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Analysis of Protein O-GlcNAcylation by Mass Spectrometry

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Abstract

O-linked β -D-N-acetylglucosamine (O-GlcNAc) addition (O-GlcNAcylation), a post-translational modification of serine/threonine residues of proteins, is involved in diverse cellular metabolic and signaling pathways. Aberrant O-GlcNAcylation underlies the initiation and progression of multiple chronic diseases including diabetes, cancer, and neurodegenerative diseases. Numerous methods have been developed for the analysis of protein O-GlcNAcylation, but instead of discussing the classical biochemical techniques, this Unit covers O-GlcNAc characterization by combining several enrichment methods and mass spectrometry detection techniques (including collision-induced dissociation (CID), higher energy collision dissociation (HCD), and electron transfer dissociation (ETD) mass spectrometry).

Keywords

BEMAD; CID; enrichment; ETD; HCD; mass spectrometry; O-GlcNAc; O-GlcNAcome; O-GlcNAcomics; site mapping

INTRODUCTION

As a ubiquitous post-translational modification, protein O-GlcNAcylation is a crucial mechanism regulating protein function in many cellular processes (Hart, 2014; Bond and Hanover, 2015). So far, myriads of intracellular proteins (mainly nucleocytoplasmic and mitochondrial proteins), are known to be O-GlcNAcylated (Ma and Hart, 2014; Ma et al., 2015). UDP-GlcNAc, the end product of hexosamine biosynthesis pathway, serves as the donor substrate for the O-GlcNAc transferase (OGT). Besides the intracellular UDP-GlcNAc level, the intricate balance between OGT and O-GlcNAcase activities regulates the cycling of O-GlcNAc on proteins (Figure 1).

During the past three decades, a number of approaches have been developed for the analysis of O-GlcNAcylated proteins (Wang and Hart, 2008; Chalkley et al., 2009a; Zachara et al., 2011a; Ma and Hart, 2013). Biochemical approaches, including galactosyltransferase-catalyzed UDP-[^3H]-galactose labeling followed by autoradiography or immunoblotting with O-GlcNAc specific antibodies or lectins, have been used to probe O-GlcNAcylation of individual proteins (please see Unit 12.8 and [Ma and Hart, 2014] for more details). As an

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alternative, mass spectrometry has emerged as a very powerful tool for the detection of protein O-GlcNAcylation. Different from biochemical approaches, mass spectrometry provides a unique capacity for determining the O-GlcNAc status in a site-specific and semi-quantitative manner. Moreover, mass spectrometry affords high-throughput O-GlcNAc analysis at the proteomic scale. To date, a number of mass spectrometric techniques using alternative fragmentation methods including collision induced fragmentation (CID), higher energy collision dissociation (HCD), or electron transfer dissociation (ETD)) have been applied to the detection of protein O-GlcNAcylation.

Prior to mass spectrometry detection, highly efficient and robust enrichment is essential for the comprehensive analysis of protein O-GlcNAcylation at the proteomic level, largely due to the substoichiometric nature of O-GlcNAcylation, severe ion-suppression of O-GlcNAcylated peptides by unmodified peptides, as well as the complexity of the cellular proteins. To this end, recent years have seen the evolving of a variety of enrichment methods. Basically, there are two major categories of methods for O-GlcNAc enrichment: direct capture and chemical/enzymatic tagging. The simplest direct capture method is affinity purification by using resins immobilized with O-GlcNAc specific antibodies (e.g., CTD 110.6 and RL2) and/or lectins (e.g., wheat germ agglutinin). Although application of these direct enrichment approaches has demonstrated some success in several large-scale studies, the rather weak retention between the O-GlcNAc moiety and the resins often requires multiple rounds of enrichment to separate the O-GlcNAcylated glycopeptides from the mixtures. In contrast, the chemical/enzymatic tagging method renders higher specificity and selectivity toward O-GlcNAcylated proteins/peptides, enabling qualitative and quantitative analysis of protein O-GlcNAcylation in a number of biological contexts. But what should be pointed out is that the chemical/enzymatic tagging method often involves several steps and is technically more challenging.

