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Characterization of *N*-linked Glycan Structures Using Normal-phase Capillary LC-MALDI Tandem Mass Spectrometry†

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The addition of carbohydrates to proteins is one of the important post-translational modifications of proteins. Glycoproteins in many biological processes play a key role in immune defense, cell growth and cell–cell adhesion.[1,2] In addition to aiding vital functionality to proteins, glycans also play an important role in protein stability.[3,4] Glycoproteins often have very heterogeneous structures, due to the great diversity in the glycan moieties (known as glycoforms). Therefore, the full characterization of glycoproteins is a very difficult task, including the determination of glycan size, compositions, branching points and linkages of the carbohydrate monomers for each glycosylation sites. The structural characterization of glycans released from glycoproteins is extremely challenging and multiple techniques are often required for complete characterization.[5] The pool of glycans released by either chemical or enzymatic cleavages of glycoproteins is usually analyzed by high-performance anion-exchange chromatography, followed by the addition of chromophore at the reducing terminus of glycans. Mass spectrometry has extensively used for detailed characterization of *N*-linked glycans, including monosaccharide composition, as well as sequence, branching, and sometimes linkage information by tandem mass spectrometry.[6]

In this work, we characterized extensively structure of *N*-linked glycans reductively aminated by 2-aminobenzamide (2-AB), using normal-phase capillary liquid chromatography coupled to off-line matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and time-of-flight/time-of-flight tandem mass spectrometry (TOF/TOF-MS/MS). This method has been usually called LC-MALDI. The 2-AB-labeled standard glycan mixture and *N*-linked glycans, which were released from PNGase F digestion of ribonuclease B glycoprotein, were separated by normal-phase liquid chromatography on home-built amide-80 capillary column and were fractionated directly into the wells of MALDI plate at regular intervals, using automatic MALDI spotter as shown in Figure 1. Then the structure of sodium-adducted molecule, $[M+Na]^+$, of each glycan component observed in MALDI mass spectra was characterized,

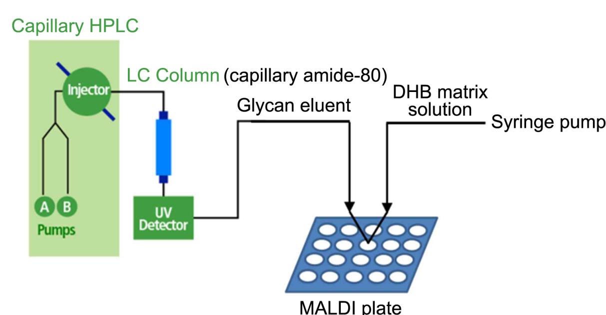

Figure 1. Schematic of an HPLC system coupled to the LC-MALDI sample-collection module.

using high-energy collision-induced dissociation (CID) tandem mass spectrometry. The product ions observed in its MS/MS spectrum provided the information about the composition, sequence, linkage and branching of the glycan.

The mixture of each 5 pmol of 2-AB-labeled mannose 6 ($Man_6GlcNAc_2$), 7 ($Man_7GlcNAc_2$), and 9 ($Man_9GlcNAc_2$) were loaded and separated on capillary amide-80 column. The top part of Figure 2 indicates the chromatogram of base peak observed in MALDI mass spectra which were obtained from the samples spotted into the first to 90th well of MALDI plate in the interval of 30 seconds. Therefore, the base peak chromatogram (BPC) in LC-MALDI measurement is similar to the chromatogram of the signal detected in liquid chromatography. Then, the number of spot well in the abscissa of BPC corresponds to the retention time in LC. The three peaks observed in the base peak chromatogram corresponding to each sodium-adducted molecule, $[M+Na]^+$, of the mannose 6, 7, and 9 were eluted at 42.0, 44.5, and 47.5 min, respectively and completely separated as shown in Figure 2. The mass spectra of glycan components showed that the abundant peaks of their sodium-adducted molecules were observed at *m/z* 1539, 1701, and 2025 in Figure 2(a), (b), and (c), respectively, including those of the minor potassium-adducted and protonated molecules. This result demonstrated that our home-built amide-80 capillary column has a sufficient separation capability for glycan mixture. A range of concentration of standard glycans down to 100 fmol could be detected by this method.

