Influence of the Labeling Group on Ionization and Fragmentation of Carbohydrates in Mass Spectrometry

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The ionization and fragmentation behaviors of carbohydrate derivatives prepared by reaction with 2-aminobenzamide (AB), 1-phenyl-3-methyl-5-pyrazolone (PMP), and phenylhydrazine (PHN) were compared under identical mass spectrometric conditions. It has been shown that the intensities of signals in MS spectra depend on the kind of saccharides investigated and reducing end labels used. PMP sialyllactose, when ionized by ESI/MALDI, produced a mixture of $[M + H]^+$, $[M + Na]^+$, $[M - H + 2Na]^+$ ions in the positive mode and $[M - H]^-$, $[M + Na - 2H]^-$ ions in the negative mode. The AB and PHN derivatives formed abundant $[M + H]^+$ and $[M - H]^-$ ions in ESI, and by matrix-assisted laser desorption/ionization (MALDI) produced abundant $[M + Na]^+$ ions. PMP- and reduced AB-sialyllactose produced only Y-type fragment ions under both MS/MS sources. In the electrospray ionization (ESI)-MS/MS spectrum of PHN-sialyllactose, abundant ions corresponded to B, Z cleavages and in its MALDI-MS/MS spectrum, the abundant ions were consistent with Y glycosidic cleavages with the concurrence of B, C, and cross-ring fragment ions. In the MALDI-MS spectra of oligosaccharides acquired immediately after derivatization, it was possible to detect only PHN derivatives. After purification, spectra of all three types of derivatives showed high signal-to-noise ratios with the most abundant ions observed for AB reduced saccharides. $[M + Na]^+$ ions were the dominant products and their fragmentation patterns were influenced by the type of the labeling and the kind of oligosaccharide considered. In the MALDI-PSD and -MS/MS spectra of AB-derivatized glycans, higher m/z fragment ions corresponded to B and Y cleavages and the loss of bisecting GlcNAc appeared as a weak signal or was not detected at all. Fragmentation patterns observed in the spectra of hybrid/complex PHN and PMP glycans were more comparable—higher m/z fragments corresponded to B and C glycosidic cleavages. For PHN glycans, the abundance of ions resulting from the loss of bisecting GlcNAc depended on the number of residues linked to the 6-positioned mannose. Also, PHN and PMP derivatives produced cross-ring cleavages with abundances higher than observed in the spectra of AB derivatized oligosaccharides. For high-mannose glycans, the most informative cleavages were provided by AB and PHN type of labeling. Here, PMP produced dominant Y-cleavages from the chitobiose while other ions produced weak signals. (J Am Soc Mass Spectrom 2005, 16, 683–696) © 2005 American Society for Mass Spectrometry

Saccharides, either free or as constituents of glycoproteins, proteoglycans, and glycolipids, are mediators for extracellular and intracellular recognition, cell differentiation and all proliferation [1, 2]. Glycosylation is also highly sensitive to alterations of cellular function, and altered protein glycosylation is diagnostic of a number of disease states including, for example, rheumatoid arthritis and cancer [3]. Therefore,

the growing interest in understanding the biological functions of carbohydrates has stimulated the development of methods for their improved analysis.

In order to detect saccharides by UV/fluorescence and also to ease their characterization by mass spectrometry (MS), introducing a chromophore into the molecules has received increasing attention. In this respect, a large number of derivatization procedures for mono- and oligosaccharides have been described in the literature, most of which have been detailed in a recent review [4]. For example, 2-aminoacridine (2-AMAC) as a sensitive fluorophore, has been used for the detection of monosaccharides, and of neutral and charged oligosaccharides by electrospray ionization (ESI) and matrix-

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Scheme 1. Structures of aminobenzamide (AB) (a), pyrazolone (PMP) (b), and phenylhydrazone (PHN) (c) derivatives of oligosaccharides.

assisted laser desorption/ionization (MALDI) MS [5, 6]. Another reagent, 3-acetamido-6-aminoacridine (AA-Ac), has been used for analysis of N-linked glycans at the picomole level and yielded in higher sensitivities than AMAC [7]. Also, the 2-(diethylamino)ethyl ester of 4-aminobenzoic acid was shown to increase the sensitivity 1000-fold relative to underivatized glycans under MALDI-MS conditions [8]. As another example, 9-aminofluorene was investigated as a label for linear and branched oligosaccharides, and the smallest amount that could be detected was 5 pmol by photodiode array UV [9]. Among other derivatization agents frequently used are 2-aminopyridine (AP) [10, 11], 4-aminobenzoic acid ethyl ester (ABAE) [12], 2-aminobenzamide (AB) [13, 14], and 2-aminobenzoic acid (ABA), the latter producing good negative ion MALDI spectra [15]. Each derivatization procedure has its advantages and inconveniences, depending on the class of carbohydrates investigated and on the amount/complexity of the sample, among other factors.

