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In-gel β-elimination and aqueous-organic partition for improved O- and sulfoglycomics

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Abstract

SDS-PAGE is a widely used technique for protein separation and in-gel tryptic digestion of resolved protein bands has enhanced the resolution of protoeomic analysis. To augment this technology and expand its usefulness for glycoproteomics, we have developed and improved methods to release and recover O-linked glycans from proteins resolved in SDS-PAGE gels for subsequent analysis by mass spectrometry (MS). Gel pieces containing target proteins are washed to remove contaminants. O-linked glycans are released through reductive β-elimination by hydrating gel pieces in base and adding reductant. Following straightforward sample cleanup, this simple treatment of glycoprotein gel pieces produces material suitable for MS analysis. We have applied this method to the analysis of mucin-type glycoproteins that are expected to carry high densities of sialylated and sulfated O-linked glycans. However, the strongly acidic nature of the sulfate moiety suppresses MS signal intensities, hampering detection and quantitative analysis. To enhance detection, we present an improved method for sulfoglycomics. A mixture of sulflo-, sialo-, and neutral glycans were permethylated and partitioned into a water-dichloromethane (DCM) solvent mixture. Sulfated glycans were selectively recovered from the aqueous phase, while neutral and sialylated glycans remained in the DCM phase. When applied to the analysis of human mucin salivary glycans, this partition method generated material of sufficient quality to identify more than sixty glycan structures by NSI-MS (LTQ-Orbitrap) in positive and negative ion modes. Also, nearly 100% of the sulfated O-linked glycans were recovered in the aqueous phase, demonstrating the feasibility of in-depth sulfoglycomic analysis using SDS-PAGE resolved proteins.

Keywords

mucin; in-gel β-elimination; O-linked glycan; sulfated glycan; mass spectrometry

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CONFLICT OF INTEREST DISCLOSURE
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INTRODUCTION

Protein glycosylation is an essential modification, contributing to the interactions between cells and the extracellular environment that influence cellular physiology, pathology, and recognition (1–3). Of all protein post-translational modifications, glycosylation is one of the most common. More than two-thirds of all proteins in the SWISS-PROT database contain potential glycosylation sites, predicting protein carbohydrate content from less than 1% to more than 90% by weight (4). Because protein glycosylation is not template-driven, glycan heterogeneity is generally the rule; two molecules of the same protein expressed in the same cell type can be modified with different glycan structures at the same glycosylation site (5,6). This microheterogeneity increases the diversity of glycoprotein structures and frequently modulates their functional interactions with other proteins or biological targets. Therefore, characterizing glycan structural variation is essential for understanding their contribution to normal tissue function and disease pathologies.

Identifying the full diversity of glycans found on a specific protein is still a challenging task, especially on proteins isolated from biological tissues under physiologic conditions, where yields can be low. In this regard, the development of robust analytical techniques that provide breadth, depth, and sensitivity to glycoprotein glycan analysis is required. Complete glycoprotein characterization entails protein identification, determination of glycosylation site usage, and profiling of glycan heterogeneity, also referred to as proteomics, glycoproteomics, and glycomics, respectively. Currently, proteomic profiles of biological samples are acquired for purified proteins, complex protein mixtures, or otherwise fractionated proteins by generating tryptic peptides that are analyzed by LC-MS/MS (7). Protein digestion can also be performed on proteins resolved by SDS-PAGE following in-gel treatment with trypsin and subsequent elution of the tryptic peptides from the gel piece (9,10). SDS-PAGE and in-gel digestion techniques enhance the depth of protein identifications in proteomic experiments by pre-fractionating the protein mixture before LC-MS/MS. Attempts to apply analogous strategies for glycomic and glycoproteomic analyses are at the forefront of current glycotechnology.

Over the past decade, significant efforts have been invested toward developing glycan analysis methods that take advantage of the sensitivity and structurally rich data of mass spectrometer (10). In order to profile glycoprotein glycans most sensitively, they must first be released from the polypeptide backbone. For glycans linked to asparagine residues (N-linked), enzymatic release with protein-N-glycanase (PNGaseF or A) is highly effective and has been successfully adapted for in-gel and on-blot use (11–14). For glycans linked to serine or threonine residues (O-linked), a broad-specificity enzyme capable of releasing anything other than the simplest O-linked glycan is currently unavailable. Fortunately, well-characterized chemical release techniques have been established (15–18). Reductive or non-reductive β-elimination, or hydrazinolysis are the most commonly used chemical methods to release O-linked glycans from glycoproteins (19). A method for the reductive β-elimination of O-linked glycans from glycoproteins resolved by SDS-PAGE, either in-gel or after blotting to PVDF, has been recently reported (20–22). This previously reported method generates free, underivatized (designated native) O-linked glycans with alditols at their reducing end, which were subsequently fractionated and analyzed by LC-MS/MS using and

electrospray interface (ESI). MS analysis in negative mode allowed the authors to identify anionic glycans carrying sialic acid or sulfate.

ESI-MS and MSn analyses are widely applied to the structural analysis of glycans released from glycoprotein. Under most circumstances, ionization of underivatized glycan is poor and highly influenced by specific structural features, including the extent of modification with anionic substituents. Permethylation of released glycans significantly increases the sensitivity of MS analysis and also tends to equalize the molar signal response across a broad range of glycan structures, providing opportunities for quantification of glycan amount. Furthermore, permethylation imparts unique masses to terminal and substituted monosaccharides, which allows the elucidation of key structural features; MS or MS/MS-based analysis of native glycans is unable to unambiguously discern positional information (23–26). In this report, we have endeavored to combine the high value of in-gel release techniques with the deep information obtainable from MSn analysis of permethylated glycans in order to significantly enhance glycomic analysis of O-linked glycoprotein glycosylation.

The major aim of this study is to improve O-glycomic methodology in order to explore the composition of O-linked glycans released from glycoproteins separated on gels. To that end, we address a critical issue related to in-gel release and subsequent glycomic analysis: removal of gel-derived contaminants that interfere with glycan profiling. While previous reports relied on HPLC separations to achieve sample clean-up during LC-MS, we now present a simple, quick, and efficient sample workflow that ends with shotgun MSn analysis and eliminates the need for in-line HPLC separation prior to MS. An additional advantage of our complete workflow is that we efficiently separate sulfated permethylated glycans from sialyalted and neutral permethylated glycans with a quick phase partition prior to MS. Thus, structural features can be probed by MS in both positive and negative modes to maximize the yield of useful data. We have also improved the recovery of O-linked glycans from SDS-PAGE gel pieces in order to be able to detect minor glycan components and thereby explore the complete diversity of O-linked glycan structures.

