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A rapid sample preparation method for mass spectrometric characterization of N-linked glycans

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A rapid method for analysis of glycans of glycoproteins is presented. This method comprised deglycosylation, sample cleanup and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of glycans. The enzymatic deglycosylation of N-linked glycoproteins was enhanced in terms of speed and reproducibility using an enzyme-friendly surfactant. The released glycans were desalted using a micro-scale solid phase extraction (SPE) device packed with a hydrophilic interaction chromatography (HILIC) sorbent. Hydrophilic glycans were well retained by SPE, while salts and surfactants were removed from the sample. The glycans were eluted using 25–50 μL of solvent and analyzed directly without derivatization using MALDI-MS. MALDI quadrupole time-of-flight (Q-Tof) instrumentation was utilized for glycan profiling and structure characterization by tandem mass spectrometry (MS/MS). The presented method allows sensitive analysis of glycans benefiting from optimized deglycosylation reactions and efficient sample cleanup. Copyright © 2005 John Wiley & Sons, Ltd.

Glycosylation is considered to be the one of the most common type of post-translational modifications (PTMs) of proteins.[1] It is estimated that about 50% of all proteins are glycosylated;[2] the carbohydrates are known to have a profound influence on the biological functions.[3,4] Due to the high degree of glycan heterogeneity, their characterization is a difficult task. Despite the recent progress in the development of mass spectrometry (MS) instrumentation that has enabled more rapid and sensitive characterization of carbohydrates, their MS analysis remains challenging; this is in part due to the poor ionization efficiency. A typical sample preparation method for MS analysis of glycans of glycoproteins involves a chemical or enzymatic cleavage of glycoproteins from protein, followed by removal of protein, salts and surfactant from the released glycans.[5,6] Native glycans can be directly analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) without loss of sensitivity, while glycan derivatization is typically conducted prior to liquid chromatography (LC)/MS analysis in order to enhance ion signals.[7] Although the efficient deglycosylation process is a key requirement for a successful and sensitive glycan analysis, quantitative glycan release (e.g. using enzymes) is rarely achieved for those glycoproteins with glycosylated sites obstructed by the protein secondary and tertiary structure. This makes the glycoprotein and glycan analysis more difficult when the sample amounts are limited or the reproducibility of the analysis is critical.

In this manuscript we describe a sample preparation method that employs an enzyme-friendly surfactant, RapiGest™ SF (RapiGest), to denature the proteins prior to enzymatic deglycosylation of N-linked proteins using PNGase F. This detergent was shown previously to enhance the proteolytic digestion of proteins. It does not inhibit the activity of the investigated enzymes, in contrast with other tested denaturants such as sodium dodecyl silfate (SDS), urea and organic solvents.[7] The goal was to develop a rapid and efficient deglycosylation method combining RapiGest with peptide-N-glycosidase F (PNGase F) for deglycosylation, combined with novel micro-scale hydrophilic-interaction chromatography (HILIC) solid-phase extraction (SPE) media for a rapid sample cleanup prior to MALDI-MS analysis.

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EXPERIMENTAL

Materials and reagents

Acetonitrile (MeCN), folate binding protein (FBP), chicken ovalbumin, polyclonal human IgG and PNGase F were purchased from Sigma (St. Louis, MO, USA) and used without purification. Surfactant RapiGest™ SF (RapiGest) and ultra-pure MALDI matrix 2,5-dihydroxybenzoic acid (DHB) were supplied by Waters Corporation (Milford, MA, USA). The DHB matrix was recrystallized in MeCN by the supplier. The 96-well HILIC micro-elution plate packed with 5 mg of aminopropyl silica sorbent in each well was obtained from Waters.

Deglycosylation of N-linked proteins

The glycoproteins (10–50 μg) were solubilized in 100 μL of 0.1% (w/v) RapiGest solution prepared in 50 mM NH_4HCO_3 buffer, pH 7.9. Protein samples were reduced with 10 mM dithiothreitol (DTT) for 45 min at 56°C, and alkylated with 20 mM iodoacetamide in the dark for 1 h (ambient temperature). The enzyme PNGase F (2.5–5 units) was added to the samples, and protein solutions were incubated for 24 h at

37°C; the reaction mixture was sampled as appropriate over this time. Deglycosylation was nearly complete after 2 h.

