Characterization of Hydrolysis and Transglycosylation by Testicular Hyaluronidase Using Ion-Spray Mass Spectrometry†

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ABSTRACT: Various oligosaccharides from hyaluronic acid, which were fluorescence-labeled and blocked by pyridylamination at the reducing terminal, were incubated as substrates or acceptors with bovine testicular hyaluronidase. Fluorescence-labeled reaction products in the reaction mixture were monitored selectively and directly by ion-spray mass spectrometry without chemical derivatization. As a result, several features of the relationship between oligosaccharides, substrates, and testicular hyaluronidase were clarified. When hexasaccharides or larger oligosaccharides having D-glucuronic acid at the nonreducing terminal were used as substrates, they were hydrolyzed sequentially to disaccharides from the nonreducing terminal, and these disaccharides were then transferred to other hexasaccharides. On the other hand, when heptasaccharides or larger oligosaccharides having *N*-acetyl-D-glucosamine at the nonreducing terminal were used as substrates, trisaccharides were released from the nonreducing terminal, and then also transferred to other hexasaccharides, thus forming nonasaccharides. Thus, the relationship between hydrolysis and transglycosylation reactions with testicular hyaluronidase was characterized using ion-spray mass spectrometry.

Bovine testicular hyaluronidase is an *endo*-β-*N*-acetyl-D-hexosaminidase that hydrolyzes the internal β-*N*-acetyl-D-hexosaminidic linkage contained in hyaluronic acid and chondroitin sulfate to D-glucuronic acid (Meyer *et al.*, 1960; Meyer, 1971). Besides this hydrolytic reaction, it is also known that hyaluronidase catalyzes the reverse reaction, transglycosylation (Weissman, 1955; Hoffman *et al.*, 1956; Schechter & Berger, 1966; Highsmith *et al.*, 1975; Rodén *et al.*, 1989). For this reason, when hyaluronic acid is digested with testicular hyaluronidase, the final reaction products consist mainly of tetrasaccharides but also a substantial proportion of hexasaccharides and smaller amounts of di- and octasaccharides. Since a hexasaccharide is the smallest susceptible substrate, it is known that this enzyme first hydrolyzes hexasaccharides into disaccharides and tetrasaccharides, and then the disaccharide is transferred to another hexasaccharide to form an octasaccharide while the tetrasaccharide remains intact. However, precise details of reactions with this enzyme, including changes in the reaction products with time, have rarely been reported because of a lack of effective techniques for analyzing the sugar chains of glycosaminoglycans (GAGs).

For usual mass analysis, GAG must be derivatized to make it volatile, and this procedure is very cumbersome for sequential measurement of enzyme reaction products (Bruins *et al.*, 1987; Covey *et al.*, 1988; Sakairi & Kambara, 1989). However, we recently devised a new method of GAG structure determination using ion-spray mass spectrometry (Takagaki *et al*, 1992a). This technique was shown to be applicable to direct analysis of oligosaccharides from hyaluronic acid without any need for chemical derivatization. Furthermore, we succeeded in labeling the reducing terminal of GAG using a fluorogenic reagent, 2-aminopyridine (Takagaki *et al.*, 1990, 1992b; Nakamura *et al.*, 1990; Kon *et al.*, 1991). Through this fluorescence labeling, it was possible to distinguish the reducing terminal of GAG from its nonreducing terminal. Moreover, highly sensitive and selective detection of GAG with time was easily achieved. In the present study, we used our new method to analyze the relationship between hydrolysis and transglycosylation reactions with testicular hyaluronidase.

EXPERIMENTAL PROCEDURES

Materials. Bovine testicular hyaluronidase (type I) was obtained from Sigma Chemical Co. (St. Louis, MO) and further purified according to the method of Borders and Raftery (1968). It was free of β-glucuronidase or β-*N*-acetyl-D-hexosaminidase activity, as measured by the method of Barrett (1972). β-Glucuronidase (from *Escherichia coli*) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Sephadex G-15 was obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Bio-Gel P-4 and AG 1-X2 were purchased from Bio-Rad (Richmond, CA). 2-Aminopyridine was purchased from Wako Pure Chemical Co. (Osaka, Japan) and recrystallized from hexane. Sodium cyanoborohydride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Other reagents and chemicals were obtained from commercial sources.