Most reactions of saccharides with amino compounds are followed by reduction, providing open ring structures at the former reducing ends. Omitting the sodium cyanoborohydride reducing step produces less stable Schiff bases. Both types of derivatives were previously examined under ESI-MS conditions [16]. Results presented in the previous study [16] showed that the type of reducing-end derivative could affect the abundance and nature of ions formed, but that it had little effect on the fragmentation pattern. Reduced

2-AMAC derivatives yielded the most intense MALDI signals, however, this labeling method produced the least informative fragmentation patterns because of the preferential formation of Y_1 and Y_2 species. The corresponding unreduced derivatives (Schiff bases/glycosylamines) produced fragmentation patterns similar to those of the reduced, however, they were very unstable and reaction yields were low in general. In other articles, it has been shown that unreduced alkylamine derivatives of monosaccharides exist as closed-ring glycosylamines [17]. These compounds have been used to facilitate chromatographic and MS analysis of saccharides [18, 19]. Besides, closed-ring derivatives provided improved structural information on linkage and anomeric configuration. Unreduced glycosylamine derivatives were also preferred over reduced derivatives for the purposes of determining sulfation and uronic acid positions in chondroitin sulfate saccharides [20].

In the present article, three tagging methods were compared (see Scheme 1). First, aminobenzamidation was selected because of its popularity and usefulness [13], second, derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) yielding bis-PMP products [21] was chosen because of their frequent use in our laboratory [22], and third, unreduced phenylhydrazone derivatives were selected because of the simplicity of the procedure and very informative fragmentation observed under MALDI-MS conditions [23, 30]. The purpose of this study was to compare how different labeling groups at the reducing end of saccharides can

Scheme 2. Structures of the small saccharides used in this study.

influence sensitivity, ionization efficiency, and fragmentation behavior. The emphasis was placed on fragmentation data that provide specific information, such as antenna composition and possible differentiation of isomeric structures among oligosaccharides. Small saccharides—lactose, N-acetylactosamine, fucosyllactose, and sialyllactose—were studied under ESI- and MALDI-MS conditions. Large oligosaccharides, because of limited amounts, were characterized only by MALDI-MS.

Experimental

Materials

Anthranilamide, sodium cyanoborohydride, phenylhydrazine, hen ovalbumin (Grade V), β -lactose (β -D-Gal-1 $\rightarrow$ 4-D-Glc), epilactose (β -D-Gal-1 $\rightarrow$ 4-D-Man), 2'-fucosyllactose (β -L-Fuc1 $\rightarrow$ 2- β -D-Gal-1 $\rightarrow$ 4-D-Glc), sialyllactose (a mixture of 3'- and 6'-N-acetylneuramin-lactose), dimethyl sulfoxide, and 2,5-dihydroxybenzoic acid matrix (DHB) were purchased from Sigma (St. Louis, MO). Sialylated oligosaccharide SC1123 was obtained from V-LABS (Covington, LA). The reagent 1-phenyl-3-methyl-5-pyrazolone was purchased from ICN Biomedicals Inc. (Aurora, OH). N-acetylactosamine was prepared according to a published procedure [25]. Peptide-N-glycosidase F (PNGase F) deglycosylation kits and GlycoClean S cartridges were purchased from Prozyme (San Lean-

dro, CA). Solvents (acetonitrile, ethanol, ethyl acetate, chloroform, methanol) were HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade deionized distilled water was obtained with a Milli-Q plus TOC water purification system (Millipore, Bedford, MA).