Sample cleanup using 96-well micro-elution HILIC SPE plates

The glycans released from ovalbumin, FBP and human IgG were cleaned prior to MALDI-MS analysis using SPE micro-elution plates packed with HILIC sorbent. HILIC sorbent (5 mg) was packed in a micro-elution SPE device (96-well SPE plate) and operated using a vacuum manifold. The extraction was performed using the following protocol: (1) each well was washed with 200 μL of Milli-Q water and conditioned with 200 μL of 90% MeCN; (2) deglycosylated protein samples were diluted with MeCN, e.g., 180 μL of MeCN were added to 20 μL of the deglycosylated protein sample to bring the organic concentration to 90%; (3) the sample was loaded onto a SPE plate by gravity (200 μL solution takes 5–10 min to break through a well completely); (4) salts, detergents and protein residues were washed out with 200 μL of 90% MeCN in water; and (5) the glycans were eluted with 10 mM of ammonium citrate in 25% MeCN (pH ~8), using an elution volume of between 25–50 μL.

LC/MS experiments

The RP HPLC instrument (CapLC®, Waters) was equipped with a microbore RP-HPLC column (1.0 × 100 mm Atlantis® C18 column, 3.5 μm, Waters Corporation). Mobile phase A consisted of 0.1% formic acid in Milli-Q water, and mobile phase B consisted of 0.1% formic acid in 100% MeCN. A linear gradient was run from 0 to 60% B in 30 min (2% B per min). Separation was achieved with 35 μL/min flow rate; the column temperature was set at 40°C. The LC system was interfaced to a quadrupole time-of-flight (Q-Tof) mass spectrometer (Waters Micromass Q-Tof micro™) via electrospray ionization (ESI). The system was controlled by MassLynx™ software version 4.0. The multiply charged ions were converted into the molecular mass of the protein using the MaxEnt™ 1 software incorporated in the MassLynx package.

MALDI-MS experiments

DHB matrix was used for MALDI-MS analyses. The DHB matrix was reconstituted in 500 μL of pure ethanol to a final concentration of 20 mg/mL. Purified glycan solutions were mixed with DHB matrix solution in a 1:1 ratio and 1 μL was placed on a stainless steel MALDI target. The quantity of glycans used for analysis is that released from 10–50 pmol of protein. The droplet was dried at ambient temperature until crystallized. A drop of ethanol (0.8 μL) was then added to the sample spot to form a more homogeneous crystalline sample.

MALDI-TOFMS (MALDI micro MX, Waters) was used to determine the molecular masses of the released glycans. Tandem mass spectrometry (MS/MS) experiments were performed to characterize the structure of the glycans using a MALDI-Q-Tof instrument (QTof Ultima™, Waters). The MALDI-TOF instrument was equipped with a pulsed N_2 laser (337 nm) and a 2.3 m flight path, and controlled by MassLynx 4.0 software. This instrument was operated in positive reflectron mode with 15 kV acceleration voltage and 500 ns delayed extraction. The laser was operated at 10 Hz. A total of 100 shots were averaged per spectrum. The MALDI-Q-Tof was also equipped with a fixed nitrogen laser source operated at 10 Hz. Each sample well was rastered at a speed of 67 s per mm in a spiral pattern. Each sample well scan was a sum of 2 s data acquisition; a spectrum was typically accumulated for 2 min. Argon was used as the collision gas. The collision energy used was in the range 70–120 eV. MS/MS data on selected ions were submitted for database search using the GlycoSuite database, which provided structural identification of the glycans.[8]

