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Ion-spray mass spectrometric analysis of glycosaminoglycan oligosaccharides

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Oligosaccharides from hyaluronic acid and chondroitin 6-sulfate were prepared by digestion with testicular hyaluronidase and separated according to their degree of polymerization by gel-permeation chromatography. These materials were successively analyzed by negative-mode ion-spray mass spectrometry with an atmospheric-pressure ion source. An ion-spray interface was used to produce ions via the ion evaporation process, producing mass spectra containing a series of molecular species carrying multiple charges. Using two adjacent multiply charged molecular ions, the exact molecular weights up to the tetradecasaccharide were calculated with a precision of ± 1 dalton. This type of mass spectrometry was also demonstrated to be feasible for the analysis of mixtures of oligosaccharides, including tetra-, hexa-, octa- and decasaccharides, from hyaluronic acid or chondroitin 6-sulfate without separation. Ion-spray mass spectrometry was thus shown to be applicable to the structural analysis of oligosaccharides from glycosaminoglycans.

Keywords: Ion-spray mass spectrometry, glycosaminoglycans, hyaluronic acid, chondroitin 6-sulfate.

Abbreviations: HA, hyaluronic acid; Ch6S, chondroitin 6-sulfate; GAG, glycosaminoglycan; GlcA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose.

The glycosaminoglycans (GAGs) constitute a broad class of acidic polysaccharides that include chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and hyaluronic acid. These GAGs, which are located predominantly on the cell surface or in the extracellular matrix, are of particular interest because of their possible role as cellular recognition sites. Such recognition sites are involved in many fundamental processes, including cell adhesion, cellular differentiation, and regulation of cell growth [1, 2]. In addition, some GAGs are well known to have biological functions such as anticoagulant or antithrombotic activity [3–5].

An understanding of these biological processes is intimately linked to a knowledge of the structures involved. However, compared with the progress achieved in the structural analysis of other biopolymers such as proteins and nucleic acids, progress has been rather slow in the field of GAGs. This is due mainly to the complexity of the GAG structures, including molecular size, sugar components, glycosidic linkages, and sulfation. Indeed, few structures of oligosaccharides larger than disaccharides have been determined unambiguously, and larger structures which have been presented so far are often composite or average structures. Because investigation of these molecular structures is very difficult, several techniques have been used, e.g., gel filtration, paper chromatography, thin-layer chromatography, electrophoresis and ^{1}H-NMR spectroscopy [6]. However, these methods all require relative large amounts of material, or chemical derivatization.

Recently, mass spectral analysis of biological polymers has been made possible by the introduction of desorption techniques. In particular, ion-spray mass spectrometry using an atmospheric-pressure ion source has been shown to be valuable for structural analysis of polar and thermally labile molecules [7–9]. We therefore applied ion-spray mass spectrometry to the analysis of some oligosaccharides derived from hyaluronic acid and chondroitin 6-sulfate. The results indicated that ion-spray mass spectrometry will be useful for the structural determination of GAG oligosaccharide chains.

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Materials and methods

Materials

Hyaluronic acid (HA) was prepared from human umbilical cord and further purified by Dowex 1-X2 chromatography as described previously [10]. Chondroitin 6-sulfate (Ch6S), super special grade from shark cartilage, was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Bovine testicular hyaluronidase, type I-S, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bio-Gel P-4 was obtained from Bio-Rad (Richmond, CA, USA). Other reagents were of analytical grade from commercial sources.