Deglycosylation by PNGaseF Digestion [26, 27]

The glycoprotein ($\sim 500 \mu\text{g}$) was dissolved in deionized water ($36 \mu\text{L}$) in a micro centrifuge tube, and Buffer 5 $\times$ supplied with the Glyko deglycosylation kit was added ($9 \mu\text{L}$). The glycoprotein was denatured by boiling in a hot water-bath for 10 min. After cooling, PNGase F enzyme ($2 \mu\text{L}$) was added and the mixture was incubated at 37°C for 18 h. The protein was precipitated by adding four volumes of cold ethanol and the mixture was kept in ice (~ 2 h). The N-deglycosylated protein was centrifuged down to a pellet and the supernatant containing the oligosaccharides was pipetted out into another micro centrifuge tube. Ethanol was evaporated and the white residue was derivatized as described below.

2-Aminobenzamide (AB) Derivatives

(1) $^\circ$ Reduced $^\circ$ AB $^\circ$ derivatives $^\circ$ [13] $^\circ$ were $^\circ$ prepared $^\circ$ by dissolving the saccharides (up to $0.5 \mu\text{mol}$) in 2AB solution ($50 \mu\text{L}$): 2AB reagent (5 mg) and NaBH_3CN (7.5 mg) in DMSO ($500 \mu\text{L}$) and acetic acid ($200 \mu\text{L}$).

Figure 1. Positive ESI-MS spectra of sialyllactose: Native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

The reaction mixture was heated at 65 °C for 2.5 h. After incubation, the samples were cleaned using GlycoClean S cartridges according to the accompanying protocol.

(2) Unreduced AB derivatives were prepared by dissolving the saccharide (up to 0.5 μmol) in 10 μL of

water and 30 μL of reagent solution made by dissolving 2 AB (10 mg) in methanol (100 μL) and reacting at 75 °C for 3 h with frequent mixing. Then methanol was evaporated and an additional 200 μL of water was added and the solution was washed with chloroform (2 × 20 μL) to remove excess AB reagent.

Figure 2. Negative ESI-MS spectra of sialyllactose: Native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

Figure 3. Positive MALDI-MS spectra of siallylactose acquired on a Biflex-IV spectrometer for: Native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

1-Phenyl-3-Methyl-5-Pyrazolone Derivatives (PMP)

The saccharides (up to 0.5 μ mol) were dissolved in 0.3 M NaOH (30 μ L), followed by addition of 0.5 M methanolic PMP solution (30 μ L). Mixtures were heated to 70 $^{\circ}$ C for 30 min and then neutralized with 0.3 M of HCl (30 μ L) [21]. More water was added (200 μ L) and the excess PMP reagent was removed by extraction with chloroform (20 μ L) or purification was achieved using a GlycoClean S cartridge.

Phenylhydrazones Derivatives (PHN)

PHN derivatives of saccharides were prepared according to a published procedure [23,24]. Phenylhydrazine (0.5–1 μ L) was added to a water solution of saccharide (50 μ L; up to 0.5 μ mol) and the mixture was incubated for 1 h at 80 $^{\circ}$ C with frequent mixing. Then the samples were analyzed directly without removing the excess phenylhydrazine or after extraction with ethylacetate.

The ethylacetate layer was pipetted out to another tube and washed with water (3×20 μ L). The water fractions were mixed together and completed to appropriate volume for analysis.

ESI-Mass Spectrometry

The mass spectrometer used for ESI experiments was a Quattro-LC (Micromass, UK) equipped with a Z-spray ion source and a triple quadrupole analyzer. Direct injections were carried out with a 20 μ L Rheodyne loop and the mobile phase solvent was acetonitrile/water (1:1). The source block and the desolvation temperatures were set at 110 and 130 $^{\circ}$ C. The samples were sprayed using a 3.5 kV needle voltage and the cone voltage was set to 20 V. Mass spectra were recorded in the positive and in the negative ion modes with a scan rate of 300 Us^{-1} . MassLynx software. CID tandem MS experiments were conducted with argon, at a gas pressure of 3×10^{-3} torr and collision energy of 28 eV. For all experiments, 5 μ L aliquots were injected.

Figure 4. Negative MALDI-MS spectra of sialyllactose acquired on a Biflex-IV spectrometer: Native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

Figure 5. Comparison of ESI-MS ionic abundances observed for small derivatized carbohydrates in positive (+) and negative (–) modes. For each derivative, seven reactions were set up and ten measurements were taken on each product. The averages are reported. Percentage errors were on the order of $\pm 5\%$. Values were obtained by adding $[M + H]^+$, $[M + Na]^+$ signals in the positive mode, and $[M - H]^-$, $[M - 2H + Na]^-$ in the negative mode.

