Improved Profiling of Sialylated *N*-Linked Glycans by Ion Chromatography-Orbitrap Mass Spectrometry

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ABSTRACT

Results from experiments performed to obtain enhanced silalylated glycan profiling using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) coupled to a Q Exactive mass spectrometer are reported here. Ion chromatography supports simultaneous separation and detection of neutral and sialylated (charged) glycans without the need for derivatization. The chromatographic resolution of glycans is based on the number of sialic acid units, branch and positional isomers, and the presence/absence of core or outer arm fucose. We show that some of these structural features can lead to different observations than conventionally believed under different elution conditions. The results obtained here can be exploited to greatly enhance the power of HPAE-PAD to profile glycans.

INTRODUCTION

Good profiling of a recombinant glycoprotein's asparagine-linked (*N*-linked) glycans requires high resolution separation and reliable identification of released glycans. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established, powerful technique for glycan analysis. HPAE-PAD is especially effective for separating sialylated glycans, but there is opportunity to increase resolution and improve glycan identification. By evaluating changes to commonly used HPAE-PAD conditions we can achieve improved resolution[1].
Given the interest in glycoproteins as therapeutics there have been attempts[2,3] to use HPAE-PAD to create a glycan database. A technique suitable for building such a database needs to be convenient and cost effective. HPAE-PAD fits both these criteria as it enables derivatization-free separation with high sensitivity and resolution. Lacking the ability for fast determination of glycan structure, such attempts can still be cumbersome. Here, we have coupled a HPAE chromatography system to an Orbitrap mass spectrometer to harvest synergy between these two techniques to facilitate rapid structure determination.

Fragmentation of glycans in the negative mode by HCD provided information-rich MS^2 spectra dominated by glycosidic and cross-ring fragments that frequently revealed linkage information. The possible glycan structures were first identified by high throughput search and score function. The structures were confirmed by annotating the diagnostic fragmentation patterns observed in MS^2 spectra. Comparison of structures identified under different sets of conditions was performed to identify whether glycan resolution is improved for previously unresolved structures. Moreover, changes in elution conditions allowed correlation of glycan structure with observed elution behavior. Here we show that in some cases, changed elution conditions can be used to resolve different glycan structures. This approach may be an effective way to quickly screen the impact of changes in cell culture conditions on sialylation.

MATERIALS AND METHODS

Sample preparation

Glycans were released from glycoproteins enzymatically using PNGase F. After sample preparation samples were dried and dissolved in DI water prior to injection.

High Performance Anion Exchange Chromatography

The glycans were separated on a Thermo Scientific™ Dionex™ CarboPac™ PA200 BioLC™ Analytical Column (3 x 250 mm) attached to a Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ dual IC system. The system was equipped with a Thermo Scientific™ Dionex™ ERD 500 electrolytically regenerated desalter.

Mass spectrometry

A Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer was used in negative mode ESI. Full mass scan: *m/z* 400-2000, resolution: 60,000 (FWHM) at *m/z* 200, AGC: $1x10^6$, maximum IT: 200 ms. Data dependent MS^2 using Top 10. Data were analyzed using SimGlycan software (Premier Biosoft).

Coupling HPAE to MS

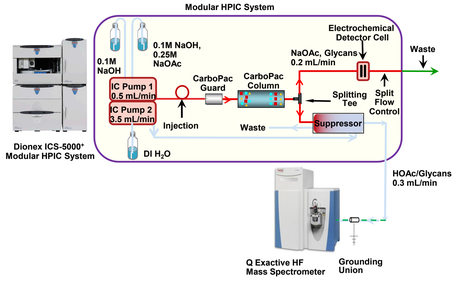
The Dionex ICS-5000+ HPIC system is configured for electrochemical detection was first connected to a Dionex AS-AS autosampler, and Q Exactive mass spectrometer modules using the HPAE-PAD/MS Assembly Kit. For detailed instruction on system configuration see Technical Note 72478[4].

RESULTS

Setting up and initial HPAE-MS experiment

Figure 1 shows schematic of this work flow. The column effluent containing separated glycans was passed through a salt exchanger to remove sodium ions contained in the effluent prior to mass spectrometry. A Q Exactive Orbitrap mass spectrometer used in negative electrospray mode was coupled to the ion chromatography system. Fragmentation of glycans in the negative mode by HCD provided information-rich MS^2 spectra dominated by glycosidic and cross-ring fragments that frequently revealed linkage information. The possible glycan structures were first identified by SimGlycan high throughput search and score function. The structures were confirmed by annotating the diagnostic fragmentation patterns observed in MS^2 spectra.