EXPERIMENTAL SECTION

Materials and Reagents

Sodium hydroxide (50%) was obtained from Fisher Scientific and AG-50W-X8 cation exchange resin (H^+) from Bio-Rad. Lewis^a (Galβ1-3(Fucα1-4)GlcNAc, Le^a) and 3′-sulfated Lewis^a (3′-O-(SO_3H)-Galβ1-3(Fucα1-4)GlcNAc, sulfo-Le^a) were purchased from Prozyme. Reversed phase octadecyl (C18) disposable extraction column were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Isomaltotriose (Dp3) and stachyose tetrahydrate (Dp4) were purchased from Supelco (Bellefonte, PA). Sulfatide and neutral glycolipid mixture were purchased from Matreya, Inc. (Pleasant Gap, PA). All other reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO).

SDS-PAGE and In-Gel O-glyan Release by Reductive β-Elimination

Five μg of bovine submaxillary mucin (Sigma) were resolved by SDS-PAGE on a 7.5% TGX pre-cast gel (Bio-Rad). The gel was run at 200 V for 5 min and stained with either

R250 Coomassie Brilliant Blue (Bio-Rad) or Silver Stain (Pierce). Protein bands were excised, cut into small pieces with a scalpel and placed into a glass tube (13 × 100 mm) with a Teflon-lined screw top. Gel pieces were washed with 1ml of 25 mM NH_4HCO_3 followed by dehydration with 100% acetonitrile (ACN) for 30 min; this was repeated for a total of four washes. Gel pieces were further washed with ethyl acetate at 4°C overnight or 3 times for 30 min each at room temperature. Gels were washed once more with ACN and then dried under nitrogen stream. O-linked glycans were released from gels by β-elimination under reductive conditions. The dried gels in the glass tube were re-hydrated by adding 250 μl of 100 mM sodium hydroxide and waiting approximately 30 sec to allow the gel pieces to swell before adding 250 μl of 2 M sodium borohydride in 100mM sodium hydroxide (final concentration 1M sodium borohydride in 100 mM NaOH). The tubes were sealed with a Teflon-lined screw top and incubated for 18 hrs at 45 °C. The tube was then placed on ice and neutralized with 10% acetic acid (AcOH). The neutralized sample was loaded onto a column of AG-50W-X8 cation exchange resin (1 ml bed volume, H^+ form) to desalt. Released glycans were eluted from the resin with three bed volumes of 5% AcOH and lyophilized to dryness. To remove borate from the sample, a solution of 10% AcOH in methanol was added and the sample was dried under a stream of nitrogen gas at 37 °C; this was repeated for a total of five times. The dried sample was resuspended in 5% AcOH and loaded onto a C18 cartridge column (J.T. Baker Co.) that was previously washed with ACN and pre-equilibrated with 5% AcOH. Flow-through from the column was collected during loading and the column was then washed a total of five times with 5% AcOH (5 ml total wash volume). The run-through and washes were combined and evaporated to dryness.

Glycan Permethylation and Enrichment of Sulfoglycans by Phase Partition

Lewis^a glycan (Le^a), sulfated Lewis^a glycan (sulfo-Le^a), and bovine mucin were used as standards (27). The standard glycans (0.5μg) and the glycans released from 1 μg of bovine mucin were permethylated to enhance sensitivity and structural determination by mass spectrometry. Permethylation reactions were carried out according to a standard method (28). After neutralizing the reaction with 1 ml of 10% AcOH on ice, the permethylated glycans were either cleaned-up by C18 Sep-Pak to prepare total glycans or partitioned with water:dichloromethane (W:DCM::1:1) to separate charged and neutral glycans. For total glycans, the neutralized reaction mixture was directly applied to a C18 Sep-Pak column that had been pre-washed with ACN and pre-equilibrated with 5% AcOH. Hydrophilic salts and contaminants were removed by washing the column with 15 ml of water and permethylated glycans were subsequently eluted with 2 ml of 85% ACN in water. For separation of charged from neutral glycans by phase partition, 2 ml of DCM were added to the neutralized permethylation reactions. After mixing and centrifugation, the upper water phase was transferred into a new glass tube and adjusted to 5 ml total volume with water. The diluted water phase was then applied to a pre-washed and pre-equilibrated C18 Sep-Pak column. After washing the column with 15 ml of water to remove contaminants and salts, permethylated glycans were eluted with 2 ml of 85% ACN in water, and dried under N_2 stream. The lower DCM phase was washed with water, and dried under N_2 stream (18).

Glycan Mass Spectrometry

Nanospray ionization mass spectrometry (NSI-MS) was performed on native and permethylated O-linked glycans. For MS of native sulfo-Le^a and permethylated sulfo-Le^a, approximately 0.5 nmol of native Le^a were reconstituted in 50 μl of methanol/2-propanol/1-propanol/13 mM aqueous ammonium acetate (16:3:3:2 by volume) for infusion and analyzed in negative ion mode (29). For MS of permethylated O-linked glycans, approximately 50 pmol of permethylated O-linked glycans were dissolved in 50 μl of 1 mM sodium acetate in methanol/water (1:1) for infusion (24, 25). Both preparations were analyzed by direct infusion into a linear ion trap mass spectrometer (LTQ-Orbitrap Discovery; Thermo Fisher Scientific) using a nanoelectrospray source at a syringe flow rate of 0.40 μl/min and capillary temperature set to 210°C. Automated acquisition of MS^2 fragmentation was achieved using the Total Ion Mapping (TIM) functionality of the Xcalibur software package (version 2.0, Thermo Fisher Scientific) (18, 30). Native sulfated glycans were analyzed in negative mode and permethylated sulfo-Le^a and Le^a were analyzed in positive mode. The instrument was tuned with permethylated glycan standards for positive ion mode and with native or permethylated sulfo-Le^a for negative ion mode. For fragmentation by collision-induced dissociation (CID) in MS/MS and MSn, a normalized collision energy of 35% to 40% was used. Quantification of individual glycans was accomplished by referencing sample signal intensities to the signal intensities of 10 pmol of deuteride-labeled permethylated maltooligosaccharide (Dp3 and Dp4) which were spiked into the resuspended sample.

