

Video Article

Improved In-gel Reductive β -Elimination for Comprehensive O-linked and Sulfo-glycomics by Mass Spectrometry

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Abstract

Separation of proteins by SDS-PAGE followed by in-gel proteolytic digestion of resolved protein bands has produced high-resolution proteomic analysis of biological samples. Similar approaches, that would allow in-depth analysis of the glycans carried by glycoproteins resolved by SDS-PAGE, require special considerations in order to maximize recovery and sensitivity when using mass spectrometry (MS) as the detection method. A major hurdle to be overcome in achieving high-quality data is the removal of gel-derived contaminants that interfere with MS analysis. The sample workflow presented here is robust, efficient, and eliminates the need for in-line HPLC clean-up prior to MS. Gel pieces containing target proteins are washed in acetonitrile, water, and ethyl acetate to remove contaminants, including polymeric acrylamide fragments. O-linked glycans are released from target proteins by in-gel reductive β -elimination and recovered through robust, simple clean-up procedures. An advantage of this workflow is that it improves sensitivity for detecting and characterizing sulfated glycans. These procedures produce an efficient separation of sulfated permethylated glycans from non-sulfated (sialylated and neutral) permethylated glycans by a rapid phase-partition prior to MS analysis, and thereby enhance glycomic and sulfoglycomic analyses of glycoproteins resolved by SDS-PAGE.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51840/>

Introduction

Glycosylation is an essential protein post-translational modification, contributing to organismal physiology, tissue pathology, and cellular recognition¹⁻³. Despite major advances in analytical glycoscience, characterizing the complete diversity of glycans on a specific protein remains an extremely challenging task, especially on proteins isolated from primary biological sources. Nonetheless, the microheterogeneity of glycoprotein glycans frequently affects functional interactions with other proteins. Therefore, characterization of glycan diversity is essential for understanding the physiological significance of cellular and tissue glycosylation^{4,5}. In order to understand the contribution of glycoprotein glycosylation to tissue physiology and pathophysiology, robust, sensitive, and comprehensive glycomic analytical techniques have become increasingly important. In proteomic analysis, protein identifications are generally achieved by LC-MS/MS analysis of tryptic peptides⁶. Protein digestion can be carried out using a purified protein or proteins resolved by SDS-PAGE following in-gel digestion with proteases such as trypsin⁷⁻⁹. Pre-enrichment of the protein mixture by SDS-PAGE enhances the depth and accuracy of protein ID. The development of analogous strategies for glycomic analysis of glycoprotein glycosylation lies at the forefront of glycoscience.

The two major classes of glycans are attached to protein backbones through either N-linkage or O-linkage. N-linked glycans are attached to asparagine (Asn) residues found as part of a sequon defined as Asn-X-Ser/Thr/Cys (X is any amino acid except proline), and can be released by enzymatic digestion with peptide-N-glycanase (PNGaseF or A), either in solution, in-gel, or on-blot¹⁰⁻¹². O-linked glycans are mainly attached to serine (Ser) or threonine (Thr) residues. However, only one enzyme has been identified that is capable of releasing O-linked glycans from glycoprotein and it has an extremely limited glycan specificity, releasing only the simplest O-linked glycans. Chemical release strategies remain the method of choice for comprehensive release of O-linked glycans from glycoproteins. Reductive or non-reductive β -elimination, or hydrazinolysis are well-characterized chemical release techniques and are currently the most commonly used approaches for releasing O-linked glycans from glycoproteins^{13,14}. Although reductive β -elimination has been used to release O-linked glycans from glycoproteins separated by SDS-PAGE, previous approaches required HPLC separation for subsequent analysis¹⁵⁻¹⁷.

Multidimensional MS (MSⁿ) analysis currently provides the richest source of structural data for characterizing glycans released from glycoproteins isolated in the amounts expected from most biological sources. The depth of MS-based structural characterization is greatly facilitated by permethylating the released glycans prior to their analysis. Permethylation enhances ionization and tends to equalize molar signal responses across a broad range of glycan structures^{18,19}. In addition, permethylation unambiguously tags terminal and substituted monosaccharide moieties with distinctive masses, thereby enhancing structural elucidation²⁰⁻²³. For example, acidic glycans are generally

difficult to detect as non-permethylated species by MS. Although acidic glycans can be detected in negative ion mode by MS, it is impossible to detect both acidic and neutral glycans in the same ion mode. A major advantage of glycan permethylation is that all of the free hydroxyl groups (OH) on a glycan's monosaccharide substituents will be capped with a methyl group (OCH₃ or OMe), thus a sialylated glycan's charges are neutralized, making them as detectable as permethylated neutral (asialo) glycans. However, the hydroxyls of sulfate moieties on sulfoglycans are resistant to permethylation, resulting in retention of anionic charge, which suppresses ionization and decreases sensitivity. This suppression currently prevents comprehensive glycomic analysis of very complex glycoproteins such as mucins, which carry a high abundance of sulfated glycans²⁴⁻²⁶.

Recent reports on purifying sulfated glycans used charged, reverse-phase chromatography to purify and separate permethylated glycans prior to MALDI analysis. This method relies on complete separation of sulfated and non-sulfated glycans using different mobile phases for elution, which we have found to be less stringent than organic phase partitioning. Therefore, new techniques suitable for the detection and enrichment of sulfoglycans are presented here. These techniques allow for the quantitative recovery of sulfated glycans in the aqueous phase following water:DCM (dichloromethane) extraction, which is routinely performed at the end of glycan permethylation reactions²⁷. Importantly, this robust separation of permethylated sulfoglycans from a mixture of permethylated non-sulfated glycans concomitantly enriches for charged species while also simplifying MS² fragmentation patterns. A comprehensive protocol for improved in-gel O-linked glycan analysis is also presented. The improved protocol enhances glycan recovery, increases the structural information obtainable through MSⁿ analysis of permethylated glycans, and improves the sensitivity of sulfoglycomic analyses applied to essential glycoproteins isolated from biological sources.

