Large-scale preparation, purification, and characterization of hyaluronan oligosaccharides from 4-mers to 52-mers

Akira Tawada, Takahiro Masa, Yuji Oonuki, Atsushi Watanabe, Yuji Matsuzaki, and Akira Asari
Seikagaku Corporation, Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo 207-0021, Japan
Received on December 27, 2001; revised on March 3, 2002; accepted on March 5, 2002

Hyaluronan (HA) was depolymerized by partial digestion with testicular hyaluronidase and separated into size-uniform HA oligosaccharides from 4-mers to 52-mers by anion exchange chromatography after removal of the hyaluronidase. The purity and size of each HA oligosaccharide was confirmed by using HPLC analyses, FACE, and ESI-MS. 1H and 13C NMR assignments and elemental analyses were obtained for each HA oligosaccharide. Endotoxins, proteins, and DNA were absent or in trace amounts in these HA oligosaccharides. Gram/mg-scale hyaluronan oligosaccharides were obtained from 200 g of HA starting material. These pure, size-uniform, and large range of HA oligosaccharides will be available for investigating important biological functions of HA, such as for the determination of the size(s) of HA oligosaccharides that induce angiogenesis or mediate inflammatory responses, and to interact with HA-binding proteins and receptors both in vitro and in vivo studies.

Key words: hyaluronan/oligosaccharides

Introduction

Hyaluronan (HA) molecules are long, unbranched chains of variable length that consist of a disaccharide, glucuronic acid-β1, 3-N-acetylglucosamine-β1, 4-, repeated several thousand times.

HA plays a crucial role in dynamic cellular processes such as embryonic development, tissue regeneration, and tumorigenesis (Toole, 1981, 1991). Consequently, evidence was sought and obtained for the presence of HA receptors on the surface of cells. Subsequent investigations led to the molecular characterization of two classes of cell surface HA receptors, namely, CD44 and RHAMM (Toole, 1991; Sherman et al., 1994; Kincade et al., 1997; Entwistle et al., 1996).

It has been shown that high-molecular-weight hyaluronan suppresses angiogenesis (Feinberg and Beebe, 1982), as well as the production of IL-1beta (Takahashi et al., 1997) and PGE2 (Asari et al., 1997, 1998). Conversely, low-molecular-weight HA, including oligosaccharides, stimulates angiogenesis (West et al., 1985) and induces many kinds of inflammatory factors (Noble et al., 1998). Toole (1991) showed that treatment with HA oligosaccharides suppresses the growth of tumors both in vitro and in vivo. These events suggest that HA obtains new activities and functions after depolymerization.

Weissman et al. (1954) first separated HA oligosaccharides in testicular hyaluronidase digests by anion exchange; Hascall and Heinegård (1974) used molecular sieve procedures for the same purpose. We have been able to obtain each HA oligosaccharide with two to seven repeats (4-mers to 14-mers), so we tried to isolate larger sizes of HA in the present study. HA oligosaccharides were subsequently purified and partially characterized by nuclear magnetic resonance (NMR) or mass spectrometry (Takagaki et al., 1992; Toffanin et al., 1993; Suzuki et al., 2001). However, in these studies, HA oligosaccharides were prepared in small amounts and characterized by a limited number of methods. In this report, we describe a large-scale isolation and purification of HA oligosaccharides and their subsequent analyses by a number of physical and chemical procedures. These can now be used as standards for a variety of cell biological studies and medical applications.

Results

HA oligosaccharides were isolated and purified from testicular hyaluronidase digests of ~200 g of rooster comb HA as described in Materials and methods. Testicular hyaluronidase is an endo-GlcNAc hydrolase that generates a ladder of HA oligosaccharides that vary by one repeat disaccharide. The anion exchange procedure that was used successfully separated HA oligosaccharides with 2 to 26 repeats (4-mers to 52-mers) that were of high purity and free of protein, DNA, and endotoxin contaminants.