This Unit mainly provides three mass-spectrometry-based protocols for the profiling of protein O-GlcNAcylation. Specifically, we introduce a protocol for probing O-GlcNAcylation with CID/HCD mass spectrometry (Basic Protocol 1). We then present two protocols for the mapping of O-GlcNAc sites, by combining the chemical/enzymatic tagging with either direct ETD mass spectrometry (Basic Protocol 2) or the mild beta-elimination and Michael addition with dithiothreitol (BEMAD) followed by CID/HCD mass spectrometry (Basic Protocol 3). For certain samples with huge complexity (e.g., whole cell lysates or tissue extracts), it is advantageous to fractionate the peptides before and/or after enrichment by using chromatographic approaches (e.g., high pH reverse phase HPLC, Support Protocol 1), which could be easily coupled to any of the Basic Protocols. It should be noted that, although mass spectrometry-based identification is powerful, other approaches are beneficial for unambiguous O-GlcNAc identification and confirmation. For example, mass spectrometry itself cannot distinguish O-GlcNAc and O-GalNAc, highly specific enrichment approaches (e.g., by using GalT1 labeling, as shown in Basic Protocol 2) assure the identified peptides are truly O-GlcNAc modified. Moreover, O-GlcNAc Western blotting can also serve as a complementary way to confirm the O-GlcNAc status of specific proteins.

BASIC PROTOCOL 1---- DETECTION OF O-GlcNAcylation BY USING CID/HCD MASS SPECTROMETRY

This protocol describes a procedure for detection of O-GlcNAc status of peptides with CID/HCD mass spectrometry. Although the biochemical approaches (e.g., antibody-based Western blotting) may provide convincing data on the O-GlcNAcylation status of proteins of interest, mass spectrometry provides a definitive method to show whether the proteins are really O-GlcNAcylated. This is achieved by observing the diagnostic ions in CID/HCD spectra. Upon CID/HCD fragmentation, O-GlcNAc and its oxonium ions (Figure 2) will show up since the O-GlcNAc moiety readily falls off before the fragmentation of peptide bone. This is especially prominent under HCD conditions which provide better fragmentation of GlcNAc. Thus, these diagnostic ions serve as one useful way to confirm the O-GlcNAc status of peptides. This approach is accessible due to the general availability of CID/HCD mass spectrometers, the most commonly used type in many labs. Moreover, this approach is quite straightforward since it can be performed without enrichment of O-GlcNAc proteins/peptides. In addition, under optimized conditions (e.g., using lower collision energy), a small percentage of peptide fragments may still carry the O-GlcNAc group, enabling the unambiguous assignment of O-GlcNAc sites on certain peptides. Although this protocol only described the analysis of protein O-GlcNAcylation in cultured cells, proteins from other resources (e.g., biological fluids or tissue extracts) could be analyzed similarly as well.

Materials

Cells of interest (in culture) and appropriate culture medium

Phosphate-buffered saline (PBS)

15-ml or 50-ml conical tubes

1.5-ml Eppendorf tubes

Cell lysis buffer (e.g., 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM beta-glycerophosphate, and 2 μ M PUGNAc, and 1 $\times$ Protease Inhibitor Cocktail), ice cold

Probe-tip sonicator (e.g., Branson)

Urea

100 mM ammonium bicarbonate

0.5 M dithiothreitol (DTT)

0.5 M iodoacetamide (freshly prepared)

Protein standards (e.g., 20 μ g alpha-crystalline) or peptide standards undergoing the same processing as the samples)

BCA assay kit or Bradford assay kit and additional reagents and equipment for measuring protein concentration

LC-MS grade Trifluoroacetic acid (TFA)

LC-MS grade formic acid (FA)

LC-MS grade H₂O

LC-MS grade acetonitrile

Trypsin, proteomic grade (Promega)

C18 spin column (The Nest Group) or C18 Sep-Pak column (Waters)

Vacuum centrifuge or SpeedVac

Trap column

Magic C18 AQ, 5 μ m, 100 $\text{\AA}$ (Michrom Bioresources)

75 μ m $\times$ 15 cm C18 analytical column with a 15 μ m emitter

Solvent A (0.1% formic acid)

Solvent B (0.1% formic acid, 98% acetonitrile)

LTQ-Orbitrap Velos (or other mass spectrometers) coupled with nanoHPLC system

Cell culture and protein extraction

1 Culture cells of interest in appropriate medium.

Depending on experimental purposes, different cell culture conditions might be used. For example, if the experiment is designed for comparing O-GlcNAcylation of proteins in different biological status, stable isotope labeling by amino acids in cell culture (SILAC) can be performed by using media supplemented with stable isotopes (e.g., ¹³C6-L-lysine and ¹³C6-L-arginine; please refer to [Ma and Hart, 2014] for details). If the experiment is designed to identify as many O-GlcNAc proteins as possible in a cell culture, it would be helpful to treat cells with O-GlcNAcase inhibitors (e.g., 1 μ M Thiamet G for 2–4 h) to globally increase the O-GlcNAc stoichiometry of proteins.

2 Harvest the desired number of cells (e.g., 10⁸ cells). For adherent cells, aspirate the medium, wash the cells once with 10 ml PBS. After adding 5 ml PBS, scrap cells on ice and collect the cell suspensions and centrifuge at 500 g for 5 min at 500 g at 4 °C, and wash the cells again with 10 ml PBS by pipetting up and down. For suspension cells, directly centrifuge cell suspensions 5 min at 500 g at 4 °C, and then wash the cell pellet once in 10 ml of PBS.