This protocol is intended for O-linked glycan analysis of whole glycoprotein extracts or of a specific glycoprotein of interest resolved by SDS-PAGE and is composed of three experimental procedures; A) gel clean-up, B) in-gel reductive β -elimination, and C) glycan permethylation. The goal is to obtain comprehensive O-linked glycomic data for glycoproteins harvested from primary sources of biological interest (**Figure 1**). Glycoproteins separated by SDS-PAGE are visualized by staining and bands of interest are excised and the resulting gel band is sliced into small pieces. The gel pieces are destained and subjected to ethyl acetate washes to remove gel contaminants (**Figure 2A**). Glycan release is achieved by in-gel reductive β -elimination (**Figure 2B**) and the released glycans are permethylated. Aqueous-organic extraction following permethylation quantitatively partitions the anionic sulfated glycans away from non-sulfated neutral glycans (**Figure 2C**). In-gel reductive β -elimination coupled to aqueous-organic extraction enables the characterization of O-linked glycans and sulfoglycans released from small amounts of glycoprotein separated by SDS-PAGE. The strategic overview is summarized in **Figure 1** and the details are shown in **Figure 2**. In addition, a portion of the destained and washed gel pieces can be used for protein ID by standard LC-MS/MS proteomic techniques.

Protocol

NOTE: Lab Safety Concerns

In keeping with standard laboratory best practices, observe the following. Store all organic solvents in appropriate locations. Keep all waste materials in chemical waste containers with clear labeling of chemical compositions. As several reagents used in these protocols are potential carcinogens or generate volatile combustible gases, handle all reagents in a fume hood with ventilation. Wear personal protective equipment such as gloves, lab coat, and eye protection when working with organic solvents.

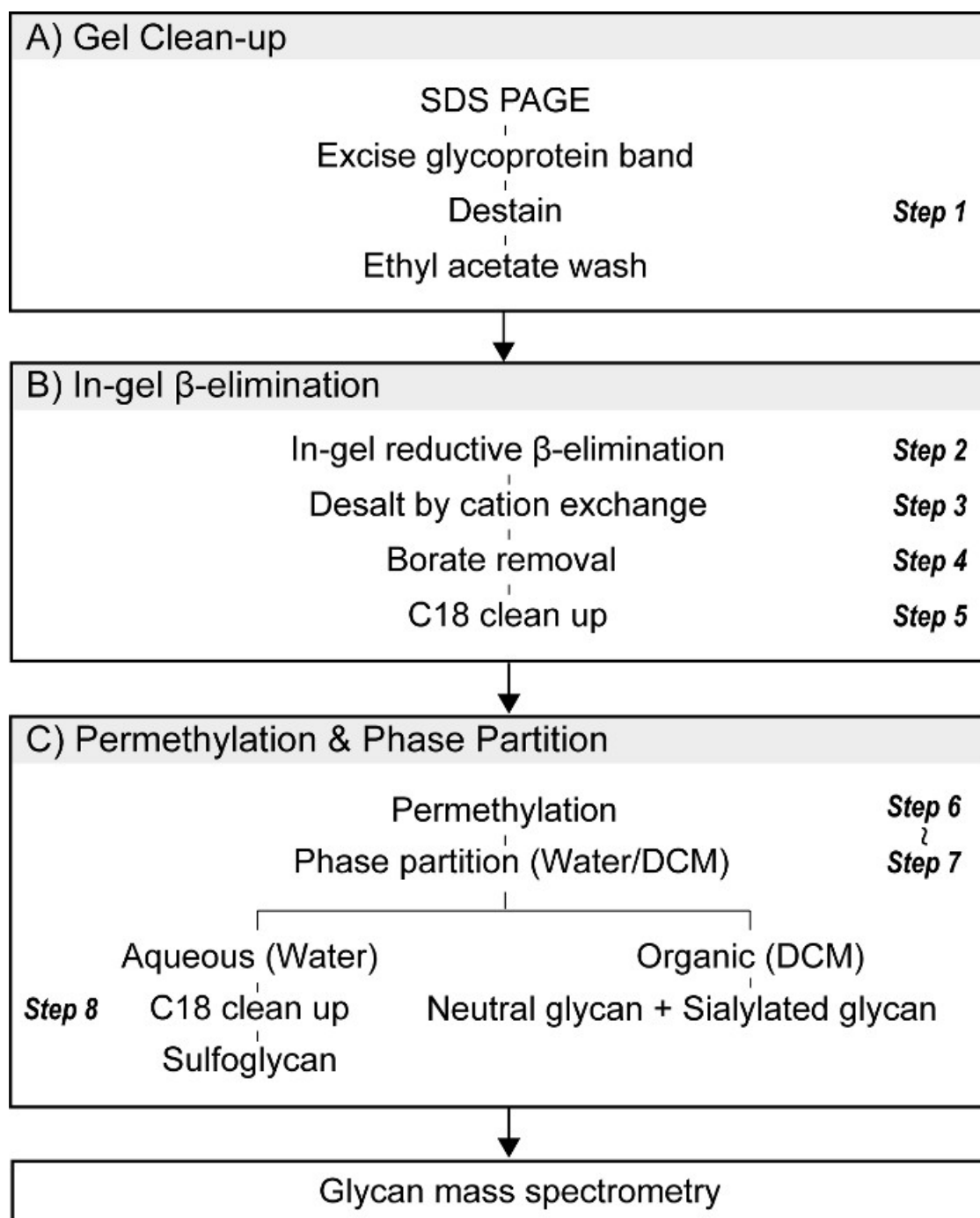


Figure 1. Procedure for in-gel O-glycomics. **(A)** Proteins of interest are resolved by SDS-PAGE, detected by appropriate staining procedures (Coomassie or silver), and excised. Excised gel pieces are sliced into small cubes, destained, and washed with ethyl acetate to reduce contaminants that interfere with subsequent MS analysis. A portion of the gel slice can be reserved for in-gel tryptic digestion and subsequent proteomic characterization by LC-MS/MS. **(B)** O-linked glycans are released from resolved glycoproteins by in-gel reductive β -elimination. Essential steps include desalting and borate removal by azeotrope with methanol. **(C)** O-linked glycans released by reductive β -elimination are permethylated and subsequently partitioned into aqueous and organic phases. Sulfoglycans are quantitatively recovered in the upper (aqueous) phase while neutral and sialylated glycans partition into the lower (DCM) layer.

