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Small-Scale Analysis of O-Linked Oligosaccharides from Glycoproteins and Mucins Separated by Gel Electrophoresis

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A technique with subpicomolar sensitivity was developed for analyzing O-linked oligosaccharides released from glycoproteins separated by gel electrophoresis. The protocol involves gel electrophoresis, electroblotting to poly(vinylidene fluoride) membrane, reductive β-elimination, and analysis of released oligosaccharides by liquid chromatography coupled to negative ion electrospray mass spectrometry. It was also found that N-linked oligosaccharides could be recovered under the same conditions, found both as free oligosaccharides and as distinct glycopeptides created from reductive cleavage of the protein backbone, giving some information on site-specific glycosylation. The method was used to demonstrate that the difference between human α-2HS-glycoprotein isoforms separated by 2D-gel electrophoresis was partially due to sialylation of both O-linked and N-linked oligosaccharides. It was also shown that both acidic and neutral oligosaccharides could be recovered and analyzed simultaneously from high molecular mass (200 000−5 000 000 Da) highly glycosylated mucin glycoproteins collected from small intestine and saliva and separated by sodium dodecyl sulfate−agarose/polyacrylamide composite gels. Mass spectrometric data not only gave information about the mass distribution of the heterogeneous mixtures of oligosaccharides from $[M - xH]^{x-}$ ions but also gave information about the isomeric heterogeneity of the oligosaccharides from their resolution by porous graphitized carbon chromatography. Tandem mass spectrometry was explored as a technique for distinguishing between oligosaccharide isomers with different sequences and also between oligosaccharides with the same sequence but with different linkage configurations.

Analysis of protein glycosylation is a topic that both fascinates and frustrates researchers in the area of protein research. Glycoproteins are involved in many biological processes, and glycosylation of proteins has been shown to be a dynamic event, changing during lactation,[1] infection,[2] and cancer development.[3,4] Analysis of glycoprotein oligosaccharides has traditionally used a combination of techniques such as mass spectrometry, ^{1}H NMR, monosaccharide composition analysis, and methylation linkage analysis. These techniques require not only purification of glycoproteins but also subsequent isolation of individual oligosaccharide components. This is both time-consuming and requires milligram quantities of glycoprotein. Recent developments in both electrospray and MALDI mass spectrometry have led to reductions in the amount of material needed for oligosaccharide analysis, and methods have been developed that not only give accurate mass determination but also provide structural information. These methods involve either the use of MS/MS[5,6] or sequential degradation with exoglycosidases followed by mass spectrometry.[7]

The sensitivity of mass spectrometry has increased to a point were it is possible to detect oligosaccharides released from glycoproteins separated by gel electrophoresis (from picomole quantities of protein).[8] While the techniques for analysis of N-linked oligosaccharides are becoming routine, the same is not true for the analysis of O-linked oligosaccharides. There are several reasons for this, the major being that N-linked oligosaccharides can be released reliably with *N*-glycosidases and then chemically labeled at their reducing terminus. Commonly used derivatization techniques increase the sensitivity for positive ion mass spectrometry, allowing efficient detection of many neutral oligosaccharides. Additional problems arise for mass spectrometric detection of highly acidic oligosaccharides in positive ion mode, which can be at least partly overcome by desialylating samples before analysis[9] or performing further derivatization of the sialic acid residues, thereby neutralizing their negative charge.[10] As there is no enzyme that efficiently releases all O-linked oligosaccharides from the polypeptide backbone, only chemical methods are available. Such chemical methods include hydrazinolysis[11] and

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nonreductive β-elimination using amines[12,13] or hydroxide.[14] The most common and reproducible method for release of O-linked oligosaccharides from serine and threonine residues is reductive β-elimination, which uses dilute base in the presence of sodium borohydride. The disadvantage of reductive β-elimination is that oligosaccharides are recovered as alditols, and it is therefore not possible to employ reducing end derivatization to increase sensitivity, as it is with N-linked oligosaccharides. However, sensitive mass spectrometry enables detection of underivatized oligosaccharides.

The biological significance of O-linked glycosylation has been identified on glycoproteins involved in processes such as cellular recognition, regulation, and disease.[15−17] We have developed a reliable method for analyzing O-linked oligosaccharides from glycoproteins separated by gel electrophoresis, using either standard one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS−PAGE) or 2D-gel electrophoresis. In addition, we have improved the use of agarose/polyacrylamide composite gels[18] in order to be able to electrophoretically separate the extremely highly glycosylated, high molecular mass (200 000-5 000 000 Da) glycoproteins known as mucins. Mucins are found at mucosal surfaces, and their glycosylation is important in mediating intermolecular and intercellular interactions.[19] They are involved in lubrication of mucosal surfaces, help prevent viral and bacterial invasion, and also protect other tissues from the hostile and degrading environment that is found in the gastrointestinal tract and at other epithelial surfaces. The oligosaccharides that are present on these proteins have been implicated as being important in the immune response[2] and inflammation.[20]

High-performance liquid chromatography (HPLC) of oligosaccharides using a porous graphitized carbon stationary phase has been shown to be a powerful separation technique,[21] and graphitized carbon withstands pH extremes, as opposed to traditional silica-based materials. Graphitized carbon HPLC coupled to electrospray mass spectrometry has been used for analysis of both neutral and acidic oligosaccharides, detected as their $[M - xH]^{x-}$ ions using volatile buffers at pH >10.[22] This approach was adopted here for analyzing O-linked oligosaccharide alditols, since sensitive detection could then be carried out without requiring further derivatization.

EXPERIMENTAL SECTION

Materials and Reagents. Rat small intestinal Muc2 mucin was prepared as described[2] from outbred Sprague−Dawley rats, and human plasma and saliva were obtained from healthy individuals. Lacto-*N*-tetraose (LNT) and Lacto-*N*-neotetraose (LNnT) were purchased from Dextra Laboratories (Reading, U.K.). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

1D SDS−PAGE of Bovine Fetuin. Bovine fetuin (0.5−600 pmol) was dissolved in sample loading buffer (10% glycerol, 0.325 M Tris-HCl pH 8.8, 1% SDS, 0.005% Bromophenol blue, and 10 mM tri-*n*-butylphosphine) and reduced at 95 °C for 15 min. The samples were then alkylated by adding iodoacetamide to a final concentration of 25 mM and incubating at room temperature for 1 h. 1D SDS−PAGE premade gels (4−12% polyacrylamide) were run according to the manufacturer's instructions (Novex, San Diego, CA). Proteins were then electroblotted to poly(vinylidene fluoride) (PVDF) Immobilon P^{SQ} membrane (Millipore, Bedford, MA) using the semidry method of Khyse-Anderson,[23] transferred at 4 mA/cm^2 for 40 min. The membrane was stained with 0.008% Direct Blue in 40% ethanol and 10% acetic acid for 7 min and destained in 40% ethanol and 10% acetic acid.