The protein amount needed for a O-GlcNAcomic analysis is largely depending on the O-GlcNAc level as well as the sensitivity of enrichment and detection approaches. Since the O-GlcNAc stoichiometry for many proteins is quite low, we recommend to start with a higher amount of proteins (e.g., 10 mg) from cells or tissues. However, the protein amount needed can be adjusted according to the O-GlcNAc level which varies in different tissues (e.g., brain is regarded to be one of the O-GlcNAc-enriched organs) and cells (including cell type and nutritional status).

- 3 Add the cell lysis buffer to pellets, mix and incubate for 20 min at 4 °C.

There are many types of cell lysis buffers to lyse cells. And different lysis buffers may have different yields of proteins (e.g., RIPA may extract a higher proportion of hydrophobic proteins including plasma membrane proteins). According to the experimental design, cellular fractionation (e.g., cytosol/nucleus separation) can be performed. If a particular study focuses on a few O-GlcNAc proteins of interest, an appropriate buffer should be considered for the cell lysis so that specific protein(s) can be directly immunoprecipitated by antibodies in that buffer. Of note, PUGNAc (e.g., 2 uM) and/or a certain amount (e.g., 40 mM) of GlcNAc should be included in the lysis buffer to block the activity of O-GlcNAcase and lysosomal hexosaminidases during protein extraction.

- 4 Sonicate the cell suspension on ice with a Probe-tip sonicator for 20 sec (with 2 pulses for 10 sec each and a 10 sec rest between each pulse).

Sonication is a popular method to improve releasing proteins from cells. It can also be used to homogenize cells or tissues in a detergent-free lysis buffer. Alternative methods (e.g., high pressure-induced cavitation and syringe-assisted disruption) can be used to enhance protein extraction efficiency as well.

- 5 Centrifuge the cell lysates for 10–20 min at 13,000 g, 4 °C.

- 6 Transfer the supernatant to a fresh 1.5-ml tube, take an aliquot to estimate protein concentration by using BCA assay or Bradford assay.

The amount of O-GlcNAcylated proteins in the whole proteome varies among cells and tissues. Normally a large-scale experiment requires 1–5 mg total protein from cell lysate. But a higher amount of starting materials might have to be used since some tissues might have relatively lower level of O-GlcNAcylation. To test the sample preparation, it is recommended to save an aliquot of the sample for a Western blot with O-GlcNAc antibodies (e.g., the CTD 110.6 antibody) after SDS-PAGE separation.

Protein digestion

- 7 Add 4 volumes of chilled acetone to the supernatant (Step 6), keep at -80°C for at least an hour.
- 8 Spin down the precipitates at 500 g for 5 min at 4°C . Wash with chilled acetone two more times.

Other protein precipitation approaches, e.g., the chloroform/methanol method, can be used instead.

- 9 Carefully remove the liquid above the protein precipitates, leaving the cap open to allow air dry.
- 10 Resuspend the protein pellet in 8 M urea and 50 mM NH_4HCO_3 (pH 8.0), and add DTT in 50 mM NH_4HCO_3 (final concn.: 10 mM), 37°C for 30 min.

If SILAC is not used for in vivo labeling and other isotopic amine-based labeling approaches (e.g., iTRAQ, TMT) are going to be performed at the peptide level, it is recommended to replace NH_4HCO_3 with triethylammonium bicarbonate as the buffer.

- 11 Add freshly prepared iodoacetamide (final concn., 30 mM), RT for 30 min in dark.
- 12 Quench remaining iodoacetamide with the addition of the same amount of DTT in 50 mM NH_4HCO_3 (as in Step 10) at 37°C for 30 min.
- 13 Dilute to urea concentration to $<1\text{ M}$, and then add trypsin (trypsin/protein = 1/50 (w/w)), with gentle shaking at 37°C overnight.

For complete digestion of very complex samples, another dose of trypsin (trypsin/protein = 1/100 (w/w)) can be added after overnight digestion followed by incubation at 37°C for 6 h. Lys/Trypsin can be used in combination as another efficient way for digestion of protein mixtures.

- 14 Acidify the peptide solution with 10% TFA (final pH: ~ 3), and desalt with C18 column, according to the manufacturer instructions.
- 15 Dry down with a Vacuum centrifuge or SpeedVac.

If SILAC is not performed, other isotopic labeling (e.g., iTRAQ, TMT) can be carried out on the desalted peptides prepared in this step for quantification purposes (refer to manufacturer's instructions).

