Glycopeptide Analysis Using Electron Transfer Dissociation and Porous Graphite Chromatography

Rosa Viner, Terry Zhang, Vlad Zabrouskov

Thermo Fisher Scientific, San Jose, CA

Abstract

Purpose: N-glycopeptide analysis by nano-LC/MS^n using Electron Transfer Dissociation (ETD).

Methods: Glycoprotein digests were analyzed by nano-LC ESI using different types of HPLC columns and a Thermo Scientific LTQ XL™ mass spectrometer equipped with an ETD source option.

Results: A graphitic carbon column (Hypercarb™, Thermo Scientific) demonstrated excellent capabilities for glycopeptide analysis especially in the case of short hydrophilic peptides containing bi- or tri-antennary glycan chains without any enrichment. Formation of metal adducts on the Hypercarb column promotes higher charged species and, as a result, promotes ETD fragmentation of glycopeptides. The combination of porous graphite chromatography and ETD-MS/MS is demonstrated to be a useful and flexible tool for studying glycosylation and identifying PTM sites.

Introduction

Glycosylation plays a key role in controlling numerous biological processes. It is one of the most widespread and complex post-translational modifications (PTMs) found on proteins and its characterization remains a great analytical challenge. LC MS/MS is the most powerful and versatile technique for glycopeptide structure elucidation. However, commonly used collisional-induced dissociation (CID) has limitations for determining the modification site due to the labile nature of glycan modifications. As a new dissociation technique, Electron Transfer Dissociation (ETD) preserves labile PTMs and provides a new and powerful tool that makes the identification of modification sites possible. Since glycosylated proteins and their resulting peptides are most often highly heterogeneous, high quality liquid chromatography is critical for glycopeptide analysis.

In this study, two reasonably well-characterized glycoproteins, bovine α1–acid glycoprotein and human α1–acid glycoprotein were analyzed using nano-LC MS/MS with ETD. Liquid chromatography separation conditions were systematically optimized for glycopeptide analysis with mass spectrometry using different stationary phase columns. Two reversed phase columns, C8 and C18, and a graphitic carbon column were evaluated.

Materials & Methods

Samples:
Reduced and alkylated enzymatic digests of bovine and human α1–acid glycoproteins. Glycoproteins were purchased from Sigma.

LC/MS:

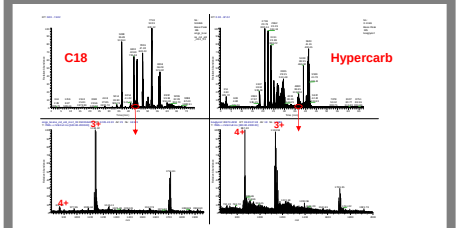
HPLC System:	Surveyor™ MS Pump with a flow splitter
Columns:	Agilent® ZORBAX® 300SB C8 column (75 µm x 5 cm) Microtech C18 column (150 µm x 10cm) Thermo Scientific Hypercarb column (75 µm x 5cm)
Mobile Phase:	A: Water, 0.1% formic acid; B: Acetonitrile, 0.1% formic acid
Gradient:	5-50% B in 30 minutes
Mass Spectrometer:	Thermo Scientific LTQ XL linear ion trap mass spectrometer equipped with ETD and nano-ESI source
Spray Voltage:	2 kV
Capillary Temp:	160 °C
Capillary Voltage:	35 V
Tube Lens:	125 V
MSn Target:	1e4
Mass range :	50-2000 *m/z* or 100-4000 *m/z*
Anion Reagent:	Fluoranthene
Anion Reagent Isolation:	On
Anion Target:	2e5
Max Anion Injection Time:	50 ms
ETD Reaction Time:	100 ms
Mass Spectrometer	Thermo Scientific LTQ Orbitrap XL™
Mass range	400-2000 m/z, resolution 100000
Full MS Target	5e5
Survey Scan	Source CID at 65 V for *m/z* 204

Data Processing:
Data were processed using BioWorks™ 3.3.1 with SEQUEST®. The Xtract program (Thermo Scientific) was used for deconvolution of multiply charged precursors. The GlycoMod tool from the Swiss-Prot website was used to assign possible oligosaccharide structures and compositions.

Results

Figure 1 shows the results of the LC/MS analysis of bovine α1–acid glycoprotein on C_{18} and Hypercarb columns. The top panels are the base peak chromatograms and the bottom panels are the bi-antennary glycopeptide 91-99 MS profiles. One pmol of protein digest was injected into the C_{18} column versus 500 fmol on the Hypercarb column resulting in a C_{18} base peak intensity three times higher than the one from the Hypercarb column. However, as shown in the bottom panel of Figure 1, chromatography on the Hypercarb column promotes additional higher charge state precursor ions than C_{18} which is instrumental in obtaining high quality ETD MS/MS. Overall intensity of glycopeptide precursor ions with the Hypercarb column is the same as, or higher than with the C_{18} column.

