Improving ETD analysis of N, O-glycopeptides by using the isobaric labeling approach with tandem mass tags (TMT).

Terry Zhang; Rosa Viner; Vlad Zabrouskov

Thermo Fisher Scientific, San Jose, CA, USA

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Overview

Purpose: To improve ETD analysis of N,O-glycopeptides by using Tandem Mass Tag (TMT) isobaric labeling approach.

Methods: 10 standard protein mix digest, labeled with TMT6 was analyzed using an LTQ Orbitrap XL ETD mass spectrometer.

Results: Labeling with basic TMT groups increased the average charge state of the precursors from 3.4 to 4. It also improved ETD fragmentation of glycopeptides even for the same charge state precursors. In addition, as reported previously, ETD of TMT6 labeled peptides generates four unique reporter ions: 114 (from tags 126 and 127); 116 (from tags128 and 129);118 (from tag 130) and 119 (from tag 131) which allowed for simultaneous relative peptide quantification.

Introduction

Electron Transfer Dissociation (ETD) preserves labile post-translational modifications (PTMs)[1,2], and allows direct mapping of peptide/protein modifications which made this type of mass spectrometry fragmentation the method of choice for sequencing of N-, O-glycopeptides. However to make ETD successful high charge state precursor ions (3 and above) are preferred. In the case of N-glycopeptides, the presence of multiply negative charged sialic acid residues presents additional challenge as it significantly reduces the average precursor charge state. In this study we 1) investigated the effect of the addition of the basic TMT groups to increase average precursor charge state and as a result to improve ETD fragmentation and 2) attempted relative quantification of TMT labeled glycopeptides by ETD.

Methods

Sample

Digest of reduced and alkylated 10 standard protein mixture containing 3 glycoproteins (human serotransferrin, chicken ovalbumin and bovine alpha-lactoalbumin) from Sigma was divided into 6 aliquots and each of these was labeled according to manufacturer provided protocol with Thermo Scientific TMT6 (126, 127, 128, 129,130 and 131) labels (Figure 1). Samples were subsequently mixed in 1:1 ratio, and diluted five times with 5% formic acid before LC-MS/MS analysis [3].

LC Separation

HPLC System:	Thermo Scientific Surveyor MS Pump with a flow splitter
Column:	PicoFrit TM column (10 cm x 75 µm id), New Objective, Inc.
Mobile Phases:	0.1% formic acid in water; 0.1% formic acid in acetonitrile
Gradient:	10% B 10 minutes, 10% - 40% B in 90 minutes
Flow:	300 nL/min on column

LTQ Orbitrap ETD Settings

MS Resolution:	60000 RP at *m/z* 400
HCD MS2 Resolution:	7500 RP at *m/z* 400
OT MS target:	5e5
OT MSn target:	1e5
Alternating Top 3 Data Dependent IT CID/HCD or IT CID/ETD MS/MS events	
MS/MS scans (35% collision energy CID, 45% collision energy for HCD)	
ETD Reaction Time:	70-150 ms
Exclusion mass tolerance:	10 ppm
IT OT MS/MS:	300 ms
Full MS mass range:	380–1700
MS/MS Mass range:	100–2000

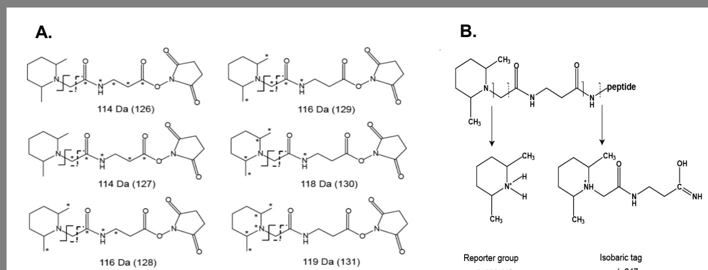
Data Analysis

The GlycoMod tool from the Swiss-Prot website was used to assign possible oligosaccharide structures and compositions.

Results

Before TMT labeling, we were able to detect 27 different precursors of glycopeptides with average charge state of 3.4 in the 10 protein mix digest . Labeling with basic TMT groups increased the average charge state of the precursors to 4 and higher charge state precursors became dominant as demonstrated in Figure 2 for human serotransferrin bi-antennary N-glycopeptide $_{622}$QQQHLFGSNVTDCSGNFCLFR$_{651}$. TMT labeled glycopeptide generated 4+ to 3+ precursor ratio 2.2 vs. 0.9 for the neat sample which would benefit to generate better quality ETD spectrum as was described previously[3]. Moreover, it also improved ETD fragmentation of glycopeptides even for the same charge state precursors as shown in Figures 3 and 4.