Separation of Mucins by 1D SDS−AgPAGE. One-dimensional sodium dodecyl sulfate−agarose/polyacrylamide gel electrophoresis (1D SDS−AgPAGE) gradient gels were made by mixing two heated solutions (both with 0.5% agarose and 0.375 M Tris-HCl pH 8.1 and one also containing 10% glycerol and acrylamide and piperazine diacrylamide (6% T, 2.5% C)). The 0.5% agarose/0−6% gradient polyacrylamide gradient gels were cast in a mini-Protean gel casting apparatus (Bio-Rad, Hercules, CA) at 50 °C after adding *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.0125%) and ammonium persulfate (0.005%) to each solution. The gels were polymerized for 1 h at 50 °C, and the agarose was then allowed to set at room temperature overnight in a humidified environment. The anode and cathode buffer was 192 mM tris-borate, pH 7.6, with 1 mM EDTA and 0.1% SDS.

Rat Muc2 intestinal mucin and saliva were reduced and alkylated in sample loading buffer as described above for 1D SDS−PAGE (except with Tris-HCl at pH 8.1), and sample equivalent to 100 μg of Muc2 and 20 μL of saliva were loaded onto 1D SDS−AgPAGE gels and electrophoresed at 100 V for 2−3 h, until the dye front migrated out of the gel. Proteins were electroblotted to PVDF as above, with methanol excluded from the anode buffer in order to prevent agarose gel dehydration. Gels were stained using periodic acid/Schiff's base (PAS)[24] or Alcian Blue (stained in 0.125% Alcian Blue in 25% ethanol and 10% acetic acid for 10 min and destained in 100% methanol for 20 min).

Separation of Human Plasma Proteins by 2D Gel Electrophoresis. Fibrinogen,[25] human serum albumin,[26] and immunoglobulin type G (protein G affinity, Amersham Pharmacia Biotech, Uppsala, Sweden) were removed from human plasma samples prior to reduction and alkylation.[27] Proteins were separated in the first dimension by isoelectric focusing on pH 4−7 immobilized pH gradient strips (Amersham Pharmacia Biotech). The strips were equilibrated with second-dimension running buffer before being applied to 6−18% polyacrylamide GelChip gels for 2D SDS−PAGE according to the manufacturer's protocol (Pro-

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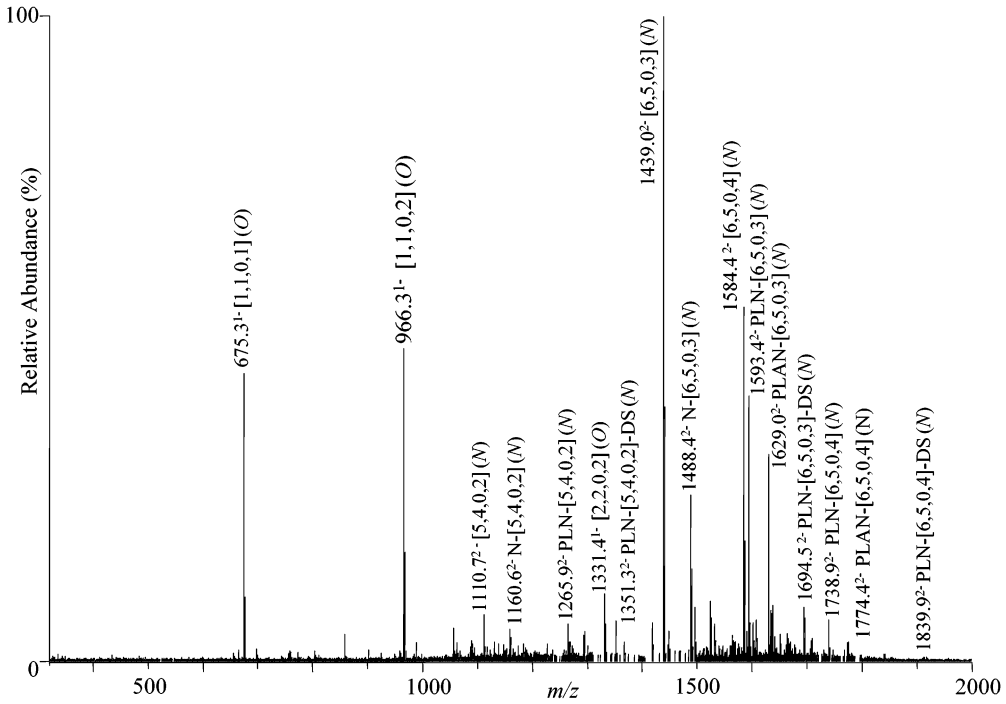

Figure 1. Total ion mass spectrum from LC/ESI-MS of oligosaccharides and glycopeptides (eluted from graphitized carbon from 10.0 to 30.0 min) released by reductive β-elimination of bovine fetuin (0.1 μg), separated by SDS−PAGE, and electroblotted to PVDF membrane. Peaks are labeled with *m*/*z*, charge state, amino acid sequence, monosaccharide composition (hexose, *N*-acetylhexosamine, deoxyhexose, *N*-acetylneuraminic acid) (for simplicity, the terminal *N*-acetylgalactosaminitol in the reduced O-linked oligosaccharides is included as an *N*-acetylhexosamine), and linkage type (O- or N-linked).

teome Systems Inc, Boston, MA). Proteins were electroblotted and stained with Direct Blue, as above.

Reductive Alkaline β-Elimination of Oligosaccharides. Oligosaccharides attached to glycoproteins separated by 1D or 2D SDS−PAGE or 1D SDS−AgPAGE, and blotted to PVDF membrane, were released by reductive β-elimination. Direct Blue- or Alcian Blue-stained bands were excised from the membrane, wetted with methanol, and incubated at 50 °C for 16 h in 20 μL of 50 mM NaOH and 0.5 M $NaBH_4$. Samples were incubated in microtiter plate wells sealed with amplification tape to prevent evaporation. The resulting solutions were neutralized by the addition of 1 μL of glacial acetic acid, before being desalted with 25 μL of AG50WX8 cation-exchange resin (Bio-Rad), laid on top of a reversed-phase μ-C18 ZipTip (Millipore), and dried in a Savant SpeedVac. Borate was removed by repeated (5 times) addition and evaporation of 50 μL of 1% acetic acid in methanol. Finally, the samples were resuspended in 10 μL of MilliQ water for liquid chromatography coupled to electrospray mass spectrometry (LC/ESI-MS) analysis.

LC/ESI-MS Analysis of Released Oligosaccharides. Desalted oligosaccharide alditols were analyzed by liquid chromatography coupled to electrospray tandem mass spectrometry (LC/ESI-MS/MS) on a homemade graphitized carbon column (7-μm Hypercarb particles (Thermo-Hypersil, Runcorn U.K.) in a 100 × 0.25 mm column) or a 150 × 0.32 mm Hypercarb column (Thermo-Hypersil). A solvent rate through the column of 5 μL/min was provided by a Surveyor LC pump (ThermoFinnigan, San Jose, CA) with flow splitting from 100 μL/min. Oligosaccharides were eluted with an H_2O/acetonitrile gradient (0−40% acetonitrile in 30 min, followed by a 3-min wash with 90% acetonitrile) containing 10 mM NH_4HCO_3. Mass spectrometry was performed using an LCQ Deca (ThermoFinnigan) in negative ion mode, with three scan events: full scan with mass range 320−2000 *m*/*z*, dependent zoom scan of the most intense ions in each scan, and dependent MS/MS scan after collision-induced fragmentation. The capillary temperature was 180 °C, the capillary voltage was 32.0 V, and the electrospray voltage was 2.5 kV. Dynamic exclusion of ions for zoom scan for 30 s was introduced after three selections within 30 s. For MS/MS, the normalized collision energy was 35%, with an activation time of 30 ms. Oligosaccharide structure and linkage were determined using a combination of analysis of LC/ESI-MS/MS data and the GlycoSuiteDB sugar database (http://www.glycosuite.com).