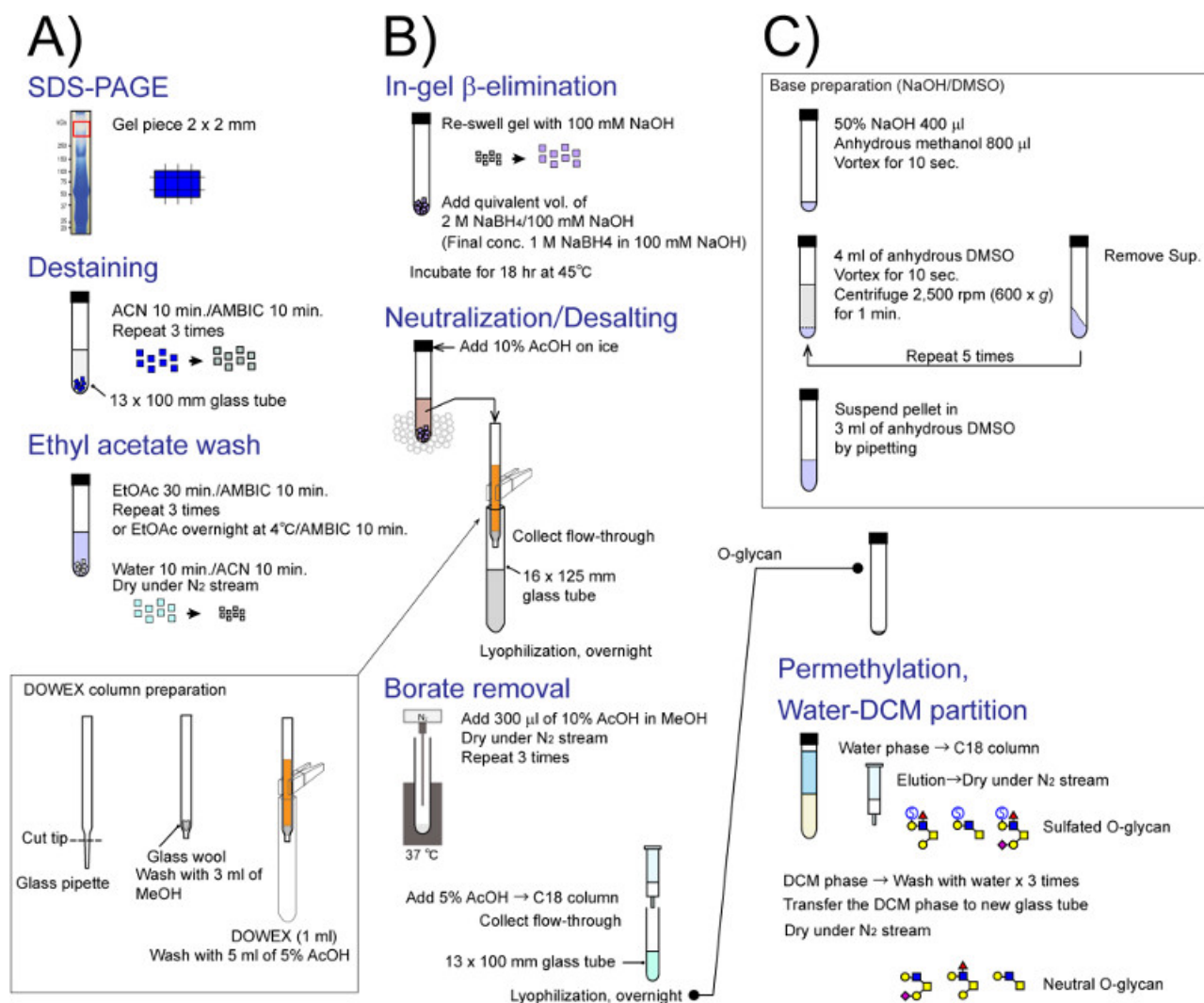


Figure 2. Detail flow chart for in-gel glycomics. Each experimental step shown in **Figure 1** is individually illustrated. **(A)** gel destaining and EtOAc extraction, **(B)** direct in-gel reductive β-elimination for O-linked glycan release, and **(C)** permethylation and phase partition. [Please click here to view a larger version of the figure.](#)

