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Comparison of Enrichment Methods for Intact N- and O-Linked Glycopeptides Using Strong Anion Exchange and Hydrophilic Interaction Liquid Chromatography

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Abstract

Heterogeneity of protein glycosylation poses great challenges for analysis that is key to understand structure and function of glycoproteins. Resolving this conundrum requires efficient and specific enrichment of intact glycopeptides for identification and quantitation. To this end, hydrophilic interaction chromatography (HILIC) has been commonly used to enrich intact N- and O-linked glycopeptides. However, its effectiveness to enrich isobarically labeled glycopeptides remains unclear. Here, we studied three different enrichment methods for enrichment of N- and O-linked glycopeptides. It was found that removal of N-glycans prior to enrichment of O-linked glycopeptides by HILIC improved identification of O-linked glycopeptides by mass spectrometry. We also compared the enrichment of intact N- and O-linked glycopeptides using other chromatography methods and found that using cartridges containing materials for strong anion exchange (SAX) chromatography increased yield and identification of N- and O-linked glycopeptides. The enrichment of O-linked glycopeptides was further improved when a Retain AX cartridge (RAX) was used. In particular, isobaric tag labeled glycopeptides after C18 desalting could be readily enriched by SAX and RAX cartridges but not by HILIC to enable quantitative glycoproteomics. It is anticipated that the use of SAX and RAX cartridges will facilitate broad applications of identifications and quantitation of glycoproteins.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b03641. Additional materials and methods (PDF)

Identification of N- and O-glycopeptides after sample preparation using HILIC, PNGase F, and β -elimination in different combinations (XLSX)

LC-MS/MS data and analysis of glycopeptides with and without prior PNGase F treatment using HILIC, RAX, and MAX (XLSX)

LC-MS/MS data and analysis of glycopeptides with and without prior PNGase F treatment using HILIC, RAX, and MAX (XLSX)

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Notes

The authors declare no competing financial interest.

Graphical abstract

Protein glycosylation, which attaches glycans to amino acids on proteins, is a common and important protein modification.¹ Although involvement of protein glycosylation in diseases is well-appreciated, the combination of hundreds of structurally different glycans in concert with thousands of glycosylation sites generates tremendous heterogeneity of site-specific glycosylation and poses great challenges for analysis.² Major types of glycosylation include N- and O-linked glycosylation with the former attaching glycans to a consensus NXS/T motif (X = proline) and the latter anchoring glycans to serine or threonine.³ On the basis of the types of attached glycans, N-glycosylation can be divided to high mannose, hybrid, and complex types whereas O-glycosylation includes O-GalNAc, O-GlcNAc, O-fucose, and O-mannose types.⁴ To study the glycoproteome, the very first step may involve glycopeptide enrichment that aims to increase the abundance of glycosylated peptides (glycopeptides).⁴ To this end, hydrophilic interaction liquid chromatography (HILIC) is often used for enrichment of intact glycopeptides based on the hydrophilic properties of glycans of glycopeptides.⁵ The global enrichment of N-linked glycopeptides (N-glycopeptides) by HILIC has been shown in different studies.^{2,6,7} However, it is argued that HILIC may not be an effective enrichment method for O-linked glycopeptides (O-glycopeptides) due to small structures of the O-glycans.^{8,9} In addition, to quantify the glycopeptides, isobaric labeling using iTRAQ or tandem mass tag (TMT) has been shown to be advantageous.¹⁰ To our knowledge, there has not been any reports of a HILIC-based method to enrich iTRAQ or TMT labeled glycopeptides following C18 desalting since C18 desalting is the final step to clean the labeled peptides as described in the manufacturer's instructions. Such a method would be highly desirable, because it could minimize variations caused during multiple enrichment processing steps and improve quantification precision. Indeed, our previous attempts of enrichments of iTRAQ labeled intact glycopeptides using ZIC-HILIC failed.^{2,7} In this study, we aim to characterize the performance of different methods for enrichment of N- and O-glycopeptides for mass spectrometry analysis with and without TMT labeling.

MATERIALS AND METHODS

Chemicals

Sequencing-grade trypsin was from Promega (Madison, WI); Sep-Pak C18 1 cm³ Vac Cartridge was from Waters (Milford, MA); PNGase F was from New England Biolabs (Ipswich, MA). Other chemicals such as urea, ammonia bicarbonate (AB), acetonitrile (ACN), trifluoroacetic acid (TFA), tris (2-carboxyethyl) phosphine (TCEP), iodoacetamide, triethylammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO).

