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Isolation and characterization by electrospray-ionization mass spectrometry and high-performance anion-exchange chromatography of oligosaccharides derived from hyaluronic acid by hyaluronate lyase digestion: Observation of some heretofore unobserved oligosaccharides that contain an odd number of units

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Abstract

Hyaluronic acid was degraded with hyaluronate lyase (E.C. 4.2.2.1, from *Streptomyces hyalurolyticus*), and the resulting oligosaccharides up to dp 16 were characterized by electrospray-ionization mass spectrometry (ESIMS) and high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). In accordance with the known regiospecificity of the enzyme, the products included even-numbered oligosaccharides of structure β-D-4en-thrHex*p*A-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A]$_n$-(1 → 3)-D-Glc*p*NAc. Minor amounts of novel and unexpected odd-numbered oligomers, having the structure β-D-4en-thrHex*p*A-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)-D-Glc*p*A]$_n$, were also isolated and characterized. This study, in addition to others beginning to appear in the literature, demonstrates the usefulness of ESIMS and HPAEC-PAD in the analysis and characterization of anionic glycosaminoglycan-type oligosaccharides. © 1997 Elsevier Science Ltd.

Keywords: Hyaluronan; Hyaluronic acid; Electrospray-ionization mass spectrometry; High-performance anion-exchange chromatography (HPAEC); Pulsed amperometric detection (PAD); Glycosaminoglycans

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1. Introduction

Numerous recent studies have established hyaluronic acid (HA) as an increasingly important bioactive polysaccharide that has potential import in medicine. Perhaps most significant are the roles it has been shown to play in cancer, as documented by several recent review articles devoted to this topic [1–3]. Numerous other reports describe its involvement in several different types of cancer, such as pancreatic, ovarian, breast, colon, prostate, and brain cancers [4–16]. As a result of its unique physicochemical properties, HA is also finding use in drug delivery, as an antiadhesive, in ophthamalic tissue replacement, and in osteoarthritis therapy applications [17–19]. In light of these circumstances, there is a need for HA fragments of precisely defined MW and end-group identity for use as biological probes in more extensive investigations of these processes.

As part of our studies on the role of HA's interaction with cancer cells during metastasis, we chose to employ oligosaccharides derived from digestion of HA with hyaluronate lyase (HA lyase, E.C. 4.2.2.1, from *Streptomyces hyalurolyticus*). This enzyme is often used to afford fragments of HA, since its eliminase activity introduces an α,β-unsaturated carboxylic acid moiety at the non-reducing terminus, thus providing oligomers with a convenient UV chromophore [20,21]. Despite their frequent use in the biochemical literature, however, we found that these digestion products have not been rigorously characterized. In addition to the obvious considerations dictated by structure–activity relationship (SAR) issues involved in our biological studies, the desire to characterize these compounds by more modern methods was further prompted by our isolation of unpredicted oligomers from the reaction mixture. As heretofore described in the literature, HA lyase is an *endo*-hexosaminidase, producing even-numbered fragments of structure β-D-4en-thrHex*p*A-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A]$_n$-(1 → 3)-D-Glc*p*NAc [22]. As detailed below, however, our results clearly show that these are accompanied by minor — but significant — amounts of the intervening odd-numbered oligomers. The precise origin of these novel products has not yet been ascertained, but could be the result of a secondary glucuronidase activity of the enzyme, or is otherwise due to anomalies such as an HA lyase isozyme or a maverick hydrolase impurity in the commercially obtained enzyme material. Studies to elucidate the origin of these oligosaccharides are, however, beyond the scope of this report.

Electrospray-ionization mass spectrometry (ESIMS) has emerged in the last few years as a powerful new tool for carbohydrate analysis [23–26]. In addition to the more usual studies on neutral oligosaccharides, studies on anionic sugars using ESIMS in the negative-ion mode have begun to appear, thus demonstrating the usefulness of this tool in important areas such as glycosaminoglycan analysis [27–30]. With respect to hyaluronic acid, Zhao and co-workers reported ESIMS-observed peptide-HA disaccharide fragments as evidence that certain serum-derived proteins are covalently bound to the polymer [31]. Arpino and co-workers studied small oligogalacturonic acid oligosaccharides of dp 4–7 under negative-ion ESIMS and queried to what extent the sugars would polyanionize as the dp increased to larger values than those they studied [32]. In this study we address that question.

