Concanavalin A Column for Analysis of Glycoproteins and Their Tryptic Glycopeptides

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Key Words

Off-Line 2D HPLC, Con A Enrichment, Reversed-Phase Peptide Mapping, UltiMate 3000 ×2 Dual Biocompatible Analytical LC System, Lectin Affinity Chromatography

Introduction

Glycosylation, one of the most common and complex post-translational modifications, is of great interest for its role in many biological processes such as molecular recognition, cell signaling, and immune defense. Additionally, glycosylation has profound effects on the properties of a glycoprotein including solubility, immunogenicity, circulatory half-life, and thermostability. These protein properties are important in today's biotechnology industry because proteins are produced for human therapeutics.

For in-depth research of a glycoprotein, peptide mapping is necessary. Several factors make glycopeptide research by mass spectrometry (MS) challenging. First, glycopeptides usually constitute a minor part of the glycoprotein's total peptides. Second, the MS signal intensities of the glycopeptides are lower relative to nonglycosylated peptides due to lower ionization efficiency and heterogeneous glycan structures on the same glycosylation site. Third, a glycopeptide's MS signal can be suppressed by nonglycosylated peptides. Therefore, enrichment of glycopeptides from a complex peptide mixture is desired and lectin affinity capture technology has been developed to fulfill this purpose.

Among all lectins, Concanavalin A (Con A) is the most well characterized and widely used. It can capture glycoproteins containing asparagine-linked (*N*-linked) high-mannose type glycans, *N*-linked hybrid type glycans, and some *N*-linked biantennary complex type glycans.

The Thermo Scientific ProSwift ConA-1S Affinity Column was designed to isolate glycans, glycopeptides, or glycoproteins from complex samples. But unlike other Con A columns, it is built upon a monolithic support and is designed to be used on a high-performance liquid chromatography (HPLC) system. HPLC compatibility and the monolithic support deliver multiple advantages: faster separation, better sample recovery, high peak efficiency, and high-throughput capability.

This approach also enables an automated configuration to identify glycopeptides using the ProSwift™ ConA-1S Affinity column to extract the glycopeptides from a peptide mixture. The isolated glycopeptides can then be separated by reversed-phase HPLC, and their identity confirmed by selected ion monitoring (SIM) of diagnostic sugar oxonium ions (e.g., *m/z* 204, 366, and 163). This method requires only a single quadrupole mass spectrometer.

Goal

The goals of this work are to:

- Use the ProSwift ConA-1S Affinity column to purify glycoproteins from commercial preparations of those glycoproteins.
- Extract glycopeptides from the tryptic digests of purified horseradish peroxidase (HRP), ovalbumin, and ribonuclease B glycoproteins.
- Identify individual glycopeptides of a tryptic digest after glycopeptide extraction using reversed-phase separation.
- Use a Thermo Scientific Dionex UltiMate 3000 ×2 Dual Biocompatible Analytical LC system to automate the experiment.

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Equipment

- UltiMate™ 3000 ×2 Dual Biocompatible Analytical LC system, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
  - TCC-3000SD Thermostatted Column Compartment
  - DAD-3000 Diode Array Detector with 13 µL flow cell
- Thermo Scientific MSQ Plus Mass Spectrometer with electrospray ionization (ESI) source
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 6.80, SR9 or higher

Reagents and Standards

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific P/N AC610010040)
- Formic acid, ~98% (Fluka P/N 94318, Sigma-Aldrich®)
- Albumin from chicken egg white (Ovalbumin), ≥98% (Sigma-Aldrich P/N A5503)
- Peroxidase from horseradish (HRP)(Sigma-Aldrich P/N P6782)
- Ribonuclease B (Worthington Biochemical P/N LS005710)
- Trypsin from bovine pancreas (Sigma-Aldrich P/N T1426)
- Endo H, 500,000 units/mL (New England BioLabs P/N P0702S)
- DL-Dithiothreitol, ≥99.0% (RT) (Fluka P/N 43819, Sigma-Aldrich)
- Iodoacetamide, ≥99% (HPLC) (Sigma-Aldrich P/N I6125