1. Gel Excision and Removal of Gel-derived Contaminants

- Before starting the procedure, prepare chemicals and reagents listed in Materials List.
- Resolve protein on SDS-PAGE gel using standard Tris-Glycine buffer systems. Stain resolved proteins with either Coomassie Brilliant Blue or Silver Stain. In general, recovery of O-linked glycans from silver stained gels is approximately 80–90% of the recovery achieved from Coomassie stained gels.
- After electrophoresis, place the gel on a glass plate and excise the region of interest using a clean scalpel or razor blade.
- To increase yield of O-glycans, transfer the gel band of interest onto another glass plate and cut the excised gel piece into approximately 2 mm cubes. Avoid making gel pieces smaller than 1 mm cubes because glycan recovery is reduced. Transfer the small gel pieces into a screw top glass tube (13 x 100 mm) with a stainless steel microspatula.
- Add 1 ml of 25 mM ammonium bicarbonate (AMBIC, NH₄HCO₃) to the sample tube, cap the glass tube with a Teflon-lined screw top cap, and gently mix by flicking the tube before letting stand for 10 min. Do not employ vigorous agitation in order to avoid ripping the gel pieces.
- Carefully remove AMBIC from the glass tube using either a Pasteur glass pipette or a pipette equipped with an extra-long plastic tip. Avoid smashing and transferring the small gel pieces while removing the AMBIC solution.
- Add 1 ml of acetonitrile (ACN) to the glass tube, cap, and gently mix by flicking tube before letting stand for 10 min. Ensure that the gel pieces are completely covered with solvent. If necessary, push gel pieces off of tube wall and into solvent with pipette tip.
- Pipette off ACN from the glass tube and repeat steps 1.5–1.7 until the bright blue color is eliminated. Repeat a minimum of five sequential AMBIC/ACN washes if necessary. For silver stained gels, use a destain kit. Proceed to the next step when the gel piece turns uniformly white while in ACN.
- Pipette off any liquid from the glass tube, recap the tube, add 1 ml of AMBIC and let stand for 5 min.
- Remove AMBIC and add 2 ml of ethyl acetate (EtOAc) to the glass tube. Recap tube with Teflon-lined cap and place at 4 °C overnight with end-over-end agitation or alternatively perform three EtOAc washes at room temperature for 30 min each. The longer the EtOAc wash, the more efficient the removal of contaminants.

11. Remove the final EtOAc wash and perform three washes each with 2 ml of H₂O. Complete removal of EtOAc will ensure a robust reductive β -elimination reaction.
12. Pipette off H₂O and dehydrate the gel by washing once with 1 ml of ACN.
NOTE: After drying, the dehydrated gels are ready for in-gel β -elimination. Dehydrated gel pieces can be stored at -20 °C until use.
13. Stop the washing process at any step and store the gel pieces at 4 °C overnight, regardless of which buffer or solution is applied to the gel (AMBIC, ACN, H₂O). Restart sample preparation anytime within the next 2 days.
14. Use a portion of the destained and dehydrated gel pieces for protein ID by standard proteomic approaches.

2. In-gel O-glycan Release by Reductive β -Elimination

1. Prepare a stock solution of 1 M sodium hydroxide (NaOH) using 50% NaOH or solid NaOH and store at 4 °C until use.
 1. Take 5.2 ml of 50% NaOH (or 4 g of solid NaOH) and adjust volume to 100 ml to make 1 M NaOH solution. Dilute the 1 M NaOH stock solution to 100 mM NaOH, which can be stored at 4 °C up to 6 months without loss of efficiency in reductive β -elimination reactions.
2. Make 2 M sodium borohydride (NaBH₄) in 100 mM NaOH. Dissolve 38 mg of NaBH₄ in 0.5 ml of 100 mM NaOH. Generally, use 0.5–1.0 ml of the borohydride solution for each sample. Vary the volume of the reaction solution on the amount of dried gel pieces prepared in step 1. Expect to use 0.5 ml of borohydride solution for 1–2 excised gel pieces (step 1.3).
3. Add 500 μ l of 100 mM NaOH to the dehydrated gel pieces and let stand for 3–5 min on ice in order to equilibrate the gel to the basic conditions which enhance glycan recovery.
4. Add 500 μ l of 2 M NaBH₄ in 100 mM NaOH, resulting in final concentrations of 1 M NaBH₄ in 100 mM NaOH. Gently mix the tube and incubate at 45 °C for 18 hr.
 1. During the first hour of incubation, gently mix the tube every 15 min. Avoid strong agitation, because it may fragment the gel pieces. Perform the reaction in a well-equilibrated incubator/oven or in a heating block. Ensure that the gel pieces are covered with reaction solution.
5. Stop the reaction by removing the sample tube from the incubator or heating block and placing it on ice.
NOTE: After allowing the β -elimination reaction to proceed for 18 hr, the desalting process must be performed within the same day.

3. Desalting on Cation-exchange Chromatography

1. While maintaining the sample tube on ice to prevent excessive heat formation, slowly add 10% acetic acid (AcOH) dropwise to neutralize the base. Gently vortex the sample tube between additions of AcOH. Be careful to add acid slowly and drop wise as the addition of acid will produce a “volcano” of bubbles. Centrifuge the tube to eliminate bubbles and prevent spillover, if necessary.
 1. In order to remove the large amount of sodium in the reaction mixture, pass the reaction mixture through a small cation-exchange column (Dowex or AG-50W-X8 resin, H⁺ form).
 2. Wash the cation exchange resin with 1 M NaOH and 1 M HCl prior to use in order to remove contaminants that interfere with MS analysis, even if the resin is of analytical grade.
 3. Soak the resin in 1 M NaOH and remove solution by decantation. Add de-ionized water, remove water, then add 1 M HCl.
2. Repeat these steps until the solution above the resin is colorless. Wash the cleaned resin with deionized water and store in 5% AcOH at 4 °C.
3. To make a small glass column, scratch and break the tip of a Pasteur glass pipette using a ceramic cutter so that the taper of the pipette tip is approximately 1 cm long. Tease apart a plug of glass wool and push towards the tip forming a support for the resin bed. Secure the pipette column with a clamp or clothespin and place over a glass test tube.
4. Wash the empty pipette with 1 ml of methanol (MeOH) and 3 ml of 5% AcOH. Swirl H⁺ Dowex or AG50 cation exchange resin slurry stored in 5% AcOH and transfer enough of the slurry to produce a 1 ml bed volume in the Pasteur pipette column.
5. Rinse the column using five volumes of 5% AcOH. Check flow through for the appearance of resin particles and do not use the column if resin is detected.
6. Place column over a new screw top glass tube (16 x 125 mm) and load sample. Collect the flow through and elute glycans with at least 3 volumes of 5% AcOH into the same tube.
7. Cover the tube with Parafilm and make tiny holes with a needle. Place the sample tube at -80 °C or on dry ice. Once frozen, dry the sample by lyophilization. Store the dried material at -20 °C until use.