Figure 1. Schematic of a HPAE system coupled to a Q Exactive Orbitrap mass spectrometer

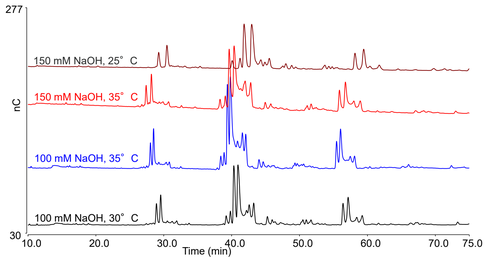


We evaluated changes to commonly used HPAE-PAD conditions to improve resolution using *N*-linked glycans released from four different glycoproteins by PNGase F. The glycoproteins used were bovine fetuin, bovine thyroglobulin, bovine fibrinogen, and human alpha 1 acid glycoprotein (AGP). We first tested HPAE-PAD separation of released *N*-linked glycans on a CarboPac PA200 column, with a typically used sodium hydroxide concentration and temperature, i.e. 100 mM with a gradient of sodium acetate at 30 ºC.

Overall resolution under high [NaOH] conditions

Potential gain in neutral glycan resolution observed with increased hydroxide concentration has been reported[4,5]. At the same time, other studies have indicated lower hydroxide concentration to be useful for improving neutral as well as sialylated glycan resolution[6]. Moreover, systematic analysis of glycan structure and its effect on observed resolution has not been performed. We have previously shown beneficial effect of higher hydroxide concentration and lower temperature on glycan resolution[4]. In continuation of that work, an effort to correlate glycan structure with elution conditions is attempted here.
Four glycoproteins that are highly sialylated were chosen for this study. This enabled availability of diverse glycans structures for the correlation. High glycan yield from these proteins also allows detection of minor glycan structures. Moreover, glycosylation patterns for these glycoproteins have also been studied using HPAE-MS[6,7]. This provides an opportunity to systematically study the effect of glycan structure on their elution behavior.
Using PNGase F released glycans for all four glycoproteins, effects of higher hydroxide concentration as well as temperature were studied on glycan separation. Sodium acetate concentration used in the eluent preparation remained unchanged for all conditions tested here. Figure 2 shows HPAE chromatograms for Fetuin glycans under different elution conditions. Higher NaOH concentration appears to improve overall glycan resolution both at 25 and 35˚ C. Moreover, lower temperature appears to be more favorable than the higher temperature.

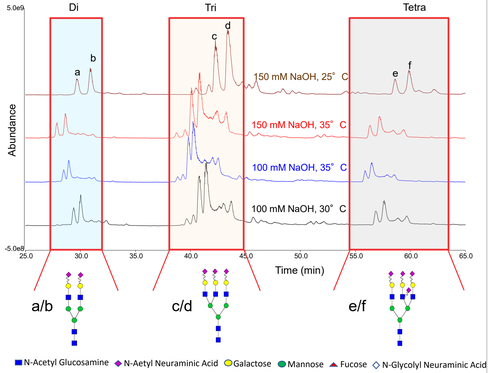
Figure 2. Separation of fetuin glycans under different elution conditions



Positional and structural isomers

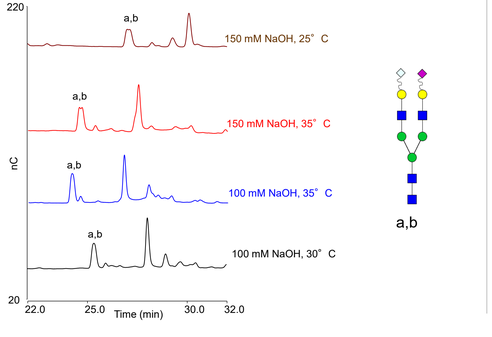
Next, we wanted to correlate the observed improved resolution with glycan structure. All major and detectable minor peaks were annotated for all four glycoproteins. Figure 3 shows glycan structures corresponding to major glycan peaks for fetuin. It appears that overall improvement in resolution achieved using higher NaOH concentration for elution is attributed to improved resolution of isomeric glycan peaks. Figure 4 shows another example for one such pair with improved resolution using higher NaOH concentration for separating fibrinogen glycans.

Figure 3. Resolution of major fetuin sialylated glycan structures under different elution conditions



[Note- Dashed lines linking sialic acids indicate presence of either α2→3 or α2→6 linkage]

Figure 4. Resolution of a fibrinogen biantennary disialylated glycan under different elution conditions



Fucosylated gylcans

It is known that fucosylated glycans elute earlier than their non-fucosylated counterparts[2] . Here, we discover that fucosylated glycans are better resolved from their non-fucosylated counterparts at 100 mM NaOH concentration as compared to 150 mM NaOH irrespective of the temperature. For example, Figures 5 and 6, show two examples of separation a fucosylated glycan from non-fucosylated analogue for fetuin and AGP respectively. Both these pairs are not resolved using 150 mM NaOH.