FIGURE 1. A: Structures of the TMT6 reagents. Bonds cleaved by ETD are indicated by solid lines and those cleaved by CID are indicated by dashed lines. B: Structures of reporter ion and isobaric tag after cleavage



ETD analysis of TMT labeled human serotransferrin bi-antennary N-glycopeptide – $_{421}$CGLVPVLAENYNK$_{433}$ precursor at charge 4+ generated a complete set of *c* ions and all important *z* ions and thus enables unambiguous mapping of N-glycosylation site as Asn432 and not as Asn430 (Figure 4,B). However, for the precursor at charge 4+ of the same but unlabeled peptide we detected fewer c/z ions as shown in Figure 3,B and most important ions *c*12 and *z*2 are missing. Interesting that TMT labeling is also improved quality of CID spectra of glycopeptides as shown in Figures 3 and 4 panels A.

It has often been reported that mapping of specific O-GlcNac glycosylation site is inherently difficult due to its labile nature[4]. Monitoring the signature ion of GlcNac- 204.09 by CAD could confirm the presence of O-linked glycopeptides[5], but not identify the exact modification site. To demonstrate this point we examined HCD and ETD spectra of chicken ovomucoid (contaminant protein in our mixture, co purified with ovalbumin) N-GlcNAc glycopeptide $_{188}$CNFCNAVVESNGTLTLSHFGK $_{210}$. As shown in Figure 5 once again TMT labeled peptide demonstrated better fragmentation (B,C) than neat peptide (A). Moreover, only ETD spectrum (B) enables GlcNac site identification as Asn198 and not as Ser197 as compared with very rich HCD spectrum or another 6 possible sites in this peptide. Existence of single N-GlcNac peptides was first demonstrated by D. Hunt [6] and again was confirmed by our study. In addition, as reported previously[3], ETD of TMT6TM labeled peptides generates four unique reporter ions: 114; 116; 118 and 119 (Figure 1) which allowed for simultaneous relative peptide quantification as shown in Figure 5.

FIGURE 2. Comparison of different charge state extracted ion chromatograms of human serotransferrin glycopeptide $_{622}$QQQHLFGSNVTDCSGNFCLFR$_{651}$ with/without TMT. Higher ratio of 4+/3+ precursor was obtained from the TMT labeled sample.

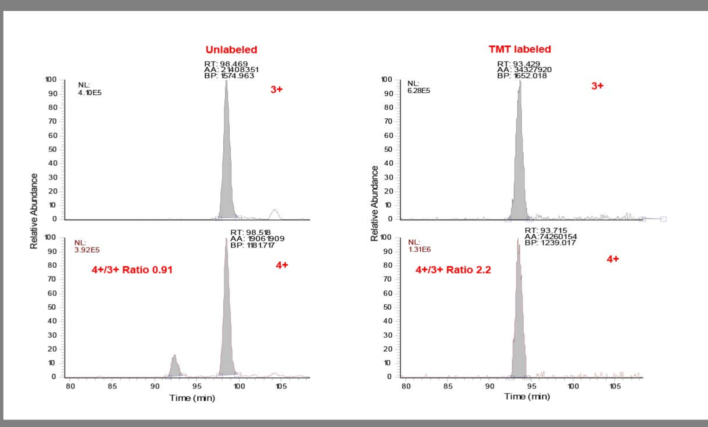


FIGURE 3. IT MS/MS CID (A) and ETD (B) spectra of human serotransferrin bi-antennary glycopeptide $_{421}$CGLVPVLAAENYNK$_{433}$ (*m/z* 920.89 , 4+)