LC-MS/MS Analysis using CID or HCD fragmentation

- 16 Resuspend peptides in 0.1% (v/v) formic acid and filter using a 0.45 micron filter.
- 17 Load sample onto a $75\text{ }\mu\text{m}$ i.d. capillary column packed with C18.
- 18 Separate sample over a 90 min linear gradient of increasing acetonitrile at a flow rate of 200–300 nL/min into the MS source. Throughout the LC gradient,

spectral data may be recorded continuously with an MS scan followed by MS/MS scans of the most intense ions (e.g., top 10). Dynamic exclusion should also be applied to prevent repetitive selection of the same ions within a preset time.

LC-MS/MS parameters should be optimized for specific instruments and specific experimental goals.

- 19 Process the mass spectra with Proteome Discoverer 1.4 or other software.

Generally, set carboxyamidomethyl on cysteine residues as a fixed modification, and set the following as variables: oxidation on Met, deamidation on residues Asn/Gln, HexNAc on residues Ser/Thr.

SUPPORT PROTOCOL 1---- High pH RPLC

Although direct LC-MS/MS analysis is applicable to tryptic digests of relatively simple samples, fractionation is often required for complex samples (e.g., a whole cell lysate) to minimize their complexity for better O-GlcNAc detection by mass spectrometry. Amongst the multiple fractionation methods developed, high pH RPLC is a very appealing one due to its orthogonality with the conventional acidic phase RPLC separation system upstream coupled with mass spectrometers.

Materials

XBridge C18, 5 μ m 4.6 $\times$ 20 mm Guard column (Waters, Milford, MA)

XBridge C18, 5 μ m 4.6 $\times$ 250 mm Analytical column (Waters, Milford, MA)

Triethylammonium bicarbonate (TEABC stock) buffer 1.0 M, pH 8.4–8.6 (Sigma)

Solvent A: add 10 ml TEABC stock to 1 L water

Solvent B: add 10 ml TEABC stock to 1 L of 90% ACN

Agilent 1100 or other HPLC systems

1. Fractionate protein digests (e.g., peptides from Step 15 in Basic Protocol 1) on the bpRPLC system using the following chromatographic parameters:

Staged gradient range and time (as an example):

0 min 0% B

5 min 0% B

10 min from 0% B to 10% B

40 min from 10% B to 35% B

45 min from 35% B to 80% B

50 min 80% B

50.5 min from 80% to 2% B

60 min 2% B

Flow rate: 1 mL/min or 0.250 mL/min

Detection: 214 or 252 nm

Column temperature: 40 °C

System running temperature: room temperature.

2. Load digests onto the RP-HPLC system.

Check out the loading capacity of the C18 column used (up to 10 mg can be loaded to the column used in this Protocol). Do not overload the column. Appropriately small size columns can be used if the sample amounts are low.

3. Collect peptide fractions in a 96-well plate, according to a fixed time frame (e.g., 1 min).

The eluates can also be collected in tubes according to the peptide absorbance, depending on sample collector used.

4. Neutralize the collected fractions with 1% formic acid.

5. Dry down the peptide fractions in a vacuum centrifuge, and combine fractions into a smaller number of pools if less fractions are desired (e.g., 12 fractions). A combinational pooling (e.g., every 12th fraction) is recommended.

The resulting fractions can be analyzed directly by LC-MS/MS or used for enrichment followed by LC-MS/MS (e.g., Basic Protocol 2 and Basic Protocol 3)

BASIC PROTOCOL 2----O-GlcNAc SITE MAPPING BY USING ETD MASS SPECTROMETRY

Although CID/HCD mass spectrometry can be used to monitor O-GlcNAc status of peptides without enrichment, most of the low-abundant O-GlcNAcylated peptides cannot be detected due to the presence of abundant non-modified peptides. This protocol describes a method to enrich the O-GlcNAc peptides by using chemical/enzymatic labeling followed by detection with ETD, a fragmentation method that preserves the O-GlcNAc moieties on peptides (i.e., minimal fragmentation of the O-GlcNAc glycosidic bond to Ser/Thr) compared to CID/HCD. Specifically, the protocol includes several steps: 1) A mutant galactosyltransferase (GalT1)-mediated enzymatic tagging of O-GlcNAc peptides; 2) Click chemistry-based reaction with a multi-functional reagent containing functional terminal alkyne, photocleavable linker, and a biotin handle (referred to as 'PC Biotin-alkyne'); 3) release of tagged O-GlcNAc peptides via photocleavage; and 4) ETD detection. This protocol is adapted from our previous one (Ma and Hart, 2016), due to the recently commercialized PC Biotin-alkyne, one key reagent used for the O-GlcNAc enrichment procedure.