Preparation of glycosaminoglycan oligosaccharides

Glycosaminoglycan oligosaccharides were prepared from HA and Ch6S by digestion with testicular hyaluronidase, respectively, according to the method of Cowman *et al.* [11]. Each sample of 50 mg was dissolved in 5 ml 0.1 M sodium acetate buffer, pH 5.0, containing 0.15 M NaCl, and then 2 mg of testicular hyaluronidase was added and the solution was covered with toluene. After digestion at 37 °C for 64 h, the reaction was terminated by boiling in a water bath for 5 min. The digests were centrifuged at 10,000 × g for 20 min to remove insoluble protein, lyophilized and redissolved in 2 ml 0.5 M pyridinium acetate buffer, pH 6.5. Then, the digested materials were separated on a Bio-Gel P-4 column (1.8 cm × 112 cm), equilibrated and eluted with the above buffer at a flow rate of 11 ml h^{-1}. Fractions of 2.8 ml were collected and screened for uronic acid. Uronic acid-positive fractions were pooled and lyophilized. The degree of polymerization of each oligosaccharide was established by analysis of the ratio of total uronic acid to the reducing terminal *N*-acetylhexosamine as described by Cowman *et al.* [11].

Chemical analysis

Uronic acid was determined by the method of Bitter and Muir [12]. Hexosamine was determined by the method of Dische and Borenfreund [13] after hydrolysis in 4 N HCl for 8 h at 100 °C. Reducing terminal *N*-acetylhexosamine was determined from the decrease in total *N*-acetylhexosamine after reduction with 0.33 M $NaBH_4$ in 0.4 M sodium carbonate buffer, pH 9.7, at 0 °C for 90 min [14]. Sulfate was determined by the method by Terho and Hartiala [15] after hydrolysis in 1 N HCl for 2 h at 100 °C.

Mass spectrometry

All mass spectra were obtained on a Sciex API-III triple-quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric-pressure ionization source. An ion-spray interface was used to effect ionization from a continuous stream of analyte. In this system, ions in the gas phase are drawn into the vacuum of the mass spectrometer through a 100 μm ID orifice. The atmospheric side of the orifice is bathed with a curtain of high-purity, dry nitrogen gas. The nitrogen curtain acts as a barrier, preventing contaminants and solvent vapor from entering the mass spectrometer vacuum. High vacuum in the analyzer region of the mass spectrometer is achieved using cryogenically cooled surfaces, maintained at 19–20 K, surrounding the quadrupoles. During determination, the indicated vacuum was 2×10^{-5} Torr. The samples (15 μM) were introduced in 0.5 mM ammonium acetate–acetonitrile (50:50 by vol). A Jasco Familic 100 N micro HPLC syringe pump was used to deliver the samples at a flow rate of 2 μl min^{-1}.

Results and discussion

Preparation and chemical analysis of oligosaccharide

Oligosaccharides obtained from HA and Ch6S by enzyme digestion were separated on a Bio-Gel P-4 column (Fig. 1). Digestion of HA and Ch6S by testicular hyaluronidase, an endo-β-*N*-acetylhexosaminidase, yields a homologous series of oligosaccharides having the repeating units (4GlcAβ1-3GlcNAcβ1-) and (4GlcAβ1-3GalNAc-(6-OSO_3H)β1-), respectively. By chemical analysis, i.e., the ratio of uronic acid to *N*-acetylhexosamine at the reducing terminal, the number of disaccharide units for each oligosaccharide obtained from HA and Ch6S was estimated for $n = 1$–6 and $n = 1$–7, respectively (Table 1). Also, the results showed that each oligosaccharide from Ch6S contained *N*-acetylgalactosamine and sulfate in a molar ratio of 1:1. Thus, considering the results of sulfate analysis, desulfation of Ch6S did not occur during the preparation of oligosaccharides.

Mass spectrometry

A 15 μM solution was introduced at a flow rate 2 μl min^{-1} during the 1 min scan (six cycles). The ion-spray mass spectra of the six oligosaccharides from HA were obtained

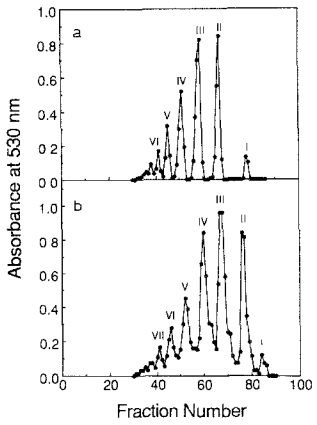

Figure 1. Separation of oligosaccharides produced by digestion of HA (a) and Ch6S (b) with testicular hyaluronidase. Approximately 50 mg of each sample was applied to a column (1.8 cm × 112 cm) of Bio-Gel P-4, and eluted with 0.5 M pyridinium acetate, pH 6.5. Peak fractions I–VI and I–VII were pooled, respectively.