Conditions	
Columns:	ProSwift ConA-1S Affinity (5 × 50 mm, P/N 074148) Thermo Scientific Acclaim Polar Advantage II (PA2), 3 µm Analytical (3.0 × 150 mm, P/N 063705)
Mobile Phase:	ProSwift Column: A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3 B: 100 mM α-methyl mannoside in mobile phase A Acclaim™ PA2 Column: A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	ProSwift Column: 0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B Acclaim PA2 Column: 0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	ProSwift Column: 0.5 mL/min Acclaim PA2 Column: 0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV absorbance at 214 nm MS SIM mode at *m/z* 163, 204, and 366
Sample Preparation:	Protein Samples: Dilute protein stock solution (2 mg/mL in DI water) in mobile phase A to 1 mg/mL before injection Peptide Samples: After tryptic digestion, dilute the peptide sample in mobile phase A to 1 mg/mL before injection

MSQ Plus™ Mass Spectrometer Conditions	
Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temperature:	400 °C
Needle Voltage:	3.5 kV
Detection Mode:	SIM at *m/z* 163, 204, and 366
Dwell Time:	0.5 sec
Cone Voltage:	140 V
Nebulizer Gas:	Nitrogen at 75 psi

Preparation of Solutions

Buffers for Tryptic Digestion

For detailed methods for preparing buffers for tryptic digestion, refer to Dionex (now part of Thermo Scientific) Application Update (AU) 183.[1]

Buffers for Deglycosylation

The buffers for deglycosylation are provided by New England Biolabs, the Endo H manufacturer. The buffer compositions are as follows: 10X Denaturation Buffer [5% sodium dodecyl sulfate (SDS), 0.4 M dithiothreitol] 5X Reaction Buffer [0.5M sodium phosphate, pH 5.5].

Protein Digestion Procedure

Tryptic Digestion

Reduce, alkylate, and dialyze HRP extensively against 50 mM sodium bicarbonate. Digest the resulting HRP with trypsin overnight. For detailed procedures, refer to AU 183.

Deglycosylation

1. Add 40 µg of glycoprotein to an Eppendorf tube. Prepare a 2 mg/mL solution by adding 20 µL DI water.
2. Add 2 µL 10X denaturation solution to the tube and heat at 100 °C for 10 min.
3. Cool, then add 2 µL 5X reaction buffer to the tube.
4. Add 2 µL of Endo H to the reaction. Incubate overnight at 37 °C.

Results and Discussion

Separation of a Glycoprotein from Its Nonglycosylated Counterpart and/or Nonglycosylated Impurities

Ovalbumin, ribonuclease B, and HRP are analyzed here as glycoprotein models. Figure 1A shows that roughly 80% of the commercial ovalbumin can be captured by the ProSwift ConA-1S Affinity column. A literature search shows that ovalbumin has one *N*-linked glycosylation site with approximately equal amounts of hybrid- and high-mannose type oligosaccharides that can be recognized by Con A. The other 20% of ovalbumin unbound to Con A can likely be attributed to the contaminant glycoproteins in ovalbumin that mainly have complex type glycan structures.[2]

Approximately 50% of ribonuclease B can be captured by the ProSwift ConA-1S Affinity column (Figure 1B). Ribonuclease B is reported to have a single glycosylation site with high-mannose type oligosaccharide chains. The ribonuclease B used in this study was labeled by the manufacturer to be "a mixture of ribonuclease A and ribonuclease B". This result indicates that the commercial ribonuclease B has roughly equal amounts of nonglycosylated ribonuclease A and glycosylated ribonuclease B. As shown in Figure 1C, most if not all of the HRP was captured by the ProSwift ConA-1S Affinity column. This observation agrees with the fact that HRP has nine potential glycosylation sites of which at least eight sites are occupied by heterogeneous high-mannose type oligosaccharides.[3]