In conjunction with the mass-spectral characterization, we sought to develop a convenient screening protocol to assess the identity and purity of fractions from our preparative-scale gel-filtration chromatography of HA digests. The requisite method should also be suited for use in future planned work with bovine testes- and leech-derived hyaluronidases [33] whose digestion products do not possess the convenient α,β-unsaturated acid UV chromophore. Although other HPLC methods have been developed for the separation of HA oligosaccharides [34,35], we selected high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) due to its well-known excellent sensitivity and resolution [36,37]. Acidic oligosaccharides have been less well-studied by this technique, perhaps because the high pH conditions typically required cause the materials to be strongly retained on the stationary phase. Nevertheless, several reports have documented progress in this area [38–43].

Described herein are data from these two methods characterizing the series of even-numbered and novel odd-numbered hyaluronic acid oligosaccharides of dp 3–16 derived from HA.

2. Experimental

Materials.—Hyaluronate lyase (cat. No. H-1136, E.C. 4.2.2.1) and sodium hyaluronate (cat. No. H-1751) were purchased from Sigma Chemical Co. (St.

Louis, MO). Water was deionized and filtered using a Barnstead Nanopure 550 system.

Enzyme degradations and preparative column chromatography.—For the digestions, 50 mg of sodium hyaluronate was suspended in 10 mL of aqueous buffer (200 mM in NaCl and 50 mM in NaOAc, adjusted to pH 6.0), and the mixture was stirred overnight at 0°C, resulting in a clear solution to which HA lyase (1000 units) was added, with stirring being continued at 37°C. Aliquots (50 μL) were periodically removed and monitored at 232 nm on a Shimadzu 2101PC spectrophotometer until absorbance began to level off (~ 2–4 h). The mixture was then boiled for 10 min, diluted with 10 mL of water, and lyophilized. The resulting white powder was subjected to gel-filtration chromatography (Bio-Gel P-30), using 250 mM HCO_2NH_4 as eluent. Collected fractions were lyophilized five times and analyzed as described below.

High-performance anion-exchange chromatography.—The analytical system consisted of a Dionex DX-500 system (Sunnyvale, CA) equipped with a CarboPac PA-1 guard precolumn (25 × 3 mm), CarboPac PA-1 column (250 × 4 mm), and a type II PAD (with gold working electrode and silver/silver hydroxide reference electrode) interfaced to a Hewlett–Packard 3390A integrator. The electrochemical detector was operated in the pulsed amperometric mode at 3 μA sensitivity. The triple pulse sequence used for amperometric detection included the following potentials and durations: $E_1 = -0.15$ V (210 ms, integrating from 81–210 ms), $E_2 = +0.75$ V (180 ms), $E_3 = -0.35$ V (360 ms). To facilitate pulsed amperometric detection and minimize baseline drift, 400 mM NaOH was added post-column at a flow rate of ~ 0.5 mL/min. HA oligosaccharide samples (25 μL of solutions ~ 1–50 μg/mL in analyte, as estimated by UV measurements assuming $\epsilon = 5900$ [44]) were eluted at a flow rate of 1.0 mL/min. Acetate buffers at the concentrations listed in Table 2 were obtained by adjusting the system pump to mix the appropriate proportion of 1 M NaOAc (made up from ~ 1 L of 1 M HOAc adjusted to pH 7.5 with 50% NaOH) with deionized water.

Electrospray - ionization mass spectrometry.—Negative-ion electrospray mass spectra were obtained using a Micromass Quattro II triple quadrupole instrument, with a nominal mass range to 4,000 Da. Data acquisition and manipulation was accomplished using the Micromass MassLynx software package, including MaxEnt data-analysis software. Sample solutions (~ 16 μg/mL in 1:1 MeOH–water containing 0.3% Et_3N) were infused at a flow rate of 4 μL/min. Nitrogen was used as the nebulizing (20 L/h) and drying gas (300 L/h) while maintaining the source temperature at 80 °C. Capillary- and cone voltages were 2.5 kV and 25 V, respectively. Spectra were acquired in multi-channel-acquisition mode from scans encompassing *m/z* 50–1750 at 10 s/scan (and averaging of 10 such scans per spectrum). Reported masses are the averaged value for the isotope cluster.