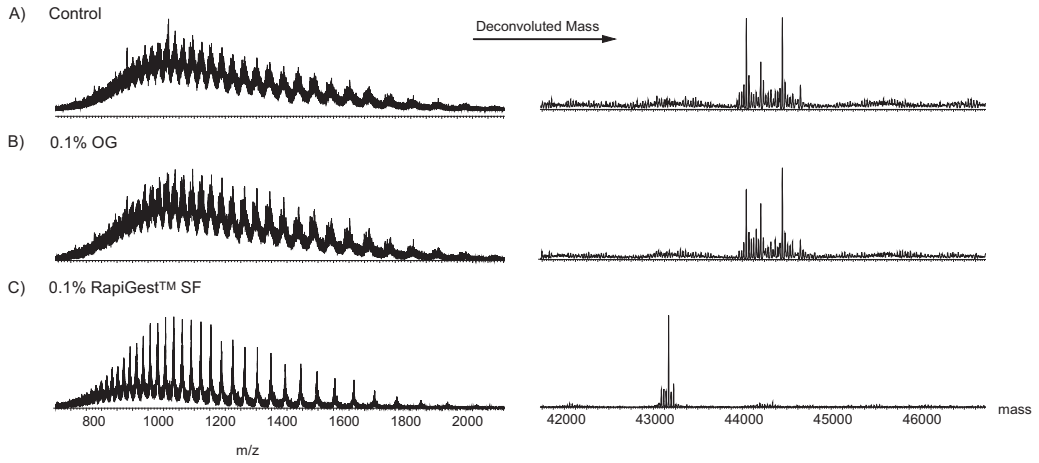

Figure 1. LC/MS spectra of deglycosylated ovalbumin. The amount of ovalbumin loaded onto the LC column was 20 pmol. (A) The ovalbumin was solubilzed without use of denaturant, and was not deglycosylated. (B) Ovalbumin was denatured using 0.1% OG and deglycosylated for 2 h. (C) Ovalbumin was denatured in 0.1% RapiGest solution and deglycosylated for 2 h. The MS scans were deconvoluted to give the MW of the protein. Complete deglycosylation was observed for the RapiGest-solubilized ovalbumin, while little to no deglycosylation was observed for the OG-solubilized protein.

RESULTS AND DISCUSSION

Protein deglycosylation under denaturing conditions

In earlier reports we described the use of the surfactant-assisted digestion of proteins using RapiGest, a mild and enzyme-friendly surfactant.[7] It was found that this surfactant improves the speed and completeness of proteolysis, most noticeably for globular and membrane proteins.[9] Other reports suggest that RapiGest also reduces the number of mis-cleaved and incompletely digested peptides.[10] Therefore, we investigated the impact of RapiGest on the enzymatic deglycosylation of glycoproteins by utilizing the surfactant, in conjunction with PNGase F, for the enzymatic release of N-linked glycans. The results were compared to those obtained using a non-ionic surfactant, n-octyl-β-glycopyranoside (OG), and a control reaction (no surfactant and enzyme added).

The progress of the deglycosylation reaction was tested using chicken ovalbumin. Ovalbumin was solubilized in either 0.1% RapiGest or 0.1% OG in solutions buffered with 50 mM ammonium bicarbonate at pH 7.9. The samples were reduced and alkylated as described in the Experimental section and deglycosylated with PNGase F for 2 h. The activity of PNGase F was quenched by boiling the samples for 5 min. A control ovalbumin sample was prepared using the same protocol without the addition of either surfactant or enzyme.

Figure 1 compares the LC/MS analyses of all three samples. The molecular mass of the protein was determined from the ESI spectra (deconvoluted mass spectra are shown in the right panel in Fig. 1). Ovalbumin has 385 amino acid residues with a calculated isotope-averaged molecular weight of 42 750 Da.[11] Its post-translational modifications include one glycosylation (N-linked), two phosphorylations and an acetylation.[11] Taking into account the deglycosylation and carboxymethylation of six Cys residues modified during the sample preparation step, the final predicted ovalbumin molecular mass is 43 300 Da.