Identification of a High Molecular Weight Human Salivary Glycoprotein by Mass Spectrometry

To demonstrate the application of in-gel β-elimination and aqueous-organic partition, a high molecular weight protein of human saliva was resolved by SDS-PAGE and subjected to glycomic analysis. The permethylated non-sulfated and sulfated O-linked glycans from human saliva were analyzed by NSI-MS in positive and negative ion mode, respectively. In addition, the protein was identified by proteomic analysis using standard LC-MS/MS techniques (31). Briefly, excised gel pieces were washed sequentially with 40 mM ammonium bicarbonate and ACN, followed by reduction with 10 mM dithiothreitol for 1 h at 55°C and carboxyamidomethylation with 55 mM iodoacetamide in the dark for 45 min. The gels were trypsinized (Promega) and the peptides were extracted and cleaned-up onto a C18 silica MicroSpin Column (The Nest Group Inc., MA). LC-MS/MS analysis was performed on LTQ-Orbitrap Discovery equipped with a nanospray ion source by data-dependent scan. The resulting data were searched against the human proteome database (Uniprot, retrieved on June, 2012) using the SEQUEST algorithm (Proteome Discoverer 1.1, Thermo Scientific). SEQUEST parameters were set to allow 50 p.p.m. of precursor ion mass tolerance and 0.8 Da of fragment ion tolerance with monoisotopic mass. Tryptic peptides were allowed with up to two missed internal cleavage sites and differential modifications were allowed for carboxyamidomethylation of cysteine and oxidation of methionine. The resulting peptide data was filtered by charge vs. Xcorr to give a strict false discovery rate (<0.01).

RESULTS AND DISCUSSION

General Strategy

The general strategy employed in this study is designed to obtain glycomic and proteomic data on glycans of interest harvested from biological material (Fig. 1). Glycoproteins were separated by SDS-PAGE and visualized by either Coomassie or silver staining. Target proteins of interest were then excised and the gel was sliced into small pieces. Following destaining and ethyl acetate wash of the gel pieces, O-linked glycans were released from the gel slices by in-gel β-elimination. The ethyl acetate wash proved essential for removing SDS and polyacryl contaminants which otherwise interfere with MS analysis. Released O-linked glycans were then analyzed by NSI-MS following permethylation. Aqueous-organic extraction of the permethylation reaction generated quantitative partition of sulfated and neutral glycans into the aqueous and organic phases, respectively. The in-gel β-elimination and aqueous-organic extraction strategy enabled characterization of O-linked glycans with and without sulfate harvested from small amounts of glycoprotein separated by SDS-PAGE.

Gel Wash Procedures Impact Glycan Detection

Bovine submaxillary mucin (5μg) was used as a test glycoprotein to investigate if reductive β-elimination can be used as a standard method for releasing O-linked glycans from biological materials resolved by SDS-PAGE. After excising the mucin band from an SDS-PAGE gel, O-linked glycans were directly released from gel slices by reductive β-elimination. Blank gels and mucin O-linked glycans obtained from gels without washing with ethyl acetate exhibited polymeric and detergent-like contaminant peaks across the low molecular weight range (Fig. 2A, B). The repeating contaminant peaks were spaced 44 mass units apart, suggesting that they could be derived from SDS, polyacrylamide, or another detergent-like component of the separation matrix. Ethyl acetate, acetone, 2-propanol, methanol, diisopropyl ether, and dichloromethane were tested for their ability to extract contaminants away from the gel pieces without reducing recovery of glycan. Of those tested, ethyl acetate effectively removed the contaminants. The O-linked glycan profile produced by in-gel β-elimination is comparable to that generated by conventional solution-phase β-elimination (Fig. 2C, D).

Optimization of Glyoprotein Glycan Recovery

To assess the efficiency of O-linked glycan release and recovery from gel pieces, β-elimination was performed on bovine mucin using the in-gel method or using conventional solution-phase chemistry. Previous proteomic studies have demonstrated that recovery of tryptic peptides from small gel pieces was ~10-fold higher than recovery from large gel pieces (32). Therefore, prior to in-gel β-elimination, excised SDS-PAGE gel slices containing resolved bovine mucin were cut into large pieces (~5 × 5 mm size) or small pieces (~2 × 2 mm size). In parallel, conventional solution-phase reductive β-elimination was performed on the same amount of bovine submaxillary mucin. Released O-linked glycans were permethylated and supplemented with a known amount of a mixture of permethylated external glycan standards (Dp3 and Dp4) to allow for relative quantification of glycan recovery. O-linked glycan amounts released from small gel slices exhibited ~10-fold stronger signals than those of large gel slices (Fig. 3A). Gel staining methods can also

adversely affect the recovery of peptides following in-gel tryptic digestions for proteomic analysis. Since the recovery from large gel pieces is more likely to be impacted by staining than recovery from small gel pieces, we assessed whether two common staining methods differentially affected glycomic analysis; slightly lower glycan recovery was noted for silver stain relative to Coomassie (Fig. 3B). Based on this data and on the greater recovery of glycan from small gel pieces, all subsequent analyses were performed on small gel pieces. We assessed the reproducibility of in-gel β-elimination across three separate experiments, comparing in-gel and solution-phase methods. The recovery of total O-linked glycans eluted from gels was approx 90.5 ± 12.5% compared to that of the same amount of native mucin glycoprotein treated with the solution-phase method (Fig. 3C). Similar recoveries were achieved for each of the analyzed O-linked glycan structures, despite nearly 10-fold differences in their individual abundances (Fig. 3D). Therefore, with appropriate gel handling practices, in-gel β-elimination reveals glycan diversity and recovery comparable to solution-phase reactions without the need for HPLC-based sample clean-up prior to MS analysis.