3. Results and discussion

Previous reports [20–22] led us to expect as the products a series of even-numbered hyaluronan fragments from the HA lyase digestion. However, as shown in Fig. 1, the UV trace of our preparative gel-filtration chromatography column suggested the presence of two series: the expected products resulting from glucosaminidase cleavage (larger peaks), and intervening odd-numbered oligomers, apparently resulting from a glucuronidase-type cleavage (smaller peaks) of unknown origin. As suggested by the observed UV activity, and as unambiguously shown by

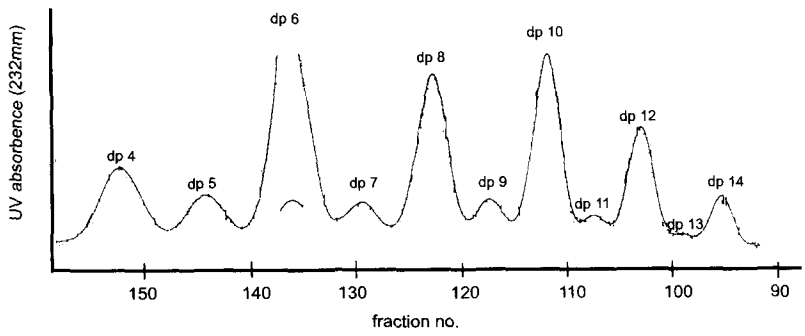

Fig. 1. Partial gel-permeation chromatogram (BioRad P-30, UV Absorbance at 232 nm) of hyaluronate lyase digest. Fraction volume = 10 mL.

Table 1
Observed vs. predicted ESIMS *m/z* values for HA lyase oligomers [a]

Oligomer (No. CO_2^- groups)	Observed *m/z*	Relative intensity (%) [b]	Possible ions (charge) predicted *m/z*
2-mer [c] (1)	378.2	100	(−1) 378.2
3-mer (2)	554.1	81	(−1) 554.1
	276.6	100	(−2) 276.5
4-mer (2)	757.3	9	(−1) 757.2
	378.2	100	(−2) 378.1
5-mer (3)	933.3	3	(−1) 933.2
	466.1	100	(−2) 466.1
6-mer (3)	– [d]	–	(−1) 1136.3
	567.9	95	(−2) 567.6
	378.3	100	(−3) 378.1
7-mer (4)	–	–	(−1) 1312.3
	655.6	28	(−2) 655.6
	437.0	100	(−3) 436.7
	–	–	(−4) 327.3
8-mer (4)	–	–	(−1) 1515.4
	757.2	20	(−2) 757.2
	504.5	100	(−3) 504.4
	378.2	20	(−4) 378.1
9-mer (5)	–	–	(−1) 1691.4
	845.4	6	(−2) 845.2
	563.3	47	(−3) 563.1
	422.3	100	(−4) 422.1
	–	–	(−5) 337.5
10-mer (5)	–	–	(−1) 1894.5
	946.9	6	(−2) 946.7
	631.0	41	(−3) 630.8
	473.1	100	(−4) 472.9
	378.2	6	(−5) 378.1
11-mer (6)	–	–	(−1) 2070.6
	1034.9	3	(−2) 1035.0
	689.6	26	(−3) 689.5
	517.1	100	(−4) 517.0
	413.5	98	(−5) 413.3
	–	–	(−6) 344.2
12-mer (6)	–	–	(−1) 2273.6
	–	–	(−2) 1136.3
	757.4	14	(−3) 757.2
	567.9	83	(−4) 567.7
	454.1	100	(−5) 453.9
	378.2	6	(−6) 378.1
13-mer (7)	–	–	(−1) 2449.7
	–	–	(−2) 1224.3
	816.0	4	(−3) 815.9
	612.1	20	(−4) 611.7
	489.4	100	(−5) 489.1
	407.5	57	(−6) 407.4
	349.0	4	(−7) 349.1

Table 1 (continued)

Oligomer (No. CO_2^- groups)	Observed *m/z*	Relative intensity (%) [b]	Possible ions (charge) predicted *m/z*
14-mer (7)	–	–	(−1) 2652.9
	–	–	(−2) 1325.9
	883.8	7	(−3) 883.6
	662.6	30	(−4) 662.4
	529.9	100	(−5) 529.7
	441.4	48	(−6) 441.3
	–	–	(−7) 378.1
16-mer (8)	–	–	(−1) 3031.9
	–	–	(−2) 1515.4
	–	–	(−3) 1009.9
	757.5	21	(−4) 757.2
	605.7	63	(−5) 605.6
	504.7	100	(−6) 504.5
	432.4	24	(−7) 432.3
	–	–	(−8) 378.1

[a] General structures: β-D-4en-thrHex*p*A-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A]$_n$-(1 → 3)-β-D-Glc *p*NAc (even-numbered oligomers), β-D-4en-thrHex*p*A-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A]$_n$ (odd-numbered oligomers). All measurements were taken with capillary and cone voltages at 2.5 kV and 25 V, respectively, as indicated in the experimental section.