Interestingly, the eluted fraction (glycoprotein fraction) peak of HRP was much sharper than the peak of ovalbumin or ribonuclease B. This observation suggests that HRP has a few dominant glycan structures with similar affinities to Con A.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH at 5.3 B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Samples:	**A**. Ovalbumin; **B**. Ribonuclease B; **C**. HRP
Sample Preparation:	1 mg/mL ovalbumin, ribonuclease B, or HRP in water/mobile phase A
Peaks:	1. Nonretained protein 2. Retained protein (nominally glycosylated) 3. Partially retained protein

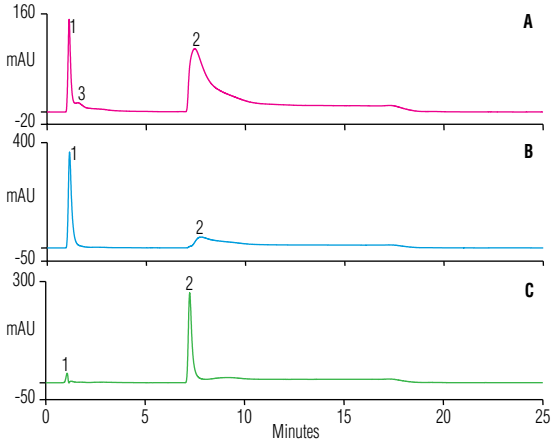

Figure 1. Glycosylated protein enrichment on the ProSwift ConA-1S Affinity column.

Separation of Glycopeptides from a Peptide Mixture

The glycoproteins discussed here were each digested with trypsin and used to test the affinity of the ProSwift ConA-1S Affinity column for glycopeptides. The separation of nonglycosylated peptides and glycopeptides by the ProSwift ConA-1S Affinity column and reanalysis of the collected fractions by reversed-phase chromatography were automated in an off-line 2D mode. The peptide fractions were collected in a 96-well plate using the fraction collector function of the WPS-3000TBFC Autosampler. The collected fractions were then loaded to an Acclaim PA2 column for peptide mapping.

Ovalbumin and ribonuclease B have only one glycosylation site, so a small fraction of their tryptic peptides will be bound to the ProSwift ConA-1S Affinity column. In contrast, HRP's multiple glycosylation sites, combined with glycan microheterogeneity on each site, predict that it will have a larger fraction of its tryptic peptides retained, which is confirmed in Figure 2C.

Figure 3 shows the peptide mapping of HRP tryptic peptides, its Con A flow-through fraction (nonglycosylated peptides), and its Con A captured fraction (glycopeptides). The UV chromatogram of the glycopeptide fraction of HRP digest shows approximately nine peaks, which may correspond to its nine glycosylation sites. Attachment of different glycans to the same glycosylation site (microheterogeneity) will have minor effects on the retention time of the peptide.[4] Therefore, a peptide with different glycans attached may be shown as a single peak in a reversed-phase chromatogram, albeit wider than a nonglycosylated peptide.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3 B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Samples:	**A**. Ovalbumin tryptic peptides **B**. Ribonuclease B tryptic peptides **C**. HRP tryptic peptides
Sample Preparation:	Tryptic peptide samples diluted with mobile phase A, 1 mg/mL solution
Peaks:	1. Nonretained peptides 2. Retained peptides (nominally glycosylated) 3., 4. Peptides with weak interaction with Con A