FIGURE 1. Comparison of Base Peak Chromatograms of Bovine α1–acid Glycoprotein using C_{18} and Hypercarb Columns



In addition, as shown in Figure 2, chromatography on the Hypercarb column promotes metal adduct formation. Figure 2 shows a high resolution deconvoluted spectrum of 91-99 bovine glycopeptides acquired on an LTQ Orbitrap XL. At least one metal adduct was observed for each glycoform. As demonstrated in Figure 1, formation of metal adducts is likely responsible for producing high charge state ions: the precursor of the 4+ charge state (867.3) was the dominant peak for glycopeptide 5 while the 3+ charge state (1138.6) was the dominant peak for glycopeptide 2 . These results can be explained by partial neutralization of the negative charge of the sialic acid by metal cations [3] and because the metal stabilizes the labile glycans and promotes formation of a higher charge state precursor [4] .

FIGURE 2. Deconvoluted Full MS Spectrum of Bovine α1–acid Bi-antennary Glycopeptide ^{91}CVYNCSFIK^{99}

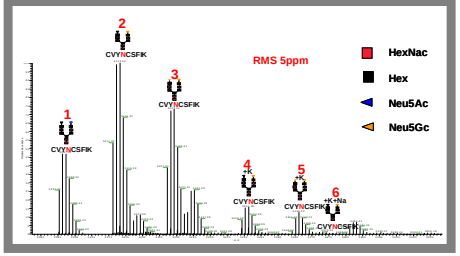


FIGURE 3. ETD spectrum of CVYNCSFIK (charge 4+, potassium adduct ion, *m/z* 863)

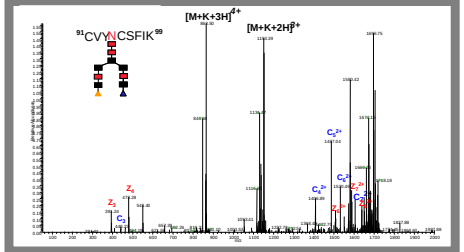
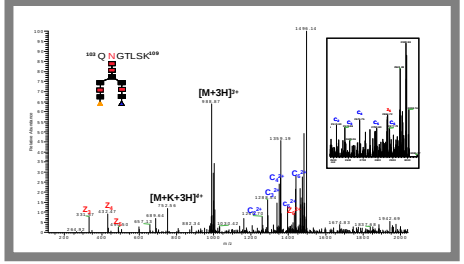


FIGURE 4. ETD spectrum of QNGTLSK (charge 4+, potassium adduct ion, *m/z* 752)

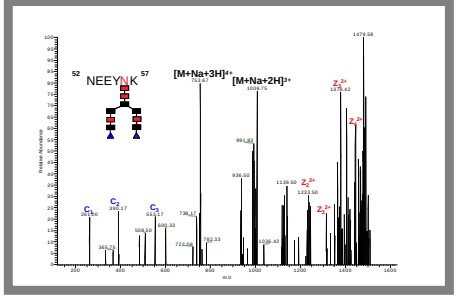


Figures 3 and 4 show ETD spectra of a potassium adduct of bovine α. Acid glycoprotein peptides 91-99 (4+ charge) and peptides 103-109 (4+ charge) are denoted as 4 on Figure 2. Glycosylation sites and peptide sequences were identified based on an almost complete series of c/z ions. No significant loss of carbohydrate was detected and, as expected, all observed glycan-containing fragments retained the metal ion. The overall difference in the performance of reversed phase chromatography and porous graphitic carbon columns can be explained by higher trapping efficiency of the graphite column for hydrophilic peptides. Bovine α1–acid glycoprotein contains five N-glycosylation sites with complex-type glycan structures [1].

TABLE 1. Bovine α1–acid glycopeptides detected by nano-LC/MS/MS

Peptides/ Type of LC column	Graphite	C_{18}	C_8
^{103}QNGTLSK^{109}	+		
^{53}NPEYNK^{58}	+		
^{91}CVYNCSFIK^{99}	+	+	+
^{128}TFMLAASWNGTK^{139}	+	+	+
^{19}QSPECANLMTVAPITNATMD LLSGK^{43}			+

FIGURE 5. ETD spectrum of NEEYNK (charge 4+, sodium-adduct ion, *m/z* 755)



Four out of five glycopeptides were detected and identified using a Hypercarb column compared to two on C_{18} and three on C_8 columns (Table 1) without any enrichment. Only the largest and most hydrophobic peptide was not detected

Similar results were obtained for human α1–acid glycoprotein which also contains five N-glycosylation sites [5]. All its glycopeptides can be detected after C_{18} [5] or C_8 chromatography but mostly as charge 2+ or 3+ species and as a result, MS^2 ETD spectra did not generate enough information for unambiguous identification. On the other hand, multiple alkali metal ion adducts of human α1–acid glycopeptides were observed after separation on the Hypercarb column at the higher charge states. Figure 5 shows an example of an ETD spectrum of a 4+ charge bi-antennary peptide 52-57 (sodium adduct), which was successfully identified by BioWorks 3.3.1. This peptide contains two asparagine residues but the site of glycosylation was still easily identified as Asn 56.

Conclusions

- **A graphitic carbon column (Hypercarb, Thermo Scientific) demonstrated excellent capabilities for glycopeptide analysis especially for short hydrophilic peptides containing bi- or tri-antennary glycan chains without any enrichment.**
- **Formation of metal adducts on Hypercarb columns promote higher charged species and as a result, promotes ETD fragmentation of glycopeptides.**
- **Optimization is required for successful glycoprotein nano-LC-ETD MS analysis.**
- **As a gentle fragmentation technique, ETD preserves labile glycans, facilitating the identification of both the peptide of interest and its site of modification.**

References

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