[b] Normalized by assigning the predominant signal to 100%.

[c] Obtained from Sigma Chemical Co.

[d] '–' Indicates ion not observed.

the mass spectral data summarized below, these oligomers have the structure β-D-4en-thrHex*p*A-(1 → 3)-[β-D-GlcN*p*Ac-(1 → 4)-β-D-Glc*p*A]$_n$, and not the other possible arrangement of residues for an odd-numbered oligomer, namely [β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A]$_n$-(1 → 3)-β-D-Glc*p*NAc. No claims are made herein regarding the presence or absence of a secondary glucuronidase activity of the hyaluronate lyase itself, as other factors such as impurities in the enzyme (used as purchased from Sigma Chemical Co.) have not yet been ruled out.

As noted above, there has been some question in the literature as to the extent of polyanionization to be expected from the larger oligomers, and the degree to which this would complicate mass spectral interpretation. This study on HA oligomers up to dp 16 [1] indicates that such multiply charged anions predominate in the spectra of the larger oligomers, thus conveniently compressing most of the observed $(M - nH)^{n-}$ signals into the *m/z* 300–1100 mass-to-charge range. One can calculate a 'fingerprint' set of expected *m/z* values for each oligomer and compare that to the observed spectrum. The calculated values are such that confusion of a species with those of half or double its MW is avoided due to key non-duplicated values. Table 1 summarizes the data for the entire sequence of oligomers. The assigned MWs are also in agreement with a MW value extracted from the raw data by the 'MaxEnt' program. Fig. 2 shows representative observed spectra, along with 'MaxEnt'-generated molecular weight information, for the 13-mer and 14-mer. Each of these is shown by the MaxEnt analysis to contain minor impurities of lower oligomers. As evident from the data in Table 1, a particular compound may not have observed signals for all possible charge states; rather, the distribution will tend to cluster in a small range of charge states. Thus, as shown graphically for the entire sequence in Fig. 3, the smaller oligomers exist predominantly as the mono- or di-anion, whereas larger ones, such as the 12–14-mers exist mainly as the pentaanion, and finally the 16-mer exists chiefly as the hexaanion. (It should be noted that charge-distribution envelopes presented in Fig. 3 were observed at a cone voltage = 25 V. At higher cone voltages, such as 40 V, the envelope for any particular X-mer is shifted somewhat to lower charge-states.) The data clearly show that anionic oligosaccharides obey a logical and ex-

[1] Under conditions of the hydrolysis, the dp 2 compound was not observed. This material was purchased from Sigma Chemical Co. (hyaluronic acid disaccharide, cat. No. H-9649) and is a product of chondroitinase action on HA.

pected pattern of behavior, and are thus readily analyzable by ESIMS. This technique should also extend to the numerous other important carboxylated and/or sulfated glycosaminoglycan oligosaccharides whose characterization is often difficult.