Figure 6. ESI-MS/MS spectra of $[M + H]^+$ ions of sialyllactose detected in the native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

MALDI-Time-of-Flight (TOF) Mass Spectrometry

MALDI spectra were recorded on a Biflex-IV spectrometer (Bruker Daltonics, Billerica, MA). Profiling of the molecular ions of native and derivatized glycans was achieved in the positive mode, using linear and reflective TOF. The accelerating voltage was 20 kV. For sensitivity studies on derivatized oligosaccharides, each glycan pool was purified and dissolved in 20 μ L of distilled water and 0.5 μ L aliquots were loaded onto the target. The sum of 200 shots was collected for each spectrum. MALDI-PSD spectra were recorded in the positive ion mode with the laser power adjusted to 80 % of its maximal intensity. MALDI-MS/MS spectra were acquired using a prototype quadrupole-quadrupole-TOF^o(QqTOF)^omass^ospectrometer^o[28].^oIn^othis^oinstru-ment, ions are produced by irradiation of the sample with photon pulses from a 20 Hz nitrogen laser (VCL 337ND, Spectra-Physics, Mountain View, CA) with 300 mJ energy per pulse. Parent ions were selected in the first quadrupole (Q1) after cooling, then broken up in the second rf-only quadrupole (q2) by CID at low energy. The collisional energy for each precursor ion was determined by applying a well-defined accelerating voltage at the entrance of the collisional cell, and values were around 50 eV per each 1000 Da. Orthogonal injection of ions from the q2 into the TOF section routinely produces a mass resolving power of $\sim 10,000$ FWHM, and accuracy within a few mDa, in both MS and MS/MS modes.

The samples were mixed with saturated matrix solution (DHB in acetonitrile/water 1:1), deposited on the surface of a MALDI target, air dried, and subjected to analysis.

Results and Discussion

AB, PMP, and PHN derivatives of saccharides were prepared according to published procedures with some variations as described in the experimental section. For example, in the preparation of unreduced AB derivatives, the use of acetic acid was avoided and the reaction was performed in a water/methanol solution at increased temperature. These conditions helped to enhance yields, however, required more frequent mixing during incubation.

Small Carbohydrates

The relative sensitivities of detection for labeled lactose and analogs (Scheme 2) were compared under ESI and MALDI-MS conditions in the positive and negative modes. Using a low cone voltage, all three types of derivatives produced $[M + H]^+$, $[M + Na]^+$, $[M - H + 2Na]^+$ ions in the positive mode and $[M - H]^-$, $[M - 2H + Na]^-$ ions in the negative mode, with relative abundances depending on the method of ionization, the kind of label, and the purification procedure used after derivatization. While AB and PHN derivatives of lac-

Figure 7. MALDI-MS/MS spectra of $[M + Na]^+$ ions of sialyllactose acquired on a MALDI-QqTOF spectrometer and detected in the native form (a), derivatized with PHN (b), AB-unreduced (c), AB-reduced (d), and PMP (e).

tose, epilactose, N-acetyllactosamine, fucosyllactose, and sialyllactose ionized mainly as $[M + H]^+ / [M - H]^-$ ions by ESI-MS and $[M + Na]^+ / [M - H]^-$ ions in MALDI-MS, PMP-sialyllactose in both ESI and MALDI arose as mixtures of $[M + H]^+$, $[M + Na]^+$, $[M - H + 2Na]^+$ ions in the positive mode and $[M - H]^-$, $[M - 2H + Na]^-$ ions in the negative mode, regardless on the choice of purification procedure (Figures 1, 2, 3, and 4). MS sensitivities depended not only on the label used but also on the kind of carbohydrate investigated. Epilactose produced the highest signal with PMP (~10% over AB and PHN), but lactose and fucosyllactose showed comparable sensitivities for all three derivatives. Figure 5 shows the values of added $[M + H]^+$, $[M + Na]^+$ signals (positive mode) and $[M - H]^-$, $[M - 2H + Na]^-$ signals (negative mode). For N-acetyl lactosamine, stronger signals were observed for unreduced AB and PHN derivatives than for their reduced analogs. In the case of sialyllactose, again AB and PHN yielded better sensitivity in the ESI-MS spectra in the positive and negative modes, however, no significant differences were observed between the AB unreduced and reduced forms (Figures 1 and 2). When derivatives