Materials

Calf intestinal phosphatase (CIP; New England Biolabs)

Peptide:N-glycosidase F (PNGase F; New England Biolabs)

TBTA (Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine, Anaspec)

tert-Butanol (Sigma)

20 mM CuSO₄

Sodium ascorbate (Sigma)

Click-iT[®] O-GlcNAc Enzymatic Labeling System (Invitrogen)

PC Biotin-alkyne (Jena Biosciences; protect from light)

SCX Column (Nest Group)

200 mM NaH₂PO₄ / 300 mM sodium acetate (pH 3.0)

5 mM KH₂PO₄, 25% ACN (pH 3.0)

5 mM KH₂PO₄, 25% ACN (pH 3.0) + 400 mM KCl

High Capacity Neutravidin Agarose (Thermo Fisher Scientific)

UV lamp (Blak-Ray Lamp, Model XX-15; UVP, Upland, CA).

LTQ Orbitrap Velos ETD mass spectrometer or other ETD-enabled mass spectrometers.

GalT1 labeling

- 1 Resuspend tryptic peptides (e.g., fractionated peptides from Support Protocol 1) in 100 µl of 10 mM HEPES with a pH of 7.9.
- 2 Add 22 µl MnCl₂, 25 µl UDP-GalNAz (from the O-GlcNAc labeling kit from Life Technologies), mix well.
- 3 Add 15 µl GalT1 (from the O-GlcNAc labeling kit from Life Technologies) and PNGase F (500 U) into the reaction mixture, pipet up and down for 10 times.
- 4 Incubate at 4°C overnight.
- 5 Add 20 U CIP (2 µl) and incubate at room temperature for 3 hours
- 6 Clean-up with a C18 column according to manufacturer's instructions.

Click chemistry and Neutravidin capture

- 7 Add 8 µl of PC-PEG-Biotin-Alkyne, 8 µl 50 mM sodium ascorbate (freshly prepared!), 22 µl 1.7 mM TBTA (in 4:1 of *tert*-butanol:DMSO) sequentially into the peptides, vortex briefly and spin down.

- 8 Add 4 μ l freshly prepared 20 mM CuSO_4 , vortex briefly and spin down.
- 9 Cover with aluminum foil and incubate overnight at room temperature.
- 10 Pre-wet the SCX column with 100% MeOH and immerse in 200 mM NaH_2PO_4 / 300 mM sodium acetate (pH 3.0); cover with Parafilm and let sit overnight at room temperature.
- 11 Spin the rest of the 0.2 M NaH_2PO_4 / 300 mM sodium acetate through.
- 12 Wash SCX column 3 μ l with 200 μ l 5 mM KH_2PO_4 / 25% ACN (pH 3.0).
- 13 Add 80 μ l 5 mM KH_2PO_4 / 25% ACN (pH 3.0) to the peptide sample.

This step and the following steps 14–20 should be performed in a dark room or other reduced light environment such as a lab with the lights off and blinds closed.

- 14 Load diluted sample (~100 μ l) onto SCX column and spin through (2000 g)–repeat once.
- 15 Elute with 130 μ l of 5 mM KH_2PO_4 , 25% ACN (pH 3.0) + 400mM KCl.
- 16 Neutralize sample with 0.2% NH_4OH (final pH 7).
- 17 Put 500 μ l high capacity neutralization agarose resin into a 15 mL conical tube; wash with 10 mL cold PBS for 5 times.
- 18 Load beads to the neutralized sample, cover with aluminum foil, and put on wheel for 2 hours at room temperature.
- 19 Wash the beads 10 $\times$ with cold PBS, 2 $\times$ with H_2O , 1 $\times$ with 20% MeOH, 1 $\times$ with 70% MeOH.
- 20 Split the beads into ~4 PCR thin-wall tubes (with a final volume of ~100–150 μ l in 70% MeOH).

UV-cleavage of O-GlcNAc-tagged peptides

- 21 Put PCR tubes ~2 in. from UV source (365 nm), rotate for 25 minutes.
- 22 Vortex briefly and pulse spin down beads, collect and pool supernatant from PCR tubes into one 1.5 mL tube.
- 23 Pulse spin down and collect supernatant a 2nd time.
- 24 Dry peptides to completion with a SpeedVac.

LC-ETD MS/MS Analysis

- 25 Resuspend the enriched peptides in 0.1% formic acid, and load onto a nano-LC-ETD MS/MS system.

It is recommend to inject 10% of the sample for an initial run to evaluate the abundance of enriched peptides. LC conditions (e.g.,

gradient and flow rate) and ETD parameters should be optimized to achieve the best separation and detection.

- 26 Open Mass Spectrometry Search Algorithm (OMSSA) or other software can be utilized to search c- and z-type fragment ions present in ETD MS/MS spectra.

