Glycopeptide Enrichment of Human Serum Using Cellulose-Based Column

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Overview
Purpose: Development of a workflow for comprehensive site-specific glycopeptide analysis in human serum.

Methods: Protein samples from human serum were enriched at the glycoprotein and glycopeptide level using a combination of Concanavalin A (ConA) and a cellulose-based column. Previously, glycopeptides were Tandem Mass Tag (TMT)-labeled and analyzed by a novel strategy on the LTQ Orbitrap Velos ETD mass spectrometer that triggers high-energy collisional dissociation (HCD)-accurate mass product ion dependent electron transfer dissociation (ETD) scans.

Results: Human serum protein samples were enriched at the glycoprotein level using ConA. Upon enrichment, glycoproteins were enzymatically digested and labeled with TMT-126™. Samples were loaded onto a cellulose column and different fractions were removed by step-gradient elution with incremental water content. The described enrichment strategy significantly improved the rate of success of ETD analysis and simplified the overall glycopeptide analysis workflow.

Introduction
Protein glycosylation is an area of active research in the realm of biological sciences. It is widely accepted that glycan moieties are important modifications that play significant roles in biochemical processes ranging from fine-tuning of protein folding to receptor site recognition in various signal transduction pathways. However, structural characterization of glycopeptides remains a challenging task.

Previously, we described a comprehensive workflow that combines cellulose-based column chromatography/tandem mass spectrometry, as a novel method for efficient extraction of glycopeptides ranging from a single glycoprotein digest to a digest of a 10-protein mixture with negligible loss of glycopeptide material or carry-over of non-glycosylated peptides into the glycopeptide fraction and subsequent ETD analysis.1 Here we expand this approach for enrichment of glycopeptides from human serum. We added the use of basic TMT groups to increase average precursor charge state, and improvement of ETD fragment ion yield was also explored in these experiments.

Additionally, we developed a novel feature on the LTQ Orbitrap Velos ETD mass spectrometer termed an HCD-accurate mass product ion dependent ETD scan that simplifies spectral interpretation for glycopeptide analysis.

Methods
Sample Preparation
Glycoproteins from human serum were isolated using ConA per manufacturer’s suggestion. Isolated glycoproteins were reduced, alkylated, and subjected to enzymatic digestion. Samples were TMT labeled (TMT-126) according to the manufacturer’s protocol. TMT-labeled glycopeptides were enriched on in-house cellulose column. In-house glycopeptide extraction columns were constructed according to a previously described method.1 Protein digest samples (100-500 μL) were loaded onto the column under ambient conditions, without vacuum assistance. The column was washed twice with 1 mL of 0.1% TFA in H2O to desalt the sample and to ensure that all peptides were bound to the C18 material. Peptides were eluted with 5 mL of 0.1% TFA in 7:3 H2O/MeOH solution as “glycopeptides” fractions. All fractions were lyophilized and reconstituted in 0.1% TFA in H2O to volumes equal to those of the initially loaded samples.

LC-MS
A Thermo Scientific MS Pump with a flow splitter and a Picofrit™ ProPep™ C18 10 cm × 75 μm i.d. column (New Objective, Woburn, MA) was used. Gradient elution was performed from 5%-45% ACN in 0.1% formic acid over 90 min at a flow rate of ~300 nL/min. The samples were analyzed with a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer with ETD. The following MS and MS/MS settings were used: FT: MS/MS Target = 56k; MS/MS MS+1 = 1000, 100000 resolution at m/z 400; MS Target = 160; MS/MS MS+1 = Top Five Data-Dependent™ acquisition HCD/Top Five Product dependent acquisition ETD; Dynamic Exclusion = Repeat count 1, Duration 30 sec, Excluded ion list 60 sec; HCD Parameters: Collision Energy = 35%; resolution 7500. MS1 Target Ion Trap 1 = 14, 24, TMT-126 labeled products, and 73 ions with average charge state >2 were detected at each scan. 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.6 Prior to enrichment on the cellulose column, enzymatically digested peptides were labeled with TMT-126 to improve ETD fragmentation efficiency. Figure 3 displays HCD spectrum of TMT-labeled haptoglobin glycopeptide T236-251 precursor from human serum.

Results
We also introduce a novel instrumental control within the LTQ Orbitrap Velos™ system termed HCD-accurate mass data-dependent product ion ETD analysis to minimize user input for glycopeptide identification. In this method setup, ETD spectra are acquired only when specific product ions are detected by MS+2 HCD, for example oxonium ions of HexNAc at m/z 204.087. This approach increases overall productivity for MS analysis of glycopeptides by acquiring ETD spectra only when a glycopeptide is detected. Additionally, this approach minimizes overall file size and the number of ETD spectra that are extraplated to characterize glycopeptides.

Figure 2 shows an extracted ion chromatogram (XIC) of characteristic oxonium ions of HexNAc at m/z 204.087 from MS+2 HCD spectra of human serum analyzed by the LTQ Orbitrap Velos system. HCD fragmentation of D-glycerated ions were measured in the Orbitrap™ mass analyzer with high mass accuracy, which allows for unambiguous assignment. As well, it shows the retention-time in the chromatogram where glycopeptides are eluting allowing for extrapolation of MS/MS spectra for structural characterization.

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Conclusion
A comprehensive glycopeptidomics workflow was developed that combines enrichment at the glycoprotein and glycopeptide level using lectin chromatography and cellulose solid-phase material. A novel instrumental control approach within the LTQ Orbitrap Velos mass spectrometer called HCD-accurate mass product dependent ETD analysis increases the overall productivity for MS analysis of glycopeptides.

References

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FIGURE 1. Schematic representation of glycoproteomics workflow.

FIGURE 2. HCD XIC of HexNAc oxonium ion at m/z 204.087 from human serum glycopeptide digest fractions eluted from a cellulose-based column.

FIGURE 3. LC-MS Orbitrap Velos HCD spectrum of TMT-labeled haptoglobin glycopeptide T236-251 precursor from m/z 1112.777 (+4) from human serum.

FIGURE 4. LC-MS Ion trap ETD spectrum of TMT-labeled haptoglobin glycopeptide T236-251 precursor from m/z 1112.777 (+4) from human serum.

By employing the strategy outlined in Figure 1, we were able to identify a total of 173 human serum proteins, and 24 of them are glycoproteins with multiple glycopeptides/glycoforms. Glycopeptide identification was done manually and is probably not complete. Due to the complexity of the data generated, we are currently employing a software prototype for ETDbased glycopeptide sequencing, GlycoMaster from Bioinformatics Solution, to characterize additional glycoprotein data from human serum samples.

Our results are in agreement with previous data reported by Harazono5 for the ConA-enriched human serum glycopeptides, but obtained via a direct data-dependent, not targeted, approach.

DThe corresponding ion trap ETD spectrum is shown in Figure 4. The ETD spectrum at precursor charge +4 generates almost complete c- and z-ions, thus enabling unambiguous mapping of the N-glycosylation site at Asn241.