RESULTS AND DISCUSSION

Bovine Fetuin Oligosaccharides. Bovine fetuin was used as a test protein to investigate whether reductive β-elimination could be used as a standard method for releasing O-linked oligosaccharides from SDS−PAGE-separated glycoproteins for analysis by LC/ESI-MS. Apart from two sites substituted with O-linked oligosaccharides, fetuin also has three sites substituted with complex N-linked oligosaccharides (http://www.expasy.ch). Interestingly, when the oligosaccharides released by β-elimination from the major 40-kDa fetuin isoform, blotted onto PVDF membrane after 1D SDS−PAGE, were analyzed by negative ion LC/ESI-MS, both singly charged pseudomolecular ions from O-linked oligosaccharides and doubly charged pseudomolecular ions from larger N-linked oligosaccharides were detected (Figure 1). The singly charged pseudomolecular ions of *m*/*z* 675.3, 966.3, and 1331.4 correspond respectively to the NeuAc(α2−3)Gal(β1−3)GalNAcol, NeuAc(α2−3)Gal(β1−3)[NeuAc(α2−6)]GalNAcol, and NeuAc(α2−3)Gal(β1−4)GlcNAc(β1−6)[NeuAc(α2−3)Gal-

(β1−3)]GalNAc species that have been reported to be O-linked oligosaccharides present on bovine fetuin.[28,29] The major N-linked oligosaccharides reported from bovine fetuin (disialylated biantennary and tri- and tetrasialylated triantennary) were found as doubly charged pseudomolecular ions at *m*/*z* 1110.7, 1439.0, and 1584.4, respectively.

It is generally believed that efficient release of N-linked oligosaccharides in alkaline conditions requires a higher concentration of alkali than is required for release of O-linked oligosaccharides, as the mechanism is a nucleophilic substitution that converts the glycosylated asparagine into aspartic acid, rather than β-elimination. However, release of N-linked oligosaccharides in conditions similar to those used here has been previously reported.[30,31] In the mass spectra of the released N-linked oligosaccharides from bovine fetuin, the isotopic distribution of the pseudomolecular ions showed a mixture of two species—one with the expected distribution and one minor component starting at 1 amu lower than the expected monoisotopic value (data not shown). The spectra of the released O-linked oligosaccharides had normal isotopic distributions. The presence of a mixture of species of released N-linked oligosaccharides in the alkaline reducing conditions is possibly due to incomplete hydroxyl replacement of the nitrogen at C-1 of the *N*-acetylglucosamine residue previously linked to asparagine.

Also present in the mass spectrum (Figure 1) are additional doubly charged pseudomolecular ions corresponding to the masses of N-linked oligosaccharides attached to varying numbers of amino acid residues. These glycopeptides presumably result from reductive cleavage of the peptide bonds by sodium borohydride and reduction of the carboxy terminus to an alcohol.[32,33] Three sites of N-linked glycosylation are reported in bovine fetuin, and glycopeptides from two of these were detected. These detected glycopeptides contained N-99 and N-156, respectively, sites that both contain a proline residue shortly before the glycosylated asparagine (PLANCSV and PLNDSR, respectively). The third reported glycosylation site in bovine fetuin, N-176, is contained in the sequence AESNGSY and was not detected. Species were also detected consisting of only a glycosylated asparagine converted into its corresponding alcohol. The data suggest that a peptide bond containing a secondary amide (proline) will be more susceptible to reductive cleavage by sodium borohydride than a primary amide. The series of glycopeptides produced by reductive cleavage in these cases can provide details of site-specific N-linked glycosylation.

Detection Sensitivity. Bovine fetuin was also used to establish that the sensitivity of detection of released O-linked oligosaccharides by negative LC/ESI-MS was high enough for the amount of glycoprotein generally loaded onto SDS−PAGE gels and detected by standard Coomassie-based protein stains (usually in the low-picomole range). The pseudomolecular ion of *m*/*z* 1331.4 was less abundant than the *m*/*z* 966.3 and 675.3 ions, and therefore, more protein was required for its detection (Figure 2). All three species could be reliably detected after release from across the measured range of 0.5−11.5 pmol of fetuin, separated by 1D SDS−PAGE and electroblotted to PVDF, indicating that the theoretical limit of detection is between 0.5 and 0.7 pmol depending on the specific oligosaccharide. The response from the ion trap analyzer was also shown to be linear over the range analyzed, with the relative intensity of each ion varying less than 2% over the range. This is a prerequisite for the method to be able to detect small glycosylation differences between samples.

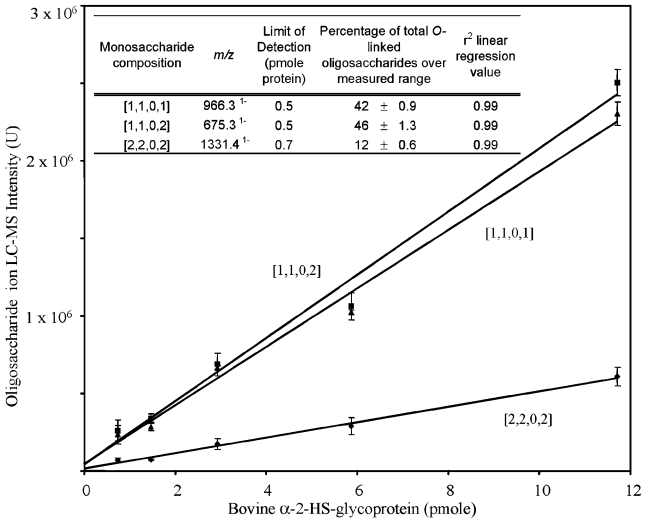

Figure 2. Mass spectrometric response of O-linked oligosaccharide alditols released from bovine fetuin subjected to 1D SDS−PAGE and electroblotted to PVDF membrane. Monosaccharide compositions labeled as hexose, *N*-acetylhexosamine, deoxyhexose, and *N*-acetylneuraminic acid (including the terminal *N*-acetylgalactosaminitol in the reduced O-linked oligosaccharides as an *N*-acetylhexosamine). Error bars are equal to the standard deviation for each data point ($n = 2$). Limits of detection are calculated as the amount of protein giving three times the signal of the *y*-intercept (signal/noise of 3:1). Percentages of total O-linked oligosaccharides are calculated as the fraction of each oligosaccharide of the sum of the mass spectrometric intensities of the three O-linked oligosaccharides detected, averaged over the concentration range 0.5−11.5 pmol of protein.