The high-energy (> 1 keV) CID tandem mass spectra of

†This paper is to commemorate Professor Myung Soo Kim's honourable retirement.

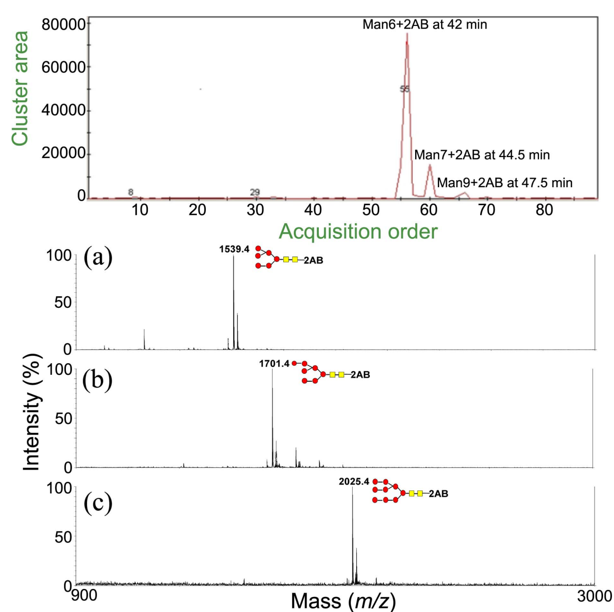

Figure 2. Base peak chromatogram of sodium-adducted molecules, $[M+Na]^+$, observed in off-line LC-MALDI mass spectra of the standard glycan sample, which was mixed with 2-AB derivatives of mannose 6 (a), 7 (b) and 9 (c). They were eluted at 42.0, 44.5, and 47.5 min, respectively.

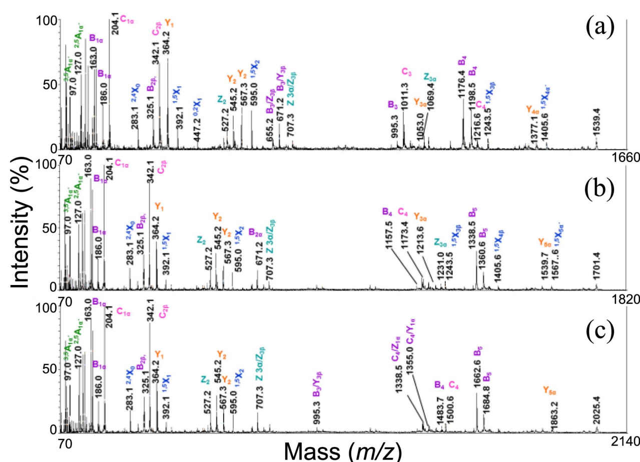

Figure 3. High-energy CID tandem mass spectra of $[M+Na]^+$ ions of 2-AB-labeled mannose 6 (a), 7 (b), and 9 (c), which were observed at *m/z* 1539, 1701, and 2025 in each MALDI mass spectra, respectively.

sodium-adducted molecules provided more information about the glycan structure than the low-energy (< 100 eV) CID tandem mass spectra, including sequence, branching and sometimes linkage information. The fragmentation of $[M+Na]^+$ ions using the MALDI-TOF/TOF mass spectrometer generate the product ions indicative of complex spectral pattern. Figure 3 showed the high-energy CID tandem mass spectra of the sodium-adducted molecules of the *N*-linked glycans observed in MALDI-TOF mass spectra of the mannose 6, 7, and 9 labeled with 2-AB, which were separated by normal-phase capillary LC. Our notation follows the common nomenclature for carbohydrate fragmentations proposed by