The general database searching parameters include the following: product ion mass tolerance, ± 0.35 dalton (a tighter mass error window is recommended for instruments with higher mass accuracy), up to 3 missed cleavages, in addition to variable modifications [(e.g., oxidized methionine (+15.9949), alkylated cysteine (+57.0215 Da), tag on serine and threonine (+502.2024). Isotopic labeling on specific residues (e.g., light/heavy labeled arginine/lysine) should be considered as variable modifications if these are present. The quantification of proteins/peptides can be obtained by using MaxQuant, Proteome Discoverer or other software available.

BASIC PROTOCOL 3----O-GlcNAc SITE MAPPING BY USING β -elimination Michael addition with dithiothreitol (BEMAD) FOLLOWED BY CID/HCD MASS SPECTROMETRY

ETD represents an useful way for direct detection of O-GlcNAc modification sites, but it also suffers from several drawbacks: 1) ETD tends to perform well on species with >2 positive charges, 2) ETD yields a overall lower number of total identifications due to its relatively slower scan rate and lower fragmentation efficiency, and 3) its availability is still quite limited especially in comparison to the widely adopted CID/HCD mass spectrometers. To make full use of CID/HCD mass spectrometers for site mapping, one alternative approach is to convert the labile O-glycosidic linkage to a more stable bond. β -elimination Michael addition with dithiothreitol (BEMAD) is one such approach, in which O-GlcNAc is β -eliminated from the peptide backbone using very mild alkali and then dithiothreitol is added back in a Michael addition-type reaction. In addition to being more chemically stable, the resulting DTT-substitute allows corresponding peptides to be captured by thiol affinity chromatography. Several critical points with this approach are that, 1) phosphatase treatment should be performed and control peptides included, and 2) only mild BEMAD conditions should be used to minimize or eliminate potential false positive identifications. This protocol describes an O-GlcNAc site mapping procedure that combines BEMAD, thiol affinity enrichment, and CID/HCD mass spectrometry.

Materials

Basic BEMAD buffer (1.5% triethylamine, pH 12.5): 1 mL triethylamine + 40 mL H₂O, adjust pH to 12.5, bring the volume to 50 mL.

Working BEMAD buffer: 6.2 mg DTT + 1.2 mL basic BEMAD buffer (1.5% triethylamine, pH 12.5) + 400 μ L of EtOH, adjust the pH to 12.5, bring up the volume to 2 mL.

Thiopropyl-Sepharose resin (sigma)

Thiol column buffer (i.e., PBS with 1 mM EDTA, freshly prepared!)

Thiol elution buffer (i.e., PBS, 1 mM EDTA, and 20 mM DTT, freshly prepared!)

LTQ-Orbitrap Velos (or other mass spectrometers) coupled with nanoHPLC system

1. To desalted and dried protein digest (e.g., fractionated peptides from Support Protocol 1), add 100 µl of Working BEMAD buffer (synthetic O-GlcNAc peptides should be used as positive control).

Isotopic DTT (e.g., d6-DTT) can be used for quantification purposes.

2. Seal the tube and place in 50 °C for 4 h.
3. Acidify the peptide solution to ~5% TFA (the pH should be around 3), dry down.
4. Clean up the peptides with a C18 column.
5. Dry down the eluted peptides with a SpeedVac.
6. Resuspend the peptides in 100 µl thiopropyl-Sepharose resin (50% slurry in thiol column buffer).
7. Rotate for 4 h at room temperature.
8. Spin down and wash the resin 3 times with 0.5 ml of thiol column buffer and once with 40% ACN.
9. Add 100 µl of thiol elution buffer, 37 °C for 30 min, spin down, collect the supernatant.
10. Elute the peptides two more times with 50 µl of thiol elution buffer and twice with 60% ACN.
11. Acidify the eluted peptides by adding TFA to a final concentration of 1% (v/v).
12. Dry down and purify the peptides using a C18 column.
13. Dry down the peptides in a SpeedVac.
14. Resuspend peptides in 0.1% (v/v) formic acid.
15. LC-CID/HCD MS/MS (as shown in Basic Protocol 1).

For the enriched peptides, set oxidation (Met), d0-/d6 DTT (Ser/Thr/Cys), and carboamidomethylation (Cys), and deamidation (Asn/Gln) as variable modifications.

COMMENTARY

Background Information

Since its discovery ~30 years ago (Torres and Hart, 1984; Holt and Hart, 1986), protein O-GlcNAcylation has drawn increasing attention. As a highly dynamic and reversible post-translational modification, O-GlcNAcylation of proteins is an important nutrient-sensing regulatory mechanism for orchestrating various biological processes (Hart, 2014; Bond and

Hanover, 2015). While traditional studies in biochemistry and molecular biology have led to discovery of thousands of O-GlcNAc events in eukaryotic systems, our understanding of protein O-GlcNAcylation at a proteomic level is far from complete (Ma and Hart, 2014). From a proteomics perspective, there are two major bottlenecks that impede O-GlcNAcomic profiling: efficient enrichment and sensitive O-GlcNAc detection.