Human α-2-HS-glycoprotein Glycoforms. As with many proteins separated by 2D-gel electrophoresis from human plasma, α-2-HS-glycoprotein appears as a train of distinctive isoforms, as shown in Figure 3A. Since both N- and O-linked oligosaccharides could be released by reductive β-elimination, it was possible to perform semiquantitative analysis of the full glycosylation profiles of the different isoforms of human α-2-HS-glycoprotein. Ions corresponding to N-linked oligosaccharide alditols and glycopeptides were again observed, along with the released and reduced O-linked oligosaccharides. Figure 3B shows the changes in the proportions of N- and O-linked oligosaccharides released from different p*I* isoforms of the α-2-HS-glycoprotein. As the isoform p*I* increases, the proportion of monosialylated O-linked oligosaccharides and disialylated N-linked oligosaccharides increases, with a concomitant decrease in the proportion of disialylated O-linked and trisialylated N-linked oligosaccharides. This suggests that differential glycosylation, especially sialylation, of both N- and O-linked oligosaccharides is at least partly responsible for the presence of multiple human α-2-HS-glycoprotein p*I* isoforms.

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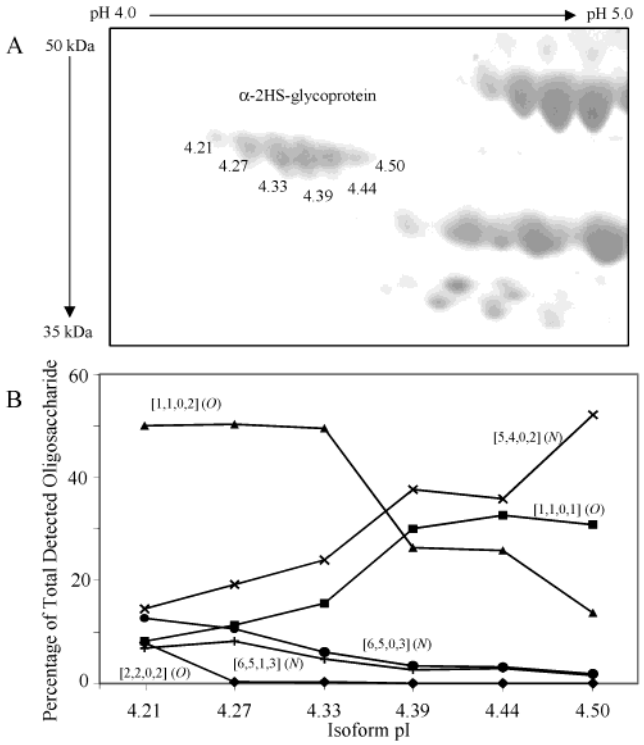

Figure 3. (A) Human α-2-HS-glycoprotein from plasma separated by 2D-gel electrophoresis, electroblotted to PVDF membrane, and stained with Direct Blue. Estimated protein isoform p*I* is indicated. (B) Glycosylation profile variation as a function of isoform isoelectric point. Monosaccharide compositions labeled as hexose, *N*-acetylhexosamine, deoxyhexose, and *N*-acetylneuraminic acid (including the terminal *N*-acetylgalactosaminitol in the reduced O-linked oligosaccharides as an *N*-acetylhexosamine) and protein linkage type indicated (O- or N-linked).

Analysis of Rat Muc2 Intestinal Mucin Oligosaccharides. Mucins are glycoproteins that are typically larger than 200 kDa and are predominantly glycosylated with O-linked oligosaccharides, which constitute up to 80% of their total molecular weight. Traditionally, characterization of oligosaccharides from mucins is carried out after isolation of mucin fractions by isopycnic centrifugation, followed by gel and anion-exchange chromatography.[34] As a final step, the oligosaccharides are released, isolated, and characterized using mass spectrometry, monosaccharide composition analysis, and ^{1}H NMR.[35,36] The approach taken here uses a highly resolving agarose−polyacrylamide gradient composite gel for isolation of mucin fractions, since the high molecular weight of most mucins makes them unsuitable for traditional SDS−PAGE. Analysis of oligosaccharides released from these electroblotted gel-separated mucin subpopulations, using high-resolving LC coupled to ESI-MS can characterize the glycosylation of small quantities of mucin (less than 100 μg of a crude mucin fraction, compared with several milligrams of purified mucin for traditional analysis).

The glycosylation of rat Muc2 intestinal mucin has been previously well characterized,[37,38] and the utility of the method described here for glycosylation analysis of mucins separated by gel electrophoresis was determined by comparison with the previously reported Muc2 mucin glycosylation profile. 1D SDS−AgPAGE separation of rat Muc2 mucin showed several distinct PAS staining bands with approximate molecular masses of 1−5 MDa in the gel. Alcian Blue was used to stain the blotted mucins for glycosylation analysis, as PAS staining oxidizes the carbohydrates, thereby rendering them unsuitable for characterization. After electroblotting, only the three lower bands could be detected by PAS and Alcian Blue staining (Figure 4A). Attempts to recover released oligosaccharides after in-gel β-elimination was unsuccessful, due to degradation of the agarose component of the AgPAGE gels in the reductive alkaline conditions, leading to increased background noise in subsequent oligosaccharide analysis by mass spectrometry.

Oligosaccharide alditols released from the major Alcian Blue-staining mucins on PVDF membrane were analyzed using porous graphitized carbon LC/ESI-MS. The total ion mass spectrum from one isoform of the rat Muc2 mucin is shown in Figure 4B and summarized in Table 1. The glycosylation profiles of all of the analyzed Muc2 protein isoforms separated by 1D SDS−AgPAGE were similar, differing only in the relative intensities of individual oligosaccharide species (data not shown).

Oligosaccharides that have been previously reported as present on the rat Muc2 mucin are catalogued in the GlycoSuiteDB sugar database (http://www.glycosuite.com), and the method described here identifies the majority of these oligosaccharide structures, as well as identifying the composition of many more. Some smaller species, such as a single GalNAcol and Gal(β1−3)GalNAcol, were not retained on the graphitized carbon LC column and were therefore not detected, while many of the acidic (sialylated and sulfated) oligosaccharides detected here have never been identified in this tissue before. Given the agreement between the methods in identifying several neutral and smaller sialylated oligosaccharides, the additional strength of this method lies in the detection of larger and more acidic oligosaccharides. This is probably due to the use of negative ion electrospray mass spectrometry for oligosaccharide detection, which provides somewhat better sensitivity for larger and negatively precharged molecules.