Tryptic Digestion, Removal of Glycans from Glycopeptides and TMT Labeling

In total, 30 mg of human serum (Sigma-Aldrich) and 1 mg of a pool of both basal (P32) and luminal (P33) breast cancer xenograft tumor tissue were denatured in 8 M urea/1 M AB buffer and reduced in 10 mM TCEP at room temperature (RT) for 1 h. Proteins were alkylated in 10 mM iodoacetamide at RT for 30 min in the dark. Samples were diluted 8-fold using 0.2 M AB buffer to decrease the concentration of urea and proteins. Trypsin (0.5 $\mu\text{g}/\mu\text{L}$, enzyme/protein ratio of 1/40 w/w) was added to digest proteins at 37°C overnight. After digestion, peptides were desalted by a C18 cartridge according to the manufacturer's instructions. For removal of N-glycans from glycopeptides, peptides were dissolved in 100 mM Tris-HCl pH 8.0 with 3 μL of PNGase F. The mixture was incubated in 37°C overnight and then desalted by C18 cartridge. To remove O-glycans from glycopeptides, speed-vac dried peptides were dissolved in 28% ammonia and incubated at 50°C for 16 h. Labeling of peptides using TMT 10plex was conducted according to the manufacturer's instructions.

Enrichment of Glycopeptides

After C18 desalting, peptides eluted in 60% ACN (v/v), 0.1% TFA (v/v) were adjusted to 80% ACN (v/v), 1% TFA (v/v) for glycopeptide enrichment using ZIC-HILIC (The Nest Group, Inc. or 95% ACN (v/v), 1% TFA (v/v) or using SAX (HyperSep Retain AX Cartridges (RAX), particle size 30-50 μm , 30 mg sorbent per cartridge (Thermo Fisher Scientific) and Oasis MAX Cartridge particle size 30 μm , 30 mg sorbent per cartridge (Waters)). For HILIC, cartridges were conditioned in water for three times followed by 1 mL of 80% ACN (v/v), 1% TFA (v/v) for three times, the samples were then loaded, washed for three times in 1 mL of 80% ACN (v/v), 1% TFA (v/v) and eluted in 400 μL of 0.1% TFA. For RAX and MAX columns, the columns were equilibrated in 1 mL of ACN for three times, 100 mM triethylammonium acetate for three times, water for three times, and finally 95% ACN (v/v), 1% TFA (v/v) for three times. The samples were loaded and washed by 1 mL of 95% ACN (v/v), 1% TFA (v/v) for three times. Finally, bound glycopeptides were eluted in 400 μL of 50% ACN (v/v), 0.1% TFA (v/v). Concentration of the eluted peptides were determined using Nanodrop spectrophotometer to measure 280 nm absorbance for tryptophan and tyrosine. Yield of the enriched peptides were calculated from the concentration. The samples were dried in speed-vac prior to LC-MS/MS analysis. Additional Materials and Methods are described in the Supporting Information.

RESULTS AND DISCUSSION

Enrichment of Intact O-Glycopeptides Using HILIC

We sought to determine the capability and selectivity of HILIC-based methods for enrichment of N- and O-glycopeptides. First, to determine the utility of HILIC for enrichment of O-glycopeptides, a systematic study was conducted using human serum that was trypsin-digested followed by sample processing with HILIC columns, PNGase F, and β -elimination in different combinations. In the HILIC enrichment, sample solutions were adjusted to 80% ACN/1% TFA where ACN-rich solution facilitates enrichment of hydrophilic compounds and 1% TFA enables ion-pairing to further improve enrichment of glycopeptides from nonglycosylated peptides.¹¹ We explored eight different workflows (Figure 1), and afterward 1 μg of peptides from each strategy described in the workflows