HPLC

Figure 1 shows anion exchange high-performance liquid chromatography (HPLC) analyses of HA oligosaccharides ranging from 4-mers to 52-mers along with a sample of an unfractionated digest. The profiles demonstrate the purity of each. Both anion exchange chromatography–HPLC and gel permeation chromatography (GPC)–HPLC analyses (data not shown) clearly demonstrated that each HA oligosaccharide was isolated more than 93% purity.

FACE

Fluorophore-assisted carbohydrate electrophoresis (FACE) analyses of a fluorotagged sample of unfractionated HA oligosaccharides showed a ladder (Figure 2, lane 1). Each of the fluorotagged purified HA oligosaccharides showed a single...
band at a unique position (Figure 2, lanes 2–7) corresponding to one of the bands in the ladder. The distance each band migrated in the gel correlated inversely with the size of the oligosaccharide, with the 4-mers migrating ahead of the 6-mers, the 6-mers ahead of the 8-mers, and so on. The ladder shows clear resolution of bands into the 20-mers under these FACE conditions. The ladder also shows a minor band (indicated by the asterisk) that migrates faster than the 4-mers and, in a position expected for a HA-disaccharide, a known minor product from digestion of hyaluronan with testicular hyaluronidase.

NMR spectroscopy, ESI-MS, and element analysis

The NMR spectra of HA oligosaccharides were assigned by a combination of correlation spectroscopy, total correlation spectroscopy, and heteronuclear single quantum coherence spectroscopy. The 1H NMR data sets and the 13C NMR data sets for HA oligosaccharides ranging from the 4-mers to the 52-mers are shown in Figures 3 and 4, respectively. The data for the 4-mers is described in detail as a representative example for the HA oligosaccharides obtained in the present study.

The characteristic anomic signals of the 4-mers (see Figure 3a) were observed for the protons: H-1α (reducing end GlcNAc), H-1β (reducing end GlcNAc), H-1c (interior GlcNAc), H-1β (interior GlcA), H-1β (interior GlcA), and H-1d (non-reducing terminal GlcA) at δ 5.144, 4.705, 4.555, 4.502, 4.460, and 4.453 ppm, respectively.
The singlet signals from 2.004 to 2.019 ppm correspond to the N-acetyl methyl signals of the GlcNAc residues. The $^{13}$C NMR data of the 4-mers (see Figure 4a) showed characteristic signals for the carbons: C-1bα (interior GlcA), or C-1bβ (interior GlcA), or C-1d (nonreducing terminal GlcA); and for the C-1c (interior GlcNAc), C-1aα (reducing end GlcNAc), and C-1aβ (reducing end GlcNAc) at $\delta$ 105.80, or 105.84, or 105.95; 103.44, 97.61, and 93.92 ppm, respectively.

Data for the NMR for each HA oligosaccharide, for the electrospray ionization mass spectrometry (ESI-MS) for 6-, 14-, 52-mers and for the elemental analysis for 14-mers are noted as representative examples for the HA oligosaccharides obtained on the present study. Figures 3 and 4 show data for the NMR for 4-, 14-, and 52-mers. Figures 5 and 6 show data for the ESI-MS for 4-, 6-, 14-, and 52-mers. Table 1 shows data for the ESI-MS and the elemental analysis for each HA oligosaccharide.

**Impurity: endotoxin, DNA, and protein**

The contents of endotoxin, DNA, and protein were negligible or not detected in each HA oligosaccharide, 4-mers to 14-mers.

**Discussion**

The HPLC and FACE methods confirmed the purity in each HA oligosaccharide prepared in the present study. The molecular size of each, determined by ESI-MS, coincides with their theoretical calculated mass (Table I) and those reported by Takagaki et al. (1992). Moreover, the NMR spectra of the HA oligosaccharides agree well with those reported by Toffanin et al. (1993). These data, and the negligible contents of endotoxins, DNA and protein, indicate that the HA oligosaccharides are pure, size-uniform for a wide variety of sizes. Thus, the procedures used, hyaluronidase digestion and anion exchange chromatography, are suitable for preparing gram scale amounts of highly purified HA oligosaccharides for use in biological applications.