Analysis of Salivary Mucin Glycosylation. The utility of the system was further challenged by using 1D SDS−AgPAGE to isolate two differently glycosylated mucins—MUC5B[36,39] and MUC7[40]—from unfractionated human saliva and demonstrating that the obtained glycosylation profiles from LC/ESI-MS could be used to detect glycosylation differences. Two major glycosylated proteins were found in 1D SDS−AgPAGE-separated saliva, electroblotted to PVDF, both of which were detected with PAS staining (not shown) and Alcian Blue staining (Figure 5A). The total ion mass spectra of released and reduced oligosaccharides from each mucin are shown in Figure 5B and C. Comparison of these spectra shows substantial differences in the glycosylation

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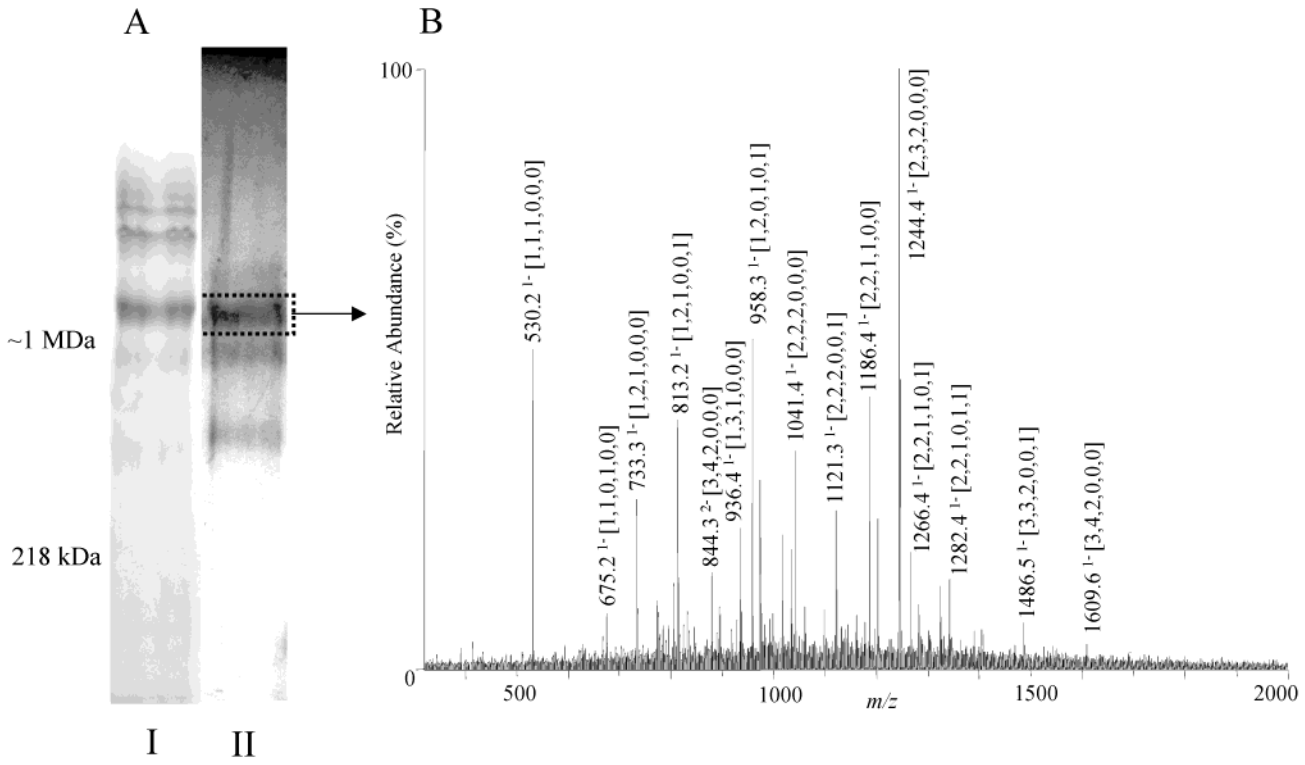

Figure 4. (A) Rat Muc2 intestinal mucin isoforms, (I) separated by 1D SDS−AgPAGE and stained with PAS or (II) separated by 1D SDS−AgPAGE, electroblotted to PVDF membrane, and stained with Alcian Blue. (B) Graphitized carbon LC/ESI-MS total ion mass spectrum of O-linked oligosaccharide alditols released by reductive alkaline β-elimination from one 1D SDS−AgPAGE-separated Muc2 isoform. Peaks are labeled with *m*/*z*, charge state, and monosaccharide composition (hexose, *N*-acetylhexosamine, deoxyhexose, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, sulfate) (including the terminal *N*-acetylgalactosaminitol as an *N*-acetylhexosamine).

profiles of these two mucins, with features including increased sialylation in the MUC7-containing band and increased fucosylation and sulfation in the MUC5B-containing band. This agrees with previously reported glycosylation profiles of these mucins.[41,42]

MS/MS Analysis of Oligosaccharides. High-resolving graphitized carbon LC/ESI-MS of oligosaccharides not only allows identification of oligosaccharides by mass but also provides insight into the isomeric heterogeneity of the sample. On-line liquid chromatography coupled to ion trap mass spectrometry can potentially differentiate structural isomers by MS/MS. Automatic selection of detected ions from LC/ESI-MS for MS/MS experiments provided interpretable information about oligosaccharide linkage and sequence, which allowed for structural analysis of oligosaccharide isomers. The base peak chromatogram of the total oligosaccharides released from rat Muc2 (Figure 6A) illustrates separation by LC of a complex mixture of oligosaccharides. Figure 6B shows separation of the two structural isomers dHex-Hex-3[HexNAc-6]HexNAcol (23.35 min) and dHex-Hex-HexNAc-3Hex-NAcol (15.91 min) from rat Muc2, detected as $[M - H]^-$ ions of *m*/*z* 733.3, and their MS/MS spectra are shown in Figure 7. These MS/MS spectra illustrate the typical fragmentation pattern of neutral oligosaccharides in negative ion mode, in which all spectra are dominated by intense fragment ions arising from cleavage of glycosidic bonds. Depending on which side of the glycosidic bond cleavage occurs, fragments either gain (Y- and C-cleavages) or lose (Z- or B-cleavages) protons. This is illustrated in Figures 7−9, where Y or C cleavages give negative ions equal to the mass of illustrated fragment ions and Z or B cleavages give negative ions two mass units less. Analysis of MS/MS spectra was in agreement with the sequence or composition of oligosaccharides already detected from rat Muc2[37,38] or on salivary mucins.[41,42]

Oligosaccharides with the same sequence but different linkage configurations were also separated on the graphitized carbon column and exhibited different MS/MS spectra. Analysis of the MS/MS spectra of the three isomers of the $[M - H]^-$ ion of *m*/*z* 1244.4 showed that all three had the sequence dHex-Hex-HexNAc-3(dHex-Hex-HexNAc-6)HexNAcol (Figure 8) but differed in Hex-to-HexNAc linkage configurations. This linkage has been reported to be either a type 1 chain (Gal(β1−3)GlcNAc(β1-)) or a type 2 chain (Gal(β1−4)GlcNAc(β1-)). MS/MS experiments were performed with standard substances in order to identify possible fragment ions that could be diagnostic for either type of linkage. Using lacto-*N*-tetraose (Gal(β1−3)GlcNAc(β1−3)Gal(β1−4)Glc) and lacto-*N*-neotetraose (Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc), it could be shown that the ^{0,2}A-fragment ion (*m*/*z* 280.8) and the ^{0,2}A-H_2O fragment ion (*m*/*z* 262.8) were only formed with lacto-*N*-neotetraose (type 2 chain; Figure 9). This suggests that the fragment ion from a ^{0,2}A-cleavage with concomitant loss of water from a GlcNAc is favorably formed when the GlcNAc is substituted on C-4 rather than C-3.