The insets are enlargements of the first 10 min of each separation

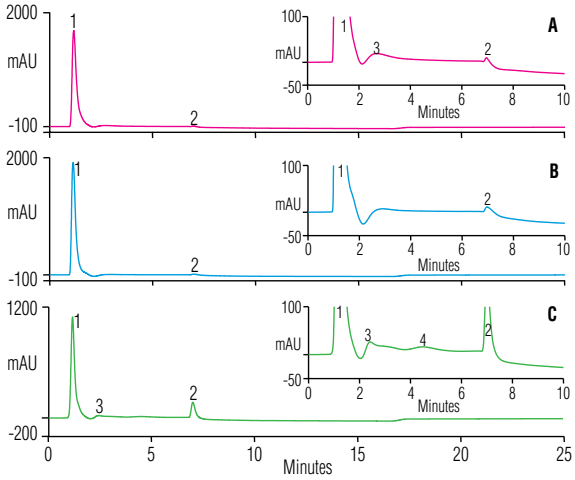

Figure 2. Glycosylated tryptic peptides enrichment on the ProSwift ConA-1S Affinity column.

Column:	Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase:	A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	0–5.0 min, 0% B; 5.0–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Sample Preparation:	HRP tryptic peptides diluted with mobile phase A,1 mg/mL solution

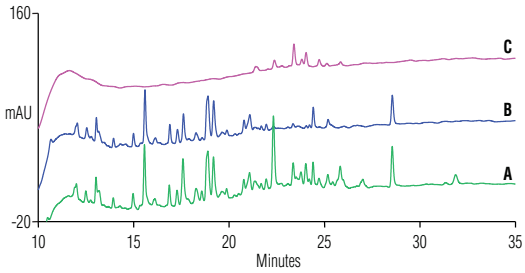

Figure 3. Peptide mapping of (A) HRP tryptic peptides, (B) HRP tryptic peptides ProSwift ConA-1S Affinity column flow-through fraction, and (C) HRP tryptic peptides ProSwift ConA-1S Affinity column eluted fraction.

Selective Monitoring of Glycopeptides by Monitoring Oxonium Ions

SIM scanning of glycan diagnostic oxonium ions and precursor ion scanning are two frequently used methods for selective detection of peptides with a post-translational modification such as glycosylation and phosphorylation.[4] Without the ability to do precursor ion scanning, scanning oxonium ions is the choice when using a single quadrupole mass spectrometer. It is reported that *m/z* 163, 204, 292, and 366 are marker ions for glycosylation.[4] Production of marker ions is controlled by the extent of collisional excitation, which depends on the voltage applied to the sampling cone. Maximum yield of marker for glycosylation is reportedly generated at a cone voltage of 140 V, which was applied in this study.

As Figure 4 shows, peak number, shape, and retention time in the mass spectroscopy traces for *m/z* 204 and 366 are equivalent to the UV chromatogram of the Con A captured fraction of the HRP tryptic peptides, offering further evidence that they are indeed glycopeptides. The sensitivity of peaks in the ion chromatogram is much higher than shown in the UV chromatogram. The extracted trace of ion *m/z* 163 can serve as a glycopeptide diagnostic ion in a less sensitive way.

In this work, the SIM trace of ion *m/z* 292 is a poor match for the UV trace (data not shown). It is known that oxonium ion 292 is from sialic acids (NeuAc+); therefore, the observation that *m/z* 292 is a poor diagnostic ion for these experiments may indicate lack of sialic acid containing oligosaccharide structures in the captured HRP tryptic glycopeptides.

The cone voltage is critical because diagnostic ion peaks under lower cone voltage, such as 100 V and 65 V in mass spectrum, do not match the UV chromatogram. Although *m/z* 204 or 366 can be generated from nonspecific fragmentation of peptide backbone, simultaneous detection of both *m/z* 204 and 366 provides strong evidence that the peptide is glycosylated. When comparing MS traces for *m/z* 204 and 366 with the UV chromatogram of the Con A captured fraction, the peptides in the captured fraction can be identified as glycopeptides.

