Protein and Glycoprotein LC/MS Using HILIC

Barry Boyes[1, 2, 3]; Ron Orlando[2, 3]; Stephanie Schuster[1]; Joseph DeStefano[1]

[1]Advanced Materials Technology Inc., Wilmington, DE; [2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA; [3]GlycoScientific, Inc., Athens, GA

Outline

- HILIC separations have utility in glycoscience applications
- Polar interactions for glycans, glycopeptides and glycoproteins lead to useful separations
- Materials and methods of use for HILIC separations are improving, but HILIC retention and band-broadening mechanisms remain poorly understood
- This poster describes examples of HILIC for protein separations based on glycoform structure, with conditions that allow MS detection methods

Introduction

In recent years Hydrophilic-Interaction Liquid Chromatography (HILIC) has become a method of choice for separation and analysis of PNGase F released protein N-linked glycans. In addition, there is a growing interest in the use of HILIC LC/MS methods for analysis of glycopeptide mixtures, taking advantage of the ability of the separation method to resolve glycopeptides that vary in the structure of the attached glycans. The high separation selectivity of HILIC polar interactions allows resolution of subtle differences in glycan structure, even at the level of variants in glycan branch structures, and even in the context of intact glycopeptide amino acid sequence.

Thus, HILIC methods have proven useful for analysis of protein glycosylation, allowing resolution of released glycans, as well as glycans attached to protein tryptic fragments. Our interest has been to extend this approach to resolution of glycosylation variants at the level of intact protein structures.

The use of HILIC methods for protein separations has been limited in practice, compared to reversed-phase (RP) methods. Various useful separations of proteins have been described by Alpert and colleagues, by Carrol et al., (P.N.A.S. U.S.A. 103 (2006), 16170), and more recently by Tetaz and co-workers (J. Chromatogr. 1218 (2011) 5892). Much of these previous efforts have been focused on membrane proteins and the like, but there have also been indications of utility for PTM analysis.

In this poster, we present some details on conditions for the use of HILIC for separations of aqueous soluble proteins. Based on these observations, consensus conditions for using HILIC for protein separations are described, and these are applied to the LC/MS analysis of a simple glycoprotein mixture, to explore the utility of the approach.

Materials and Methods

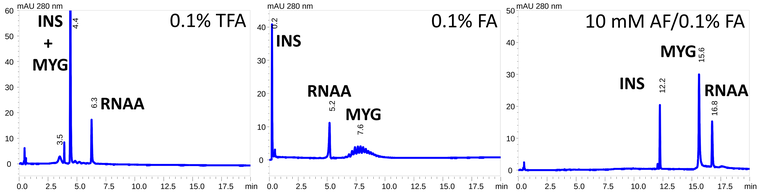
Columns of HALO PentaHILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous FusedCore ™ silica particles of with 2.7 µm diameter, a solid core of 1.7 µm diameter, and a shell thickness of 0.5 µm. Tested materials have included 400, 160 and 90 Å pore diameters based on nitrogen adsorption. Proteins were used as supplied by Sigma ((St. Louis, MO). Analytical protein separations used the Shimadzu Nexera LC-30 components (40 µL mixer), with the SPD20 Absorbance detector, fitted with a high sensitivity semi-micro flow cell, or the RF20-Axs fluorescence detector with semi-micro flow cell. The MS-2020 quadrupole MS was operated in series using +4.5 kV capillary potential, scanning between 500-2000 m/z at 0.5 sec/scan, with identified selected in monitoring (SIM, 0.5 sec/event). Preparative separations employed the Agilent 1200 LC, with automated fraction collection. Infusions of proteins in the Orbitrap (Orbitrap Velos Pro, ThermoScientific, Inc.) were conducted at 3 µL/min., with the IonMax ESI interface at 3.8 kV potential. Deconvolution of MS spectra used MagTran v1.02, based on ZScore (Zhang and Marshall (JASMS 9 (1998) 225).