This fragmentation pattern would give the ^{0,2}A-H_2O fragment ion of *m*/*z* 409 (428 − H − H_2O) in Figure 8B and C, indicating

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Table 1. O-Linked Oligosaccharides Detected from Rat Muc2 Intestinal Mucin Using the Method Described Here Compared with Those Previously Reported

calcd neutral reduced mass	predominant $[M - xH]^{x-}$ ion	monosaccharide composition[a]						intensity (‰)	isomers	structure previously identified[b]
		Hex	HexNAc	Deoxyhex	NeuAc	NeuGc	Sulf			
Neutral Oligosaccharides										
223.1			1					nd[c]	1	+
385.2		1	1					nd	1	+
426.2	425.2^{-1}		2					1.2	1	+
531.2	530.2^{-1}	1	1	1				55.4	1	+
588.2		1	2					nd	1	+
629.3	628.3^{-1}		3					0.6	1	+
734.3	733.3^{-1}	1	2	1				26.7	3	+
896.3	895.3^{-1}	2	2	1				6.3	3	+
791.3		1	3					nd	2	+
937.4	936.4^{-1}	1	3	1				8.4	2	+
1042.4	1041.4^{-1}	2	2	2				103.0	2	+
1099.4	1098.4^{-1}	2	3	1				2.4	1	+
1245.5	1244.4^{-1}	2	3	2				219.0	3	+
1610.6	844.3^{-2}	3	4	2				24.7	1	
Sialylated Oligosaccharides										
676.2	675.2^{-1}	1	1		1			20.4	1	+
692.2	691.2^{-1}	1	1			1		6.5	1	+
879.3	878.3^{-1}	1	2		1			6.8	1	+
895.3	894.3^{-1}	1	2			1		22.1	1	+
1041.4	1040.4^{-1}	2	2		1			4.2	1	+
1057.4	1056.4^{-1}	2	2			1		5.1	1	+
1187.4	1186.4^{-1}	2	2	1	1			68.7	1	+
1203.4	1202.4^{-1}	2	2	1		1		34.4	1	+
1332.5	1331.5^{-1}	2	2		2			5.9	1	
1390.5	1389.5^{-1}	2	3	1	1			7.0	1	
1406.5	1405.5^{-1}	2	3	1		1		4.0	1	
1552.6	775.3^{-2}	3	3	1	1			16.3	1	
1568.6	783.4^{-2}	3	3	1		1		10.5	1	
1755.6	876.7^{-2}	3	4	1	1			2.5	1	
Sulfated Oligosaccharides										
668.2	667.2^{-1}	1	2				1	1.4	1	+
709.2			3				1	nd	1	+
814.2	813.2^{-1}	1	2	1			1	64.0	1	+
830.2		2	2				1	nd	1	
871.3	870.3^{-1}	1	3				1	1.8	3	+
959.3	958.3^{-1}	1	2		1		1	54.3	1	
975.3	974.3^{-1}	1	2			1	1	40.5	1	
976.3	975.3^{-1}	2	2	1			1	4.3	2	+
1017.3	1016.3^{-1}	1	3	1			1	68.7	3	+
1033.3		2	3				1	nd	2	+
1121.3	1120.3^{-1}	2	2		1		1	4.5	1	
1122.4	1121.4^{-1}	2	2	2			1	41.6	1	+
1179.4		2	3	1			1	nd	1	+
1267.4	1266.4^{-1}	2	2	1	1		1	13.6	1	
1283.4	1282.4^{-1}	2	2	1		1	1	4.5	1	
1325.4	1324.4^{-1}	2	3	2			1	6.3	2	+
1341.4	1340.4^{-1}	3	3	1			1	9.0	2	+
1487.5	1486.5^{-1}	3	3	2			1	13.1	1	+
1544.5	1543.5^{-1}	3	4	1			1	1.4	1	+
1690.6	1689.6^{-1}	3	4	2			1	9.0	1	

[a] Hex, hexose; HexNAc, *N*-acetylhexosamine (including the terminal *N*-acetylgalactosaminitol in the reduced O-linked oligosaccharides); Deoxyhex, deoxyhexose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Sulf, sulfate. [b] + = yes; = GlycoSuiteDB (http://www.glycosuite.com). [c] nd, not detected.

that these isomers contain type 2 chains. These are not seen with the 20.86-min isomer (Figure 8A), which shows fragment ions of m/z 407.2 (408 − H) and 389.2 (392 − 3H). These fragments are probably formed by a double cleavage of the glycosidic linkage of one of the branches of the HexNAcol and the glycosidic bond between its adjacent HexNAc and either a C-3 or C-4 substituent (Figure 8D). This fragmentation will occur in both type 1 and 2 chains, but in a type 2 chain it will compete with the formation of the m/z 409 ion, because both involve cleavage at the same monosaccharide residue, and would therefore be less intense.

It could be speculated that oligosaccharide linkage conformations on rat Muc2 are similar to oligosaccharides on glycosphingolipids from rat small intestine. The C-6 of the branching galactose of the major blood group H decaglycosylceramides from glycosphingolipids contains either blood group H type 1 or blood group H type 2 sequences, while the C-3 branch has only been