Aqueous-organic partition following permethylation separates neutral and acidic glycans

Acidic glycans, such as those containing sialic acid, are generally difficult to detect as non-permethylated (native) species by MS. Permethylation of sialylated glycans neutralizes their charge, making them detectable at the same sensitivity as permethylated neutral (asialo) glycans in positive ion mode. However, the sulfate moieties of the sulfoglycans do not loose their anionic charge upon permethylation, suppressing ionization and decreasing sensitivity of MS-based detection. This characteristic is of particular concern when analyzing mucin glycoprotein glycosylation since many mucins exist as sulfated glycoforms that carry very strong negative charges (33). In order to enhance the detection and analysis of sulfoglycans, we hypothesized that sulfoglycans could be quantitatively recovered in the aqueous phase resulting from the water:DCM extraction routinely performed at the end of the glycan permethylation reaction (28, 30). To assess the relative extraction behaviors of sulfoglycans and neutral glycans, permethylated Le^a and sulfo-Le^a were partitioned into water:DCM and both the aqueous and organic phases were analyzed by MS (Fig. 4A). Permethylated Le^a ($[M + Na]^+ = 692.35$ in positive ion mode) was quantitatively recovered in the DCM (Fig. 4B) and permethylated sulfo-Le^a ($[M-H]^- = 780.27$ in negative ion mode) was quantitatively recovered in the water phase (Fig. 4C). Additionally, a mixture of sulfo-Le^a and bovine mucin was subjected to conventional solution-phase β-elimination and the released O-linked glycans were permethylated (Fig. 5A). Following post-derivatization work-up by C18 Sep-Pak (34) or by aqueous-organic partition, glycan recovery was quantified relative to an external standard (Dp3 and Dp4) spiked into the sample. Again, the sulfoglycan (sulfo-Le^a) was quantitatively recovered in the aqueous phase while the permethylated sialylated glycans derived from the mucin glycoprotein partitioned into the DCM phase. For both the sulfated and the neutral glycans, recovery by aqueous-organic partition was comparable to the C18 Sep-Pak clean-up method (Fig. 5B). In addition to the speed and simplicity of the method, the phase partition completely fractionates sulfated and non-sulfated glycan species, facilitating subsequent analytic approaches.

We also examined the extent to which a negatively charged sulfate group suppresses sensitivity for permethylated sulfoglycans detected by MS in positive ion mode. When known amounts of permethylated sulfo-Le^a and Le^a were mixed in a 1:2 molar ratio and analyzed by MS in positive mode, the permethylated sulfoglycan generated approximately 10% of the signal intensity of permethylated Le^a, indicating significant suppression (Fig. 6A). The 1:2 permethylated glycan mixture was then subjected to solvolysis for desulfation (35), repermethylated with deuterated methyl iodide (CD_3I), and reanalyzed by MS. The signal intensity associated with the desulfated and repermethylated sulfo-Le^a was increased approximately 5-fold compared to the starting material, bringing the signal close to that expected for the input (Fig. 6B). A strength of this approach is that the elimination of suppression allows desulfated, repermethylated sulfoglycans to be quantitatively compared to permethylated neutral glycans. Furthermore, the use of CD_3I during permethylation imparts a mass tag to glycans that were originally sulfated in the biological sample. The extent of sulfation on N-linked and O-linked glycans varies significantly across different tissues and different sample types. Except for glycosaminoglycans and a few other specialized contexts (36–38), glycoprotein sulfoglycan abundance is limited. However, the technical considerations described here may have previously limited the sensitivity for detecting glycoprotein glycan sulfation. Enhanced recovery and chemical derivatization may provide new opportunities for understanding the functional roles of sulfoglycans.

Sulfoglycomics of human salivary mucin

To assess the ability of our combined in-gel β-elimination and aqueous-organic partition method to capture the O-linked glycan diversity of a glycoprotein resolved from a complex biological matrix, we analyzed the O-linked glycome and sulfoglycome of human salivary mucin. Saliva was collected from a healthy individual (blood type-O) and the salivary proteins were precipitated by ice-cold acetone. Proteins equivalent to 40 μl of saliva were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (G-250). A protein band at MW ~600 kD, suspected to be MUC5B, was excised from the gel (Supplement fig. 1). A portion of the gel slice was subjected to in-gel tryptic digestion followed by LC-MS/MS based proteomic analysis (31), which validated the assignment of this band as MUC5B (21, 33). The remainder of the gel slice was subjected to in-gel β-elimination and the released, permethylated O-linked glycans were partitioned into (W:DCM::1:1). The aqueous and organic phases were analyzed by direct infusion NSI-MS in the positive mode (for both the DCM- and the water-phases) and negative mode (for the water-phase). Full MS profiles and automated MS^2 fragmentation by TIM analysis revealed the existence of 70 total glycan compositions, including 38 non-sulfated glycan compositions (neutral and sialylated) and 32 sulfated glycan compositions including mono-, di-, and tri- sulfated species (Supplemental Table I and II). The non-sulfated permethylated O-linked glycans were almost exclusively recovered from the organic phase although a small amount of these glycans were also detected in the aqueous phase. Essentially all of the permethylated sulfoglycans were recovered from the aqueous phase (Fig. 7).

The robust separation of permethylated sulfoglycans from neutral glycans by aqueous-organic partition simplifies the interpretation of MS^2 fragmentation patterns. For example, the predicted masses (*m/z*) of glycan structures (NeuAc-6(Fuc-Gal-3(Fuc-

GlcNAc-3)GalNAc-ol and (SO_3^-)-Gal-GlcNAc-6(Fuc-Gal-GlcNAc-3)GalNAc-ol are 1488.8 $[M+Na]^+$ and 1490.7 $[M+2Na-H]^+$, respectively (positive ion mode). Automated MS^2 acquisition (TIM) collects fragment ions from 2.2 mass unit windows (18, 30). Therefore, MS^2 data at *m/z* 1490 (1487.8–1492.2) would contain molecular ion from *m/z* at 1488.8 and 1490.7, producing a complex pattern of fragments arising from both parents. However, separation of the sulfoglycan and the neutral glycan into aqueous and organic phases, respectively, produces simplified MS^2 fragmentation patterns, allowing clear structural assignments (Fig. 8). The importance of being able to separately fragment permethylated sulfoglycans and neutral glycans is even more clearly demonstrated by our analysis of detected molecular ions that differ by only 0.1 mass units ($Fuc_1Hex_3HexNAc_2GalNAc$-ol, 1606.8, $[M+Na]^+$ and $(SO_3)_1NeuAc_1Fuc_1Hex_2HexNAc_1GalNAc$-ol, 1606.7 $[M+2Na-H]^+$) (Supplement Fig. 2). MS^2 fragmentation at *m/z* 1608 supports the presence of multiple isobaric structures consistent with these compositions. Again, physical separation of the sulfoglycan species from the neutral glycan species by phase partition greatly simplifies the interpretation of the isobaric possibilities for each class of glycan. Finally, the yield and enrichment of sulfoglycans achieved by in-gel β-elimination and aqueous-organic phase partition is sufficient to allow in-depth queries of novel and minor glycan modifications, including sulfation position (Supplement Fig. 3) and multiple sulfation isomers (Supplement Fig. 4).