of sialyllactose were examined by MALDI-MS in positive mode, on average the AB derivatives yielded the most intense signals, however, the most abundant ions corresponded to the loss of sialic acid (m/z at 484.9, Figure 3d). In negative mode MALDI-MS spectra, non-reduced AB and PHN derivatives of sialyllactose yielded the best signals for this compound, with no loss of sialic acid (Figure 4).

Next, the MS/MS fragmentation patterns of these derivatives were compared. In the ESI-MS/MS spectra of $[M + H]^+$ ions of PMP- and AB-sialyllactose (reduced and unreduced forms), abundant Y-type fragment ions were observed (nomenclature of Domon and Costello [29]). In the spectrum of PHN-sialyllactose, the most abundant ions corresponded to B and Z cleavages (Figure 6). Under MALDI-MS/MS conditions, all three derivatives fragmented by Y_2 cleavages of sialic acid (Figure 7). In the spectra of unreduced AB and PHN, abundant $Y_2 / 0,2A_3$ internal fragment ions at m/z 305.2 appeared. A previous study on the fragmentation of chondroitin sulfates showed the advantage of using unreduced AB derivatives over pyridylamidation (PA) to avoid acid residue losses subsequent to B fragmen-

Figure 8. MALDI-MS spectra of the glycans released from ovalbumin: (a) Native forms, (b) PHN, (c) AB, and (d) PMP derivatives.

tations° [20].° In° our° MS/MS° spectra° of° AB° and° PMP derivatized sialyllactose, however, no fragment ions appeared to have retained sialic acid. Only the spectra of native and PHN sialyllactose showed B₁ ions at m/z 292, B₂ at m/z 454, and C₂ at m/z 492 (Figure 6a,b).^{d,2} A₃ cross-ring cleavages were observed at m/z 596 in the MALDI-MS/MS spectra of unreduced AB and PHN derivatives (Figure 7b,c).

Large Oligosaccharides

In the MALDI-MS spectra of N-linked oligosaccharides from ovalbumin loaded onto the target immediately after derivatization, i.e., without further cleaning, it was possible to detect oligosaccharides only in the case of PHN derivatives. After purifying procedures, the spectra of all three types of derivatives had good signal-to-noise ratios, with the best observed for AB reduced glycans (Figure 8). [M° + °Na]⁺ ions dominated and, under MALDI-PSD and -MS/MS conditions, fragmentation through glycosidic and cross-ring cleavages to some extent, depending on the nature of the tag used. MALDI-MS/MS spectra of AB derivatives showed fragmentation patterns similar to those obtained by ESI-MS/MS [16]. Higher m/z fragment ions corresponded to B and Y cleavages, whereas for PHN and PMP deriva-

tives, only B and C glycosidic cleavages were observed. B ions formed by loss of chitobiose seemed to undergo easier losses of residues from 3-linked mannose than from 6-linked mannose. Also, in the spectra of PHN derivatives, the abundance of ions corresponding to the cleavage of bisecting GlcNAc depended on the number of residues attached to the 6-antenna [30]. Three GlcNAc residues attached directly to 6-mannose caused the most extensive loss of bisecting GlcNAc. Residues at the 6-position favored the loss of bisecting GlcNAc in the following decreasing order: (GlcNAc)₃Man-, Gal(GlcNAc)₃Man/(GlcNAc)₂Man-, GlcNAcMan-, Man-.

Differences were also noted in the abundances of cross-ring cleavage ions. While in all MS/MS spectra of PHN and PMP derivatives these types of ions were relatively abundant, corresponding peaks were either small or missing in the spectra of hybrid and complex AB glycans. Only MS/MS spectra of high mannose glycans contained cross-ring cleavage ion peaks of significant heights.