Operational Conditions for Separations of Proteins

- Simple proteins mixture separations (b. Insulin (INS); e. apo-myoglobin (MYG); b. RNase A (RNAA)) were applied to compare mobile phase modifiers, indicating that a mix of Ammonium Formate/Formic Acid (AF/FA) is attractive, compared to Formic Acid (FA) alone, showing narrow and symmetrical peaks, improved load tolerance (linear isotherm), and good compatibility with online ESI-MS detection (McCalley, J. Chromatogr. A, 1038 (2004), 77; Schuster, Boyes, Wagner, Kirkland, J. Chromatogr. A, 1228 (2012), 232; Johnson, Boyes, Orlando, J.Biomol.Tech. 24 (2013), 187)
- FA mobile phases can exhibit complex elution profiles, which we attribute to the presence of folding intermediates, and/or electrostatic interactions with the stationary phase surface, or self-associations (eg., MYG profile below).
- TFA mobile phases exhibited excellent peak shape, reduced retention, but were not pursed further due to MS detection needs.
- AF/FA mobile phases were examined at a variety of concentrations, and with the use of various mixtures of organic solvents, including acetonitrile (AcN), *i*-propanol (IPA), *n*-propanol, trifluoroethanol (TFE), and hexafluoro-*i*-propanol (HFIP). The consensus conditions shown below were selected for mobile phase modifiers, based on chromatographic and MS detection considerations.
- Pore size effects have been compared, with data shown for a simple protein mixture. Retention was progressively reduced as the pore size increased (specific surface area decreased). Modest improvements in peak widths were observed for intermediate pore size materials, but at the expense of retention.
- Moderately increased column temperature (40-60 C) improves peak shape and recovery, but high temperature (80-100 C) exhibited poor recoveries of certain proteins.
- Adjusting sample solvent is crucial – addition of FA to 1-2% resolves many solubility problems for proteins, which otherwise will precipitate from the high organic solvent mixtures used during injection.

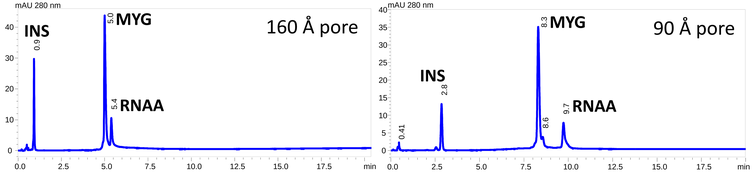
Effect of Acid Modifier

2.1 x 100 mm Halo Penta-HILIC 90 Å pore size, 0.5 mL/min at 50°C; Gradient - 90% to 50% AcN/20 min (-2%AcN/min).
Gradient conditions: A – 0.1% acid; B – 90% Acetonitrile in A.



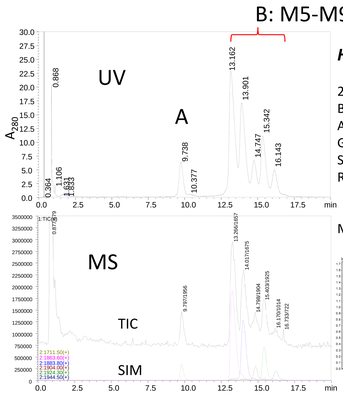
Consensus Mobile Phase & Pore Size

2.1 x 100 mm Halo Penta-HILIC, 0.5 mL/min at 50°C; Gradient - 90% to 55% B in 20 min.
B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA



Resolution of RNase B Glycoforms

- Bovine RNase is a mixture of glycosylated variants, with RNase A lacking glycosylation, and RNase B exhibiting N-linked glycosylation with a series of the high mannose structure glycans, with 5-9 mannose units (Man5 – Man9), attached at a single glycosylation site – Asn 34 (for a detailed discussion, see Prien, et al., . J.A.S.M.S. 20 (2009) 539).
- High resolution RP HPLC allows modest separation of RNase A and B, but no resolution of the RNase B glycoforms.
- High performance HILIC separations using the consensus mobile phase conditions with EITHER 90 or 160 Å pore size column packing materials permits resolution of RNase A and B, and RNase B glycoprotein variants.
- Retention is progressive with Man5 through Man9 structures retained longer, as determined by online MS .
- The glycoprotein variants are labeled as M5 – M9 proteins, based on the attached glycan variants.
- Deconvolution of the MS1 data correctly identified masses of the glycosylated protein variants.
- High resolution MS analysis of protein variants confirms the on-line SQ analysis, and identified probable oxidation (+16.0 amu) of the proteins under the handling conditions for these experiments (seen for all 5 RNase B glycovariants).