Enrichment of O-GlcNAcylated proteins/peptides—O-GlcNAc is generally substoichiometric. Moreover, severe ion suppression occurs with the presence of non-modified peptides. There are two major categories of enrichment methods: direct capture and chemical tagging. Generally, antibodies are the primary choice for the enrichment of post-translationally modified peptides. However, traditional O-GlcNAc specific antibodies (e.g., CTD 110.6) only show limited affinity when pulling down O-GlcNAc proteins/peptides (Wang et al., 2007; Zachara et al., 2011b). Several newly developed antibodies provide some promise for enhanced O-GlcNAc enrichment efficiency (Teo et al., 2010; Zhao et al., 2011), but their applicability toward complex samples needs to be further verified. Lectin capture (especially wheat germ agglutinin), a tool that is commonly used in the glycobiology field, has proven to be a useful method for the enrichment of O-GlcNAc peptides (Vosseller et al., 2006; Trinidad et al., 2012; Nagel et al., 2013), although the specificity and selectivity could be improved. In contrast to these direct capture strategies, chemical tagging has evolved as another promising approach. By using intracellular salvage pathways, feeding cells with sugar analogs (e.g., azido-substitute Ac4GlcNAc) provides another way to facilitate O-GlcNAc tagging (Vocadlo et al., 2003; Nandi et al., 2006; Boyce et al., 2011; Hahne et al., 2013). However, the efficiency of these *in vivo* labeling methods is poor since natural donor substrates are preferred by OGT. In spite of the relatively inert chemistry of the O-GlcNAc moiety, *in vitro* GalT1-mediated enzymatic labeling offers another convenient approach for the efficient ‘activation’ of O-GlcNAc peptides (Khidekel et al., 2004, Khidekel et al., 2007, Wang et al., 2010a, Wang et al., 2010b, Parker et al., 2011, Alfaro et al., 2012). These tagging techniques enable facile enrichment with finely designed chemical reactions (e.g., via the highly efficient and orthogonal Copper-catalyzed azide-alkyne cycloaddition (CuAAC or click chemistry)).

Detection of O-GlcNAcylated peptides—O-GlcNAc detection was challenging for a very long time and has largely benefited from the rapid advances in the field of modern mass spectrometry in recent years. CID/HCD mass spectrometry provides a series of diagnostic ions, indicating the presence of O-GlcNAc on peptides. By replacing the labile O-GlcNAc group with relatively stable DTT on peptides, BEMAD offers an indirect way to detect O-GlcNAc sites with commonly used CID/HCD mass spectrometers, although appropriate controls must be included to avoid unwanted side reactions (Wells et al, 2002; Vosseller et al, 2005; Wang et al, 2009; Ma et al, 2015; Ramirez-Correa et al., 2015). As a technology breakthrough, ETD mass spectrometry affords a direct way to detect O-GlcNAc modification sites (Syka et al., 2004; Chalkley et al., 2009b). Of note, with the further development of mass spectrometers, combined mass spectrometry techniques (e.g., HCD/ETD) offer even better O-GlcNAc characterization since they can provide complementary information (Zhao et al., 2011).

Critical Parameters and Troubleshooting

The amount of O-GlcNAcylated proteins in the whole proteome varies among cells and tissues. Western blotting with O-GlcNAc specific antibodies (e.g., CTD 110.6) can be used as the 1st step to determine the O-GlcNAc level of a sample. Normally a large-scale O-GlcNAcomic experiment requires milligrams of total protein from cell lysate or tissue homogenate for O-GlcNAc enrichment followed by site mapping and quantification (e.g., Basic Protocol 2 and Basic Protocol 3). Moreover, inhibitors (e.g., PUGNAc) should be included into the lysis buffer to block the activity of O-GlcNAcase and lysosomal hexosaminidases during protein extraction.

Depending upon research goals (identification and/or quantification), different experimental strategies can be designed by combining the available enrichment techniques and mass spectrometers. Since there is not a single analytical method that can fit all our needs, new methods (especially enrichment methods) still need to be developed. Peptide fractionation (e.g., by high pH reverse phase HPLC), which could be performed on pre-enriched or post-enriched samples, represents an effective way to decrease the sample complexity, enabling better detection of low abundant O-GlcNAc peptides. Although we did not describe the isotopic labeling methods (e.g., SILAC, iTRAQ, and TMT) in detail herein, these can be applied for the quantification of O-GlcNAc proteins/peptides in a straightforward way as per the manufacturer's instructions.