Figure 5 shows that scanning for diagnostic oxonium ions is a selective and sensitive method to monitor glycopeptides in a peptide mixture that has not been passed through the ProSwift ConA-1S Affinity column. Major peaks in the *m/z* 204 SIM spectrum of unseparated HRP tryptic digest fit with peaks in the spectrum of the Con A captured fraction. Notice that some peaks shown in HRP tryptic digest cannot be found in the Con A captured fraction. These peaks may be lost in minor peaks (very wide peaks such as peaks 3 and 4, shown in Figure 2, with retention times of ~2.5 min and ~3.5 min) that elute just after the flow-through peak. This fraction probably has glycan structures that are not recognized by Con A. Published literature shows that HRP does have a minor glycan structure, Fuc(1-3)GlcNAc-, that could bind to Con A, though very weakly.[5]

Column: Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase: A: Water with 0.05% formic acid
B: Acetonitrile with 0.04% formic acid
Gradient: 0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate: 0.425 mL/min
Inj. Volume: 20 µL
Temperature: 30 °C
Detection: **A**. UV at 214 nm **C**. *m/z* 204 in SIM mode
B. *m/z* 366 in SIM mode **D**. *m/z* 163 in SIM mode
Sample Preparation: Tryptic peptides diluted with mobile phase A, 1 mg/mL solution

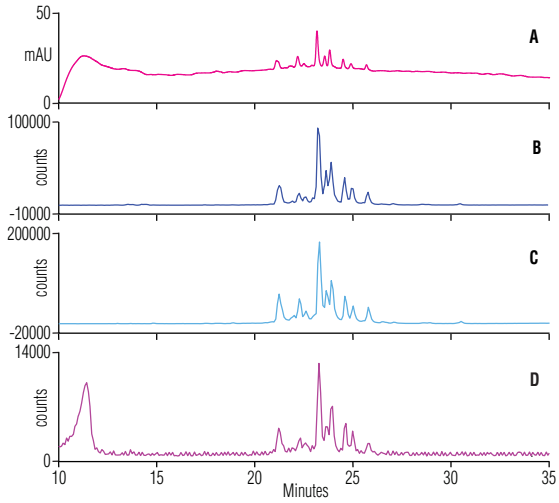

Figure 4. Peptide mapping of Con A captured fraction from the HRP tryptic digest detection by UV and MS in SIM mode.

Column: Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase: A: Water with 0.05% formic acid
B: Acetonitrile with 0.04% formic acid
Gradient: 0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate: 0.425 mL/min
Inj. Volume: 20 µL
Temperature: 30 °C
Detection: *m/z* 204 in SIM mode
Samples: **A**. HRP tryptic peptides
B. Con A captured fraction of HRP tryptic peptides
C. Con A flow-through fraction of HRP tryptic peptides

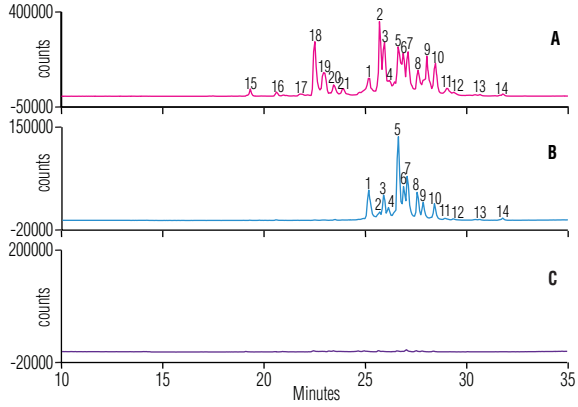

Figure 5. Peptide mapping of (A) HRP tryptic peptides, (B) Con A captured fraction of HRP tryptic peptides, and (C) Con A flow-through fraction of HRP tryptic peptides.