4. Borate Removal

1. Prepare 10% AcOH in MeOH. Take 10 ml of glacial acetic acid and adjust volume to 100 ml with methanol. Store this reagent in a glass bottle with Teflon-lined cap for up to 6 months.
2. Add 300 μ l of 10% AcOH in MeOH to the dried, lyophilized sample tube and vortex. Remove the resulting trimethyl borate by evaporation under a N₂ stream.
NOTE: Mixing borate salts with methanol under acidic conditions produces trimethyl borate which is a volatile compound.
3. Dry under N₂ stream at 37 °C. After drying, add another aliquot of AcOH in MeOH as described in step 4.2. Dry the liquid for 5 min under nitrogen (N₂) stream at 37 °C.
 1. Repeat resuspension and drying at least 3 times. Store the dried material at -20 °C until use.

5. C18 Clean-up

1. Equilibrate a C18 cartridge column (size 1 ml, 100 mg resin) using three volumes of ACN and five volumes of 5% AcOH. Accelerate passage of the equilibrating solutions by application of mild positive air pressure.

2. Add 500 μ l of 5% AcOH to the desalted and borate-free sample tube and dissolve by vortex.
3. Load resuspended sample onto the equilibrated C18 column and collect the flow through into a glass screw cap tube (13 x 100 mm). Elute O-glycans with 3 ml of 5% AcOH into the same tube.
4. Parafilm the tube, make small holes with a small needle or use loosely closed PTFE-lined cap to cover the tube, and freeze the sample at -80 °C or on dry ice. Remove solvent by lyophilization. Store the dried sample at -20 °C until use.

6. Base Preparation for Permethylation

NOTE: In order to achieve robust permethylation, prepare NaOH slurry fresh. All glassware used for permethylation reactions should be extensively cleaned.

1. To prepare the base reagent for permethylation, add 400 μ l of 50% NaOH to a clean glass screw-top tube (13 x 100 mm). Add 800 μ l of anhydrous MeOH and vortex.
2. Add 4 ml of anhydrous dimethyl sulfoxide (DMSO) and vortex to generate a white precipitate.
3. Centrifuge at 600 x g for 1 min to pellet the precipitate. Pipette off supernatant and discard. Add an additional 4 ml of anhydrous DMSO to the pellet. Vortex.
4. Repeat step 6.2 and 6.3 a minimum of three more times or until no white precipitate forms.
5. Dissolve the pelleted base in 3 ml anhydrous DMSO and gently mix by pipetting up-and-down with a clean Pasteur pipette.
6. Use the base immediately for the following permethylation reaction as it cannot be stored.

7. Permethylation of Released Glycans

1. Add 200 μ l of anhydrous DMSO to the C18-purified sample and vortex or sonicate to resuspend.
2. Add 300 μ l of the resuspended base slurry to the sample and immediately add 100 μ l of iodomethane (MeI). Seal tube with Teflon-lined cap and vigorously mix for 5 min by vortex.
3. To stop the permethylation reaction, add 2 ml of 5% AcOH on ice and vortex. Pipette solution up and down 5 times to reduce the remaining volume of MeI by evaporation.
NOTE: The AcOH neutralizes the solution and removal of MeI increases the extraction efficiency of sulfated glycans into the water phase during the phase-partition.
4. Add 2 ml of dichloromethane (DCM) and vortex. Centrifuge at 600 x g for 1 min at room temperature to separate the aqueous and organic phases.
5. Transfer the top layer (aqueous phase that contains the majority of permethylated sulfated glycans) into a new glass tube (13 x 100 mm).
6. Add 2 ml of H₂O to the organic phase and vortex. Centrifuge at 600 x g for 1 min to separate the aqueous and organic phases and combine this second aqueous phase with the first aqueous phase (step 7.5).
7. Repeat step 7.6 and 7.5 three more times, but there is no need to save the aqueous phases resulting from the subsequent partitions.
8. Remove the final top layer (aqueous phase) as much as possible from the bottom layer (organic phase) and transfer the bottom layer (organic phase) into a separate new glass tube using clean Pasteur pipette.
9. Dry the organic phase under N₂ stream at 42 °C.
10. Cover the tube with Parafilm and store at -20 °C until use.

8. C18 Clean-up of Permethylated Sulfated O-glycans from the Aqueous Phase

1. Equilibrate a C18 cartridge column with three volumes of ACN and five volumes of 5% AcOH.
2. Load the aqueous phases collected and combined from steps 7.5 and 7.6 onto the column.
3. Wash the column with 10 ml of H₂O for desalting.
4. Elute the permethylated sulfated O-glycans into a new glass tube with 2 ml of 50% ACN. Use an additional elution with 85% ACN to enhance recovery of permethylated sulfated O-glycans on larger oligosaccharides.
5. Dry eluates under nitrogen stream at 42 °C.
6. Store the dried material at -20 °C up to 6 months prior to MS analysis.

9. Mass Spectrometry of Permethylated O-glycans

1. Analyze permethylated O-glycans by direct infusion into an appropriate mass spectrometer using a nanoelectrospray source at a syringe flow rate of 0.40–0.60 μ l/min at 210 °C capillary temperature. For fragmentation by CID in MS/MS and MSⁿ of an ion trap instrument, apply 30–40% collision energy.
2. For negative ion mode, reconstitute permethylated sulfated O-glycans in 50 μ l of methanol/2-propanol/1-propanol/13 mM aqueous ammonium acetate (16:3:2 by volume). For positive ion mode, reconstitute permethylated neutral/sialylated O-glycans in 50 μ l of 1 mM sodium hydroxide in methanol/water (1:1).
3. Use the total ion mapping (TIM) and neutral loss scan (NL scan) functionality of the Xcalibur software package version 2.0 for MSⁿ analysis to characterize individual O-glycan structures^{13,19}.

