Polysialic Acid Analysis: Separating Polymers with High Degrees of Polymerization

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Key Words
HPAE-PAD, Polymer of *N*-Acetylneuraminic Acid, Colominic Acid
Dionex CarboPac PA200 Column

Introduction

Polysialic acid is a collective name for linear polymers of sialic acid that are covalently bound to proteins as a post-translational modification.[1] It is widely expressed in nature in bacterial capsules, fish, sea urchin eggs, embryonic tissues, amphibians, animal and human brains, and in a variety of cancers.[2] These sugar chains modulate cell-cell interaction (mainly during embryonic growth), neural plasticity, and tumor metastasis.

The major carrier of polysialic acids in mammals is the neural cell adhesion molecule (a glycoprotein that belongs to the immunoglobulin superfamily). As a linear oligo/homopolymer (n = 8 to >100) of sialic acid, these cell surface glycans are highly expressed during embryonic brain development. In postnatal and adult animals, their expression is restricted to regions capable of neuronal and synaptic plasticity. Polysialic acid is considerably anionic. This strong negative charge gives this modification the ability to change the protein's surface charge and binding ability.[3]

The degree of polymerization (DP) of polysialic acid chains is postulated to be of critical importance in regulating their function, hence the need for sensitive methods to accurately determine DP for polysialic acid. This will shed light on the molecular mechanism of polysialic acid biosynthesis and its ability to regulate cell-cell interactions.

The existing methods for polysialic acid analysis can be classified into the following groups:

- Methods based on the specificity of antibodies,[4] which give neither DP information nor distribution of chains with different DPs
- Methods based on gel or capillary electrophoresis[5]
- Methods based on nuclear magnetic resonance (NMR) spectrometry[6]
- Mass spectrometric methods[7]
- High-performance liquid chromatography (HPLC)-based methods,[8-11] for which detection has been either by fluorescence (after labeling the polysialic acids with a fluorogenic reagent) or by pulsed amperometric detection

Typically, polysialic acids have been resolved on anion-exchange columns like the Thermo Scientific Dionex CarboPac PA1, PA100, DNAPac PA100, or Mono Q™ columns.

In high-performance anion-exchange with pulsed amperometric detection (HPAE-PAD)-based methods, large poly/oligosaccharides are typically separated using a gradient of sodium acetate in the presence of 100 mM sodium hydroxide. An HPAE-PAD-based method using the Dionex CarboPac™ PA100 column and acetate gradient has been shown to separate unmodified α2,8-linked 5-*N*-acetyl-neuraminic acid (Neu5Ac) polymers with DP up to 60 within 68 min. Using sodium nitrate instead of sodium acetate resulted in elution and resolution of the high polymers (maximum DP resolved was ~20 DP higher), in part because nitrate is a stronger eluent when compared to acetate.[8]

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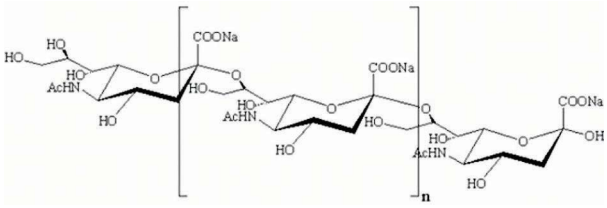

Figure 1. Colominic acid.

The Dionex CarboPac PA200 column is better able to resolve the high DP polymers using a colominic acid preparation when compared to other columns used for this application. Colominic acid (Figure 1) is a homopolymer of Neu5Ac and is produced by special strains of *Escherichia coli*.[12] The Dionex CarboPac PA200 column and has been specially developed to provide high-resolution separations of charged and neutral oligosaccharides.[13] This column has a smaller particle size for the substrate (5.5 µm) and the microbead (43 nm) compared to similar Dionex CarboPac columns, thus enabling higher-resolution separations when compared to those columns.