Employment of eluents and PAD waveforms pre-

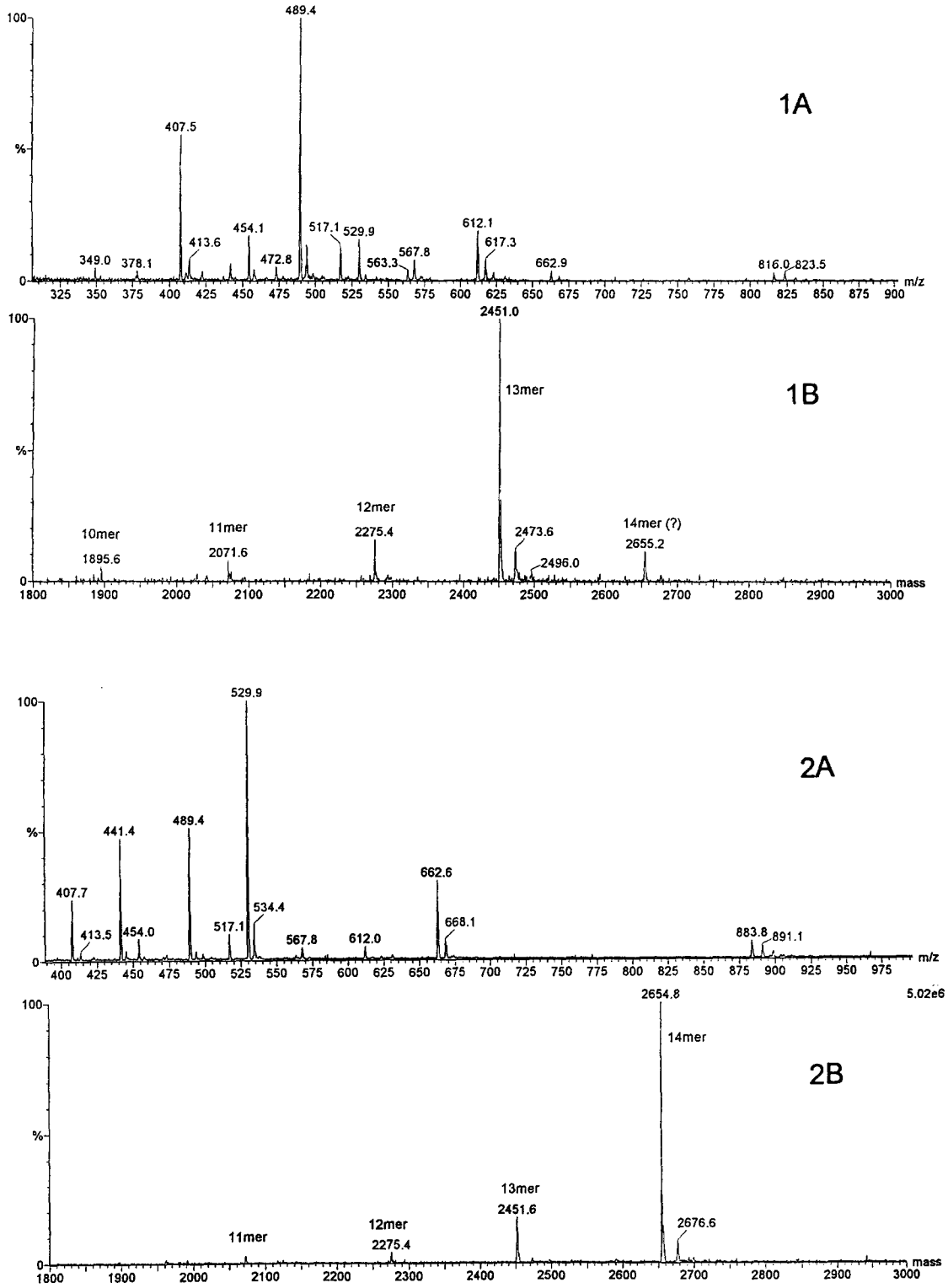

Fig. 2. Negative-ion electrospray mass spectra for two representative HA lyase-derived oligosaccharides. (1A) Observed spectrum and (1B) MAX ENT-generated MW distribution for 13-mer. (2A) Observed spectrum and (2B) MAX ENT-generated MW distribution for 14-mer.

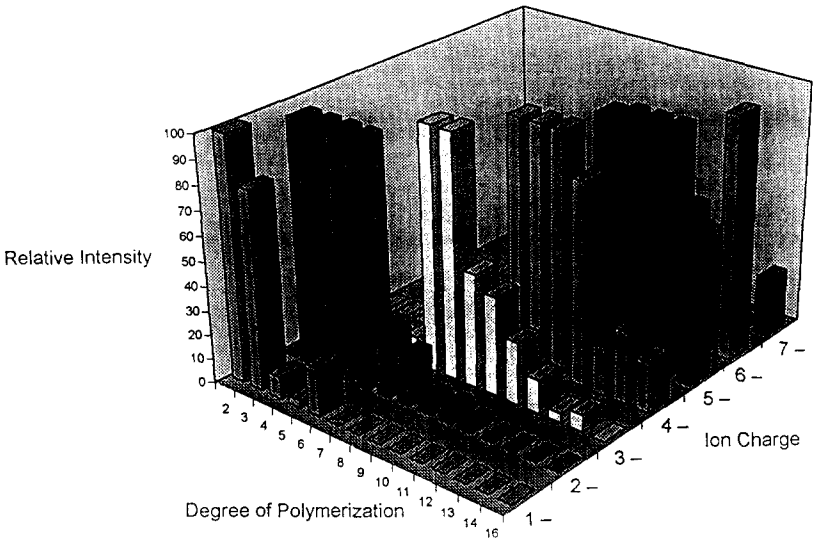

Fig. 3. Observed relative intensities of the different charge states of HA lyase-derived oligosaccharides in negative-ion ESIMS.