Preparation of Hyaluronic Acid and Its Oligosaccharides. Hyaluronic acid was purified from human umbilical cord according to the method of Nakamura *et al.* (1990). The purified hyaluronic acid was partially hydrolyzed by testicular

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hyaluronidase using the procedure described in a previous report (Takagaki *et al.*, 1992a), and then hyaluronic acid–oligosaccharides (from di- to dodecasaccharide) having *N*-acetyl-D-glucosamine at the reducing terminal were purified through a Bio-Gel P-4 column (400 mesh). Hyaluronic acid–oligosaccharides (penta- and heptasaccharides) having *N*-acetylglucosamine at both the reducing and nonreducing terminals were further purified with a Bio-Gel P-4 column after digestion of hexa- and octasaccharides with β-glucuronidase in 0.1 M ammonium acetate buffer (pH 5.0) at 37 °C for 24 h.

Fluorescence-Labeled Oligosaccharides. The reducing terminals of the purified oligosaccharides were selectively labeled with a fluorescent reagent (2-aminopyridine) using the procedure described in a previous report (Kon *et al.*, 1991) (pyridylamino (PA)-oligosaccharides).[1] Briefly, oligosaccharides (1 mg) were dissolved in 100 μL of PA solution (prepared by mixing 1.0 g of PA, 0.76 mL of concentrated HCl, and 2.2 mL of water, giving a final pH of 6.2) and reacted at 100 °C for 13 min. Next, the oligosaccharides were reacted with 6 μL of a reducing reagent, which was prepared by dissolving 10 mg of sodium cyanoborohydride in 15 μL of PA solution and 20 μL of water immediately before use, at 90 °C for 15 h for reductive amination. Fluorescence-labeled oligosaccharides were then obtained by removal of excess reagents with a Sephadex G-15 column.

Analytical Methods: Conditions for Enzyme Reactions. The hydrolytic reaction was carried out by incubating 1 nmol of oligosaccharides as a substrate dissolved in 25 μL of 0.2 M ammonium acetate buffer (pH 5.0) with 1.0 NFU of testicular hyaluronidase at 37 °C for various times. The reaction was stopped by transferring the solution to a boiling water bath at 100 °C for 3 min. The transglycosylation reaction was also performed in the presence of 2 nmol of PA-hexasaccharides as acceptors.

Mass Spectrum Measurements. All mass spectra were obtained on a Sciex API-III triple-quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source (Takagaki *et al.*, 1992a). The mass spectrometer was operated in the negative mode; the ion-spray voltage was set at −4000 V, the interface plate voltage was −600 V, and the orifice voltage was −100 V. The samples were introduced in 0.5 mM ammonium acetate–acetonitrile (50:50 by volume). A JASCO Familic 100N micro HPLC syringe pump was used to deliver the samples at a flow rate of 2 μL/min. Scanning was done from m/z 300 to 1200 during the 1-min scan (six cycles). The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas, and the collision energy was 40 eV. The CAD product ion spectrum was recorded from 200 to 1000.

HPLC Analysis. HPLC analysis was performed using a Hitachi L-6200 equipped with a fluorescence detector (F-1050, Hitachi Co., Tokyo, Japan). The reaction products were eluted through a Palpak Type S column (4.6 mm × 250 mm) under the following conditions: solution A containing 3% acetic acid solution adjusted to pH 7.3 with triethylamine and acetonitrile at a ratio of 35:65 and solution B containing the same agents at a ratio of 50:50 were prepared; the column was equilibrated with solution A, and the ratio of solution B to solution A was increased linearly to 100% over 50 min after sample injection; the flow rate was fixed at 1.0 mL/min; the column temperature was 40 °C; PA was detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

[1] Abbreviations: GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; PA, pyridylamino; CAD, collisionally activated dissociation.