Representative Results

Effect of Ethyl Acetate Treatment Prior to In-gel Reductive β -Elimination

A representative mass spectrum of permethylated O-linked glycan samples released from bovine mucin using in-gel reductive β -elimination is shown in **Figure 3**. The EtOAc wash of the gel pieces effectively removes SDS and polyacryl contaminants which interfere with subsequent MS analysis²⁷.

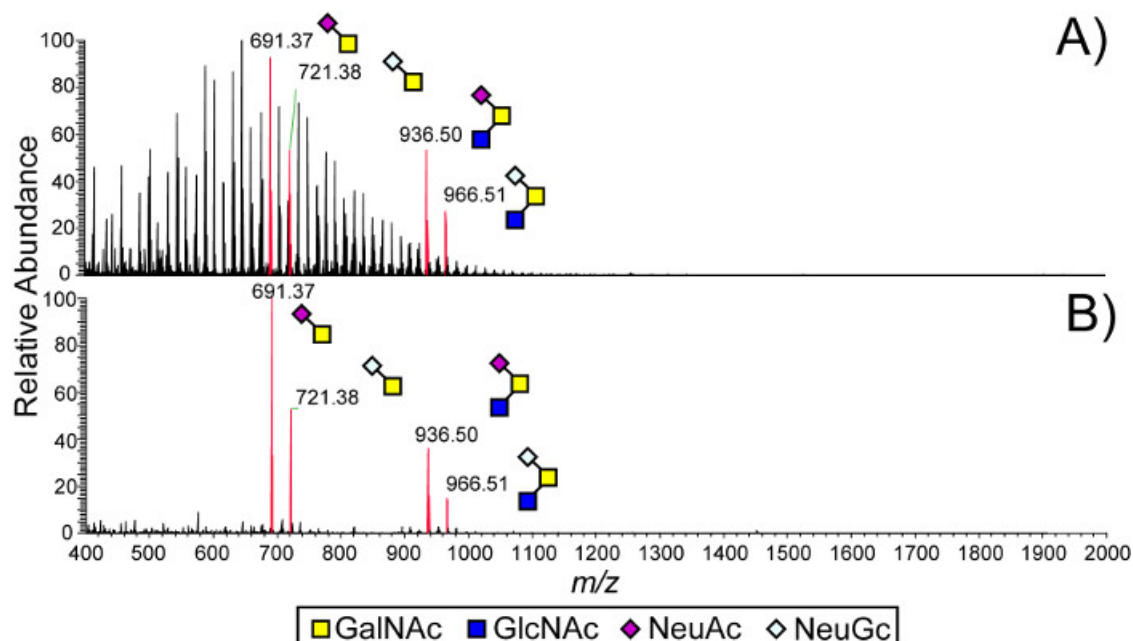


Figure 3. Ethyl acetate wash of polyacrylamide gel piece prior to in-gel reductive β -elimination enhances detection of released glycans. (A) Without wash with ethyl acetate, the mass spectrum of permethylated O-linked glycans released by in-gel β -elimination from bovine submaxillary mucin is dominated by an abundance of a polydisperse contaminant, which almost completely obscures the MS signals associated with O-linked glycans. (B) Washing the gel piece with ethyl acetate eliminates the contaminant peaks, allowing sensitive detection of glycans. Modified from Kumagai²⁵.

Recovery of O-linked Glycan is Greater from Small Gel Slices

O-linked glycans were released from bovine submaxillary mucin by in-gel reductive β -elimination using gel pieces that were either sliced small ($\sim 2 \times 2$ mm) or large ($\sim 5 \times 5$ mm). Gel pieces smaller than $\sim 2 \times 2$ mm were not efficiently recovered through the washing steps. Following permethylation, a known amount of a permethylated external glycan standard (maltotri- and maltotetrasaccharide, Dp3 and Dp4) was added to each to facilitate quantification of glycan recovery²⁷. The recovery of O-linked glycans from small gel pieces was almost 10-fold greater than from large gel slices (**Figure 4**).

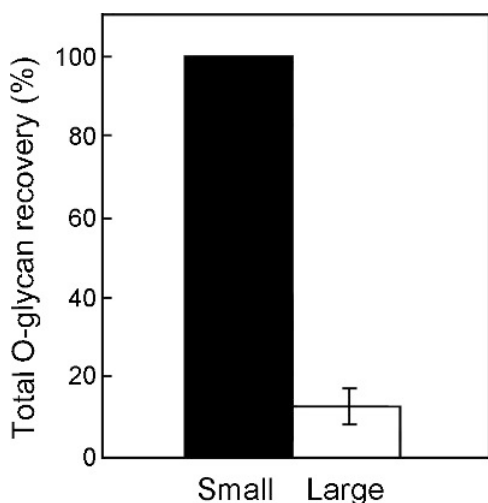


Figure 4. Glycan recovery from small gel pieces is more efficient than from larger pieces. Small pieces ($\sim 2 \times 2$ mm) yielded more glycan than larger cubes ($\sim 5 \times 5$ mm). Bars show relative signal intensities for the sum of all O-linked glycan structures normalized to the signal detected for an external standard (maltotrisaccharide Dp3, set to 100%), which was added to the released glycan before MS analysis. Values are mean standard deviation for n=3. Modified from Kumagai K²⁵.

Enrichment of Sulfoglycans by Phase Partition

A permethylated sulfated glycan standard (sulfo-Le^a) was completely recovered in the aqueous phase. Permethylated sialylated glycans released from the mucin glycoprotein partitioned into the DCM phase (**Figure 5**). The recovery of each permethylated glycans by aqueous-organic partition was comparable to that of the previously characterized C18 Sep-Pak clean-up method²⁷. The efficiency and simplicity of the phase partition method greatly facilitates sample throughput and subsequent analytic approaches.