was analyzed by LC–MS/MS. The intact glycopeptides were identified using the GPQuest software with 1% FDR filter as described in the Materials and Methods.^{12,13} In the global peptide analysis without HILIC enrichment, 134 unique N-glycopeptides and 47 unique O-glycopeptides were identified (Figure 1 and Table S1). After HILIC enrichment, the number of identified N-glycopeptides increased significantly to 313 (Figure 1 and Table S1). However, O-glycopeptides did not seem to be further enriched with the result of 45 O-glycopeptides identified (Figure 1 and Table S1). This observation is not surprising because O-glycans consist of short sugar chain with low hydrophilicity for the HILIC enrichment compared to N-glycopeptides during the affinity enrichment. It could also be possible that the O-glycopeptides were low abundant and they were shadowed by other competing and abundant peptides such as N-glycopeptides during LC-MS/MS analysis. To distinguish these two situations, we therefore used PNGase F to remove N-glycans before and after the HILIC enrichment. In the case of PNGase F treatment after HILIC enrichment, the number of identified O-glycopeptides slightly decreased to 36 while the number of identified N-glycopeptides significantly reduced to 74 (Figure 1 and Table S1). We reasoned that the PNGase F treatment after HILIC enrichment did not sufficiently remove interfering abundant peptides from N-glycopeptides but only removed the N-glycans from N-glycopeptides. Therefore, an additional HILIC enrichment was used (Figure 1) that, however, was found to further decrease the number of identified N- and O-glycopeptides probably due to sample loss from a very limited sample amount in this extra enrichment step. Noticeably, when PNGase F was used before HILIC enrichment, the number of identified O-glycopeptides increased to 66 (Figure 1 and Table S1). Next, we applied β -elimination treatments at the end of each workflow and showed that the identified O-glycopeptides were susceptible to β -elimination treatment (Figure 1 and Table S1). Together, our analysis suggested that HILIC was able to enrich both N- and O-glycopeptides and removal of N-glycans facilitated enrichment of O-glycopeptides.

Improved Performance to Enrich and Identify Glycopeptides by Strong Anion Exchange Columns

Owing to the smaller size of O-glycans compared to their N-linked counterpart, the HILIC column may not efficiently enrich O-glycopeptides. Therefore, we sought to examine the use of SAX columns in HILIC mode for enrichment of N- and O-glycopeptides. To test whether different column materials may confer different enrichment preference for glycopeptides, 1 mg of human serum peptides with or without PNGase F treatment were enriched by ZIC-HILIC column, HyperSep Retain AX (RAX), and Oasis MAX columns following the protocol as described in the Methods and Materials. We used the Nanodrop spectrophotometer to estimate the amount of enriched peptides. After enrichment, it was noted that HILIC was able to enrich approximately 24 μg from the total 1 mg of peptides, achieving a yield of 2.4% (Table 1). In sharp contrast, peptide yield from RAX and MAX columns were approximately 92.4 and 129.2 μg , reaching yields of 9.2% and 12.9% of the total 1 mg of peptides, respectively (Table 1). The enriched peptides were dried and resuspended in different volume of 0.2% formic acid to make 1 $\mu\text{g}/\mu\text{L}$ and 1 μL was analyzed from each sample. LC profile and total ion current (TIC) supported that the input amount of peptides was similar (Figure S1). MS/MS revealed that all three columns efficiently enrich glycopeptides such that over 90% of MS/MS spectra contained oxonium

ions (Table 1). Among these spectra, 481, 578, and 605 unique N-glycopeptides were identified while 46, 58, and 51 unique O-glycopeptides were identified using HILIC, RAX, and MAX columns, respectively (Table S2). Noticeably, the number of identified glycopeptides was higher in RAX and MAX than in HILIC column under the same LC-MS/MS setting. After removal of N-glycopeptides by PNGase F, it was found that both yield and number of identified N-glycopeptides was drastically reduced (Table 1 and Table S2). However, some N-glycopeptides were identified because of, in part, resistance of N-glycopeptides with glycosylation site at the peptide N-termini to PNGase F treatment such as N*ISDGFDPDNVDAAALALPAHSYSGR listed in the top five glycopeptides with the highest PSMs in the three enrichments (Table S2). This observation is consistent with previous reports that such N-glycopeptides are resistant to PNGase F treatment.¹⁴ The residue N-glycopeptides and O-glycopeptides were enriched and MS/MS showed that 55, 78.6, and 74.7% of MS/MS spectra contained oxonium ions using HILIC, RAX, and MAX, respectively (Table 1). The data were searched and identified 68, 140, and 81 unique O-glycopeptides from 12, 31, and 13 peptide backbones in HILIC, RAX, and MAX, respectively (Table S2). It appeared that RAX column showed improved enrichment for O-glycopeptides. O-Glycopeptides with peptide backbones of HYTNPSQDVTVPVCPVSTPPTPSPSTPPTPSPSCCHPR and TPLPPTSAHGNVAEGETKPDVDVTER from the Ig alpha-1 chain C region and protein hemopexin have highest PSM in the three enrichment methods suggesting their preferential enrichment by the three enrichment methods.