The results of the FACE analyses for the HA oligosaccharides fluorotagged with disodium 8-aminonaphtalene-1,3,6-trisulfate (ANTS) indicate that each migrates to a unique position as a direct function of its length (mass). This is in contrast with results when 2-aminoacridone (AMAC) was used as the fluorotag and the FACE analyses were done with the O-linked profiling gels used in our study. In this case, the AMAC-derivatized oligosaccharides migrated only slightly into the gel.

![Fig. 4. 125-MHz $^{13}$CNMR spectra of D$_2$O solution of HA oligosaccharides: (a) HA 4-mers, (b) HA 14-mers. The numbers and letters in the spectra of HA 4-mers (a) refer to corresponding residue in the structure.](image-url)

**Table I. Results of ESI-MS and the elemental analysis for HA oligosaccharides**

<table>
<thead>
<tr>
<th>HA oligosaccharide</th>
<th>Observed multiply charged ions and molecular mass obtained by deconvolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-mers</td>
<td>[M-H]$^{-}$ $m/z$ 775.1, [M-2H]$^{2-}$ $m/z$ 387.2</td>
</tr>
<tr>
<td>6-mers</td>
<td>[M-H]$^{-}$ $m/z$ 1154.2, [M-2H]$^{2-}$ $m/z$ 576.8, molecular mass 1155.6</td>
</tr>
<tr>
<td>8-mers</td>
<td>[M-H]$^{-}$ $m/z$ 1534.0, [M-2H]$^{2-}$ $m/z$ 766.3, molecular mass 1534.7</td>
</tr>
<tr>
<td>10-mers</td>
<td>[M-H]$^{-}$ $m/z$ 1913.1, [M-2H]$^{2-}$ $m/z$ 955.9, molecular mass 1913.8</td>
</tr>
<tr>
<td>12-mers</td>
<td>[M-2H]$^{2-}$ $m/z$ 1145.7, [M-3H]$^{3-}$ $m/z$ 763.4, molecular mass 2293.4</td>
</tr>
<tr>
<td>14-mers</td>
<td>[M-2H]$^{2-}$ $m/z$ 1335.4, [M-3H]$^{3-}$ $m/z$ 889.8, molecular mass 2672.5</td>
</tr>
<tr>
<td>48-mers</td>
<td>[M-5H]$^{5-}$ $m/z$ 1823.5, [M-6H]$^{6-}$ $m/z$ 1519.5, [M-7H]$^{7-}$ $m/z$ 1302.2, [M-8H]$^{8-}$ $m/z$ 1139.0</td>
</tr>
<tr>
<td>50-mers</td>
<td>[M-9H]$^{9-}$ $m/z$ 1012.3, [M-10H]$^{10-}$ $m/z$ 911.2, [M-11H]$^{11-}$ $m/z$ 828.3, [M-12H]$^{12-}$ $m/z$ 758.9</td>
</tr>
<tr>
<td>52-mers</td>
<td>[M-13H]$^{13-}$ $m/z$ 700.4, molecular mass 9121.8</td>
</tr>
</tbody>
</table>

Calculated for C$_{98}$H$_{142}$N$_{7}$Na$_{7}$O$_{78}$: C, 41.64; H, 5.06; N, 3.47; Na, 5.69. Found: C, 41.44; H, 5.36; N, 3.37; Na, 5.20.

*The elemental analysis*
even when voltages higher than 1000 V were used (data not shown). AMAC interacts with the reducing ends of sugar chains in same manner as ANTS but has no anionic charge. Therefore, the additional three negative charges provided by the trisulfate moiety of ANTS on the fluorotagged HA oligosaccharides is the major contributor to their migration under these FACE conditions. This permits them to interact with the sieving meshwork of the highly cross-linked gel (36% polyacrylamide) and separate on the basis of their mass.

In this respect, the results differ from those described by Calabro et al. (2000). In their study, they used AMAC as the fluorotag and MONO gels (Glyco), which contain borate, as did the running buffer. Fluorotagged HA oligosaccharides in this case also sorted effectively as a function of molecular size, but only for 8-mers and larger. Fluorotagged HA oligosaccharides between the 6-mers and 2-mers showed inversions in mobility, with the 6-mers migrating in the same position as the 8-mers, the 4-mers with the ~14-mers, and the 2-mers with the ~36-mers. They suggested that this inversion was the result of differential interaction of borate with the smaller as compared to the larger fluorotagged oligosaccharides. Because borate was not involved in the electrophoresis buffer used in the present study, no inversion in mobility was observed.

