

Glycopeptides Analysis Using Cellulose-Based Separation Cartridges and LTQ Orbitrap ETD

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Overview

Purpose: Development of novel workflow for comprehensive site-specific glycan/glycopeptides analysis.

Methods: Cellulose powder packed into a small plastic solid-phase extraction reservoir tube was used as the glycopeptide extraction medium. Purified glycopeptides were analyzed by an LTQ Orbitrap XL with electron transfer dissociation (ETD).

Results: Protein digest mixtures were loaded onto a custom-made cellulose column and different fractions were removed by a step-gradient elution with incremental water content. The described glycopeptide enrichment strategy significantly improved the rate of success of ETD analysis and simplified the overall glycoproteomics workflow.

Introduction

Protein glycosylation is an area of active research in the realm of biological sciences. It is widely accepted that oligosaccharide moieties are important modifications that play significant roles in many biochemical processes ranging from fine-tuning of protein folding to receptor site recognition events in some key signaling pathways¹. Much of the current research in the field of glycoproteomics is focused on development of methods aimed at the enrichment of the glycopeptides/glycans and their sensitive detection, with particular emphasis on accurate quantitative analysis and detailed structural characterization². ETD MS/MS fragmentation³ is the method of choice to characterize this type of PTM. In this work, we describe a novel workflow, which combines a new method for efficient extraction of glycopeptides from a complex digest mixture with nearly negligible loss of glycopeptide material or carryover of non-glycosylated peptides into the isolated glycopeptide fraction. This procedure is followed by ETD analysis.

Methods

Sample Preparation and Glycopeptide Enrichment

Digests of a reduced and alkylated 10 standard protein mixture containing 3 glycoproteins (human serotransferrin, chicken ovalbumin and bovine alpha-lactalbumin) and of bovine fetuin (Sigma) were used in this study. The mixture was divided into 6 aliquots and labeled with Thermo Scientific Tandem Mass Tags (TMT) – 126, 127, 128, 129, 130 and 131 TMT tags⁴ respectively – according to the manufacturer's protocol. Upon labeling, samples were mixed one to one and dried.

In-house glycopeptide extraction columns were constructed according to the illustration in Figure 1. Bulk cellulose (~10 g) was washed 3 times with 50 mL of water and 3 times with 50 mL of MeOH, successively, in order to remove any soluble impurities. The liquid portion of the resulting suspension was removed by centrifugation each time. Cellulose material was then loaded into a 8 mL solid-phase extraction tube plugged with a frit as a suspension in MeOH to the net volume of ~2 cm³ (after settling). C₁₈ material was then added as a suspension in MeOH to form a final column packing volume of ~2.5 cm³. The packing material was then gently compressed with a second (top) frit, which was left in the column to prevent any disturbance of the packing material during elution. The cellulose column was conditioned by washing with 10 mL of 0.1% TFA in water (2 mL at a time). Protein digest samples (100–500 µL) were loaded onto the column under ambient conditions, without vacuum assistance. The column was then washed twice with 1 mL of 0.1% TFA in water 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 1:9 water/MeOH solution as a "peptides" fraction. Glycopeptides were eluted with 5 mL of 0.1% TFA in 7:3 water/MeOH solution as a "glycopeptides" fraction. All fractions were lyophilized and re-suspended in 0.1% TFA in water to volumes equal to those of the initially loaded samples.

LC/MS

A Thermo Scientific Surveyor MS Pump with a flow splitter and PicoFrit[®] ProteoPep[™] 2 C₁₈, 5 or 10 cm x 75 µm i.d. column (New Objective, Woburn, MA) or Thermo Scientific Hypercarb 5 cm x 180 µm i.d. column was used. Gradient elution was performed from 5–45% acetonitrile in 0.1% formic acid over 60 min at a flow rate of ~300 nL/min. The samples were analyzed with a Thermo Scientific LTQ Orbitrap XL hybrid mass spectrometer equipped with ETD. The following MS and MS/MS settings were used: FT: MSn Target = 1e5; MS/MS = 1 µscans, 300 ms max ion time; MS = 400–2000 m/z, 100000 resolution at m/z 400, MS Target = 5e5; MS/MS = Top Three Data Dependent[™] acquisition HCD/ Top Three Data Dependent acquisition ion trap ETD; Dynamic Exclusion = Repeat count 1, Duration 30 sec, Exclusion duration 60 sec; HCD Parameters: Collision Energy = 45%, resolution 7500, MSn Target Ion Trap = 1e4, 3 µscans, ETD anion AGC target=2e5, charge-dependent ETD reaction time was used. Peptide masses were determined by deconvolution of isotopically resolved spectra using Thermo Scientific Xtract software.