Comparison of Preference and Specificity of HILIC and Strong Anion Exchange Enrichment Methods for Enrichment of Glycopeptides

To determine whether different enrichment methods have different enrichment preference to peptide backbones or glycans, the glycopeptides identified by different enrichment methods were compared. When peptide backbones from identified N-glycopeptides enriched by different columns were compared, 96 among the total 163 of identified N-linked glycopeptide backbones (59%) were identified by all three enrichment methods. Thirty glycopeptide backbones were identified by both RAX and MAX enrichment methods but not in HILIC indicating that RAX and MAX enriched an additional set of N-glycopeptides although the mechanism was not clear (Figure 2A, left panel). A total of 8 or 13 peptide backbones of N-glycopeptides were unique to each column (Figure 2A, left panel). Consistently, RAX and MAX enriched additional N-glycans that were not enriched by HILIC (Figure 2A, middle panel). Both N-glycopeptides with sialylated and nonsialylated glycans were enriched equally well by all three affinity methods (Table S2). Interestingly, RAX appeared to enrich O-glycopeptides more effectively comparing to MAX and HILIC, with an additional number of 14 O-glycopeptide backbones enriched (Figure 2A, right panel). This suggested that RAX has favorable affinity for these O-glycopeptides. The performance similarity of the columns was revealed by unsupervised hierarchical clustering using spectral counting (Figure 2B). The clustering analysis showed that RAX and MAX had similar performance overall and were different from HILIC (Figure 2B). Finally, specificity of the enrichment methods was determined by calculating percentage of PSMs of all glycopeptides to the total PSMs of peptides and glycopeptides. We observed that, without PNGase F treatment, HILIC, RAX, and MAX had comparable specificity reaching 69, 72,

and 66%, respectively. After PNGase F treatment, the specificity decreased to 32, 43, and 31% for HILIC, RAX, and MAX, respectively. The reduction of specificity after PNGase F treatment suggested that removal of N-glycans in the sample resulted in compromised specificity for the enrichment methods.

Reproducibility of HILIC and Strong Anion Exchange Enrichment Methods

To validate the improved performance of SAX columns for enrichment of glycopeptides, serum sample was analyzed using the same workflow as described in the above sections. We confirmed the increased yield of enrichment for RAX and MAX reaching 97 and 121 μg , respectively; in contrast, HILIC enriched 21 μg of glycopeptides from the same starting amount of 1 mg of serum peptides. LC-MS/MS analysis and data searching identified 166, 183, and 230 N-glycopeptides and 51, 106, and 50 O-glycopeptides using HILIC, RAX, and MAX, respectively (Table S3). After PNGase F treatment, 5, 40, and 39 N-glycopeptides and 51, 142, and 92 O-glycopeptides were identified using HILIC, RAX, and MAX, respectively (Table S3). This repeated experiment demonstrated the trend that RAX and MAX enabled improved yield and identification of glycopeptides than HILIC. MAX and RAX columns have improved enrichment and identification for N- and O-glycopeptides, respectively. Finally, removing N-glycopeptides prior to enrichment facilitated identification of O-glycopeptides.

Enrichment of Glycopeptides Labeled with Isobaric Tags by Strong Anion Exchange for Quantitative Glycopeptide Analysis