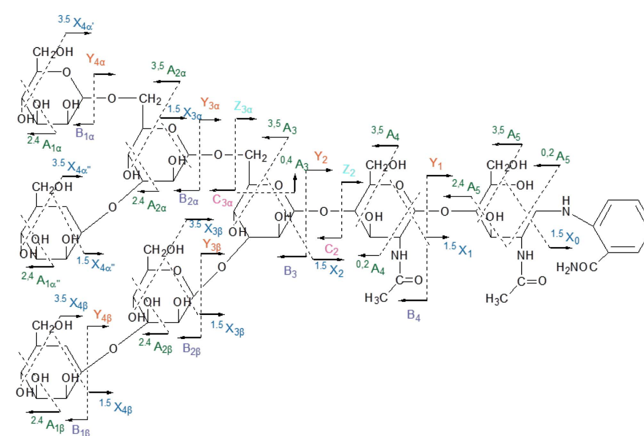

Figure 4. The fragmentation pathways for the product ions observed in high-energy CID tandem mass spectrum of $[M+Na]^+$ ion (*m/z* 1539) of 2-AB derivative of standard mannose 6. The notation follows the common nomenclature for carbohydrate fragmentations proposed by Domon and Costello.

Domon and Costello.[7] The B, C, Y and Z ion series, from glycosidic bond cleavages between the sugar rings informative about the sequence of carbohydrate were observed commonly. The A and X ion series, generated by cross-ring cleavages of sugar rings provided the information about the linkage (1 → 3 or 1 → 6) and branching of the carbohydrate structure as shown in Figure 4.

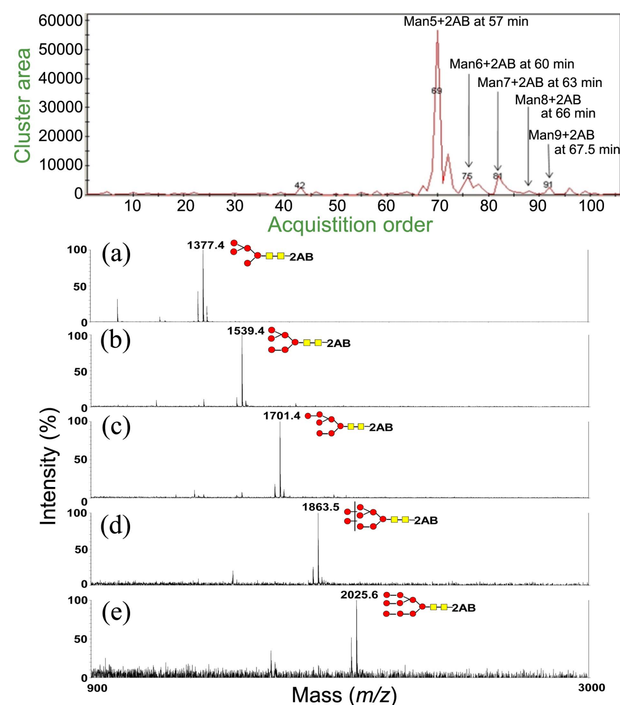

Figure 5. Base peak chromatogram of sodium-adducted molecules, $[M+Na]^+$, observed in off-line LC-MALDI mass spectra of the 2-AB derivatves of *N*-linked oligosaccharides, which were released from ribonuclease B. They were eluted at 57, 60, 63, 66, and 67.5 min and observed at *m/z* 1377 (a), 1539 (b), 1701 (c), 1863 (d), and 2025 (e), respectively. They had structures of high-mannose type and identified as Man5, 6, 7, 8, and 9, respectively.