Goal

To develop an HPAE-PAD-based method to separate polymers of polysialic acid with higher DPs than existing methods can separate

Equipment

- Thermo Scientific Dionex ICS-5000 system including:
  - SP Single Pump or DP Dual Pump module
  - DC Detector/Chromatography compartment
  - ED Electrochemical Detector (P/N 061718)
  - Gold on PTFE Disposable Electrode (P/N 072042)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - AS or AS-AP Autosampler
- Thermo Scientific Dionex Chromeleon Chromatography Data System software
- EO Eluent Organizer, including 2 L plastic bottles and pressure regulator
- Vial Kit, 0.3 mL Polyprop with Caps and Septa (P/N 055428)
- Polypropylene Microcentrifuge Screw Cap Tubes, 1.5 mL (Sarstedt® P/N 72.692.005 or equivalent)
- Nalgene Lab Quality Narrow-Mouth Bottles, HDPE (P/N 2002-0032)
- Nalgene™ Rapid-Flow Sterile Disposable Filter Units with Nylon Membrane (P/N 154-0020)
- MFC-1 Metal-Free Column (P/N 037017)

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or above, filtered through a 0.2 µm filter immediately before use
- Sodium Acetate Salt (Preweighed Reagent) (P/N 059326), recommended for carbohydrate analysis
- Sodium Nitrate (Fisher Scientific P/N S343-500)
- Colominic Acid (Sigma-Aldrich® P/N 27698)
- *N*-Acetylneuraminic Acid, Trimer (Nacalai USA P/N 0064194)
- *N*-Acetylneuraminic Acid, Pentamer (Nacalai USA P/N 0064374)

Conditions

Method	
Columns:	Dionex CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896) Dionex CarboPac PA200 Guard, 3 × 50 mm (P/N 062895)
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Column Temperature:	30 °C
Cell Temperature:	30 °C
Backpressure:	3000 psi
Detection:	PAD
Background:	30–50 nC
Working Electrode:	Gold on PTFE Disposable Electrode (P/N 066480)
Reference Electrode:	Mode: Ag/AgCl mode Noise: 10–30 pC

Carbohydrate Waveform

Carbohydrate 4-Potential Waveform for the ED

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required on Dionex ICS-3000/5000 systems but not used for older Dionex systems

Preparation of Solutions and Reagents

General Advice on Preparing Samples Containing Oligo/Polysaccharides for HPAE-PAD Analysis

Dissolve samples in water or 100 mM sodium hydroxide that has been reconstituted just prior to analysis. Filter cloudy samples using a 0.45 µm syringe filter. If samples are injected that are still cloudy even after filtration, they can damage the guard column and clog the injector, injection valve, injection loop, or tubing leading to the guard column. For additional advice on sample considerations for Dionex CarboPac columns, see the Dionex CarboPac column manual.[14]

Eluent Solution

100 mM sodium hydroxide

To make 0.1 M NaOH, add 8.0 g (5.2 mL) of 50% (w/w) NaOH to 1 L of degassed DI water by removing the NaOH aliquot from the middle of the stock solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where sodium carbonate precipitate may have fallen, and only prepare eluent from a bottle of 50% sodium hydroxide that still contains at least a third of its original volume. Place the tip of the pipette containing the aliquot of NaOH ~1 in. (2.54 cm) below the surface of the water and dispense the NaOH. If properly prepared without stirring, most of the concen-trated sodium hydroxide will stay in the lower half of the container and the rate of carbon dioxide adsorption will be much lower than that of a homogenous solution.

Seal the container after the sodium hydroxide transfer is complete. Immediately replace the cap on the 50% hydroxide bottle as well. Mix the contents of the tightly sealed container holding the 0.1 M hydroxide.

1 M sodium acetate/100 mM sodium hydroxide or 1 M sodium nitrate/100 mM sodium hydroxide

To make 1 L of 100 mM sodium hydroxide containing 1.0 M sodium acetate or nitrate, dispense approximately 800 mL of DI water into a 1 L volumetric flask. Vacuum degas for approximately 5 min. Add a stir bar and begin stirring. Weigh 82.0 g anhydrous, crystalline sodium acetate or 85.0 g of sodium nitrate. Add the solid acetate/nitrate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. Once the salt has dissolved, remove the stir bar with a magnetic retriever.

Add DI water to the flask to bring the volume to the 1 L mark. Vacuum filter the solution through a 0.2 µm nylon filter. This can take some time because the filter may clog with insoluble material from the sodium acetate/nitrate. Using a plastic tip volumetric pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide solution from the middle of the bottle. Dispense the sodium hydroxide solution into the acetate/nitrate solution ~1 in. (2.54 cm) under the surface of the acetate solution and then mix in the same manner as the 100 mM NaOH above. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than ~1 week.