The following section shows and compares MALDI-MS/MS spectra of hybrid, complex, galactosylated, and sialylated oligosaccharide derivatives. Fragmentation patterns observed in the tandem mass spectra of [M + Na]⁺ of hybrid glycans (MW_{native} 1640.5) indicated the presence of three isomers (Figure 9). Y₄ ions were

Figure 9. MALDI-MS/MS spectra of $[M + Na]^+$ ions of hybrid glycans with MW_{native} 1663.5, obtained from ovalbumin and detected as (a) PHN, (b) AB-reduced, and (c) PMP derivatives. Fragment ions are sodiated.

consistent with the cleavage of a GlcNAc residue from the nonreducing end, and appeared as a large peak at m/z 1580.6 only in the spectrum of AB derivatives (Figure 9b). For Structure 1, the loss of bisecting GlcNAc (-221 u) from $B_3/Y_{3\beta}$ ions appeared at m/z 653.2 in the spectra of all three types of derivatives and were the most abundant for PHN-glycans (Figure 9a). In Figure 9b (AB derivatives), this peak was masked by preferential loss of water from $B_3/Y_{3\alpha}$ ions (m/z 671.3) in Structure 3, which has no bisecting moiety. In the case of Structure 2, the loss of bisecting GlcNAc appeared at m/z 491.1. The presence of this latter isomer is also supported by cross-ring $^{3,5}A_3$ ions at m/z 599.3, detected in all three spectra.

Figure 10 presents spectra of complex glycans with MW_{native} 1722.5. Abundant Y_4 ions appeared in the spectrum of AB derivatives at m/z 1662.6 (Figure 10b), while in the other cases abundant ions corresponding to glycosidic cleavages from the reducing end were observed, B_4 at m/z 1524.5 and B_3 at m/z 1321.4 (Figure 10a, c). Peaks at m/z 1159.3 could arise from Structures 1 and 2, according to cleavage patterns observed in the case of AB derivatized glycans (Figure 10b). However the large

peak at m/z 938.3 in the spectrum of PHN-glycans is consistent with a loss of bisecting GlcNAc from m/z 1159.3 ions (Figure 10a) and could be assigned to Structure 3. These ions with low abundances were also detected with PMP derivatives (Figure 10c). Structure 3 is also supported by the observation of $C_{2\alpha}$ ions at m/z 812.4 in the spectra of PHN and PMP derivatives. A PSD spectrum acquired after fractionation of the whole PHN derivatized glycan pool [30] provided evidence for the presence of three isomers for $(\text{GlcNAc})_6\text{Man}_3$ glycans.

The MALDI-MS/MS fragmentation patterns of all three types of derivatized, galactosylated complex glycans with MW_{native} 2290.8 are shown in Figure 11. In the spectra of PMP and PHN glycans, higher m/z ions again corresponded to B, C, and cross-ring fragments $^{3,5}A_5$ at m/z 1963.7, $^{0,2}A_5$ at m/z 2009.7, and $^{0,2}A_6$ at m/z 2212.8 (Figure 11a, c). The spectrum of AB derivatives contained dominant Y-type ions (Y_4 at m/z 2230.7). Further loss of GlcNAc or hexosyl from the nonreducing end produced ions at m/z 2027.6 and 2068.6 (Figure 11b). The presence of bisecting GlcNAc in both isomers was apparent from the fragmentation pattern observed in

Figure 10. MALDI-MS/MS spectra of $[M + Na]^+$ ions of complex PHN-glycans with $MW_{\text{native}} 1745.5$ obtained from hen ovalbumin and detected as (a) PHN, (b) AB-reduced, and (c) PMP derivatives. Fragment ions contain sodium.

the spectrum of PHN derivatives, where the loss of 221 u from B_3/Y_3 ions appeared at m/z 1100.4 and 938.3. These cleavages were also present in the spectra of the other derivatives, although difficult to detect.

In the MS/MS spectra of high-mannose glycans (Scheme 3) other differences were noticed; for instance, B and C ions were absent from the spectra of AB derivatives. Instead, abundant Y_1 and Y_2 ions appeared (m/z 364.1 and 567.2). Very abundant and diagnostically useful $B_3/Y_{3\beta}$ ions, corresponding to the loss of chitobiose core and of the 3-antenna (m/z 671.3), were observed in the spectra of AB and PHN glycans. For PMP derivatives, these signals were very weak and Y_1 fragments were the most abundant and dominated other ions.

