for the preparation of chondroitin by solvolytic desulfation of chondroitin sulfates. *J. Biochem.* **86**, 1323–1329
28. Takagaki, K., Kojima, K., Majima, M., Nakamura, T., Kato, I., and Endo, M. (1992) Ion-spray mass spectrometric analysis of glycosaminoglycan oligosaccharides. *Glycoconj. J.* **9**, 174–179
29. Saito, H., Yamagata, T., and Suzuki, S. (1968) Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* **243**, 1536–1542
30. Takagaki, K., Munakata, H., Nakamura, W., Majima, M., and Endo, M. (1998) Ion-spray mass spectrometry for identification of the nonreducing terminal sugar of glycosaminoglycan. *Glycobiology* **8**, 719–724
31. Takagaki, K., Nakamura, T., Kawasaki, H., Kon, A., Ohishi, S., and Endo. M. (1990) Determination of the reducing terminal sugars of glycosaminoglycans using 2-aminopyridine. *J. Biochem. Biophys. Methods* **21**, 209–215
32. Hase, S., Ibuki, T., and Ikenaka, T. (1984) Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins. *J. Biochem.* **95**, 197–203
33. Takagaki, K., Nakamura, T., Majima, M., and Endo, M. (1988) Isolation and characterization of a chondroitin sulfate-degrading endo-β-glucuronidase from rabbit liver. *J. Biol. Chem.* **263**, 7000–7006
34. Himeno, M., Hayashiguchi, Y., and Kato, K. (1974) β-Glucuronidase of bovine liver, purification, properties, carbohydrate composition. *J. Biochem.* **76**, 1243–1252
35. Neumann, K.W., Tamura, J., and Ogawa, T. (1996) Synthesis of a novel glycosaminoglycan pentasaccharide serine having an *N*-acetylgalactosamine residue α-linked to the core linkage tetrasaccharide. *Glycoconj. J.* **13**, 933–936
36. Takagaki, K., Nakamura, T., Kon, A., Tamura, S., and Endo, M. (1991) Characterization of β-D-xyloside-induced glycosaminoglycans and oligosaccharides in cultured human skin fibroblasts. *J. Biochem.* **109**, 514–519
37. Greiling, H., Stuhlsatz, H.W., and Tillmanns, U. (1984) Hyaluronate in *Methods of Enzymatic Analysis* (Bergmeyer, M.U., ed.) Vol. 6, pp. 45–59, Academic Press, New York
38. Ohya, T. and Kaneko, Y. (1970) Novel hyaluronidase, from streptomyces. *Biochim. Biophys. Acta* **198**, 607–609
39. Jandik, K.A., Gu, K., and Linhardt, R.J. (1994) Action pattern of polysaccharide lyases on glycosaminoglycans. *Glycobiology* **4**, 289–296
40. Baker, J.R., Yu, H., Morrison, K., Averett, W.F., and Pritchard, D.G. (1997) Specificity of the hyaluronate lyase of group-B streptococcus toward unsulphated regions of chondroitin sulphate. *Biochem. J.* **327**, 65–71
41. Hascall, V.C. and Hascall, G.T. (1981) Proteoglycans in *Cell Biolgy and Extracellular Matrix* (Hay, E.D., ed.) pp. 39–63, Penum Publishing, New York
42. Höök, M., Kjellen, L., Johansson, S., and Robinson, J. (1984) Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* **53**, 847–869