Samples

- Thermo Scientific Dionex OligoStandards Sialylated *N*-Linked Alditols (P/N 043164)

The Dionex OligoStandards™ Sialylated *N*-Linked Alditols contains 25 nmol reduced oligosaccharides purified from bovine fetuin. Add 1 mL of DI water. Run this standard every time a new column is installed and subsequently anytime it becomes necessary to troubleshoot the system.[14]

- Colominic Acid

Weigh 10 mg of colominic acid and dissolve in 2 mL of DI water to prepare a 5 mg/mL solution. Freeze the stock solution at -20 °C until needed.

- *N*-Acetylneuraminic Acid, Trimer and Pentamer

Weigh 1 mg of trimer and dissolve in 1 mL of DI water. Freeze stock solution at -20 °C until needed.

Results and Discussion

Figure 2 shows the separation of polymers in a commercial sample of colominic acid on a Dionex CarboPac PA200 column using a gradient of 200 to 1000 mM sodium acetate in 100 mM sodium hydroxide. The DP for n = 3 and 5 was identified by using the retention time of the trimer and pentamer of *N*-acetylneuraminic acid. The Dionex CarboPac PA200 column is able to resolve colominic acid homologues with DP up to 100 (Figure 2, inset) in 70 min. This is an improvement over the maximum DP (up to 60 in 68 min) that can be discerned with a Dionex CarboPac PA100 column.[8]

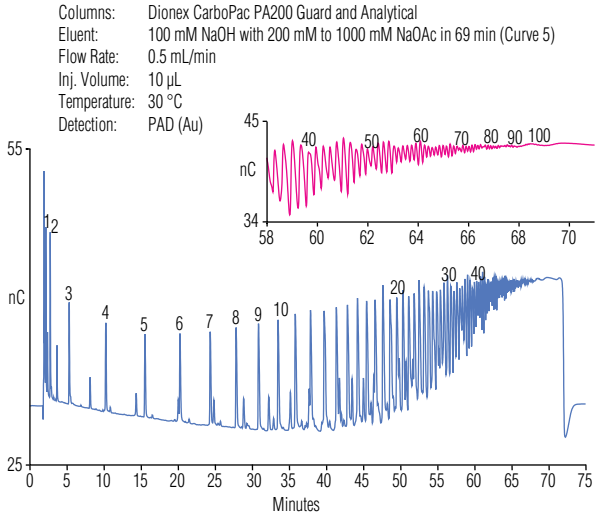

Figure 2. HPAE-PAD chromatographic profile of a commercial sample of colominic acid: 50 mg were injected on a Dionex CarboPac PA200 column and eluted with acetate as the pushing agent. Peaks are labeled with the putative n values, with the exception of the trimer (n = 3) and pentamer (n = 5), which were determined using standards. Inset shows the profile from 58–71 min.

It has been reported that using nitrate instead of acetate as the pushing agent resulted in better resolution of higher polymers.[9,10] With the current configuration, the best separation of Neu5Ac polymers (present in commercial colominic acid) obtained with sodium nitrate is shown in Figure 3. The maximum DP detected with the nitrate gradient is 140 in 90 min (Figure 3B). This is ~60 DP higher than that obtained using a Dionex CarboPac PA100 column. Note that a steeper gradient was used in the first phase of the separation, followed by a more moderate one in the second phase, to obtain a consistent peak resolution over a wide range.

A gradual drop in peak area from one injection to the next was observed when sodium nitrate was used as the pushing agent (data not shown). This loss in response was not observed when an acetate gradient was used. This is most likely due to metal contamination in the sodium nitrate (vendor specification for iron is ≤3 ppm on ACS-grade sodium nitrate), which could lead to electrode fouling.

Therefore, use a Thermo Scientific Dionex IonPac MFC-1 Metal-Free Column when sodium nitrate is used for this application. Install the MFC-1 column in the eluent line prior to the injection valve to remove trace transition metal contaminants from high-pH eluents. When this trap column was used in the eluent line, the peak area was higher than when the column was not used; and in over 35 consecutive injections, no peak area loss was observed.

With acetate as the pushing agent, changing the linear gradient (curve 5 in Figure 2) to a slightly convex gradient (curve 4) yielded the separation in Figure 4. Although no additional peaks were identified, the spacing between the early eluting peaks improved. In the case of nitrate as the pushing agent, the convex gradient was comparable to the linear gradient.