Isobaric labeling such as TMT and iTRAQ is a robust technique for quantitative proteomics.¹⁵ Isobaric tags have been used to quantify intact glycopeptides detected from global proteomics.⁷ However, we experienced failure multiple times to use HILIC to enrich isobaric labeled glycopeptides following C18 desalting, which is the final step in the manufacturer's instruction. Therefore, the three columns were examined for their capability to enrich TMT labeled glycopeptides from pool of both basal (P32) and luminal (P33) breast cancer xenograft tumor tissue.¹⁶ To do this, TMT10plex was used to label tryptic peptides from tissues and the labeled glycopeptides were enriched by the three columns. LC-MS/MS analysis revealed that HILIC failed to enrich labeled glycopeptides with only approximately 9.7% of oxonium ion containing MS/MS spectra in the raw spectral data (Table 2). In sharp contrast, RAX and MAX showed great capability to enrich labeled glycopeptides with 74.9 and 72.3% of oxonium ions containing MS/MS spectra in the data, respectively (Table 2). Consistently, RAX and MAX enriched approximately 1.5 and 8 μg (1.2% and 6.8%) from starting 125 μg peptides (Table 2). The peptides were dissolved in 0.2% formic acid to make 1 $\mu\text{g}/\mu\text{L}$. LC-MS/MS analysis of 0.8 μL (0.8 μg) of each sample supported the observation that higher yield was obtained from enrichment using MAX column. The underlying reasons for successful use of RAX and MAX but not ZIC-HILIC to enrich labeled glycopeptides remain elusive. Possible factors including different chemical groups in the bedding material of the columns, excessive TMT labeling reagents that were not efficiently removed after C18 desalting, increase of charge and hydrophobicity of labeled glycopeptides, alone or in combination may contribute to the observed capability of RAX and MAX but not ZIC-HILIC to enrich labeled glycopeptides.

In conclusion, we determined the utility of three different cartridges in HILIC mode to study N- and O-glycopeptides with and without isobaric labeling. It was found that removal of N-glycans by PNGase F treatment prior to enrichment improved the effectiveness of O-glycopeptide enrichment. When the cartridges were studied in parallel, both SAX columns demonstrated higher yield for enrichment of both N- and O-glycopeptides. This facilitates analysis of intact glycopeptides from very limited amounts of samples. Remarkably, increased identification of N-glycopeptides was seen using SAX columns. This has been recently reported studying the RAX cartridge.¹⁷ It was noteworthy that RAX appeared to be more efficient in enriching O-glycopeptides. We found that HILIC column failed to enrich isobaric tag labeled glycopeptides while SAX column could be used for efficiently enrichment of isobaric tag labeled glycopeptides. These desired features of SAX columns are anticipated to empower broad application of quantitative glycoproteomics to underpin systems glycobiology in a variety of diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Identification of N- and O-glycopeptides after sample preparation using HILIC, PNGase F, and β -elimination in different combinations. Trypsin-digested human serum was treated with different conditions and analyzed by LC–MS/MS. Intact glycopeptides were identified using GPQuest with 1% FDR.

Figure 2. Overlaps of peptide backbones of N- and O-linked peptides and N-glycans from glycopeptides enriched by HILIC, RAX, and MAX columns. (A) Venn diagram of overlapping peptide backbones and N-glycans of glycopeptides enriched using the columns. (B) Unsupervised hierarchical clustering using spectral-counting of peptide backbones for each run. Euclidean Distance was used for clustering peptide backbones and glycans of N-glycopeptides. Spearman's rank correlation was used for clustering peptide backbones of O-glycopeptides.

Table 1

Enrichment of Glycopeptides with and without Prior PNGase F Treatment by Three Different Columns in HILIC Mode

starting peptides was 1 mg for each enrichment	yield (μ g)	yield (%)	total scan	MS full scan	total MS2	oxonium ion containing spectra	ratio (%) ^a
HILIC	24	2.4	41031	12050	28981	26248	90.6
RAX	92.4	9.24	40842	12028	28814	26731	92.8
MAX	129.2	12.92	41061	11802	29259	26983	92.2
PNGase + HILIC	10	1	34882	17422	17460	9611	55.0
PNGase + RAX	28.8	2.88	41498	11565	29933	23527	78.6
PNGase + MAX	68	6.8	40882	12068	28814	21523	74.7

^aNumber of oxonium ion containing spectra/number of total MS2.

Table 2Enrichment of TMT Labelled Glycopeptides by Three Different Columns^a

starting peptides was 125 μ g for each enrichment	yield (μ g)	yield (%)	total scan	MS full scan	total MS2	oxonium ion containing spectra	ratio (%) ^b
TMT10 + HILIC	26.5	21.2	45166	12636	32530	3146	9.7
TMT10 + RAX	1.5	1.2	41319	11727	29592	22179	74.9
TMT10 + MAX	8	6.4	40691	12250	28441	20571	72.3

^aPeptides from tissue samples were labelled with TMT-10plex followed by HILIC, RAX, and MAX column enrichment.^bNumber of oxonium ion containing spectra/number of total MS2.