Results

Most glycopeptide extraction procedures found in the literature are based on the principle of hydrophilic interactions between the oligosaccharide portion of a molecule and appropriate solid-phase material⁵. On the basis of previously published work⁶ on the use of cellulose for isolation of glycopeptides from complex proteolytic digests in solution, we began to examine the use of this inexpensive material in a column format. All work was performed using in-house made columns, designed according to

FIGURE 1. Design of a cellulose-based glycopeptide extraction column.

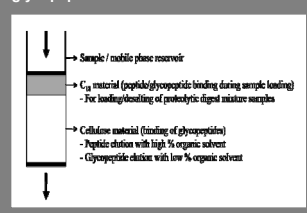


Figure 1 and described in Methods. To examine the performance of the cellulose based columns, we used moderately complex samples, an enzymatic digest of neat and TMT-labeled 10-protein mixtures. Ovalbumin, transferrin, and α-lactalbumin were the three glycoproteins in this sample. Upon applying the sample onto the column and subjecting the column to "peptide eluting" and "glycopeptides eluting" conditions, the sample was separated into 3 fractions, a "peptides" and two "glycopeptides" fractions. As shown in Figures 2 & 3, several non-glycopeptides were detected in the "glycopeptides" fractions. However, most detected peaks corresponded to glycopeptides, as was confirmed by the extracted ion chromatogram of characteristic oxonium ions of HexNAc at m/z 204.085 from MS2 HCD spectra (Figure 2A). HCD-generated ions were measured in the Orbitrap[™] mass analyzer with high mass accuracy which allowed unambiguous assignment.

FIGURE 2. MS analysis of 10-protein mix digest fractions eluted from the cellulose-based column. A) HCD XIC of HexNAc oxonium ion at m/z 204.09. B) Xtract-deconvoluted spectra of eluted species, with major glycopeptides marked as in Figure 3B.

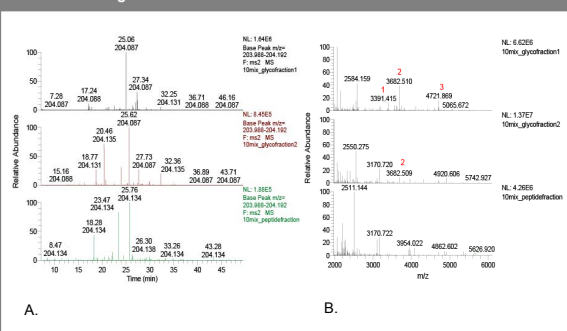
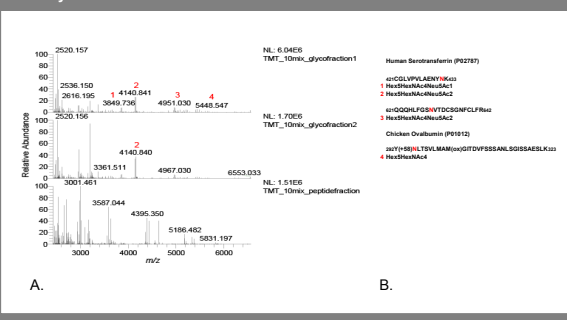


FIGURE 3. FT-MS analysis of TMT⁶-labeled 10-protein mix digest fractions eluted from the cellulose based column. A) Xtract-deconvoluted spectra of eluted species. B) Assignment of major detected glycopeptides based on mass accuracy and HCD/ETD MS2 data.



Two of the most prominent peaks in the mass spectrum of the "glycopeptides" fraction 1 (Figures 2B & 3A) correspond to both expected glycopeptides from transferrin in their dominant biennary disialylated glycoforms. This is despite the fact that transferrin constituted only ~4% of the total molar protein content of the sample. A large number of N-linked glycopeptides originating from the ovalbumin peptide T292-323 were also observed, however due to presence of 20 or more known glycoforms, all glycopeptide ion peaks related to ovalbumin were relatively small in the neat sample but became more prominent in the TMT labeled sample (Fig.3A). As we demonstrated before⁴ 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. Figure 4 displays an ion trap ETD spectrum of major glycoform of the TMT-labeled ovalbumin N-glycopeptide from Figure 3. Interestingly, this glycoform corresponds to a peptide which has its N-terminus alkylated and Met₃₀₀ oxidized. Without complete sequence information from ETD, we would not have been able to identify it. We were not able to identify any glycopeptides derived from α-lactalbumin (T36-77) and are currently investigating this further.