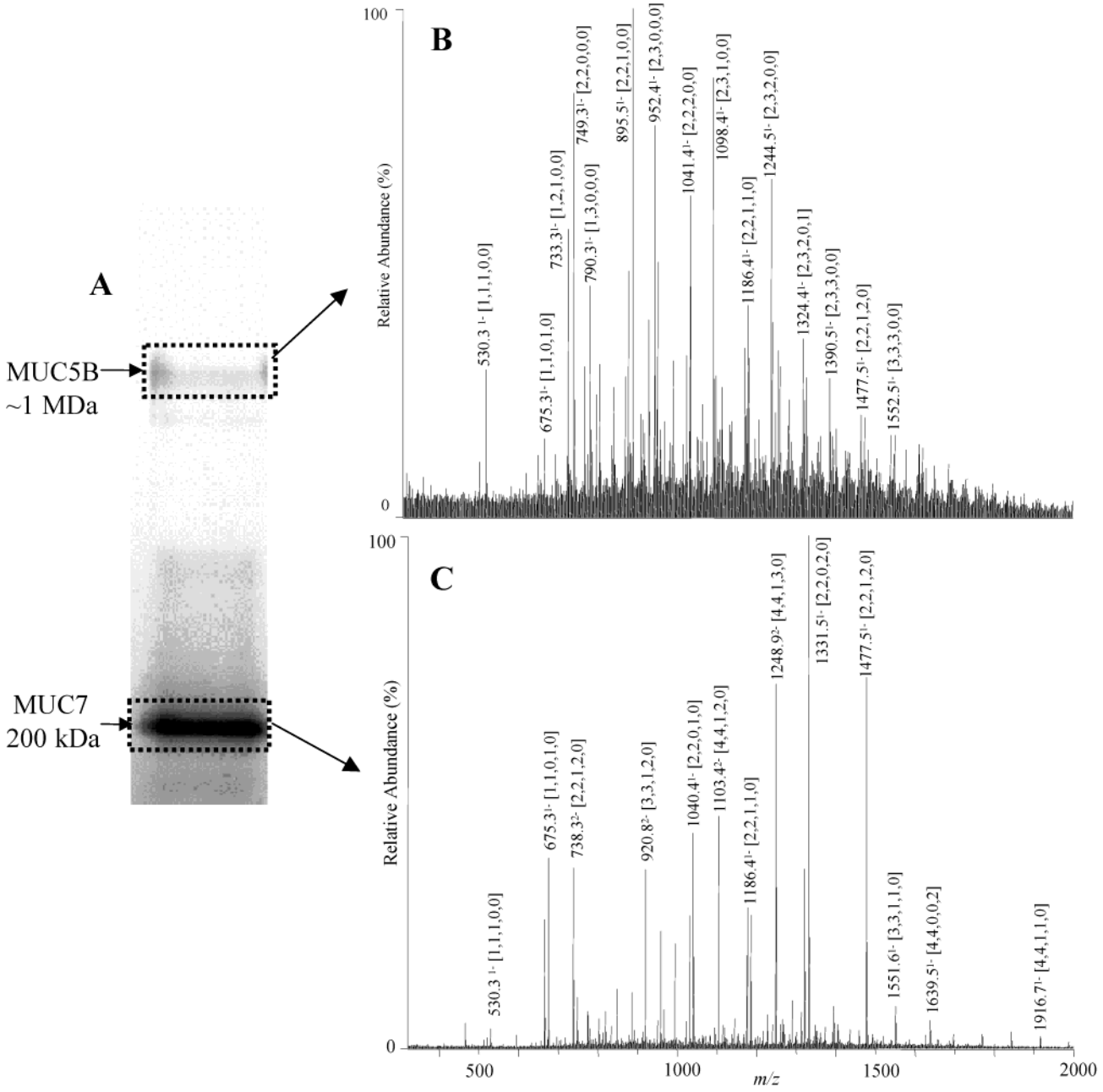

Figure 5. (A) Human salivary mucins separated by 1D SDS−AgPAGE, electroblotted to PVDF membrane, and stained with Alcian Blue. Graphitized carbon LC/ESI-MS total ion mass spectra of O-linked oligosaccharide alditols released by reductive alkaline β-elimination from (B) MUC5B- and (C) MUC7-containing bands. Peaks are labeled with *m*/*z*, charge state, and monosaccharide composition (hexose, *N*-acetylhexosamine, deoxyhexose, *N*-acetylneuraminic acid, sulfate) (including the terminal *N*-acetylgalactosaminitol as an *N*-acetylhexosamine).

found to contain the type 1 sequence.[43] The two combinations of these linkage configurations (type 1 on both the C-3 and C-6 branches of the GalNAcol (Figure 8A), and type 1 on the C-3 branch with type 2 on the C-6 branch (Figure 8B)) could explain two of the three chromatographic isomers of the $[M - H]^-$ ion of m/z 1244.4 (Figure 6C). However, the presence of a third (albeit minor) component (Figures 6C and 8C) with an MS/MS spectrum indicative of a type 2 sequence suggests that this sequence can be present on both or either of the C-3 and C-6 branches of the GalNAcol on rat Muc2.

Another significant and reproducible difference between the MS/MS spectra of the isomers of the $[M - H]^-$ ion of m/z 1244.4 from rat Muc2 is the marked differences in the ratios of the intensities of the m/z 570 and 733 fragment ions relative to the normalized m/z 715 ion. While there is no obvious explanation for this phenomenon, it must be caused by the differences in glycosidic linkages of these isomers.

MS/MS fragmentation of acidic oligosaccharides presents additional problems to neutral oligosaccharides, because any negative charge will be predominantly localized to acidic residues. Sialic acids are also readily cleaved in the fragmentation process, leaving the remaining fragment uncharged and therefore not detected. Sequencing of acidic oligosaccharides with this approach therefore relies on sensitive detection of low abundance fragment ions. This is illustrated in Figure 10, where the fragment ions of the $[M - H]^-$ ion of m/z 1331.5 from the salivary MUC7-

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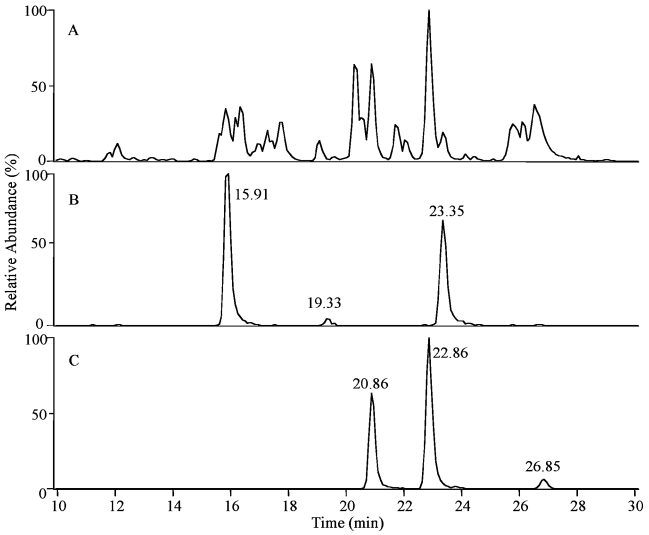

Figure 6. (A) Base peak ion chromatogram from graphitized carbon LC/ESI-MS of oligosaccharide alditols released by reductive alkaline β-elimination from a 1D SDS−AgPAGE-separated isoform of rat Muc2, and single-ion chromatogram of isomers corresponding to the composition (B) $dHex_1Hex_1HexNAc_1HexNAcol_1$ ([M − H]$^-$ ion of *m*/*z* 733.3) and (C) $dHex_2Hex_2HexNAc_2HexNAcol_1$ ([M − H]$^-$ ion of *m*/*z* 1244.4).

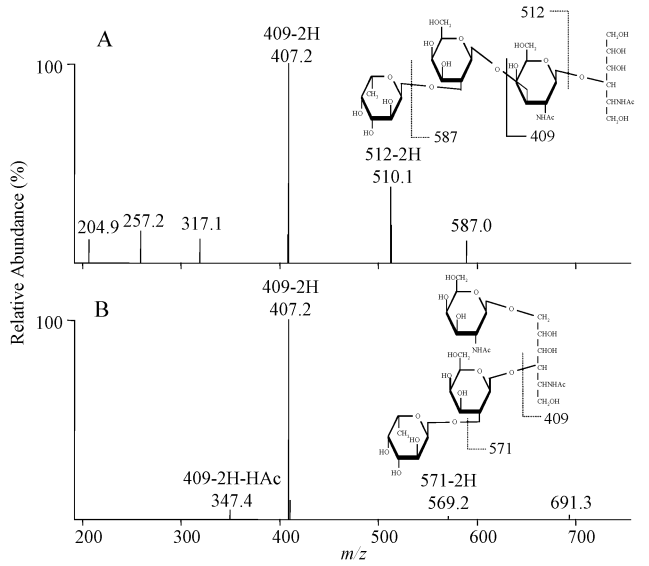

Figure 7. MS/MS spectra and proposed fragmentation of oligosaccharide structural isomers with [M − H]$^-$ ions of *m*/*z* 733.3 released by reductive alkaline β-elimination from 1D SDS−AgPAGE-separated rat Muc2 electroblotted to PVDF (Figure 6B). (A) dHex-Hex-HexNAc-3HexNAcol isomer (eluted at 15.91 min) and (B) dHex-Hex-3(HexNAc-6)HexNAcol isomer (eluted at 23.35 min).

containing band are shown. This structure could be deduced to have the same sequence as a previously described hexasaccharide NeuAc(α2−3)Gal(β1−4)GlcNAc(β1−6)[NeuAc(α2−3)Gal(β1−3)]-GalNAcol from human saliva.[44] In the spectrum, the dominating fragment ions are the loss of one and two sialic acids (*m*/*z* 1040.3 and 749.3, respectively), but additional glycosidic cleavages are seen, corresponding to the cleavages shown in Figure 10B, enabling sequencing of the structure.