viously used in the literature [38–43] resulted in no (or very weak) detector response and/or anomalous or irreproducible retention times. In order to force a more rapid elution of the highly retained carboxylated sugars, and also in light of Hicks' recent observations that sugars having 2-acetamido groups with an adjacent glycosidic linkage from the 3-position are unstable under the high-pH conditions normally used in HPAEC [45], we resorted to the use of lower pH levels, as reported by Hotchkiss and Hicks [38–43]. Furthermore, it is widely known that optimal electrochemical waveform parameters vary widely with the sugar used, and are often tedious to identify [46]. The pulse sequence we eventually developed (by trial and error), whose potentials and durations differ significantly from others reported in the literature, allowed satisfactory detection of the HA oligomers. Standard retention times are summarized in Table 2. Interestingly, we also found that the compounds do *not* elute strictly in order of increasing number of monosaccharide residues; that is, the additional residue possessed by an odd-numbered species (with respect to its next-lower even-numbered neighbor) serves to *decrease* the retention time, sometimes by a factor of almost 2. Thus, for example, whereas the hexamer elutes at 5.6 min in 280 mM buffer, the heptamer elutes at only 3.8 min. We consider that this phenomenon may be rationalized as follows. In the odd-numbered oligomers, the acidic residues outnumber the neutral residues, whereas in the even-numbered oligomers, the acidic and neutral residues are present in equal numbers. Apparently, then, although there is the usual and expected contribution by increased MW toward increased retention, there also appears to be a counterbalancing contribution *toward*

Table 2
HPAEC retention times (min) of HA lyase-derived oligosaccharides at the indicated concentration of acetate (Na^+/H^+) solution, pH 7.5 [a]

Oligomer (No. GlcA residues)	200 mM	280 mM	350 mM	400 mM
2-mer (1)	3.0	– [b]	–	–
3-mer (2)	2.7	–	–	–
4-mer (2)	5.0	3.5	–	–
5-mer (3)	3.7	2.7	–	–
6-mer (3)	11.8	5.6	–	–
7-mer (4)	8.0	3.8	–	–
8-mer (4)	–	10.3	–	–
9-mer (5)	–	6.5	–	–
10-mer (5)	–	21.3	9.1	–
11-mer (6)	–	12.9	5.8	–
12-mer (6)	–	–	15.7	8.1
13-mer (7)	–	–	9.5	5.5
14-mer (7)	–	–	29	13.7
16-mer (8)	–	–	–	22.3

[a] Dionex DX-500 system with CarboPac PA-1 guard precolumn (25×3 mm) and CarboPac PA-1 column (250×4 mm); HA oligosaccharide samples (25 μL of solutions ~ 1–50 μg/mL in analyte) eluted at a flow rate of 1.0 mL/min; Acetate buffers obtained by adjusting the system pump to mix the appropriate proportion of a 1 M sodium acetate (made up from ~ 1 L of 1 M HOAc adjusted to pH 7.5 with 50% NaOH) with deionized water.
[b] '–' Indicates not measured.