The next set of experiments was carried out with the intent to examine the efficiency of our extraction method for O-linked glycopeptides. Bovine fetuin was the subject of our experiments. The fetuin O-linked glycopeptide (T228-288) is a relatively large glycopeptide of mass 6014.124 Da (peptide only) with three potential sites of glycosylation. It was previously reported that the MS signals of these O-linked glycopeptide ions are usually much lower than the signals of the N-linked ions, mainly due to low glycosylation occupancy and higher structural heterogeneity⁷ (Figure 5A).

FIGURE 4. LC-MS ion trap ETD spectrum of TMT⁶-labeled chicken ovalbumin glycopeptide T292-323 (marked as 4 in Figure 3) precursor at m/z 1090.517 (5+). "TMT" corresponds to TMT reporter ions or c-ion of whole TMT⁶ label

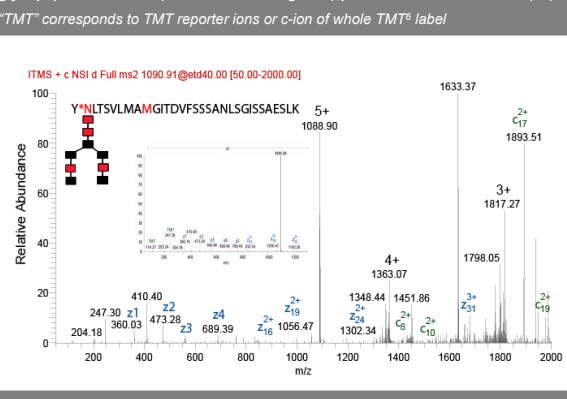
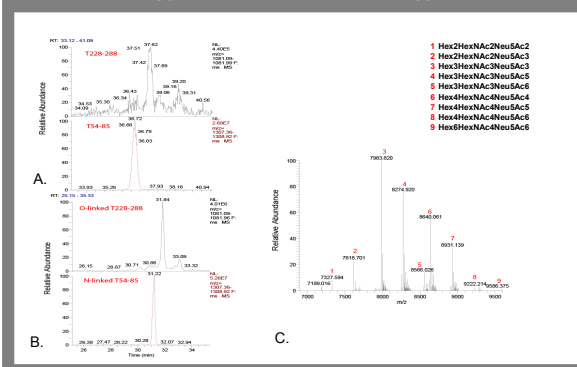
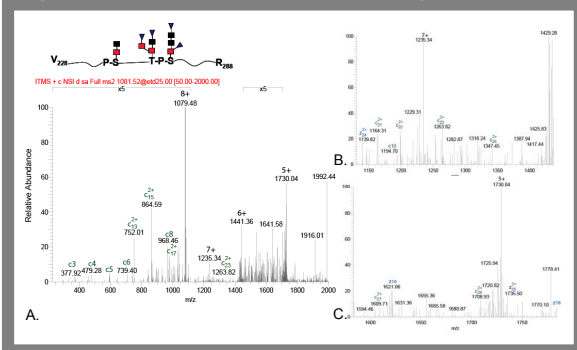


FIGURE 5. MS analysis of bovine fetuin digest fractions eluted from the cellulose-based column. XIC for major glycoforms of fetuin N-linked glycopeptide T54-85 (m/z 1307.362, 5+) and O-linked glycopeptide T228-288 (m/z 1081.139, 8+) before (A) and after (B) enrichment. Xtract-deconvoluted spectra of O-linked glycopeptide T228-288 identified in "glycofraction 1" (C).



Using our cellulose-based column, we were able to successfully enrich (Figures 5B vs. 5A) and detect multiple glycoforms – some of them not reported before – in the presence of N-linked glycopeptides, as shown in Figure 5. The identity of assigned O-linked glycopeptides, including specific glycosylation sites were confirmed by ETD analysis (Figure 6).

FIGURE 6. LC-MS ion trap ETD spectrum of bovine fetuin glycopeptide T228-288 (marked as 6 in Fig. 5) precursor at m/z 1080.887, 8+ (A). Zoom in for mass ranges m/z 1100-1430 (B) and m/z 1590-1790 (C) are displayed.



Conclusion

- A simple and efficient method was developed for on-column isolation of glycopeptides from complex proteolytic digests, using cellulose as solid-phase extraction material.
- N-, O-linked short, long, TMT-labeled glycopeptides were enriched in good yield for further glycoproteomic / glycomic studies.
- Combination of HCD and ETD MS2 analyses provided optimal information for both detection and identification of the glycan and peptide of interest and the site of modification.

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