Of note, many of the techniques described in this Unit may recognize any GlcNAc residue, and it is important to perform the described controls, such as PNGase F digestion, to demonstrate specificity. Moreover, although mass spectrometry itself cannot distinguish O-GlcNAc and O-GalNAc, highly specific enrichment approaches (e.g., by using GalT1 labeling) provides a definitive criteria for the identification of O-GlcNAcylation on proteins/peptides. In addition, immunoprecipitation of specific proteins followed by O-GlcNAc Western blotting offers another way for further confirmation of the O-GlcNAc status of proteins of interest.

Anticipated Results

Typical mass spectra by using the Basic Protocol 1, Basic Protocol 2, and Basic Protocol 3 are shown in Figure 3, Figure 4, and Figure 5, respectively. The protocols described herein have been successfully applied to confirm the O-GlcNAc status of proteins, identify and/or quantify O-GlcNAc proteins in a number of biological settings. These methods should serve as a good starting point for any global analysis of the O-GlcNAcome. However, the actual number of O-GlcNAc peptides identified in any given experiment can be affected by several factors including O-GlcNAcylation level, sample amount, enrichment method and the mass spectrometer used (as mentioned above). Higher amounts of starting materials, efficient enrichment techniques, and recently introduced mass spectrometers (e.g., Orbitrap Fusion Lumos) will yield better results. Of special note is that, large scale characterization of protein O-GlcNAcylation is not a simple task, one reason is that O-GlcNAc enrichment is quite technically challenging since intensive experience is often needed (especially for the chemical reactions adopted in Basic Protocol 2 and Basic Protocol 3). Protein level enrichment followed by peptide level enrichment might be beneficial for improved O-

GlcNAc identification. Although many methods have been developed for O-GlcNAc enrichment, highly robust and efficient approaches are still required. Last but not least, fractionation by chromatographic methods (e.g., high pH RPLC) is a useful way to reduce sample complexity, which should be used in combination with enrichment techniques.

Time Considerations

Basic Protocol 1 should take about 3–4 days or even weeks (if SILAC is applied) for cell culture; 1 to 2 days for cell lysis or tissue lysis, and protein extraction, depending on the number of samples; another day for protein digestion and cleanup for mass spectrometric analysis. Support Protocol 1 should take 1 day to finish peptide fractionation. Either Basic Protocol 2 or Basic Protocol 3 should take 2–3 days to complete enrichment of O-GlcNAc peptides, while the mass spectrometry time might vary depending on the optimization process and the number of samples. For people who have not used these techniques before, it is recommended to try out the methods on control peptides/proteins first and then move on to their favorite proteins and complex samples.

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Figure 1.

Schematic illustration of protein O-GlcNAcylation. The GlcNAc group is transferred by O-GlcNAc transferases (OGT) from UDP-GlcNAc to the side chain of a Ser/Thr residue, leading to a covalent chemical modification on target proteins. O-GlcNAcase reverses the O-GlcNAcylation process by removing GlcNAc from proteins.

	Oxonium ion (m/z)	Description	Formula
1	126.05	HexNAc-several functional groups	C ₆ H ₈ O ₂ N
2	138.05	HexNAc-several functional groups	C ₇ H ₈ O ₂ N
3	144.06	HexNAc-several functional groups	C ₆ H ₁₀ O ₃ N
4	168.06	HexNAc-2H ₂ O	C ₈ H ₁₀ O ₃ N
5	186.07	HexNAc-H ₂ O	C ₈ H ₁₂ O ₄ N
6	204.08	HexNAc	C ₈ H ₁₄ O ₅ N

Figure 2. Fragmentation patterns of HexNAc (denoted with dashed gray lines), as observed in CID/HCD (especially HCD which gives more prominent fragmentation of HexNAc) mass spectra (with the characteristic oxonium ions shown in the insert table).

B

C

Figure 3. CID (A) and HCD (B) spectra of standard O-GlcNAc modified peptide PGGSTPVSSANMM. Note: “–HexNAc” or “–H₂O” indicates the loss of HexNAc or H₂O. Zoom-in of the low m/z range HCD (C) shows the typical pattern of HexNAc fragments. (Adapted from Zhao et al., 2011, with the permission from American Chemical Society).

Figure 4.

ETD spectra of standard O-GlcNAc modified peptide YSPTgSPSK (where 'gS' represents the O-GlcNAc modified Ser) by using the PC-Biotin enrichment approach. (Adapted from Wang et al., 2010).

Figure 5.
HCD (B) spectra of O-GlcNAc modified peptide IASQVAALDLGYKPGVEAIR (where ‘S’ represents the DDT-substituted Ser) by using the refined BEMAD approach. (Adapted from Ma et al., 2015).