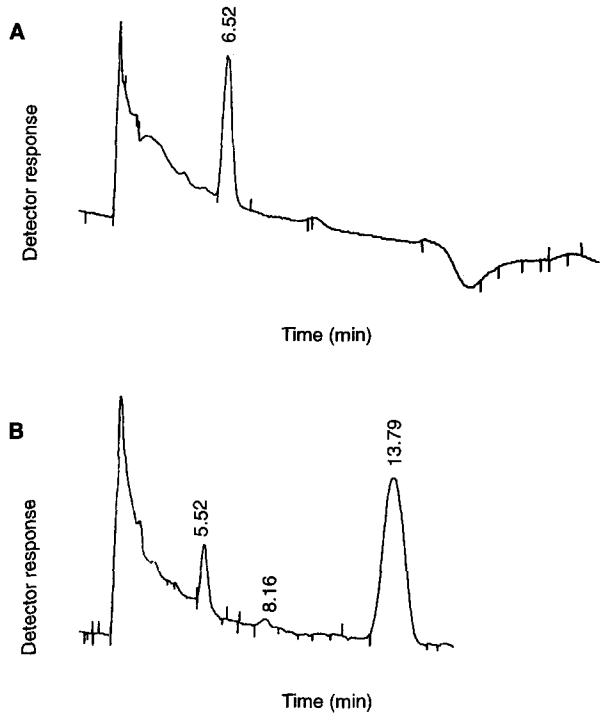

Fig. 4. HPAEC chromatograms (Dionex system, PAD) for HA oligosaccharides. (A) 9-mer (6.5 min) at 350 mM acetate buffer. (B) 14-mer (13.7 min) at 400 mM acetate buffer, with impurity 13-mer (5.5 min.) and 12-mer (8.1 min) present at approximately 50:6:1, respectively. Peak and noise following 1.8 min correspond to buffer salts, as established by control experiments.

increased mobility by additional glucuronate residues that, overall, outweighs the MW contribution.

We found that the extreme sensitivity of the PAD, as well as its imperviousness to the 250 mM ammonium formate buffer, allowed us to directly sample fractions from the preparative gel-permeation column without desalting or concentrating them. This convenience circumvents problems associated with pooling fractions based on UV traces of the column, which often do not reveal overlapping components present in individual fractions. Thus, with standard retention times correlated to mass spectral data, the HPAEC method furnished us with the desired means of rapidly screening the fractions from our preparative gel-permeation column. Representative chromatograms are shown in Fig. 4. The protocol is currently being applied to other types of digest-derived HA fragments. The developed methods demonstrate the usefulness of both ESIMS and HPAEC-PAD in the analysis of anionic glycosaminoglycan-type oligosaccharides.

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References

[1] M.S. Sy, D. Liu, R. Schiavone, J. Ma, H. Mori, and Y. Guo, *Curr. Top. Microbiol. Immunol.*, 213 (1996) 129–153.
[2] W. Knudson, *Am. J. Pathol.*, 148 (1996) 1721–1726.
[3] J. Entwistle, C.L. Hall, and E.A. Turley, *J. Cell. Biochem.*, 61 (1996) 569–577.
[4] P. Heldin, M. De La Torre, D. Ytterberg, and J. Bergh, *Oncol. Rep.*, 3 (1996) 1011–1016.
[5] P. Kogerman, M.S. Sy, and L.A. Culp, *Clin. Exp. Metastasis*, 14 (1996) 73–82.
[6] M. Goebeler, D. Kaufmann, E.-B. Broecker, and C.E. Klein, *J. Cell Sci.*, 109 (1996) 1957–1964.
[7] R. Goshen, I. Ariel, S. Shuster, A. Hochberg, I. Vlodavsky, N. De Groot, Z. Ben-Rafael, and R. Stern, *Mol. Human Reprod.*, 2 (1996) 685–691.
[8] A.R. Guenthert, J. Straeter, U. von Reyher, C. Henne, S. Joos, K. Koretz, G. Moldenhauer, P.H. Krammer, and P. Moeller, *J. Cell Biol.*, 134 (1996) 1089–1096.
[9] V. Assmann, H.F. Kern, and H.-P. Elsaesser, *Clin. Cancer Res.*, 2 (1996) 1607–1618.
[10] M.J. Gardner, J.B. Catterall, L.M.H. Jones, and G.A. Turner, *Clin. Exp. Metastasis*, 14 (1996) 325–334.
[11] T.-K. Yeo, J.A. Nagy, K.-T. Yeo, and H.F. Dvorak, *Am. J. Pathol.*, 148 (1996) 1733–1740.
[12] C. Wang, S. Zhang, and E.A. Turley, *Round Table Ser.-R. Soc. Med. Press*, 45 (1996) 3753.
[13] B.S. Mitchell, A. Whitehouse, P. Prehm, B. Delpech, and U. Schumacher, *Clin. Exp. Metastasis*, 14 (1996) 107–114.
[14] V.B. Lokeshwar, B.L. Lokeshwar, H.T. Pham, and N.L. Block, *Cancer Res.*, 56 (1996) 651–657.
[15] U.B.G. Laurent, T.C. Laurent, L.K. Hellsing, L. Persson, M. Hartman, and K. Lilja, *Acta Neurol. Scand.*, 94 (1996) 194–206.
[16] D.M. Jaworski, G.M. Kelly, J.M. Piepmeier, and S. Hockfield, *Cancer Res.*, 56 (1996) 2293–2298.
[17] K. Akima, H. Ito, Y. Iwata, K. Matsuo, N. Watari, M. Yanagi, H. Hagi, K. Oshima, and A. Yagita, *J. Drug Targeting*, 4 (1996) 1–8.
[18] T. Spruss and G. Bernhardt, *Contrib. Oncol.*, 51 (1996) 145–150.
[19] T. Pouyani, G.S. Harbison, and G.D. Prestwich, *J. Am. Chem. Soc.*, 116 (1994) 7515–7522, and references cited therein.
[20] L.E. Chun, T.J. Koob, and D.R. Eyre, *Anal. Biochem.*, 171 (1988) 197–206.