Endo H Digestion Eliminates the Con A Binding Ability of HRP

Endo H has been reported to cleave within the chitobiose core of high-mannose type and some hybrid type oligosaccharides from *N*-linked glycoproteins. Figure 6 shows that HRP cannot be retained by the ProSwift ConA-1S Affinity column after Endo H treatment. This observation confirms that main glycosylation types of HRP are high mannose and/or hybrid. Peak 3 in Figure 6 is postulated to be either the released oligosaccharides—because it is not found in the Endo H control (no HRP added)—or more likely, the fraction of glycosylated HRP that is not susceptible to Endo H (i.e., all Endo H-susceptible structures have been removed and only the nonsusceptible structures remain on the HRP). It is more likely a non-Endo H-susceptible fraction because oligosaccharides have little or no absorbance at 210 nm.

Conclusion

This work shows that the HPLC-compatible ProSwift ConA-1S Affinity column can capture glycoproteins and glycopeptides efficiently. The UltiMate 3000 ×2 Dual Biocompatible Analytical LC system can automate the entire off-line 2D process from ProSwift ConA-1S Affinity column sample enrichment and fraction collection to automatic reanalysis of collected sample by peptide mapping. Monitoring oxonium ions (e.g., *m/z* 204, 366, and 163) in a peptide mixture with a single quadrupole mass spectrometer is a selective, sensitive, and reliable method that also confirms the identity of glycopeptides captured by the ProSwift ConA-1S Affinity column.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3 B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Sample Preparation:	1 mg/mL HRP in water/mobile phase A
Samples:	**A**. Before Endo H treatment **B**. After Endo H treatment
Peaks:	1. HRP 2., 3. Deglycosylated HRP

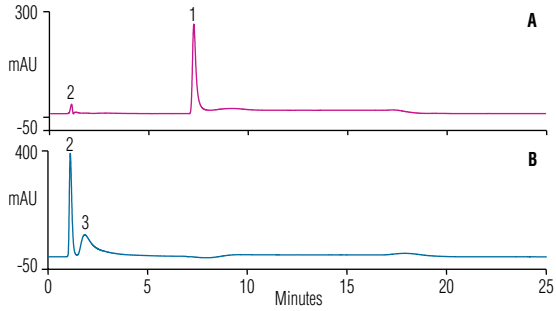

Figure 6. Glycosylated HRP enrichment on the ProSwift ConA-1S Affinity column (A) before and (B) after Endo H treatment.

References

1. Dionex (now part of Thermo Scientific) Application Update 183: Separation of Peptides from Enzymatic Digestion on Different Acclaim Columns: A Comparative Study. Sunnyvale, CA, 2011. [Online] www.dionex.com/en-us/webdocs/111297-AU183-LC-Peptides-EnzyDigest-AcclaimCompar-02Nov2011-LPN2973.pdf (accessed June 12, 2012).
2. Harvey, D.J.; Wing, D.R.; Küster, B.; Wilson, R.B.H. Composition of N-Linked Carbohydrates from Ovalbumin and Co-purified Glycoproteins. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 564–571.
3. Wuhrer, M.; Hokke, C.H.; Deelder, A.M. Glycopeptide Analysis by Matrix-Assisted Laser Desorption/Ionization Tandem Time-of-Flight Mass Spectrometry Reveals Novel Features of Horseradish Peroxidase Glycosylation: A Correlation Study. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1741–1748.
4. Bean, M.F.; Annan, R.S.; Hemling, M.E.; Mentzer, M.; Huddleston, M.J.; Carr, S.A. LC-MS Methods for Selective Detection of Posttranslational Modifications in Proteins: Glycosylation, Phosphorylation, Sulfation, and Acylation. In *Techniques in Protein Chemistry*; J. Crabb, ed.; Academic Press: San Diego, 1995; pp 107–116.
5. Wuhrer, M.; Balog, C.R.A.; Koeleman, C.A.M; Deelder, A.M; Hokke, C.H. New Features of Site-Specific Horseradish Peroxidase (HRP) Glycosylation Uncovered by nano-LC-MS with Repeated Ion-Isolation/Fragmentation Cycles. *Biochim. Biophys. Acta* **2005**, *1723*, (1-3) 229–239.

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