CONCLUSIONS

The methods that we have described here provide improved recovery, quantification, and separation of O-linked glycan classes released from glycoproteins of biological samples resolved by SDS-PAGE. The most sensitive, currently employed in-gel β-elimination approaches require purification of released O-linked glycans by in-line HPLC systems in order to separate glycans from gel and detergent contaminants. We have demonstrated that quick acetonitrile and ethyl acetate washes of SDS-PAGE gel pieces are sufficient to remove contaminants and thereby enhance MS-based detection of released O-linked glycans. The unique features of our methods are: (i) gel derived contaminants are completely removed by a simple washing process, eliminating the need for HPLC prior to MS; (ii) O-linked glycans directly released from SDS-PAGE gels can be quantified by reference to well-characterized external glycan standards that can be spiked into samples; (iii) after permethylation, sulfoglycans are quantitatively recovered in the aqueous phase following a rapid water-DCM partition; (iv) desulfation/repermethylation of sulfoglycans circumvents ion suppression and simultaneously tags the sulfation position. The combination of these methods presents new opportunities to investigate total glycoprotein O-linked glycan and sulfoglycan dynamics following gel electrophoresis, enabling targeted glycomic analysis of glycoproteins expressed at endogenous levels in biological samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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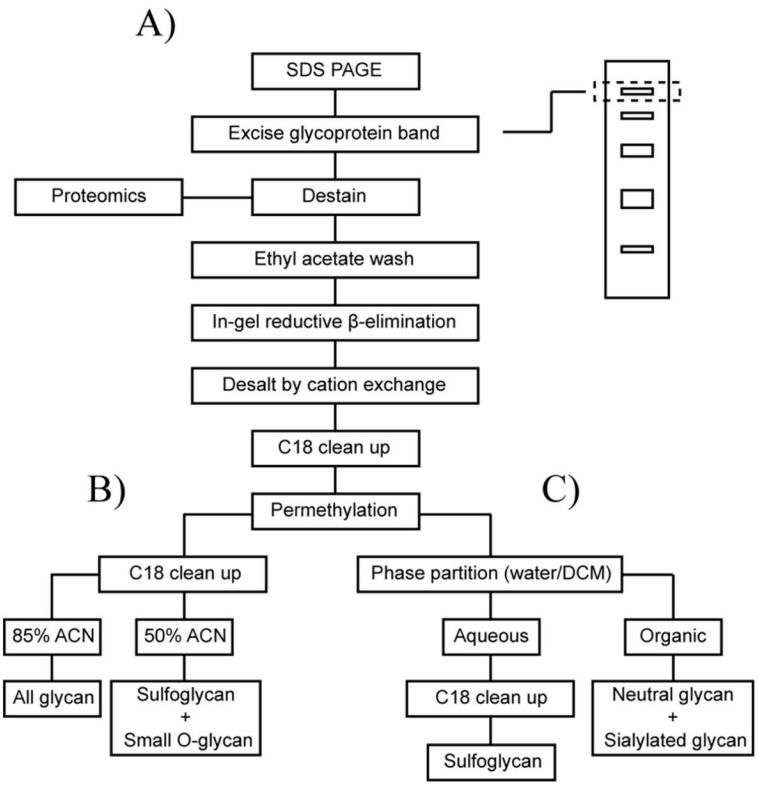

Figure 1. Analytical strategy for in-gel O-glycomics

(**A**) Purified protein, cell lysate, or tissue extract is run on SDS-PAGE gel and the band of interest excised. The gel piece is cut into smaller pieces and destained. Gel pieces can be subjected to in-gel tryptic digestion for proteomic analysis of the protein diversity. For in-gel glycomic analysis, the destained gel is washed extensively to remove contaminants before performing reductive β-elimination. Released O-linked glycans are eluted from the gel pieces and permethylated. (**B**) Following permethylation, the reaction mix can be loaded onto a C18 column for clean-up. Elution with 85% acetonitrile (ACN) gives an eluate containing all glycans. However, elution with 50% prior to 85% ACN can be used to preferentially elute small O-linked glycans and highly polar sulfoglycans. (**C**) Alternatively, the permethylation reaction can be partitioned by the addition of water and dichloromethane (DCM) to produce an aqueous phase containing sulfoglycans and an organic phase containing neutral and sialylated glycans.

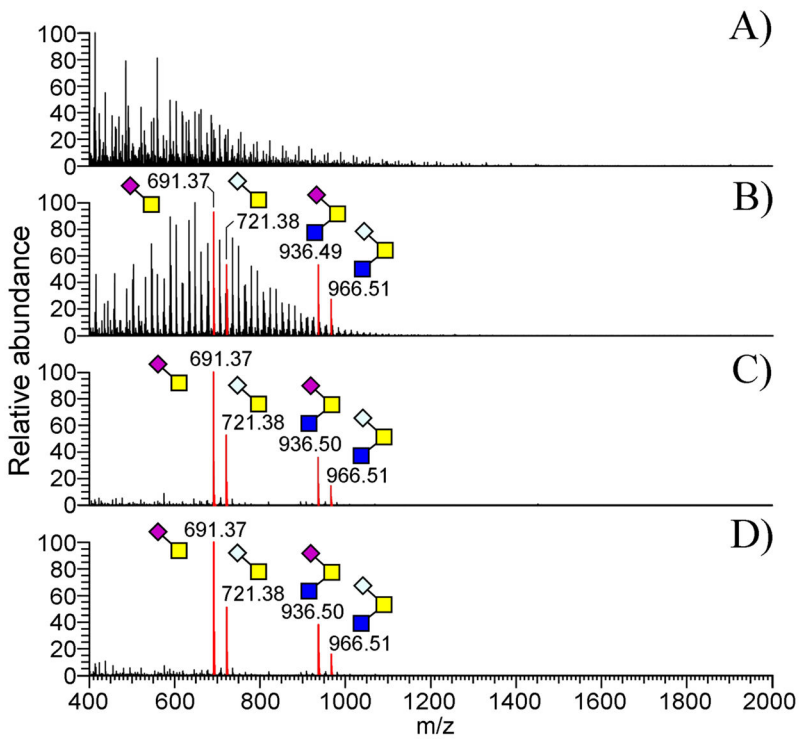

Figure 2. Wash of gel pieces with organic solvent prior to in-gel β-elimination is essential for detection of released glycans by mass spectrometry