As expected, no signal corresponding to the molecular weight (MW) of deglycosylated protein was found for the control sample (Fig. 1(A)). Interestingly, no detectable deglycosylation was observed in the OG-mediated reaction; the multiple peaks between 44–45 kDa (Figs. 1(A) and 1(B)) represent the various N-linked glycoforms of ovalbumin. These data show that the deglycosylation reaction in the OG-solubilized sample did not progress significantly within 2 h of reaction. In contrast, the reaction in the presence of RapiGest shows nearly complete deglycosylation; the protein mass shifted significantly and a prominent peak was detected at 43 297 Da which is within 70 ppm of the theoretical mass. These experimental results confirm that the multiple signals of the glycosylated protein are due to the heterogeneous glycoforms,[11] not due to major heterogeneity of ovalbumin itself. Glycan heterogeneity causes the MS signal 'broadening' observed in the ESI multiply charged states and corresponding reduction in signal (Figs. 1(A) and 1(B), left panel). The signal-to-noise ratio of the ovalbumin mass spectrum was dramatically enhanced upon deglycosylation, as is apparent in both raw and deconvoluted spectra (Fig. 1(C)). The experiment summarized in Fig. 1 suggests that RapiGest significantly improves the speed and completeness of glycoprotein deglycosylation with PNGase F. It was found that the ovalbumin deglycosylation reaction rate in the presence of 0.1% RapiGest follows pseudo-first-order reaction kinetics; the reaction rate was estimated by plotting the fractional ovalbumin deglycosylation (based on the deconvoluted mass spectra) against the deglycosylation time. The

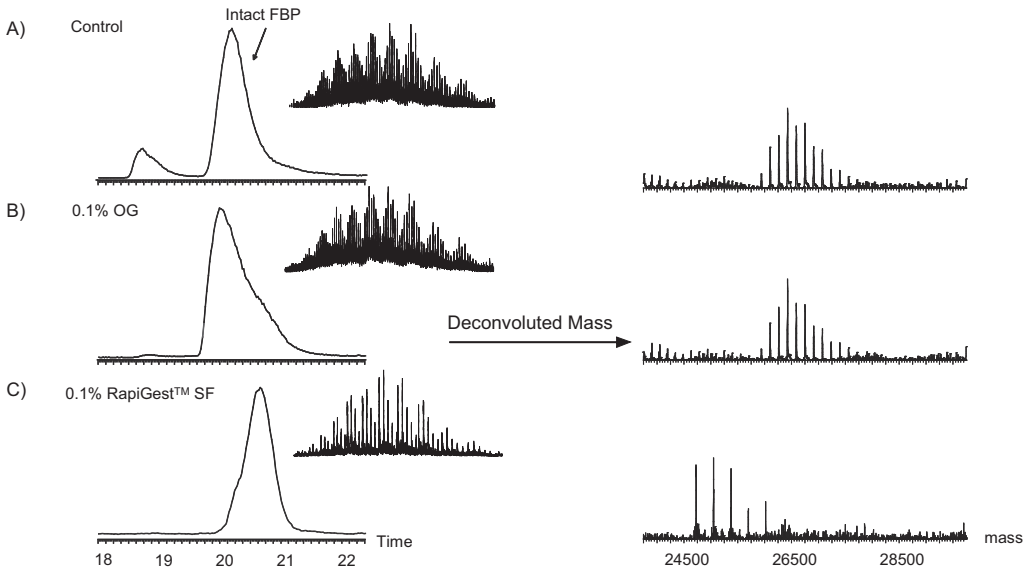

Figure 2. The left panels show the LC/MS TIC and the summed MS scans of (A) intact FBP, (B) denatured and deglycosylated FBP using OG, and (C) denatured and deglycosylated FBP using 0.1% RapiGest. The deglycosylation duration was 2 h for (B) and (C). The deconvoluted mass spectra are shown in the right panels. The amount of FBP used for each experiment was about 40 pmol.