The oligosaccharides were also enzymatically released from ribonuclease B using PNGase F, which specifically cleave the bond between *N*-acethylglucosamine at the reducing terminus of *N*-linked glycan and asparagine of glycosylation site. The released glycans were also aminated by 2-AB and then separated effectively by normal-phase capillary LC. As shown in Figure 5, BPC and MALDI mass spectra indicated that the *N*-linked glycans eluted at 57, 60, 63, 66, and 67.5 min had the same oligosaccharide compositions corresponding to Man 5, 6, 7, 8, and 9, and were identified as the sodium-adducted molecules observed at *m/z* 1377, 1539, 1701, 1863, and 2025, respectively.

Their MS/MS spectra had almost same pattern to those of standard glycans shown in Figure 3. Thus *N*-linked glycans released from ribonulease B has the structure of high mannose type as reported previously.

In summary, we reported a sensitive method for the separation and structure elucidation of reducing-end labeled *N*-glycan mixture using a combination of normal-phase capillary HPLC and off-line MALDI-TOF/TOF mass spectrometer. It was found that detection limit of 2-AB labeled high mannose type glycans by normal-phase capillary LC MALDI-MS analysis was below 50 fmol. The extensive fragmentation including glycosidic cleavages and cross-ring cleavages was shown to facilitate the detailed structural characterization of *N*-linked oligosaccharides. However, quantitative data from the peak area of BPC could not be obtained reliablely due to the difference of the ion suppression among the *N*-linked oligosaccharides.

Experimental Section

Deglycosylation of Ribonuclease B by PNGase F and Reductive Amination of *N*-linked Glycans with 2-AB. The dried sample containing 15 μg (1 nmol) of ribonuclease B (Sigma) was incubated in 40 μL of water containing 10 μL denaturing buffer at 100 °C for 10 min. The sample was added with 10 μL G7 buffer, NP40 and 60 μL water. This solution was added 1 μL PNGase F (NEB, 500 unit/μL) and incubated for 24 h at 37 °C. The released glycans were purified by porous graphitized carbon (PGC) column (Alltech) and eluted by 25% acetonitrile. Eluted glycan mixture was completely dried in speed vac. This glycan mixture and standard sample were reductively aminated by 2-aminobenzamide labeling kit (Prozyme). These derivatives were heated at 65 °C for 3 h. The labeled glycans were washed using Whatman paper (3017 915), dried and then stored at −20 °C.

Normal-phase Capillary LC-MALDI Tandem Mass Spectrometry. The normal-phase column was made by packing amide-80 resin (5 μm, TOSOH) in the fused silica capillary tubing (ODxIDxL, 360 μm × 250 μm × 17 cm) using He gas and bomb loader (Proxeon). Normal-phase capillary LC was carried out using a LC-Packings Ultimate System (LC Packings, Amsterdam). Solvent A was 10 mM ammonium formate adjusted to pH 4.4 with formic acid. Solvent B was 20% solvent A in acetonitrile.[8] The labeled glycans dissolved in 80% acetonitrile were loaded onto an amide-80 column and eluted with increasing aqueous concentration. The column was equilibrated at 5% A and the gradient was initiated 10 min at flow rate of 1.5 μL/min. after injection and increased to 52% A over 60 min, 95% A over 90 min and 5% A over 100 min. The elution fractions were directly spotted onto a 192 disposible MALDI plate at 30 sec interval using Probot spotter system (LC Packings). The sample spots were overlaid with 0.6 μL of 2,5-dihydroxybenzoic acid (DHB, LaserBio Lab) added with NaI (5 μg) and rapidly dried in vacuum. All mass spectra were obtained with 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA) and each well on the plate was irradiated with 355 nm UV light of an Nd:YAG laser with a repetition rate of 200 Hz. 1500 to 2000 laser shots were averaged to normal mass spectra and 4800 to 5500 laser shots to MS/MS spectra. The samples were analyzed at 25 kV of source acceleration voltage with two-stage reflection in MS mode. In the MS/MS experiments, collision energy, defined by the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV), was set to 1 kV.

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