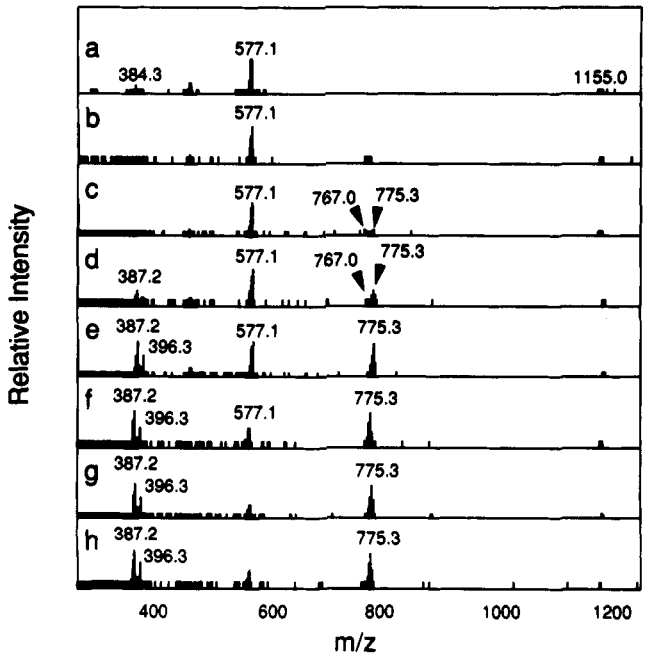

FIGURE 1: Ion-spray mass spectra of hyaluronidase digests. Hexasaccharide from hyaluronic acid was incubated with hyaluronidase for 0 min (a), 5 min (b), 30 min (c), 60 min (d), 90 min (e), 120 min (f), 150 min (g), and 180 min (h) in 0.2 M ammonium acetate solution, pH 5.0, at 37 °C. An aliquot of the reaction mixture was then subjected to ion-spray mass spectrometry in 0.5 mM ammonium acetate–acetonitrile (50:50 by volume) at 2 μL/min.

Table 1: Major Observed Ion Species of Oligosaccharides from Hyaluronic Acid

	m/z			
oligosaccharides	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	$[M-4H]^{4-}$
disaccharide	396.3			
tetrasaccharide	775.3	387.2		
hexasaccharide	1155.0	577.1	384.3	
octasaccharide		767.0	510.8	
decasaccharide		956.0	637.2	
dodecasaccharide		1144.8	763.2	572.2

RESULTS

Analysis by Mass Spectrometry. When oligosaccharides derived from hyaluronic acid were measured by ion-spray mass spectrometry, they showed characteristic multiply-charged ions produced by proton abstraction (Takagaki *et al.*, 1992a). For some hexasaccharides, for example, the spectra showed peaks of $[M-H]^-$ at m/z 1155, $[M-2H]^{2-}$ at m/z 577, and $[M-3H]^{3-}$ at m/z 384 (Figure 1a). Each oligosaccharide had more peaks than one characteristic ion species, and their major ion species are shown in Table 1. Thus, on the basis of these peaks, it was possible to distinguish and identify oligosaccharides contained in the mixture. Furthermore, each oligosaccharide was also quantified by integrating all ion intensities (Figure 2).

Hydrolysis of Oligosaccharides. Reaction products derived from some oligosaccharides by hyaluronidase digestion were analyzed by ion-spray mass spectrometry. Time-sequence changes in the mass spectra of reaction products obtained during incubation of hexasaccharide with hyaluronidase are shown in Figure 1. Although three characteristic peaks (m/z 384, 577, and 1155) derived from hexasaccharide were observed before incubation (0 min), the intensity of two of

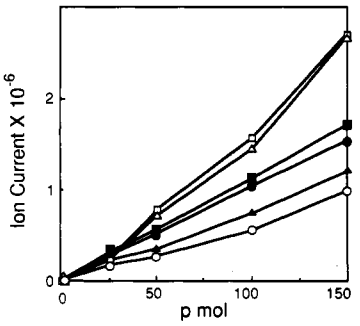

FIGURE 2: Oligosaccharide response curves. Standard oligosaccharides were prepared at concentrations ranging from 0 to 150 pmol/2 μL and then subjected to ion-spray mass spectrometry. The total intensities of the major ion species were measured for each oligosaccharide: (○) di-, (●) tetra-, (□) hexa-, (■) octa-, (△) deca-, and (▲) dodecasaccharide.