[21] J.E. Christner, M.L. Brown, and D.D. Dziewiakowski, *J. Biol. Chem.*, 254 (1979) 4624–4630.
[22] H. Kresse and J. Glössl, *Adv. Enzymol.*, 60 (1987) 217–311.
[23] G.E. Black and A. Fox, *ACS Symp. Ser.*, 619 (1996) 81–105.
[24] J. Peter-Katalinic, *Croat. Chem. Acta*, 69 (1996) 731–739.
[25] L. Garone, T. Edmunds, E. Hanson, R. Bernasconi, J.A. Huntington, J.L. Meagher, B. Fan, and P.G.W. Gettin, *Biochemistry*, 35 (1996) 8881–8889.
[26] V.N. Reinhold, B.B. Reinhold, and S. Chan, *Methods Enzymol.*, 271 (1996) 377–402.
[27] W.M.A. Niessen, R.A.M. Van der Hoeven, and J. Van der Greef, *Org. Mass Spectrom.*, 27 (1992) 341–342.
[28] J. An, M.A. O'Neill, P. Albersheim, and A.G. Darvill, *Carbohydr. Res*, 252 (1994) 235–243.
[29] U.R. Bhat, L.S. Forsberg, and R.W. Carlson, *J. Biol. Chem.*, 269 (1994) 14402–14410.
[30] K. Sugahara, Y. Ohkita, Y. Shibata, K. Yoshida, and A. Ikegami, *J. Biol. Chem.*, 270 (1995) 7204–7212.
[31] M. Zhao, M. Yoneda, Y. Ohashi, S. Kurono, H. Iwata, Y. Ohnuki, and K. Kimata, *J. Biol. Chem.*, 270 (1995) 26657–26663.
[32] M.G. Xie, D. Giraud, Y. Bertheau, B. Cassetta, and P. Arpino, *Rapid Commun. Mass Spectrom.*, 9 (1995) 1572–1575.
[33] K. Meyer, *Hyaluronidases*, in P.D. Boyer (Ed.), *The Enzymes*, 3rd ed., Vol. 5, Academic Press, New York, 1971, pp 307–320.
[34] For examples, see: N. Caram-Lelham, L.-O. Sundelöf, and T. Andersson, *Carbohydr. Res.*, 273 (1995) 71–76.
[35] P. Nebinger, M. Koel, A. Franz, and E. Werries, *J. Chromatogr.*, 265 (1983) 19–25.
[36] C. Corradini, *Ann. Chim.*, 84 (1994) 385–396.
[37] S. Mou and Z. Li, *Sepu*, 13 (1995) 320–324, *Chem. Abstr.*, 123 (1995) 358061.
[38] A.T. Hotchkiss, Jr. and K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200–206.
[39] H.-P. Lieker, K. Thielecke, K. Buchholz, and P.J. Reilly, *Carbohydr. Res.*, 238 (1993) 307–311.
[40] D.M. Balmer and D. McLellan, *Fruit Process.*, 5 (1995) 86–89.
[41] N. Kawasaki, K. Morimoto, and T. Hayakawa, *Eisei Shikensho Hokoku*, 113 (1995) 69–73, *Chem. Abstr.*, 124 (1996) 76674.
[42] S. Thurl, B. Mueller-Werner, and G. Sawatzki, *Anal. Biochem.*, 235 (1996) 202–206.
[43] A.F.A. Wallis, R.H. Wearne, and P.J. Wright, *Appita J.*, 49 (1996) 258–262, *Chem. Abstr.*, 125 (1996) 198822.
[44] E. Shimada and G. Matsumara, *Biochem. J.*, 88 (1980) 1015–1023.
[45] K.B. Hicks, *J. Chrom. Libr.*, 58 (1995) 370.
[46] D.C. Johnson, *J. Chrom. Libr.*, 58 (1995) 391–430.