Figure 5. Separation of a fucosylated and non-fucosylated fetuin glycan tested at different elution conditions

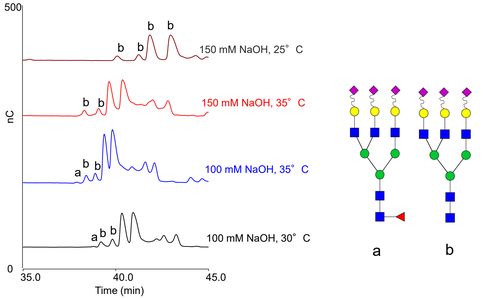
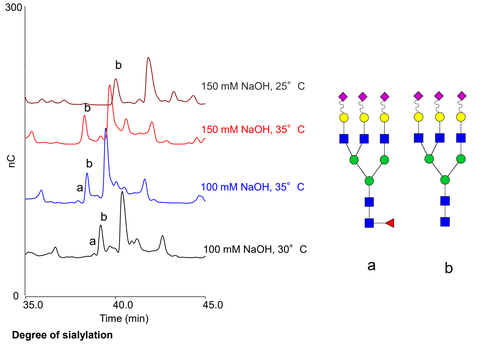


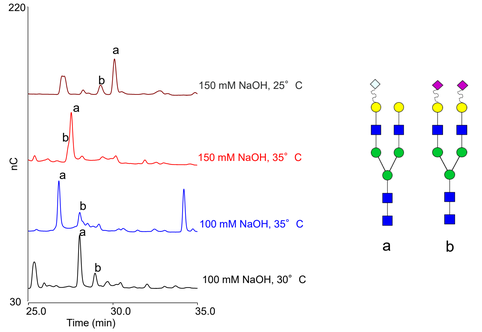
Figure 6. Separation of fucosylated from non-fucosylated AGP glycan tested at different elution conditions



Degree of sialylation

Generally, in a HPAE separation glycans of a homologous series elute in order, Although an exception to this rule is known[2]. Sialylated glycans elute in the increasing order of sialylation due to increased binding due to increased charge. So, for sialylated glycans the elution order would be, asialo < monosialylated < disialylated < trisialylated < tetrasialylated glycan indicating increased retention with charge. Here we report that at least in some cases the elution order changes using higher sodium hydroxide, for example, as shown in Figure 7, in case of Fibrinogen glycans a biantennary disialylated glycan changes elution order under high hydroxide condition. Using 100 mM NaOH the disialylated glycan elutes after the monosialylated glycan.

Figure 7. Change in elution order of a fibrinogen biantennary sialylated glycan pair under different elution conditions



CONCLUSIONS

- Hydroxide concentration and column temperature can be exploited for increasing glycan coverage.
- Higher hydroxide concentration leads to improved separation of glycan isomers.
- Lower hydroxide concentration helps resolve certain structural features such as fucosylation

REFERENCES

1. Thermo Scientific Application Update 72829 (AU72829). HPAE-PAD analysis of *N*-linked glycans: improving glycan resolution. [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/au-72829-hpae-pad-n-linked-glycans-au72829-en.pdf
2. Hermentin, P., Witzel, R., Vliegenthart, J., Kamerling, J., Nimtz, M., Conradt, H. A strategy for the mapping of N-glycans by high-pH anion-exchange chromatography with pulsed amperometric detection, *Analytical Biochemistry*, (203) 2, 1992, Pages 281-289.
3. Grey, C., Edebrink, P, Krook M., Jacobsson, S. Development of a high performance anion exchange chromatography analysis for mapping of oligosaccharides. *Journal of Chromatography B*, (877), 2009, pages 1827-1832
4. Thermo Fisher Scientific Technical Note 72478. Instrument configuration for native *N*-linked oligosaccharide characterization by HPAE-PAD/MS [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/TN-72478-ICMS-HPAE-PAD-MS-Characterization-TN72478-EN.pdf
5. Cooper, G., and Rohrer, J. Separation of neutral asparagine-linked oligosaccharides by high-pH anion exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry*, (226), 1995, pages 182-184
6. Thermo Fisher Scientific Technical Note 72478. Instrument configuration for native *N*-linked oligosaccharide characterization by HPAE-PAD/MS [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/TN-72478-ICMS-HPAE-PAD-MS-Characterization-TN72478-EN.pdf
7. Thermo Scientific Application Note 595 (AN595). Integrated LC/MS Workflow for the Analysis of Labeled and Native N-Glycans from Proteins Using a Novel Mixed-Mode Column and a Q Exactive Mass Spectrometer. [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-595-LC-MS-Labeled-Native-N-Glycans-Proteins-AN63938-EN.pdf

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