The DP information obtained from the high-resolution method will be useful in the study of biosynthesis, degradation, and structure-function correlations of polysialic acids; for the quality control of related molecules that have the bioengineering potential for increasing the stability of enzymes;[15] or for use in antiviral drugs.[16]

Conclusion

The proposed HPAE-PAD method for the analysis of a homologous series of polysialic acids achieves better resolution when compared to existing methods. The Dionex CarboPac PA200 column, specially developed to provide high-resolution separation of oligosaccharides, resolves homologues of polysialic acid in colominic acid with DP up to 100 in 70 min (with a sodium acetate gradient) and 140 in 90 min (with a sodium nitrate gradient). This is an improvement over the maximum DP (approximately 60 for acetate and 80 for nitrate) that can be achieved using other anion-exchange columns.

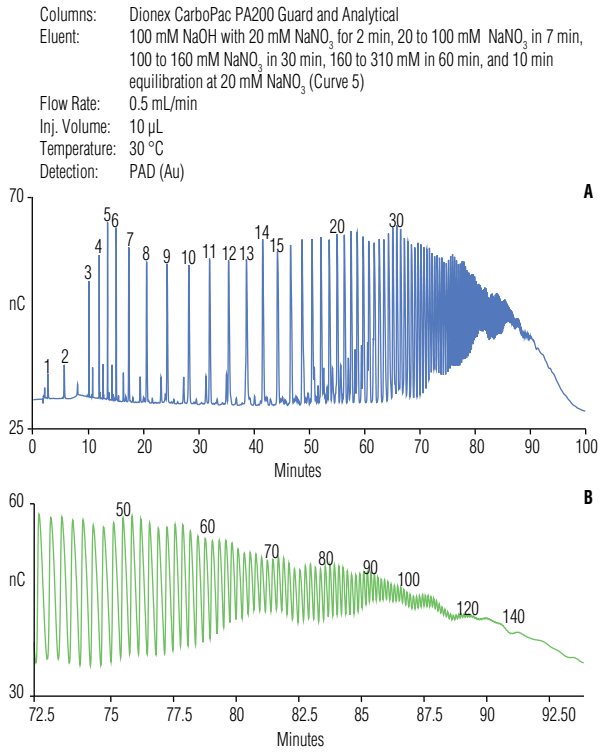

Figure 3. HPAE-PAD chromatographic profile (A) of a commercial sample of colominic acid: 50 mg were injected on a Dionex CarboPac PA200 column and eluted with nitrate as the pushing agent. Peaks are labeled with their putative n values, with the exception of the trimer (n = 3) and pentamer (n = 5), which were determined using standards. An enlarged view of the 72 to 92 min region (B) is also shown.

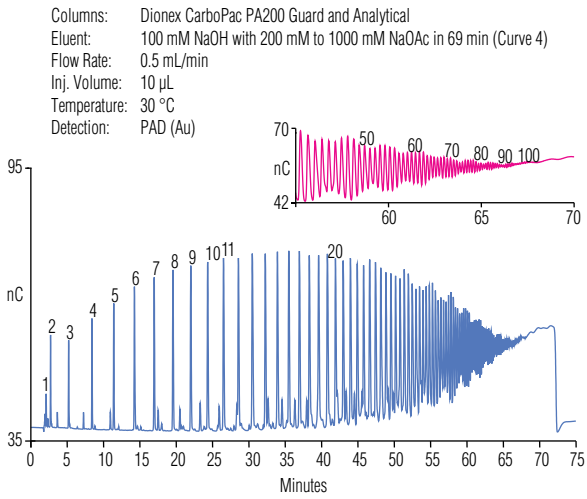

Figure 4. HPAE-PAD chromatographic profile of a commercial sample of colominic acid: 50 mg were injected on a Dionex CarboPac PA200 column and eluted with a convex acetate gradient (curve 4). Peaks are labeled with the putative n values, with the exception of the trimer (n = 3) and pentamer (n = 5), which were determined using standards. Inset shows the profile from 42–70 min.

Suppliers

Nacalia USA, 6640 Lusk Blvd., Suite A 200, San Diego, CA 94121, U.S.A., Tel: (858) 404-0403.

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A., Tel: 800-932-5000.

Sigma-Aldrich Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A., Tel: 800-558-9160.

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