Termeer et al. (2000) have reported that tetra- and hexasaccharides of HA induce immunophenotypic maturation of human monocyte-derived dendritic cells and production of the cytokines IL-1β, TNF-α, and IL-12 in dendritic cells. However, they showed no data indicating an absence of endotoxin in the HA oligosaccharides applied in the experiments, which if present, could stimulate such cells in the manner described. Moreover, they used a mixture of tetra- and hexasaccharides of HA. Hexasaccharides of HA have been used as a tool for probing the cell surface in a study of HA receptor function that showed them to be the minimum size required to compete effectively with native HA in binding to CD44 on chondrocytes (Knudson and Knudson, 1993). Tammi et al. (1998) have shown that HA bound to CD44 on keratinocytes, however, is displaced by HA decasaccharides and not by shorter oligosaccharides. Therefore, highly pure and size-uniform HA oligosaccharides are very important tools to investigate the relationship between HA and molecules that interact with HA in a wide variety of biological situations.

The pure, size-uniform HA oligosaccharides in wide variety of sizes obtained in the present study are now available for investigating biological functions and possible medical applications of HA.

Materials and methods

Materials

HA sodium salt from Rooster comb was supplied by Seikagaku Corporation (Tokyo). Hyaluronidase from bovine testicular was purchased from Biozyme Laboratories (Gwent, UK).

Preparation of HA oligosaccharides

HA was partially degraded by bovine testicular hyaluronidase. To a solution containing 200 g of HA in 10 L of 100 mM phosphate buffer (pH 5.3) containing 150 mM sodium chloride, 2 MU hyaluronidase was added, and enzymatic digestion was performed at 37°C for 6–40 h. The incubation time of the hyaluronidase varied according to the sizes of HA oligosaccharides to be obtained. The reaction was stopped by boiling for 20 min. The sample was centrifuged at 10,000 rpm for 30 min, and the supernatant was concentrated and lyophilized. The lyophilized sample was dissolved in distilled water, and each HA oligosaccharide was isolated from the parent digest by anion exchange chromatography on a Dowex 1 × 2 column. Each size-uniformed HA oligosaccharide fraction was desalted by gel-filtration on Sephadex G-10. Then pyrogen was removed from the desalted HA oligosaccharides by using a molecular weight 10,000 cut-off device (Centricon Plus 20). Then each HA oligosaccharide was lyophilized to give the final products. Gram or mg scale of size-uniformed HA oligosaccharides were obtained from 200 g of HA bulk.

HPLC analysis using YMC NH2 column

Anion exchange chromatography–HPLC equipped with YMC NH2 column (4 × 250 mm) was performed using a 60-min linear gradient of 16–800 mM NaH2PO4 under constant flow (1 ml/min) at 40°C. The eluent was monitored at 210 nm.
GPC–HPLC analysis

GPC–HPLC was done with serially combined columns of TSK-gel PWXL-4000, PWXL-3000, and PWXL-2500 under constant flow (1 ml/min) of 0.2 M NaCl at 40 °C. The eluent was monitored with refractive index.

FACE analyses

For the FACE analyses, the method of Calabro et al. (2000) was modified. Derivatization of HA oligosaccharides with ANTS was used 2 nmol of each oligosaccharide and 20 nmol (as disaccharide unit) of the unfractionated oligosaccharides. Each were lyophilized in a 0.5-ml tube until dry on a vacuum concentrator (SpeedVac, model AS160, Savant Instruments, NY). All samples were then derivatized by adding of 5 µl of ANTS reagent solution (Glyko L2, Part 50058) followed by incubation for 15 min at room temperature. Then 5 µl of sodium cyanoborohydride solution (Glyko L1, Part 50056) was added followed by incubation for 16 h at 37 °C. After incubation, all samples were centrifuged briefly and adjusted to a final volume of 20 µl with pure water. For electrophoresis, OLIGO Gel Running Buffer was dissolved in pure water and cooled on ice. O-linked oligosaccharide profiling gels were thoroughly cleaned with pure water, and the wells of each gel were rinsed extensively with running buffer just prior to use. The assembled electrophoresis apparatus containing the electrophoresis buffer and one or two gels was placed in the Glyko Gel Box and cooled on ice to equilibrate the buffer to 4 °C or less at the start of electrophoresis.