(**A**) Mass spectra (MS) acquired from β-elimination of a blank piece of acrylamide cut from an SDS-PAGE gel produces variable amounts of a polydisperse contaminant, probably a mixture of detergent and polyacrylamide fragments. (**B**) The MS signals associated with glycans released by in-gel β-elimination of bovine submaxillary mucin are almost completely obscured by contaminant peaks if the gel pieces are not first washed with acetonitrile and ethyl acetate. If the gel pieces are first washed before β-elimination, contaminant peaks are almost completely eliminated (**C**) and glycan-associated MS signals are comparable to the signals detected by β-elimination of bovine submaxillary mucin performed in solution (**D**). Glycan representations are consistent with the guidelines proposed by Molecular and Cellular Proteomics and Essentials for Glycobiology.

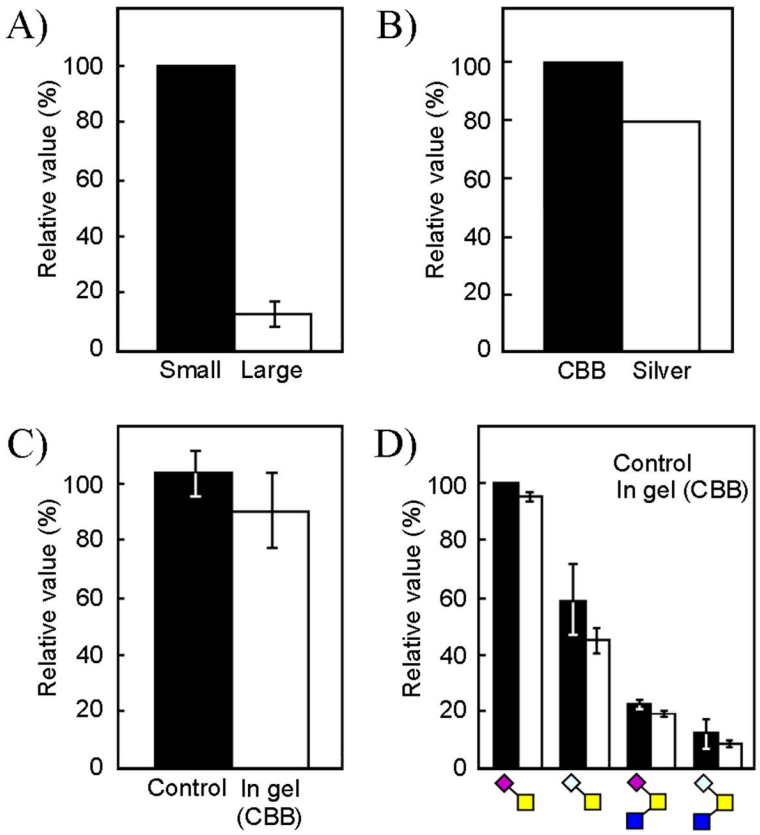

Figure 3. Recovery of glycan by in-gel method compared to solution-phase β-elimination

(**A**) Glycan recovery is enhanced by cutting the gel slice into small pieces. Small pieces (~2 × 2 mm) yielded more than 5 times the glycans as larger cubes (~5 × 5 mm). Bars show relative signal intensities for each structure when normalized to the signal detected for NeuAc1 HexNAc1 (set to 100%). (**B**) Relatively little difference in glycan recovery is detected when gels are stained with either Coomassie Brilliant Blue or silver. (**C**) Total O-linked glycan recovery from bovine mucin by the in-gel method (mean ± SEM for 3 separate determinations). (**D**) Similar recoveries are achieved for individual glycans by in-gel and solution-phase β-elimination, independent of each glycan's abundance.

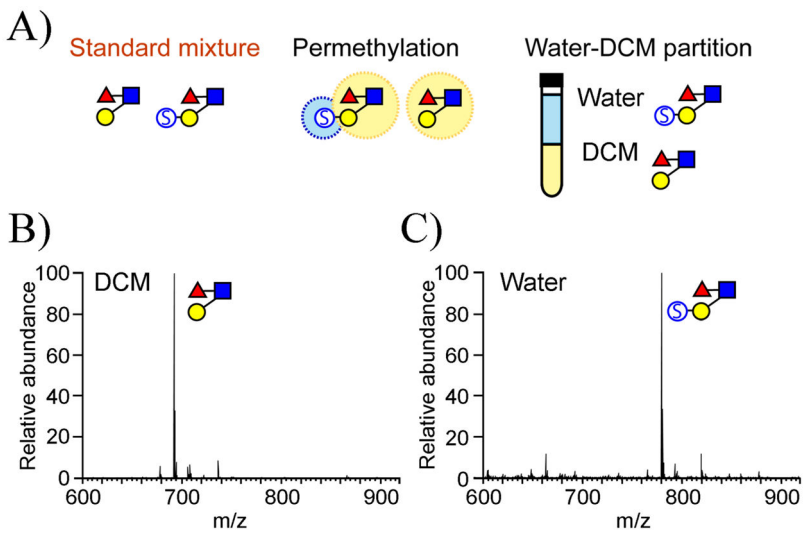

Figure 4. Separation of permethylated neutral and sulfoglycans by aqueous-organic partition

Standard Le^a and sulfo-Le^a glycans were mixed together and permethylated. The permethylation reaction was adjusted to 1:1::water:DCM and the aqueous and organic phases were analyzed separately by MS (**A**). The permethylated neutral Le^a glycan ($[M+Na]^+$ = 692.35) is recovered in the DCM phase (**B**). The permethylated sulfo-Le^a glycan ($[M-H]^-$ = 780.27) partitions quantitatively into the water phase due to its anionic sulfate moiety (**C**).