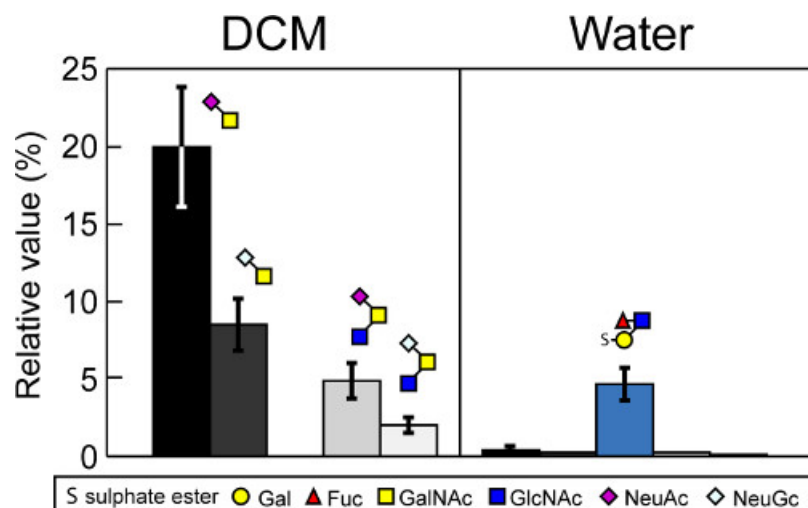


Figure 5. Differential recovery of sulfo- and sialo-glycans by phase partition. Bovine mucin glycoprotein was spiked with a known amount of a sulfo-glycan standard (sulfo-Le^a) before being subjected to reductive β -elimination. Released glycans were permethylated and the permethylation reaction was adjusted to 1:1:water:DCM. The resulting organic and aqueous phases were separated and analyzed by MS. Glycan recovery was quantified relative to permethylated external glycan standards, which were spiked into the sample before MS analysis. Glycan recoveries into the organic (DCM) and aqueous (Water) phases are shown relative to the external standard, which was set to 100%. Sulfoglycans were undetectable in the organic phase but quantitatively recovered in the aqueous phase. Results represent the mean \pm S.E. of three independent experiments. Modified from Kumagai²⁵.

Application to In-depth Proteomic and Glycomic Analyses in Biological Samples

Human saliva proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (G-250). A high molecular weight protein at MW ~600 kDa was excised from the gel and a portion of the gel piece was subjected to in-gel tryptic digestion and LC-MS/MS-based proteomic analysis, which identified this band as MUC5B^{16,24}. The remainder of the gel piece was subjected to in-gel reductive β -elimination for O-glycan analysis²⁷. Following permethylation and aqueous-organic phase partition (Water:DCM, 1:1), permethylated glycans in the aqueous and organic phases were analyzed by NSI-MS. Non-sulfated permethylated O-glycans were recovered from the organic phase and all of the permethylated sulfoglycans were recovered from the aqueous phase, which facilitates the identification and characterization of nearly isobaric sulfated and non-sulfated glycans, e.g., Fuc₁Hex₃HexNAc₂GalNAc-ol ($m/z=1606.8$, $[M+Na]^+$) and (SO₃)₁NeuAc₁Fuc₁Hex₂HexNAc₁GalNAc-ol ($m/z=1606.7$, $[M+2Na-H]^+$) differ by only 0.1 mass units and would be difficult to resolve without physically separating the two species by phase partition (**Figure 6**).