A 2-µl aliquot of each fluorotagged HA oligosaccharide was mixed with 3 µl pure water and 5 µl Glyko loading buffer, and a 2-µl aliquot of the fluorotagged unfractionated HA oligosaccharides was mixed with 2 µl Glyko loading buffer. Each of the eight lanes of a gel was loaded with 4 µl of a sample. The samples were then electrophoresed for 160 min at a constant 1000 V with an initial current of 8 and a final current of ~30 mA/gel. After electrophoresis, gels were removed from the apparatus, and the covering glass plates were thoroughly cleaned with pure water. The gels were illuminated with UV light (365 nm) and photographed.

NMR spectra were recorded with a Varian Unityinova 500 [1H (500 MHz) or 13C (125 MHz)] spectrometer. Chemical shifts are expressed in ppm downfield from the signal for internal t-BuOH (1H: 1.230 ppm, 13C:32.461 ppm), for solutions in D2O. The sample temperature was 23 °C, and the concentration was 16 mg/ml.

Mass spectrometry

Ten oligosaccharides (HA 4-mers, HA 6-mers, HA 8-mers, HA 10-mers, HA 12-mers, HA 14-mers, HA 16-mers, HA 48-mers, HA 50-mers, and HA 52-mers) were dissolved in distilled water at concentrations of 1.0–6.5 mg/ml. ESI-MS analysis was done on a Thermo Quest TSQ triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (Thermo Finnigan, San Jose, CA), scanning over the m/z range 100–2500 in 10 s/scan. Five-microliter aliquots of sample solutions were introduced into the ion source of the mass spectrometer via an autosampler of Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA). The carrier solvent used for the flow injection analysis, was 10 mM ammonium formate: methanol (80: 20, v:v) at a flow rate of 0.1 ml/min. ESI was carried out in negative ion mode with a spray voltage of 4.5 kV, and the heated capillary temperature was 350 °C. The pressure of the sheath gas was 30 psi. The molecular masses of HA oligosaccharides were obtained by the deconvolution of the multiply charged ions using Xcalibur Bioworks software supplied with the instruments.

Impurity: endotoxin, DNA, and protein

The quantities of impurity (endotoxin, DNA, and protein) were estimated as follows. Endotoxin activity was checked by Limulus amebocyte lysate assay using the Toxicolor system (Seikagaku). Protein was quantified by the Bradford method using the Biorad Protein Assay system. DNA was quantified by using the threshold total DNA analysis system as described previously (Mizusawa et al., 1991)

Acknowledgment

We are indebted to Mr. R. Rosemura, Ms. Shibata, Ms. Imai, and Mr. Tominaga at Seikagaku Corporation for their technical assistance and help in the preparation of this manuscript. We are grateful to Mr. Ken Mizutani at Seikagaku Corporation for his general support. We thank Dr. Hascall at the Cleveland Clinic Foundation for review of this manuscript. While this manuscript was under consideration, similar work was published by Mahoney et al. (2001) in Glycobiology 11, 1025–1034.

Abbreviations

AMAC, 2-aminoacridone; ANTS, disodium 8-amino-1,3,6-naphthalene trisulfonate; ESI-MS, electrospray ionization mass spectrometry; FACE, fluorophore-assisted carbohydrate electrophoresis; GPC, gel permeation chromatography; HA, hyaluronan; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

Reference


Large-scale purification of hyaluronan oligosaccharides

GPC–HPLC was done with serially combined columns of TSK-gel PWXL-4000, PWXL-3000, and PWXL-2500 under constant flow (1 ml/min) of 0.2 M NaCl at 40 °C. The eluent was monitored with refractive index.