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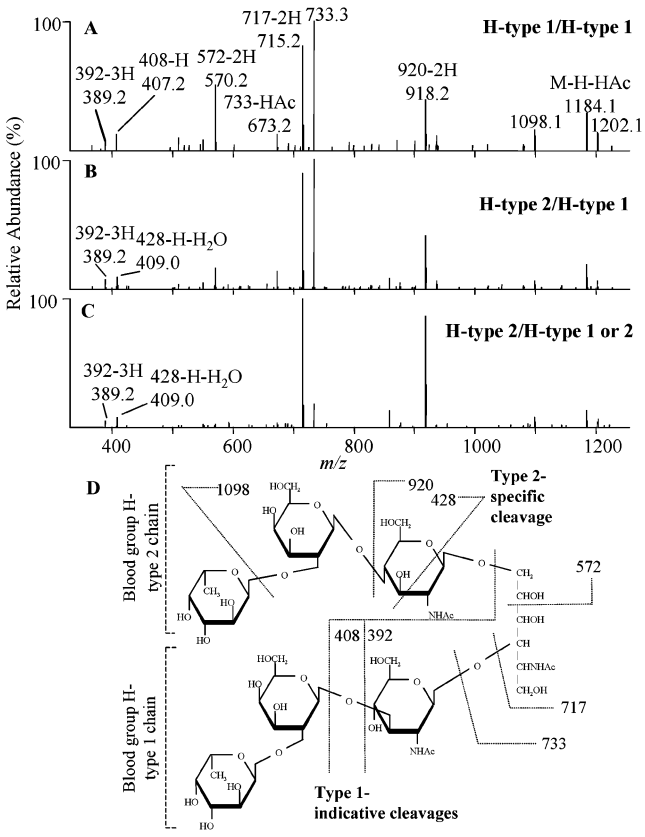

Figure 8. MS/MS spectra of three oligosaccharide linkage isomers with [M − H]$^-$ ions of *m*/*z* 1244.4 all sharing the same sequence dHex-Hex-HexNAc-3(dHex-Hex-HexNAc-6)HexNAcol, released by reductive alkaline β-elimination from electroblotted 1D SDS−AgPAGE-separated Rat Muc2 (Figure 6C), and eluted at (A) 20.86, (B) 22.86, and (C) 26.85 min; and (D) proposed fragmentation of a structure containing a blood group H type 1 trisaccharide epitope on the C-3 branch of the GalNAcol and a type 2 epitope on the C-6 branch.

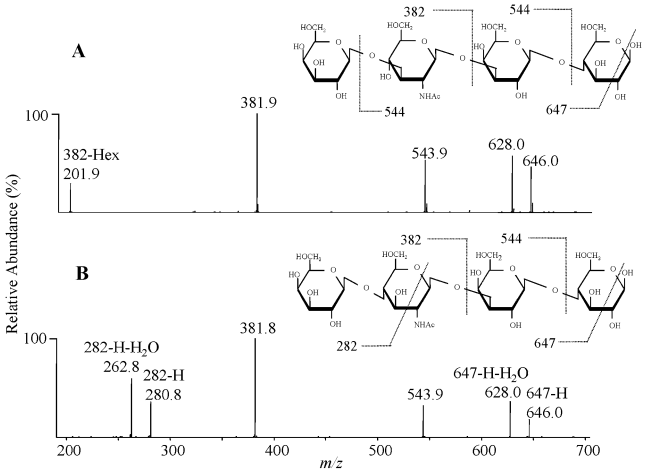

Figure 9. MS/MS spectra and proposed fragmentation of oligosaccharide isomers with [M − H]$^-$ parent ions of *m*/*z* 706.2: (A) lacto-*N*-tetraose (Gal(β1−3)GlcNAc(β1−3)Gal(β1−4)Glc); (B) lacto-*N*-neotetraose (Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc).

Currently, the precise mechanisms of collision-induced fragmentation in MS/MS are not completely understood. However, even in the absence of a complete mechanistic understanding, pattern matching of mass spectra can provide a high degree of oligosaccharide structural information. The method described

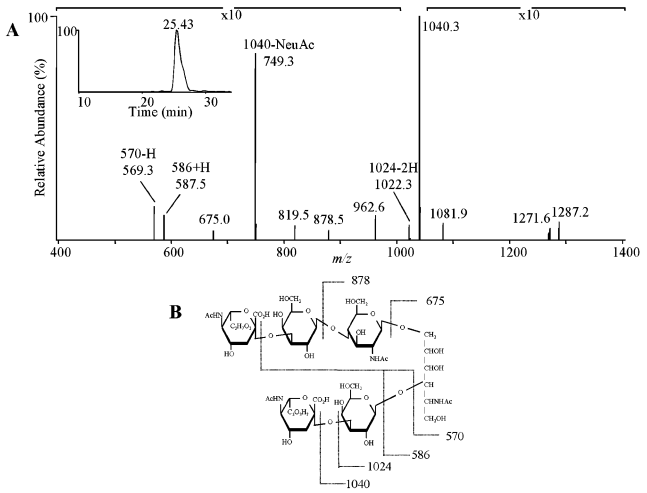

Figure 10. (A) MS/MS spectrum of the oligosaccharide $NeuAc_2Hex_2HexNAc_1HexNAcol_1$ with an $[M - H]^-$ ion of m/z 1331.5 released by reductive alkaline β-elimination from electroblotted 1D SDS–AgPAGE-separated human salivary MUC7 containing band, with an inserted single-ion chromatogram of m/z 1331.5; (B) proposed fragmentation.

here provides reproducible and specific MS/MS fragmentation patterns and chromatographic retention times, a combination of which can be used to identify oligosaccharide isomers.

CONCLUSIONS

The method described here enables semiquantitative glycoproteomic analysis of complex protein, glycoprotein, and mucin mixtures. Such analysis is not generally possible by other approaches for glycoprotein analysis, such as LC/LC–MS/MS or chromatography-based methods. Reductive alkaline β-elimination from electrophoretically separated glycoproteins electroblotted to PVDF membranes allows the electrospray mass spectrometric analysis of the compositions, structures, and linkages of released oligosaccharides from picomolar quantities of glycoproteins and mucins. The agarose–polyacrylamide composite gels used here allow the characterization analysis of glycoforms and splice variants of very high molecular weight mucin-type proteins. This allows for detailed glycosylation analysis of small amounts of glycoproteins or mucins through either a targeted or a general glycoproteomic approach.

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