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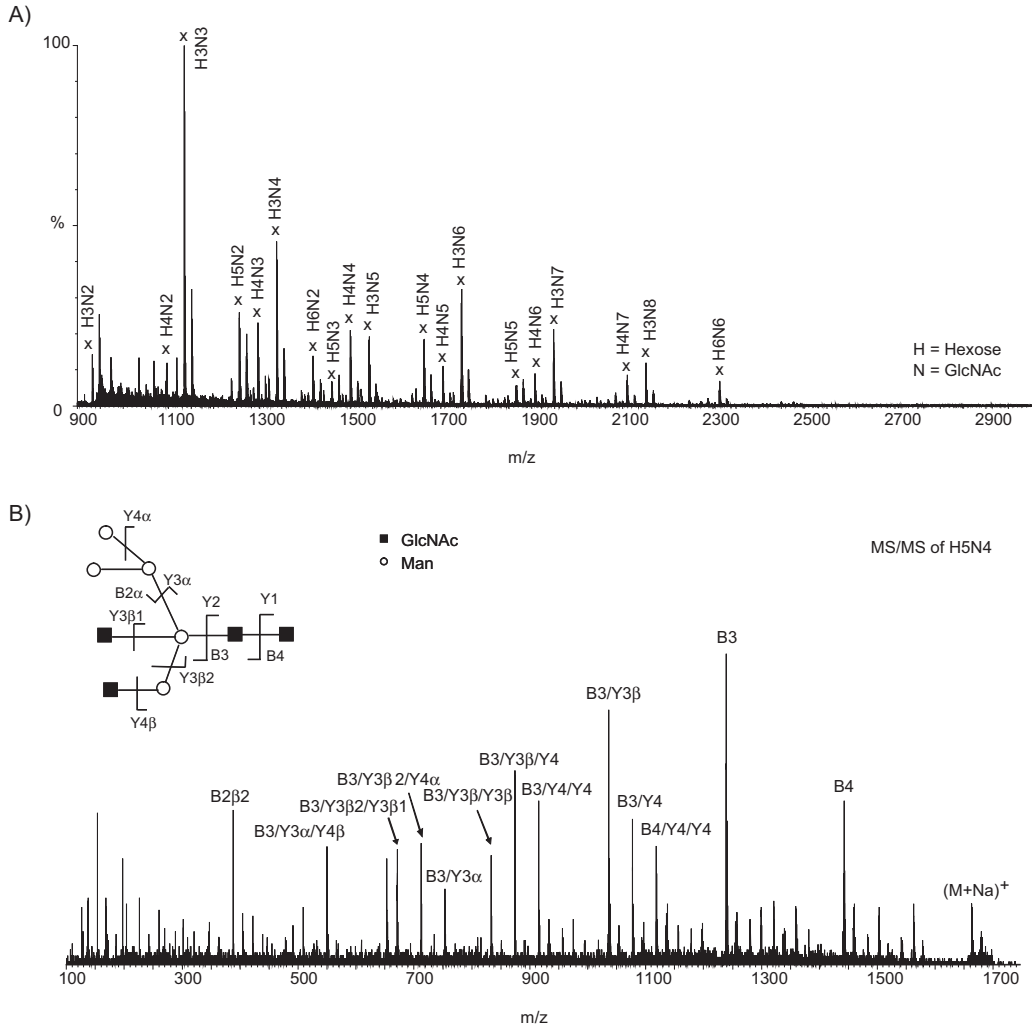

Figure 3. (A) MALDI-Q-Tof mass spectrum of oligosaccharides released from ovalbumin. The amount of glycans spotted on the MALDI target was obtained from 10 pmol ovalbumin. $[M+Na]^+$ ions corresponding to the glycan species, $H_{3\text{-}6}N_{2\text{-}8}$, are labeled. This spectrum showed that the HILIC SPE micro-elution plate was able to remove salts and other contaminants. (B) An example of MS/MS fragmentation is shown for the $[M+Na]^+$ ion of H_5N_4 (*m/z* 1663.64). The correct structure and linkages were identified via a GlycoSuite database search.

reaction rate thus determined was 0.2 mg of protein per hour per unit of PNGase F; this value may vary with the enzyme quality and other experimental factors.

Folate binding protein (FBP) was also deglycosylated using PNGase F under two different denaturation conditions. Solutions of FBP were prepared in 0.1% non-ionic OG surfactant, and another with 0.1% RapiGest. Deglycosylation was then performed as described above for ovalbumin. Both samples and the control reaction were subjected to LC/MS analysis; the summed MS spectra and deconvoluted molecular weight spectra are shown in Fig. 2. As in the previous experiment on ovalbumin, little or no deglycosylation was observed with FBP denatured in OG solution, i.e., the deconvoluted masses were essentially the same as those for the control sample. The MWs observed for the FBP-deglycosylated protein in the 0.1% RapiGest solution were shifted to lower values; however, multiple peaks spaced by 324 Da can be seen in the deconvoluted spectrum, which indicates that some degree of glycosylation is still present. It is known from the literature that certain N-linked glycosylation sites are resistant towards PNGase F. Since the peak spacing (324 Da) corresponds to two hexose units, we speculate that such a glycosylation site is present in FBP. The extended overnight deglycosylation of FBP had no impact on the pattern of MS signals, shown in Fig. 2(C), so the deglycosylation reaction did not progress to completion even under these extended conditions (data not shown).