MALDI-MS/MS spectra of biantennary sialylated fucosylated oligosaccharide $SA(Gal)_2(GlcNAc)_4(Man)_3Fuc$ ($MW_{\text{native}} 2078.9$) are shown in Figure 12. In all three spectra the most abundant ions, $Y_{6\alpha}$, resulted from loss of sialic acid, but otherwise the spectra showed similar features as discussed earlier. Although $B_5/Y_{6\alpha}/Y_{3\beta}$ ions at m/z 712.3 appeared in all spectra, only in the spectrum of the PHN derivative these ions showed favorable loss of water (m/z 694.2; Figure 12a), which could be reliably assigned to loss of mannose from the 3-antenna. X-type

fragments were present in the spectrum of the PHN derivative at m/z 1971.7, 1708.6, 1343.4, 1313.3. The spectrum of the PMP derivative showed X ions at m/z 1947.6. In the spectrum of the AB derivative, these ions were absent. Relatively abundant A cross-ring cleavage ions were observed in the case of the PHN derivatives. These ions retained sialic acid and originated from loss of the chitobiose core (m/z 1633.6 and 1806.5; Figure 12a).

Conclusions

Variations in ESI/MALDI-MS sensitivity of derivatized carbohydrates depended on the choice of label and on the type of saccharide investigated. While epilactose showed enhanced sensitivity with PMP derivatization, lactose and fucosyllactose produced comparable peak heights with all three labels. For N-acetylglucosamine and sialyllactose, best signals were observed with AB and PHN. Reduced AB derivatives of N-glycans after purification produced the highest signals. Desalting of AB and PMP derivatized mixtures after enzymatic cleavage and removal of excess derivatization agent were both very important. PHN labeling was advantageous in this view, as it allowed direct analysis, i.e., without sample cleanup. On the other hand, reduced

Figure 11. MALDI-MS/MS spectra of $[M + Na]^+$ ions of galactosylated complex glycans with MW_{native} 2313.8 from hen ovalbumin and detected as (a) PHN, (b) AB-reduced, and (c) PMP derivatives. Fragment ions contain sodium.

AB and PMP derivatives are more stable and their purification using cartridges is very handy. The cartridge cleanup procedure can be also used for unre-

duced AB and PHN derivatives, when necessary, however yields are lowered.

As opposed to results shown in a previous study [16], the type of label used influenced the fragmentation pattern observed by CID-MS/MS. In our hands, PMP- and AB-sialyllactose produced only Y-type ions under ESI/MALDI-MS/MS conditions. The ESI-MS/MS spectrum of PHN-sialyllactose was dominated by B and Z glycosidic cleavages. B_1 and B_2 fragments containing sialic acid were observed only in the spectra of native and PHN sialyllactose. In the MALDI spectrum of PHN sialyllactose, these ions appeared as B_1 , C_2 , and $^{0,2}A_6$ cross-ring cleavages. The latter type of cleavage was also detected in the spectrum of the unreduced AB derivative.

By MALDI-MS/MS, hybrid and complex N-glycans derivatized with PHN and PMP showed comparable glycosidic fragmentation patterns, while for AB-derivatives differences were observed. Here also, the most informative spectra were acquired with PHN derivatives, as B_x/Y_3 ions, consistent with losses of chitobiose core and 3-antenna, produced intense signals. These ions underwent loss of water in all oligosaccharides tested here. The abundance of ions corresponding to the cleavage of bisecting GlcNAc (-221 u) depended on the

Scheme 3. Fragmentation scheme of AB, PMP and PHN derivatives of high-mannose glycans, MW_{native} 1235 (1) and 1437 (2).

Figure 12. MALDI-MS/MS spectra of $[M + Na]^+$ ions of sialylated biantennary fucosylated oligosaccharide with $MW_{\text{native}} = 2078.7$ and detected as PHN (a), AB-reduced (b), and PMP (c) derivatives. Fragment ions contain sodium.

number of residues linked to the 6-positioned mannose. Cross-ring cleavages, important to characterize linkages, produced relatively abundant ions. Spectra of some PMP derivatives showed this feature, but in the case of AB derivatives these ions were mostly suppressed. For high-mannose N-glycans, AB and PHN produced the most useful fragmentation patterns. Highly favored Y-glycosidic cleavages from the reducing end of PMP derivatives suppressed the relative abundance of other ions.

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