Table 1. Chemical analyses of oligosaccharides.

Oligosaccharide	*Total uronic acid*	*Sulfate*	*Number of disaccharide units*
	Reducing terminal N-acetylhexosamine	*N-Acetyl-hexosamine*	
Hyaluronic acid			
I	1.06		1
II	2.04		2
III	2.78		3
IV	4.04		4
V	5.31		5
VI	6.00		6
Chondroitin 6-sulfate			
I	1.64	–[a]	1
II	2.17	1.03	2
III	3.03	1.03	3
IV	4.35	0.94	4
V	4.92	0.93	5
VI	5.56	0.98	6
VII	6.67	0.93	7

[a] Determination impossible due to interference.

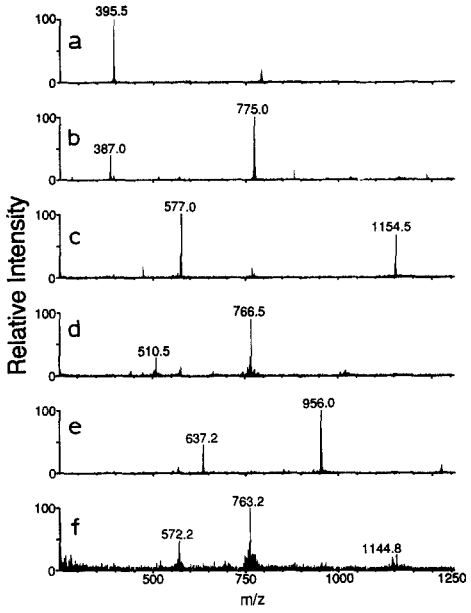

Figure 2. Ion-spray mass spectra of di- (a), tetra- (b), hexa- (c), octa- (d), deca- (e), and dodecasaccharides (f) from HA. (Continuous sample introduction at 2 μl min^{-1}; 1 min profile scan. 100 nM solution in 0.5 nM ammonium acetate (pH 7.0)–acetonitrile (50:50 by vol)).

in the accumulated scans, and are shown in Fig. 2. In the case of the octasaccharide, for example, the spectra showed doubly and triply charged molecular ions, $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ (Fig. 2d). Also, the ion-spray mass spectra of the six oligosaccharides from Ch6S are shown in Fig. 3. In the same way, ions at *m/z* 617.5, 463.0 and 369.5 in the

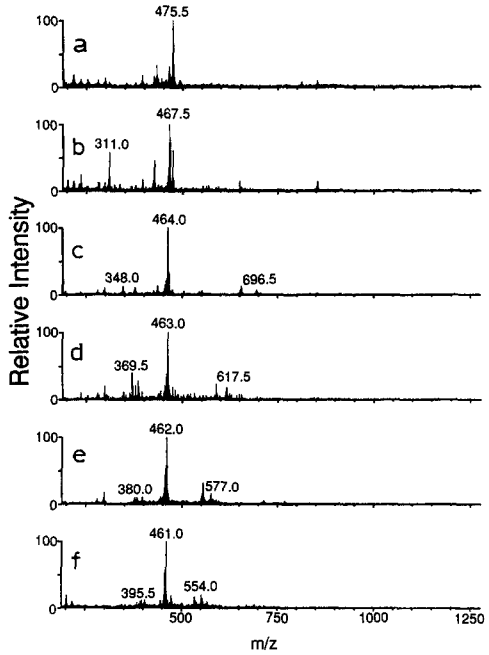

Figure 3. Ion-spray mass spectra of di- (a), tetra- (b), hexa- (c), octa- (d), deca- (e), and dodecasaccharides (f) from Ch6S. The conditions were the same as those described in Fig. 2.

negative ion spectra of octasaccharide were doubly, triply and quadruply charged molecular ions, $[M - 2H]^{2-}$, $[M - 3H]^{3-}$ and $[M - 4H]^{4-}$. These results indicated that the ion-spray, in the negative mode, produced multiply charged ions of oligosaccharides by proton abstraction.