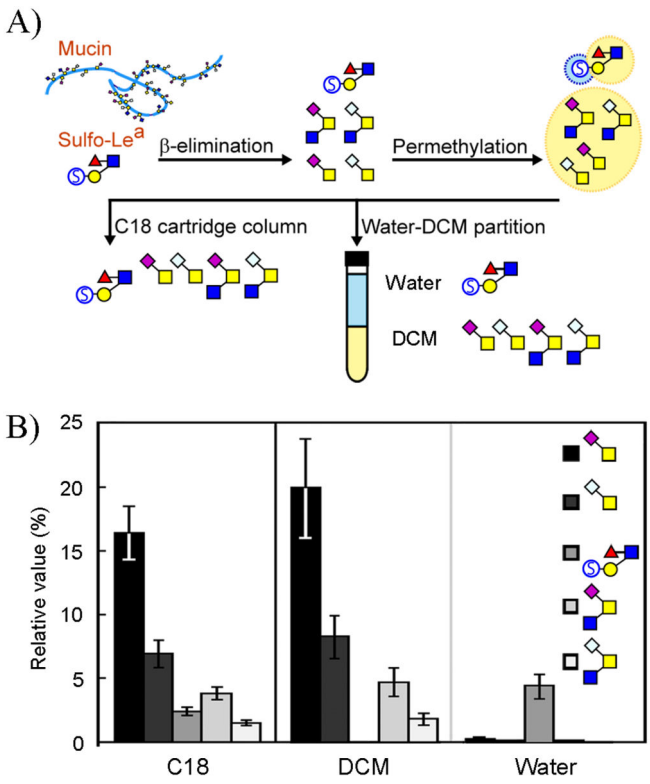

Figure 5. Separation and recovery of sulfoglycan from neutral glycans derived from a biological matrix by aqueous-organic partition

A known amount of sulfo-Le^a and bovine mucin were mixed together and subjected to β-elimination in solution. Following permethylation, equivalent reaction mixtures were cleaned-up by C18 Sep-Pak or adjusted to 1:1::water:DCM for phase partition (**A**). Recovery of the indicated glycans was quantified relative to the signal obtained for permethylated external standards (Dp3 and Dp4), which were spiked into the sample before MS analysis and set to 100%. Recovery is shown for the glycans obtained following C18 Sep-Pak clean-up (C18) and for the glycans obtained in the aqueous (Water) and organic (DCM) phases following partition (**B**). Sulfoglycan is quantitatively recovered in the aqueous phase and recovery of all glycans by phase partition is comparable to the recovery by C18 Sep-Pak. Results represent the mean ± S.E. of three independent experiments.

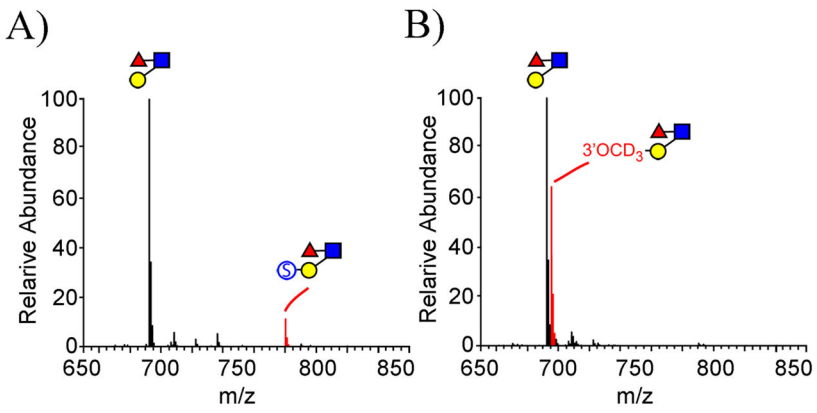

Figure 6. Desulfation/repermethylation enhances detection and quantification of sulfoglycans

Two standard permethylated glycans, sulfo-Le^a (m/z 780.27) and Le^a (m/z 692.35), were mixed together in a 1:2 ratio. (**A**) MS signal intensity associated with the sulfo-glycans (red peak) is only 10% of the neutral glycan, indicating significant suppression. (**B**) After solvolysis and repermethylation with deuterated CH_3I, the signal intensity associated with the previously sulfated glycan (red peak, m/z 695.37) is increased 5-fold, closely approximating the signal expected for the relative ratio of the two glycans in the starting material.