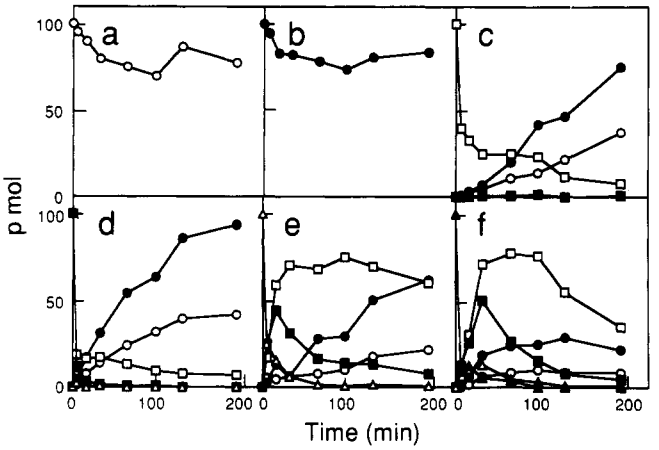

FIGURE 3: Time course of hyaluronidase digestion of oligosaccharides from hyaluronic acid. Di- (a), tetra- (b), hexa- (c), octa- (d), deca- (e), and dodecasaccharide (f) were incubated with hyaluronidase in the same buffer system at 37 °C. Aliquots were analyzed at various times as described in the text: (○) di-, (●) tetra-, (□) hexa-, (■) octa-, (△) deca-, and (▲) dodecasaccharide.

these peaks decreased with prolongation of the incubation time, while peaks at m/z 775 and 387 derived from tetrasaccharide and a peak at m/z 396 derived from disaccharide increased.

Time-sequence changes in hyaluronidase digests from di- to dodecasaccharide, which were used as substrates, and those in the amounts of the reaction products, are summarized in Figure 3. From these results, it was revealed that the amount of neither disaccharide nor tetrasaccharide was decreased (Figure 3a,b), i.e., these oligosaccharides were not digested. Hexasaccharide (Figure 3c) showed a decrease in amount, while di- and tetrasaccharide increased, indicating the digestion of hexasaccharide by hyaluronidase into di- and tetrasaccharide. In addition, octasaccharide produced by transfer of disaccharide to hexasaccharide was also detected. As octasaccharide decreased (Figure 3d), hexasaccharide showed a rapid increase and then a fall, followed by an increase of tetrasaccharide, indicating sequential release of disaccharide from octasaccharide. A similar tendency was also observed when deca- and dodecasaccharide were used as substrates (Figure 3e,f), suggesting that hyaluronidase hydrolyzed sugar chains in disaccharide units, although the amount of disaccharide in the reaction mixture was not remarkable.

Hydrolysis of PA-oligosaccharides. Using several oligosaccharides, whose reducing terminals had been labeled and blocked with the fluorescent reagent, PA, as substrates for hyaluronidase, fluorescence-labeled products in the reaction

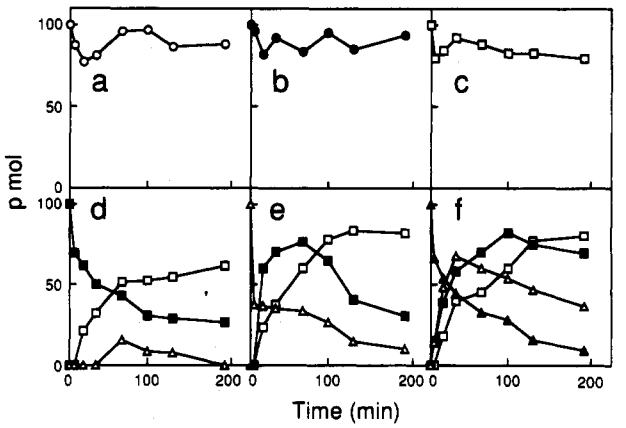

FIGURE 4: Time course of hyaluronidase digestion of PA-oligosaccharides labeled with the fluorescent reagent, 2-aminopyridine, at the reducing terminal. PA-di- (a), PA-tetra- (b), PA-hexa- (c), PA-octa- (d), PA-deca- (e), and PA-dodecasaccharide (f) were incubated with hyaluronidase, and then aliquots were analyzed described in Figure 3. Only PA-containing oligosaccharides were selectively detected: (○) PA-di-, (●) PA-tetra-, (□) PA-hexa-, (■) PA-octa-, (△) PA-deca-, and (▲) PA-dodecasaccharide.