Molecular weight of oligosaccharides

The ion-spray technique produces abundant multiply charged ions which have mass-to-charge ratios falling within the mass range of normal instruments. Therefore, the molecular weights of oligosaccharides were calculated by the method of Covey *et al.* [8]. Thus, if the molecular weight of the molecule is M, and multiply charged negative ions are formed by proton abstraction, the identities of the ions formed are $(M - n)^{n-}$, where n is the number of charge species subtracted. Hence, the observed masses, given by the mass-to-charge m, are

$$m = \frac{M - n}{n}.$$

Given two values of mass-to-charge ratio, m_1 and m_2, such that $m_2 > m_1$, and assuming that they are separated by the addition of just one charged species, it follows that

$$m_2 = \frac{M - n_2}{n_2}$$

$$m_1 = \frac{M - (n_2 + 1)}{n_2 + 1},$$

where n_2 is the number of charges on the species observed

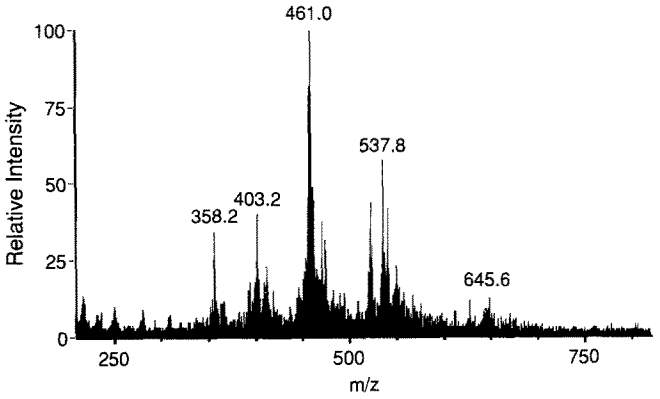

Figure 4. Ion-spray mass spectrum of the tetradecasaccharide from Ch6S. The conditions were the same as those described in Fig. 2.

at mass-to-charge ratio m_2. Hence,

$$n_2 = \frac{m_1 + 1}{m_2 - m_1}$$

and

$$M = n_2(m_2 + 1).$$

Thus, the molecular weight M can be calculated easily from any pair of adjacent peaks. Using this procedure, an attempt was made to calculate the molecular weight of the tetradecasaccharide from Ch6S. The spectrum obtained showed major peaks at m/z 403.2, 461.0 and 537.8, and relatively minor peaks at m/z 358.2 and 645.6 (Fig. 4). Table 2 lists the molecular masses calculated from the peaks present in the spectrum of tetradecasaccharide from Ch6S. In this table, m_2 and m_1 are adjacent masses, real n_2 is the number of charges for the ion of mass m_2 calculated directly, n_2 is the corresponding nearest integer value and M is the calculated molecular weight. The real n_2 values were very close to integers, so that incorrectly assigned peaks were immediately apparent. The experimentally determined molecular weight for the tetradecasaccharide was 3233.3 ± 0.5 Da, representing the mean and standard deviation of four determinations from a single spectrum. The average molecular weight, calculated using the average

Table 2. Calculation of number of charges and molecular weight from major ion series of Ch6S-tetradecasaccharide.[a]

m_2	m_1	Real n_2[b]	n_2	M
645.6	537.8	5.00	5	3232.8
537.8	461.0	6.02	6	3234.0
461.0	403.2	6.99	7	3233.6
403.2	358.2	7.98	8	3232.8

[a] See text for details.

[b] Real $n_2 = \frac{m_1 + 1}{m_2 - m_1}$.

Table 3. Experimentally determined molecular masses of oligosaccharides from HA and Ch6S.