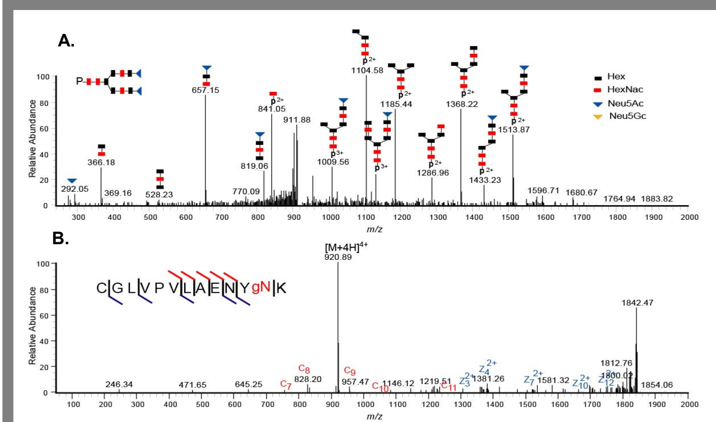
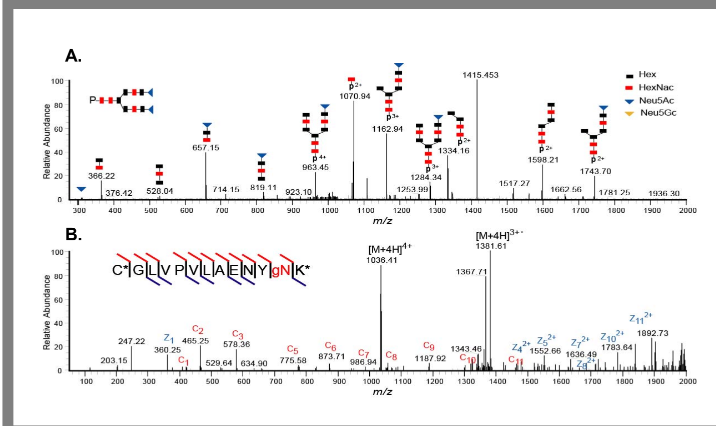


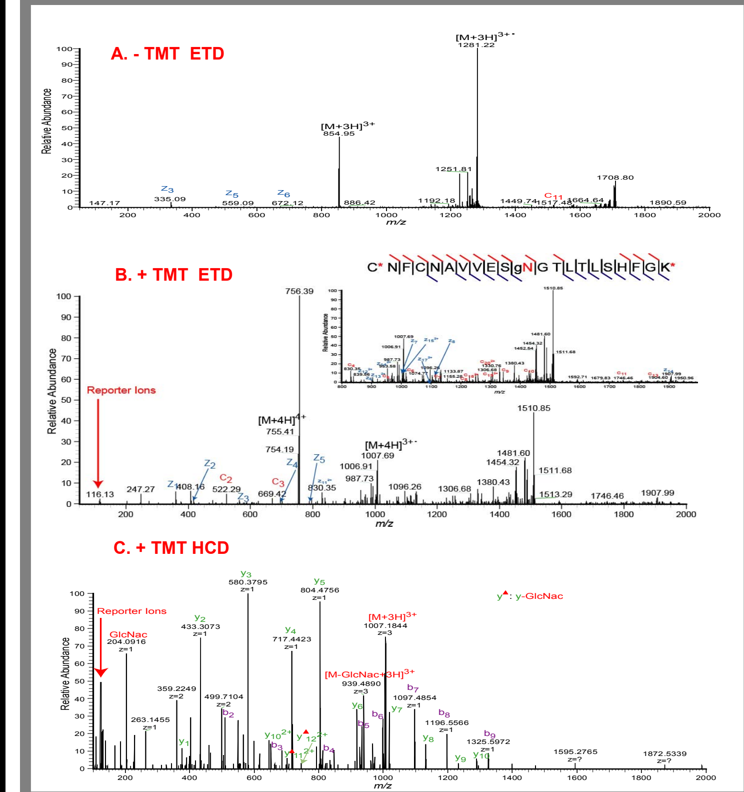
FIGURE 4. IT MS/MS CID (A) and ETD (B) spectra of human serotransferrin bi-antennary TMT labeled glycopeptide $_{421}$C*GLVPVLAAENYNK*$_{433}$ (*m/z* 1036.412, 4+)



Conclusions

- The addition of the basic TMT groups increases the average charge state of the precursors and as a result improves ETD fragmentation of acidic glycopeptides and simultaneously enables its relative quantification.
- ETD preserves labile glycans and it is the only dissociation method which can facilitating the identification of both the peptide of interest and the site of modification.
- Existence of single N-GlcNAc modification was confirmed for chicken ovomucoid glycopeptide $_{188}$CNFCNAVVESNGTLTLSHFGK $_{210}$.

FIGURE 5. Chicken ovomucoid N-GlcNAc glycopeptide $_{188}$*CNFCNAVVESNGTLTLSHFGK*$_{210}$ mass spec characterization: A. MS/MS IT ETD spectrum of unlabeled peptide at *m/z* 854.95 (3+). B. MS/MS IT ETD spectrum of TMT labeled peptide at *m/z* 755.41 (4+). C. Orbitrap HCD spectrum of TMT labeled peptide at *m/z* 1007.184 (3+).



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