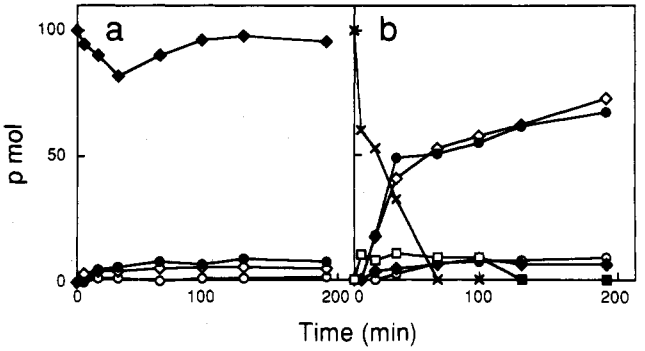

FIGURE 5: Time course of hyaluronidase digestion of oligosaccharides having *N*-acetylglucosamine at their nonreducing terminals. Penta- (a) and heptasaccharide (b) were incubated with hyaluronidase, and aliquots were analyzed as described in Figure 3: (○) di-, (◇) tri-, (●) tetra-, (◆) penta-, (□) hexa-, and (×) heptasaccharide.

solution were selectively monitored by ion-spray mass spectrometry. As a result of PA-labeling, hexasaccharide was not digested with hyaluronidase (Figure 4c), and the smallest PA-labeled substrate was found to be octasaccharide (Figure 4d). Octasaccharide increased as decasaccharide decreased, followed by a successive decrease, and then hexasaccharide showed an increase (Figure 4e). A similar tendency was also observed for dodecasaccharide (Figure 4f). This indicated that sugar chains were shortened successively in disaccharide units from the nonreducing terminal. To summarize, it was found that hyaluronidase hydrolyzed even-numbered oligosaccharides with glucuronic acid at the nonreducing terminal by successive removal of disaccharide units from the nonreducing terminal.

Next, the effects of hyaluronidase on oligosaccharides bearing *N*-acetylglucosamine at the nonreducing terminal were investigated. For this, changes in hyaluronidase digestion with prolongation of the incubation time were monitored by ion-spray mass spectrometry using penta- and heptasaccharides having *N*-acetylglucosamine at both the reducing and nonreducing terminals as substrates (Figure 5). It was found that pentasaccharide remained unchanged, while heptasaccharide was hydrolyzed into tri- and tetrasaccharides, which were GlcNAc-GlcA-GlcNAc and $(GlcA\text{-}GlcNAc)_2$, respectively. Thus it was revealed that hyaluronidase first hydrolyzed trisaccharide from the nonreducing terminal of heptasac-

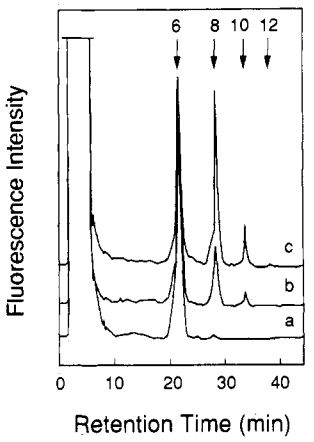

FIGURE 6: HPLC of transglycosylation products released by hyaluronidase. PA- and non-PA-labeled hexasaccharides, which were used as an acceptor and a donor, respectively, were incubated with hyaluronidase for 0 (a), 5 (b), and 30 min (c) in the same buffer system at 37 °C and then subjected to HPLC. The chromatographic conditions were as described in the text. Arrows indicate the elution positions of PA-hyaluronic acid oligosaccharide standards: 6, hexa-; 8, octa-; 10, deca-; 12, dodecasaccharide.