Glycan sample cleanup prior to MALDI-MS analysis

Complex carbohydrates can be readily retained and separated using hydrophilic-interaction liquid chromatography (HILIC).[12,13] In HILIC mode, the hydrophilic glycans are retained due to a mechanism involving partitioning between the organic mobile phase and a layer of water adsorbed on the surface of sorbent. Since a high concentration of organic solvent is necessary to ensure good retention of glycans, the samples were first diluted with MeCN to a final concentration

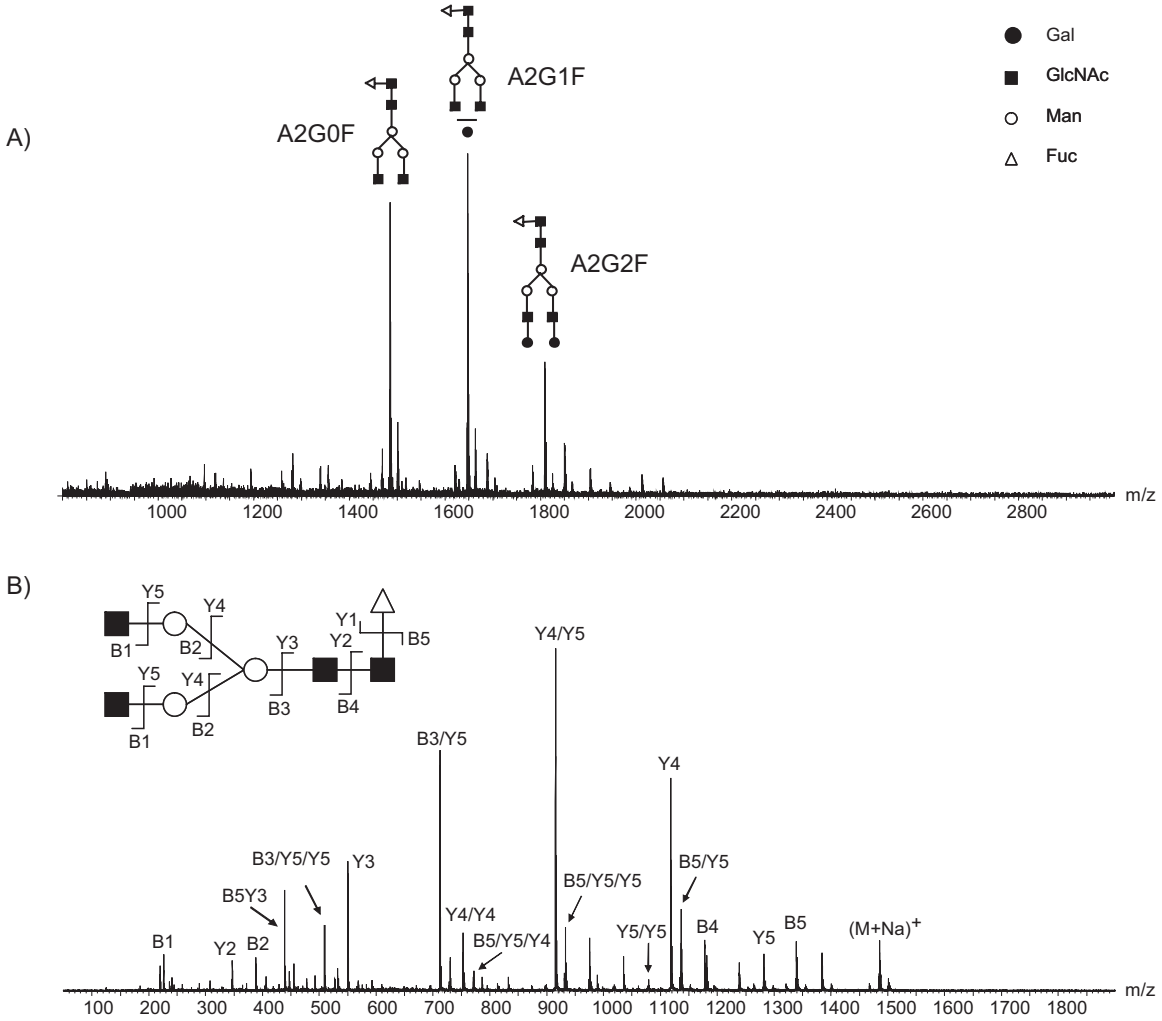

Figure 4. (A) MALDI-Q-Tof mass spectrum in positive ion mode of glycans from human polyclonal IgG extracted by means of a HILIC micro-elution plate. (B) The MS/MS spectrum of the A2G0F glycan. The fragment masses were submitted to a GlycoSuite database search. The amount of glycans loaded on the MALDI target was obtained from 10 pmol IgG.

of 80–90%; note that some precipitation of glycans may occur if they are present at high concentrations. It is not recommended to centrifuge samples prior to loading to the HILIC micro-elution plate. After plate conditioning (see 'Sample cleanup' in the Experimental section), glycan samples were loaded by gravity. The HILIC plate performance was evaluated using a maltohexose standard. Load, wash, and SPE elution fractions were quantitatively analyzed using an HPLC system equipped with a HILIC column (Sentry™ high performance carbohydrate guard column) and Sedex ELSD detector. The mass balance of maltohexose revealed no breakthrough in the sample load fraction. Most of the material eluted in the first elution. The total mass balance was 90%, and the overall recovery of glycans was estimated to be approximately 70% (data not shown).

The glycans released in the RapiGest-assisted deglycosylation of ovalbumin and FBP with PNGase F were cleaned up using the HILIC micro-elution plate. The MALDI-Q-Tof MS spectra of underivatized N-linked glycans released from 10 pmol ovalbumin (Fig. 3(A)) show that sodiated glycans were the most intense ions. The compositions of the ovalbumin glycans were derived from their MWs published in the literature.