Oligosaccharide	*HA*	*Ch6S*
Di-	397.0 (397.3[a])	477.3 ± 0.4 (477.4)
Tetra-	776.5 ± 0.1 (776.7)	936.7 ± 0.1 (936.8)
Hexa-	1155.8 ± 0.0 (1156.0)	1396.1 ± 0.1 (1396.1)
Octa-	1535.2 ± 0.4 (1535.3)	1855.4 ± 0.4 (1855.6)
Deca-	1914.4 ± 0.4 (1914.6)	2314.6 ± 0.4 (2314.6)
Dodeca-	2293.8 ± 0.1 (2293.9)	2774.1 ± 0.7 (2774.2)
Tetradeca-	N.D.	3233.3 ± 0.5 (3233.6)

[a] Average Mass.
N.D. = Not determined.

of the isotopic masses, was 3233.6 Da. Table 3 also shows the experimentally determined molecular weight of oligosaccharides from HA and Ch6S. These data indicate that each molecular weight was close to the average mass.

Furthermore, Table 4 shows the major observed multiply charged ions and their relative intensities. One interesting feature of oligosaccharides from Ch6S was that the most intense peak in the envelope of multiply charged molecular ions was observed about m/z 460 with some charge. The number of charged ions observed in the negative ion mode corresponded to the number of sulfate or carboxyl groups possessed by each oligosaccharide. However, the same feature was not observed in the oligosaccharides from HA, which had no sulfate groups. Therefore, it is reasonable to conclude that the number of charges is equivalent to the number of sulfate groups, which are acidic sites, available for proton abstraction, of oligosaccharides from Ch6S.

Mixture analysis

It was difficult to determine the exact molecular weights of the oligosaccharides from GAG. One of the reasons for this was the difficulty in purifying the oligosaccharides. Therefore, mixture analyses, involving simultaneous separation and determination, were performed. First, equal molar quantities of tetra-, hexa-, octa-, and decasaccharides from HA were re-mixed and determined. The results, shown in Fig. 5, indicated the relationships of some multiply charged ions corresponding to each oligosaccharide, from which it was possible to calculate separately the molecular weight of each oligosaccharide, i.e., 776.0 (tetra-), 1155.5 (hexa-), 1535.7 (octa-) and 1913.8 (deca-), respectively. Second, equal molar quantitites of hexasaccharides from HA and Ch6S were re-mixed and determined (Fig. 6). The results also showed that the sample consisted of two compounds, with molecular weights of 1155.4 and 1395.7, respectively.

As there are no experimental methods for studying the complex structures of oligosaccharides from GAGs, due to their polydispersity, these structures have often been represented as composites or averages. In general, less volatile oligosaccharides have been analyzed by mass

Table 4. Major observed ion species and their relative intensities.

Oligosaccharide	*m/z*								
	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	$[M-4H]^{4-}$	$[M-5H]^{5-}$	$[M-6H]^{6-}$	$[M-7H]^{7-}$	$[M-8H]^{8-}$	$[M-9H]^{9-}$
[HA]									
Di-	396(100)								
Tetra-	775(100)	387(38)							
Hexa-	1155(66)	577(100)							
Octa-		767(100)	511(27)						
Deca-		965(100)	637(45)						
Dodeca-		1145(15)	763(100)	572(38)					
[Ch6S]									
Di-	476(100)								
Tetra-		467(100)	311(56)						
Hexa-		697(6)	464(100)	348(12)					
Octa-			618(12)	463(100)	370(23)				
Deca-				577(12)	462(100)	385(5)			
Dodeca-					554(11)	461(100)	396(5)		
Tetradeca-					646(14)	538(53)	461(100)	403(40)	358(6)

spectrometry after chemical degradation or derivatization [16–19]. Barber *et al.* [20] analyzed the structure of heparin, using methylation and mass spectrometry. Recently, new mass spectrometric techniques have been developed that enable direct analysis of polar and thermally labile molecules [21]. These include secondary ion mass spectrometry [22], laser desorption mass spectrometry [23], plasma desorption [24] and fast-atom bombardment [25]. The major feature of these techniques is their ability to produce intact molecular ions of nonvolatile and generally intractable samples directly from the condensed phase, without chemical derivatization of the polar functional groups. Using fast-atom bombardment mass spectrometry, oligosaccharides have been analyzed by Reinhold *et al.* [26], Carr and Reinhold [27] and Takagaki *et al.* [28]. However, for underivatized oligosaccharides, it is difficult to determine molecular weights up to penta- or hexasaccharides.