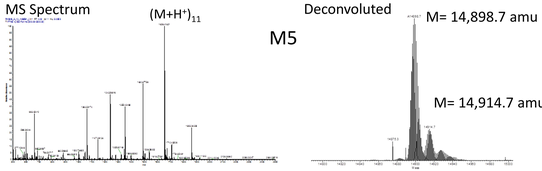
HILIC Separation of RNases

2.1 x 100 mm Halo Penta-HILIC, 0.35 mL/min at 50°C;
B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP;
A – 0.2% FA/20 mM AF/10% IPA
Gradient – 85% to 75% B in 20 min.
Sample: synthetic mixture of 10% RNase A and 90% RNase B.

MS: ESI(+) 4.8 kV; 500-2000 m/z scan (0.5 sec)
SIM as identified on chromatogram (0.5 sec)

ID	Mass (Da) Theory	Mass (Da) Observed
RNA A	13,681	13,686
RNA B M5	14,898	14,903
RNA B M6	15,060	15,068
RNA B M7	15,222	15,227
RNA B M8	15,384	15,390
RNA B M9	15,564	15,550

ESI/MS (Orbitrap) - M5 Glycoprotein Infused in 0.5% FA/50% AcN

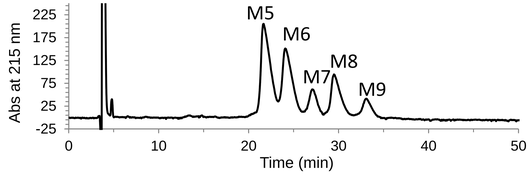


Characteristics of RNase B Glycoforms

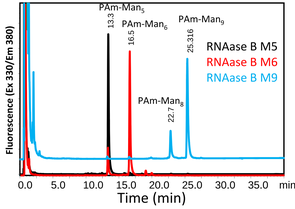
- RNase B separation was scaled up (4.6 mm ID column) to allow recovery of variant proteins.
- N-linked glycans were released with PNGase F, then subjected to reductive amination end –labeling using procainamide, with HILIC separation of the glycans. M5 was thus shown to be highly pure, and M6, slightly contaminated by M5, whereas M9 is contaminated by c. 20% M8 protein.
- The isolated RNase B glycovariants are compared for enzymatic activity using the hyperchromic shift spectrophotometric assay. The general pattern is reduced activity as glycan size is increased.

Preparation of Isolated Glycovariants

4.6 x 250 mm Penta HILIC 2.7 µm 90Å, 0.4 mL/min, at 60C; 1 mg Inject; 78-75 % B over 60 min,
B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA

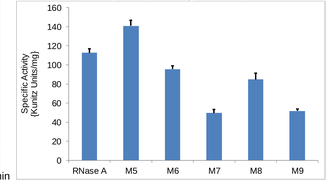


PNGase F released glycans from M5, M6 and M9 Proteins



Enzymatic Activity of Glycovariants

Assay Conditions: 50 mM NaOAc,
pH 5.0, 25°C, 0.5 mg/mL RNA, ΔA300



Conclusions

- Results with intact protein and glycoprotein LC/MS separations of smaller proteins performed well, but expanding to a broader range of proteins requires further effort on mobile phase and use conditions (sample solvent dependencies for solubilities).
- Selectivity for glycan separations, in glycoprotein structures, appears excellent.
- RNase B glycoforms can be well resolved, under conditions that retain activity, and that allow mass spectral analysis.

Acknowledgements: Dr. Jack Kirkland, William Miles and Bob Moran for advice and technical assistance. Supported in part by NIH Grant GM093747 (BEB).