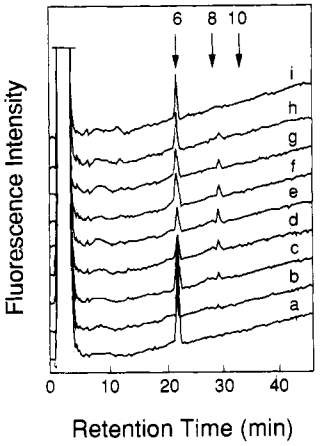

FIGURE 7: HPLC of transglycosylation products released by hyaluronidase. PA-hexasaccharide and non-PA-labeled heptasaccharide, which were used as an acceptor and a donor, respectively, were incubated with hyaluronidase for 0 (a), 5 (b), 15 (c), 30 (d), 60, (e), 90 (f), 120 (g), 180 (h), and 240 min (i) and then subjected to HPLC. The chromatographic conditions and arrows were the same as described in the legend of Figure 6.

charide and larger oligosaccharides having *N*-acetylglucosamine at the nonreducing terminal.

Transglycosylation by Hyaluronidase. Since hyaluronidase was found to have slight transglycosylation activity, as shown in Figures 3c and 4d, PA-hexasaccharide and PA-unlabeled hexasaccharide, as an acceptor and a donor, respectively, were incubated with hyaluronidase for further study. As mentioned previously, since PA-hexasaccharide was not hydrolyzed by hyaluronidase, transglycosylation from the PA-unlabeled hexasaccharide was analyzed by tracing the fluorescence of the PA-oligosaccharides by HPLC. It was revealed that PA-octasaccharide increased with incubation time and that decasaccharide increased (Figure 6), suggesting the transfer of disaccharide units to PA-hexasaccharide as an acceptor.

Next, PA-unlabeled heptasaccharide bearing *N*-acetylglucosamine at the nonreducing terminal and PA-hexasaccharide, as a donor and an acceptor, respectively, were incubated with hyaluronidase and analyzed by HPLC. One peak was found to appear more prominently at the midpoint between the standard PA-octasaccharide and PA-decasaccharide as the duration of incubation increased (Figure 7). This peak was

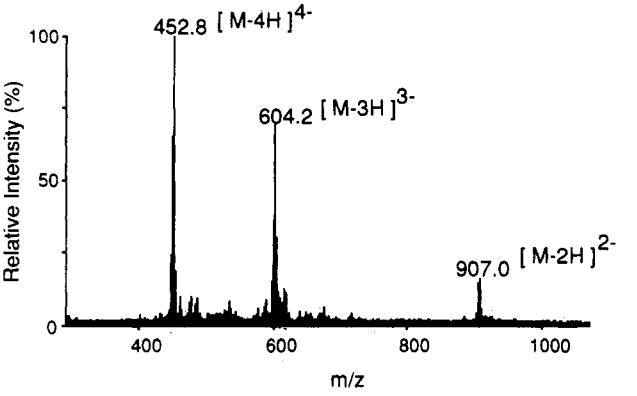

FIGURE 8: Ion-spray mass spectrum of transglycosylation products. The conditions were the same as those described in Figure 1.

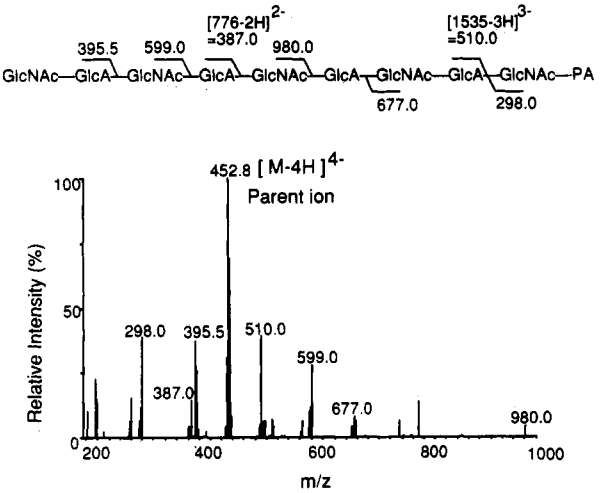

FIGURE 9: MS/MS spectrum from CAD of the quadruply charged ion (m/z 452.8) among the transglycosylation products. This spectrum was measured at a collision energy of 40 eV using argon as the collision gas.