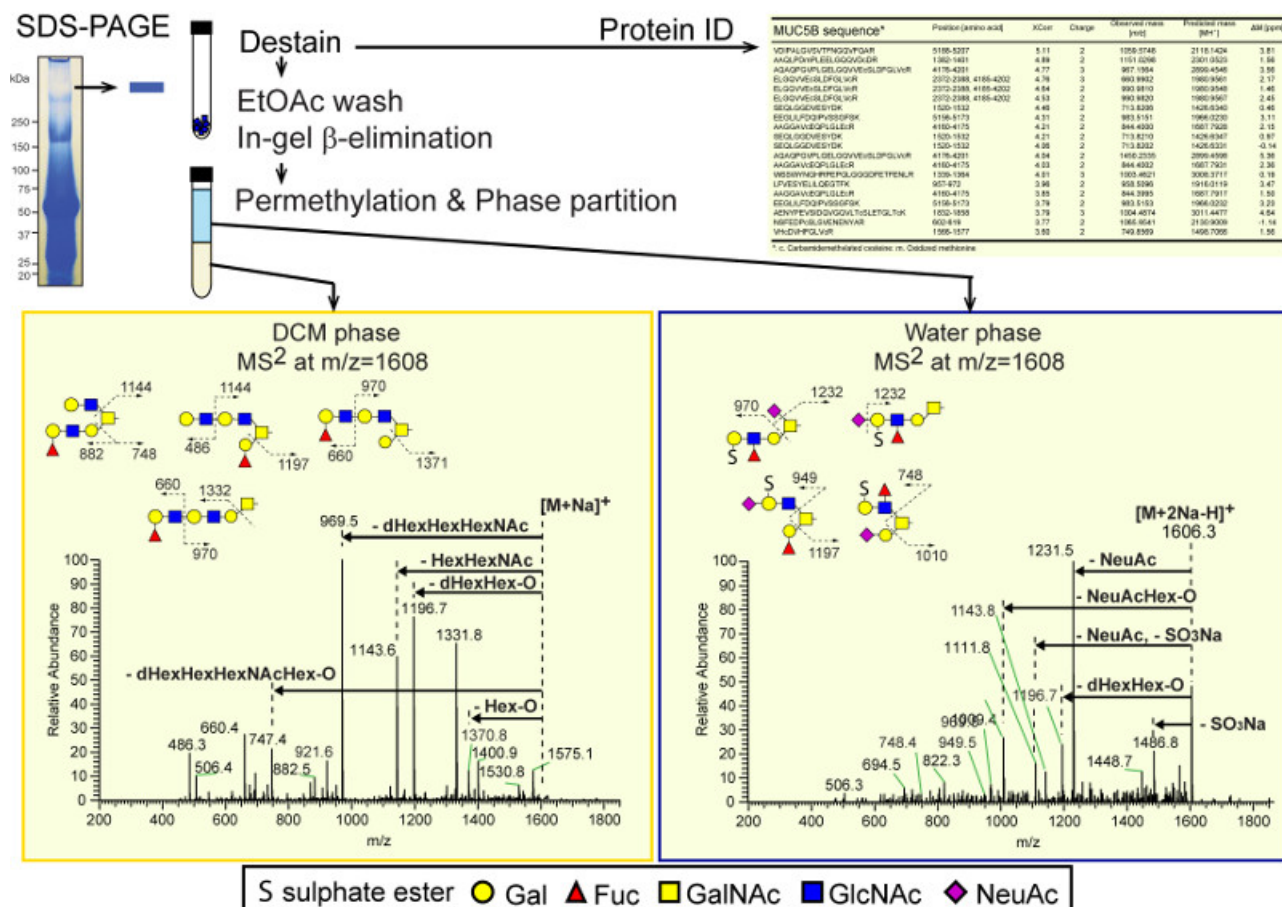


Figure 6. Detection of isobaric complexity in neutral and sulfo-glycans separated by aqueous-organic partition. MS² fragmentation patterns obtained from parent ions detected by total ion mapping (TIM) analysis of permethylated O-glycans released from human salivary mucin by in-gel β -elimination and water:DCM partition following permethylation. (A) MS² from TIM analysis of DCM phase for a 2.8 mass unit window around m/z = 1608. (B) The MS² spectrum of the same mass window for TIM analysis of the water phase. In the DCM-phase, the major fragment ions correspond to loss of Hex₁-O (m/z 1370.8), Fuc₁Hex₃HexNAc₂+Na (1331.8), loss of Fuc₁Hex₁-O (1196.7), loss of Hex₁HexNAc₁ (1143.6), loss of Fuc₁Hex₁HexNAc₁ (969.5), loss of Fuc₁Hex₂HexNAc₁-O (747.4), Fuc₁Hex₁HexNAc₁+Na (660.4), and Hex₁HexNAc₁+Na (486.3). Based on these fragment ions, a mixture of non-sulfated structures with a composition of Fuc₁Hex₃HexNAc₂GalNAc-ol is proposed as shown to the right of the spectrum. In contrast, the major fragment ions for the water phase are loss of SO₃Na (1486.8), loss of NeuAc (1231.5), loss of Fuc₁Hex₁-O (1196.7), loss of combination of NeuAc and SO₃Na (1111.8), and loss of NeuAc₁Hex₁-O (1009.4). A mixture of sulfated structures with a composition of (SO₃)₁NeuAc₁Fuc₁Hex₂HexNAc₁GalNAc-ol is proposed. Without physical separation of the neutral and sulfoglycans by phase partition, interpreting MS² spectra of such mixtures is significantly more challenging. Modified from Kumagai²⁵. [Please click here to view a larger version of the figure.](#)

Discussion

Combining in-gel reductive β -elimination with aqueous-organic extraction enhances the sensitivity and depth of structural data that can be acquired for characterizing sulfated and non-sulfated O-linked glycans harvested from small amounts of mucin-type glycoproteins resolved from other proteins by SDS-PAGE. The essential advances of the technical approaches presented in this study are: (a) facile removal of gel derived contaminants by simple washing steps; (b) quantitative recovery of permethylated sulfoglycans in the aqueous phase following a rapid water-DCM partition. The workflow described here dramatically improves the sensitivity of detection for sulfated and non-sulfated glycans released from glycoproteins expressed at physiological levels in biological samples.

While the methods described here are robust and highly reproducible when applied with careful attention to standard analytic practices, a few considerations regarding possible sources of contamination are worth a brief mention. Ensuring that all glassware is exceptionally clean and free of dust and detergent is critical. Upon noticing contaminant peaks in MS spectra, all glassware should be washed thoroughly and rinsed with MeOH prior to final drying. Another source of contamination is the use of rubber-lined screw caps in place of PTFE-lined caps. The lack of organic chemical resistance characteristic of the rubber lining results in samples contaminated with an interfering polymeric compound. Although this protocol was specifically designed with the intention of utilizing MSⁿ analysis by NSI-MS on an ion trap instrument, other types of mass spectrometers, such as MALDI-TOF/TOF can be also applied to dissecting sulfoglycoforms in biological materials using the preparative methods described here.

In comparison to other methodologies currently in use for in-gel glycan release and analysis, the techniques described here do not require HPLC separation prior to MS analysis and are applicable to permethylated glycans, producing greater structural characterization than can be extracted

from non-derivatized samples. Moreover, the methods described here are capable of separating permethylated forms of sulfated O-glycans from non-sulfated neutral and sialylated O-glycans by a simple phase partition. The optimized sample clean-up and simple phase partition methodologies minimize sample manipulation and losses that can occur through chromatography steps. Methods requiring greater sample handling may lead to an underestimate of sulfoglycan diversity due to losses during work-up. Furthermore, detection of permethylated sulfated glycans in negative mode is significantly less sensitive than detection of permethylated glycans in positive mode. Desulfation of permethylated sulfoglycans by solvolysis specifically removes sulfate groups, leaving naked hydroxyls that can be subsequently permethylated with deuterated methyl iodide, increasing MS-signal intensities approximately 5-fold and providing a unique isotopic mass tag at the site of sulfation²⁵.

Disclosures

The authors declare no competing financial interest.

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