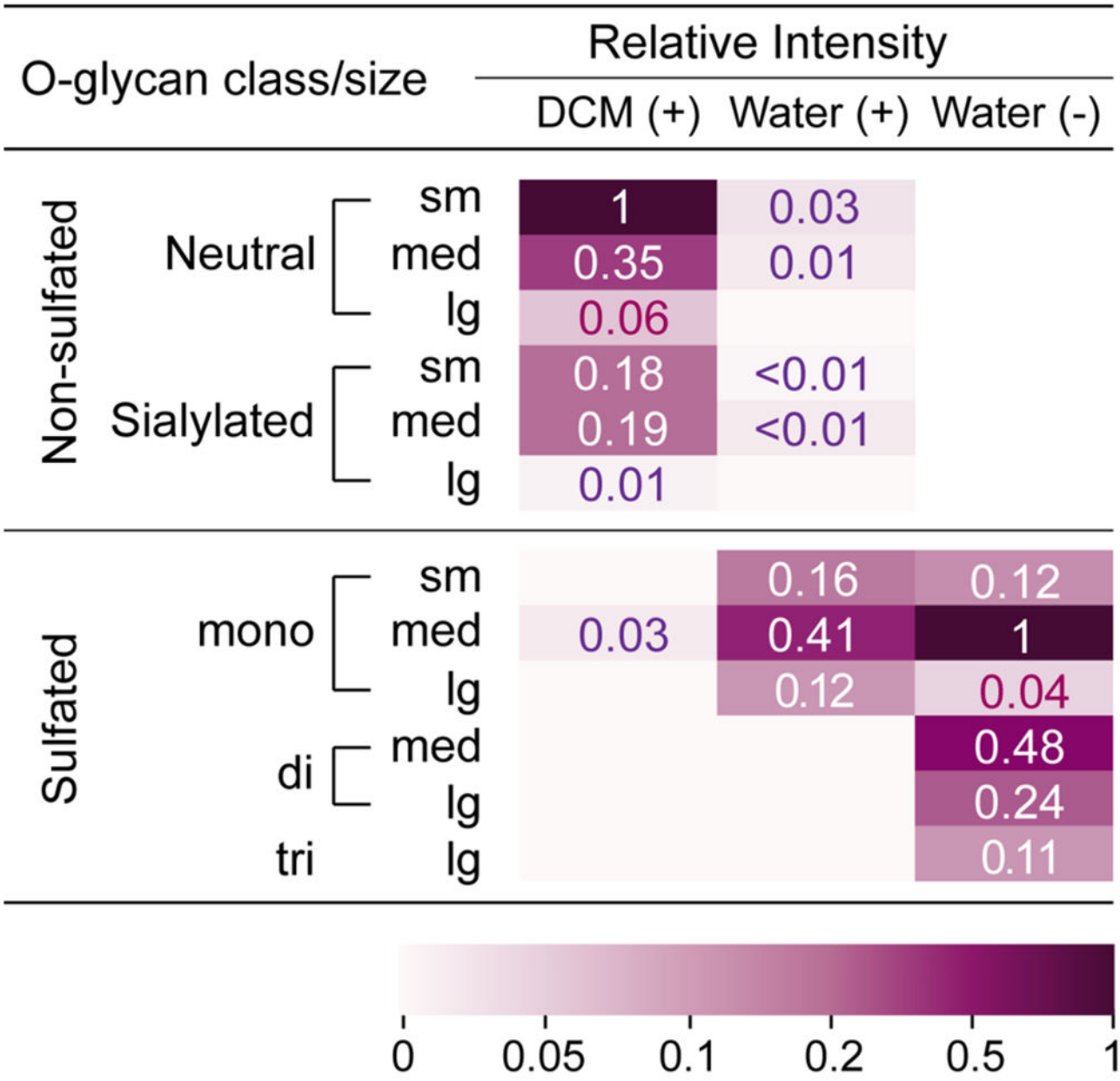

Figure 7. Partition behavior and optimal detection mode for recovery and analysis of human saliva glycans following in-gel β-elimination and aqueous-organic partition

The distribution between the organic (DCM) and aqueous (Water) phase for the indicated classes of permethylated O-linked glycans was quantified from MS signal intensities detected in the full MS profile using positive (+) or negative (−) ion modes. Small (sm, ~2–4 residues), medium (med, ~5–7 residues), and large (lg, ~8–10 residues) glycans were separately quantified within each class. For both non-sulfated and sulfated glycans, the signal intensities of the most abundant glycan subclasses (specifically, small neutrals in positive mode for non-sulfated glycans and medium mono-sulfated in negative mode for sulfated glycans) were set equal to 1 and the intensity of all other subclasses were then normalized proportionately. The normalized signal intensity values are presented for each detected subclass and are also color coded according to the scale at the bottom of the figure. Permethylated neutral and sialylated O-linked glycans were mostly recovered from the DCM phase, while the

permethylated sulfoglycans were quantitatively recovered from the aqueous phase. Negative mode ionization enhanced detection of glycans bearing more than one sulfate.

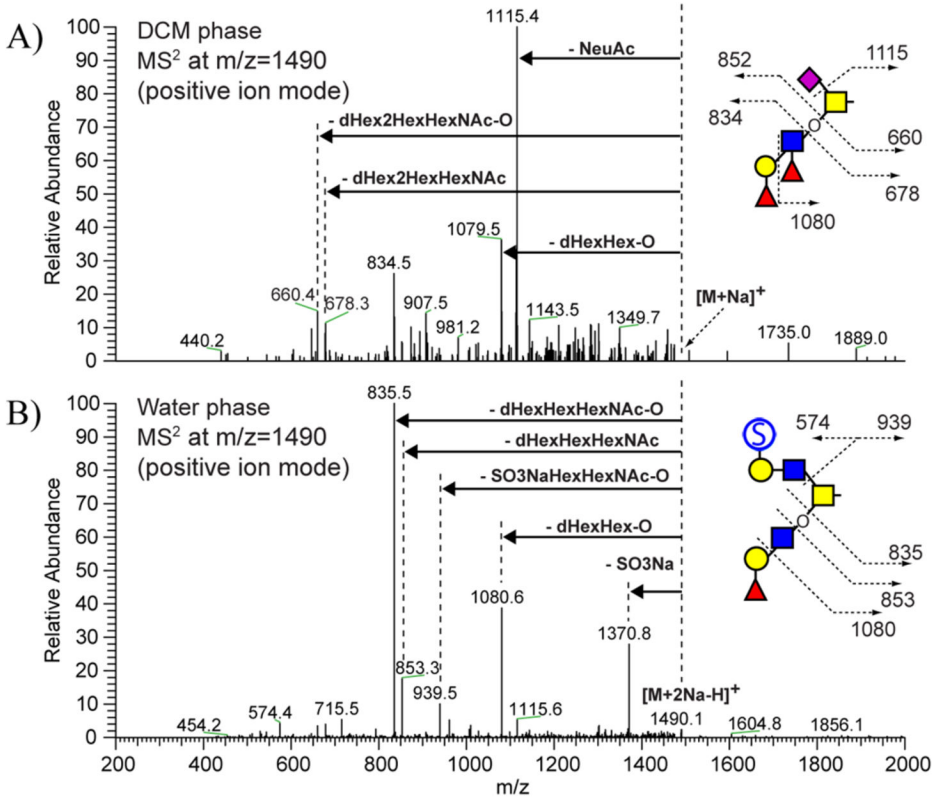

Figure 8. Aqueous-organic phase partition simplifies interpretation of MS^2 data

Distinct MS^2 fragment patterns are obtained for two molecular ions detected at around *m/z* 1490 by TIM analysis. Without separation by phase partition, the MS^2 fragmentation profile for the 2.2 mass unit window containing m/z = 1490 would contain both patterns, greatly complementing interpretation of the spectra. (**A**) Fragmentation of the proposed structure NeuAc-6(Fuc-Gal-3(Fuc-GlcNAc-3)GalNAc-ol (predicted mass = 1488.8 $[M+Na]^+$) detected as the indicated permethylated, sialylated core 2 glycan in the DCM phase. (**B**) Fragmentation of the proposed structure (SO_3^-)-Gal-GlcNAc-6(Fuc-Gal-GlcNAc-3)GalNAc-ol (predicted mass = 1490.7 $[M+2Na-H]^+$) detected as the indicated permethylated sulfoglycan in the water phase. Fragmentation schemes are shown to the right of each spectra.