[11] MS/MS spectra of selected ions were also acquired to validate their structures, using comparisons with the GlycoSuite database. For example, Fig. 3(B) shows the collision-induced dissociation (CID) of the ion of *m/z* 1663.64, a complex hybrid glycan. This ion was observed in the MS mode with low intensity, but enough fragment ions were produced in the MS/MS mode to permit determination of its structure. Two major fragment ions (B and Y ions) were generated from glycosidic cleavages; the nomenclature is that of Domon and Costello.[14] The structures of the FBP glycans are not known, and to our knowledge have not been previously reported. Our future plan is to probe their structures using MALDI Q-Tof MS/MS methodology.

MALDI-Q-Tof MS/MS of glycans released from IgG

The capabilities of the present approach combined with MS/MS methodology were evaluated using the human polyclonal

IgG. The glycans were released from 10 pmol of IgG protein, purified on a HILIC plate. The MALDI-Q-Tof analysis of the IgG glycans is shown in Fig. 4. Three major glycans (A2G0F, A2G1F and A2G2F) were observed, and the *m/z* values of the $[M+Na]^+$ ions were matched to the published results.[15] MS/MS spectra were also acquired for these three glycans to further confirm their structures. An example of these MS/MS spectra, that of A2G0F (*m/z* 1485.533), is shown in Fig. 4(B). The majority of the fragments are B- and Y-type ions generated from glycosidic cleavages. All fragment ions were first manually assigned to derive the structural information. After de-isotoping, the peak lists (*m/z* and intensity) were submitted to a GlycoSuite database search to further confirm the glycan structure.

CONCLUSIONS

We have developed a method suitable for fast and robust analysis of N-linked glycans released from glycoproteins. The method utilizes an enzyme-friendly surfactant that was shown to greatly accelerate deglycosylation reactions via glycoprotein denaturation, which makes the glycans more accessible to enzymatic cleavage. A complete deglycosylation of most proteins was achieved after a 2 h incubation with PNGase F. The HILIC SPE device was utilized to extract and desalt the glycans prior to their analysis by MS; this SPE method is fast and requires minimum sample manipulation. The purified glycans were analyzed by MALDI-MS without derivatization. Further, a MALDI-Q-Tof mass spectrometer proved to be a versatile instrument for glycoprofiling, especially for deriving structural information, i.e., CID was able to generate high-quality MS/MS fragmentation spectra from limited amounts of glycan samples.

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