then analyzed by ion-spray mass spectrometry after separation and purification, and this revealed multiply-charged ions, $[M - 2H]^{2-}$, $[M - 3H]^{3-}$, and $[M - 4H]^{4-}$, at m/z 907, 604, and 453, respectively (Figure 8). The molecular weight of the compound was computed to be 1816 based on the detection of these ions. The formula for calculating molecular weights from m/z values of multiply-charged ions have been reported previously (Takagaki *et al.*, 1992a). Next, the structure of this compound was analyzed from its fragmentation pattern on MS/MS (Figure 9). The quadruply charged ion at m/z 452.8 was selected as the precursor ion. The product ions were mainly due to fragmentation at glycosidic bonds. The locations of the fragment ions at m/z 980.0, 387.0 = $[776 - 2H]^{2-}$, 599.0, and 395.5 were consistent with the loss of each monosaccharide. In addition, the ions at m/z 510.0 = $[1535 - 3H]^{3-}$ and 298.0 indicated the presence of fragments containing (GlcNAc-GlcA)$_4$ and PA-*N*-acetylglucosamine, respectively. These results suggested that the compound was PA-nonasaccharide, of which the reducing terminal *N*-acetylglucosamine was bound to PA, glucuronic acid, and *N*-acetylglucosamine were then alternately arranged, and the nonreducing terminal was *N*-acetylglucosamine (Figure 9). From these results, it was revealed that hyaluronidase could also transfer trisaccharide when heptasaccharide with *N*-acetylglucosamine at the nonreducing terminal was used as a donor.

DISCUSSION

Although some detailed reports on time-related changes in reaction products released by hyaluronidase have been published (Weissman, 1955; Hoffman *et al.*, 1956; Highsmith *et al.*, 1975), on the basis of analysis of the products by paper chromatography or column chromatography, the oligosaccharides released by the action to this enzyme have not yet been analyzed thoroughly. In particular, the relationship between hydrolysis and transglycosylation by hyaluronidase has remained obscure.

In recent years, soft ionization, which allows mass analysis of natural-state oligosaccharides, which are thermolabile and poorly volatile compounds, has been developed (Bush & Cooks, 1982). Using this technology, Carr and Reinhold (1984), Reinhold *et al.* (1987), and Takagaki *et al.* (1991, 1992a) have successfully analyzed oligosaccharides derived from GAG sugar chains. In the present study, using an ion-spray mass spectrometer, we successfully identified and also quantified multiple oligosaccharides present among the enzyme reaction products. Through this technique, the time course of hydrolysis of hyaluronic acid by hyaluronidase could be easily traced.

From the present results, two important aspects of the hydrolytic reaction were clarified, and one new observation related to the transglycosylation of hyaluronidase was made. First, hyaluronidase was shown to sequentially hydrolyze oligosaccharides bearing glucuronic acid at the nonreducing terminal by cleavage of disaccharide units from the nonreducing terminal. Second, when heptasaccharides or larger oligosaccharides having *N*-acetylglucosamine at the nonreducing terminal were used as a substrate, trisaccharides were released from the nonreducing terminal. Testicular hyaluronidase is known to induce not only hydrolysis, but also transglycosylation, the reverse reaction, simultaneously (Weissman, 1955; Hoffman *et al.*, 1956; Highsmith *et al.*, 1975). Third, trisaccharides were also transferred when oligosaccharides bearing *N*-acetylglucosamine at the nonreducing terminal were used as a donor, besides the transfer of disaccharide units.

In recent years, it has been clarified that oligosaccharide-sized functional domains with physiological activities, such as blood-anticoagulative activity, are present on GAG sugar chains (Lane, 1984; Maimone, 1990). Since hyaluronidase is known to exert hydrolytic and glycosyltransfer actions not only on hyaluronic acid, but also on other GAGs (chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate), the enzyme is expected to play an important role in the remodeling of new bioactive oligosaccharides based on the structures and functions of these functional oligosaccharides.

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