In this study, the mass spectra of oligosaccharides with molecular weights up to the tetradecasaccharide were obtained using a quadrupole mass spectrometer equipped with an atmospheric-pressure ion source. The exact molecular weight was calculated with a precision of ± 1 Dalton. The results indicated that ion-spray mass spectrometry could be very useful for the precise determination of molecular weights of oligosaccharides from

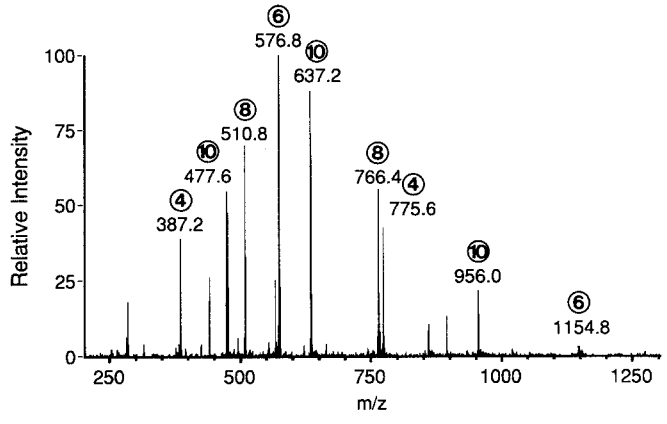

Figure 5. Ion-spray mass spectrum of a mixture of tetra-, hexa-, octa-, and decasaccharides from HA. Equal molar quantities were re-mixed and determined by ion-spray mass spectrometry. The numbers in circles indicate the multiply charged ions from tetra- (4), hexa- (6), octa- (8), and decasaccharide (10). Except for the final concentration of samples, the conditions were the same as those described in Fig. 2.

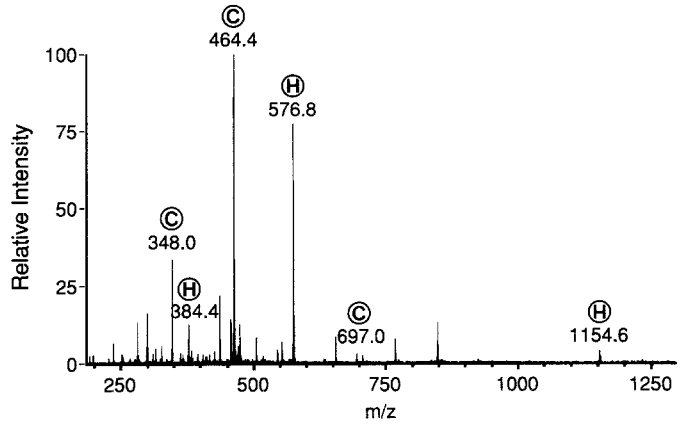

Figure 6. Ion-spray mass spectrum of a mixture of hexasaccharides from HA and Ch6S. Equal molar quantities were re-mixed and determined by ion-spray mass spectrometry. The letters in circles indicate the multiply charged ions from HA (H) and Ch6S (C), respectively. Except for the final concentration of samples, the conditions were the same as those described in Fig 2.

GAGs, and might be a valuable tool for detailed structural analysis of GAGs.

However, it is still not possible to obtain the mass spectra of high-molecular-weight GAG chains obtained from proteoglycan by endo-β-xylosidase digestion [29] or alkaline reduction [30], due to their lack of solubility in the 50% acetonitrile solution used as a mobile phase. Although their insolubility may preclude the use of some other solvent such as an aqueous base, the ion intensity of negative ions is much lower at lower concentrations of acetonitrile. Therefore, modification of the solvent will be necessary for the analysis of high-molecular-weight GAG chains.

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