Anal. Chem. **2004,** *76,* 6989−6997

Direct *N*-Glycan Profiling in the Presence of Tryptic Peptides on MALDI-TOF by Controlled Ion Enhancement and Suppression upon Glycan-Selective Derivatization

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Even though the formidably laborious and time-consuming nature of oligosaccharide analysis limits certain attempts to analyze the glycosylation profile, the significant elucidation of carbohydrate modifications is largely dependent on it. Aiming to substantially improve the sample preparation procedure, a novel protocol allowing glycan-specific detection in the presence of other species, such as tryptic peptides, on MALDI-TOF was proposed and then evaluated. The new protocol is based on the concept that the desorption/ionization efficiency of glycans could be selectively and substantially enhanced while drastically suppressing the other ion species upon glycan-selective derivatization. A series of known and novel labeling reagents, all of which carry hydrazide functionality to allow glycan-specific derivatization, were prepared and evaluated in terms of their abilities to enhance the detection sensitivity of glycans, suppress ions of other contaminants (e.g., peptides), and detect acidic oligosaccharides. Several novel reagents that possess hydrophobic residue(s) together with quaternary ammonium/pyridinium or guanidino functionalities significantly enhanced the detection sensitivity of oligosaccharides. When enzymatically deglycosylated tryptic ovalbumin digest was directly derivatized by these reagents and subjected to MALDI-TOF analysis without any prior purification, we observed that a single type of analyte ion (labeled glycan) could suppress a large majority of peptide ions while allowing a low-femtomole level detection of oligosaccharides. The efficacy of this approach was further evaluated using several other model glycoproteins, including α_1-acid glycoprotein that contains a variety of sialylated oligosaccharides.

Glycoprotein oligosaccharides play a vital role in biological processes, such as stability, protein conformation, intra- and intercell signaling, and a binding affinity to and a specificity for other biomolecules.[1,2] It is also becoming evident that glycosylation can affect the safety and efficiency of the therapeutic glycoproteins.[3,4] However, the analysis of constituent glycans has not reached the same degree of sophistication as peptides. This is because analyzing carbohydrates is much more difficult due to the problems imposed by structural complexities and their inherent difficulty in detecting chromatography or mass spectrometry in their native state. Due to its simplicity, speed of analysis and comparatively increased sensitivity, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has proven to have several advantages over other analytical techniques when analyzing carbohydrates.[5−7] However, the relatively low response to oligosaccharides (compared, for instance, peptides) often requires derivatization of oligosaccharides where highly sensitive detection is required.

Although a number of derivatization procedures have been evaluated and were proven to be efficient in improving detection sensitivity,[8−10] there are still vast challenges for glycosylation analysis on both naturally occurring and recombinant proteins. Substantial improvement in sample preparation is eagerly expected in particular. Analyzing oligosaccharides is a formidably laborious and time-consuming task. For the sensitive detection of oligosaccharides, they need to be liberated from any protein(s) or peptide(s), extensively purified from other molecular species, derivatized with a labeling reagent, and must be purified often from the excess reagent. The purification step(s) is/are typically achieved by chromatography. Indeed, many sample preparation steps are hardly integratable as an automated format, and so they raise a substantial risk to lose trace components in the sample. These inconveniences are further pronounced for the analysis of sialylated oligosaccharides, which have been implicated in a number of important biological processes. Sialylated oligosaccharides analyzed in MALDI-TOF often cause metastable fragmentation,

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10.1021/ac0492766 CCC: $27.50
Published on Web 10/30/2004

and those analyzed in a positive ion mode have yielded a mixture of cation adducts ions, giving rise to a complex mass profile. Although permethylation[10] and methyl esterification of sialic acid[12] have been demonstrated to be quite useful in stabilizing these groups and rendering them chemically equivalent to neutral oligosaccharides, they have also been proven to lower the throughput.

Aiming at partially filling these gaps, our attention was directed toward utilizing the phenomenon known as "ion suppression" in a mass spectrometric analysis.[13,14] Ion suppression is a competition effect in ionization when the sample contains a high concentration of salts, or (excess of) other analyte(s) that can be ionized in the operating condition. For instance, peak intensities of peptide mixtures, such as those obtained by tryptic digestion, differ significantly. This is considered attributable not only to the intrinsic properties of peptides, but also to ion suppression.[15,16] The magnitude of the observed suppression strongly depends on both nature and the amount of suppressing peptide. Due to this phenomenon, the direct analysis of unseparated mixtures is a particularly difficult exercise, since the suppression could have been caused by other analytes in the mixture, as well as by contaminants introduced during isolation and workup procedures. Thus, the negative aspects of ion suppression have quickly become a major concern. Consequently, many research interests have been directed to reducing this unfavorable phenomenon to enable the detection of as many peptide ions as possible, either by derivatization[17−19] or by thorough purification.[20,21] Our interest is to see whether specific ion species could be almost completely embossed in a complex mixture if the ionization efficiency of the ions of interest could be selectively and substantially enhanced.

In this article, a series of known and novel labeling reagents, all of which carry a hydrazide functionality to allow glycan-specific derivatization in a complex mixture, were evaluated in terms of their abilities to enhance the ionization efficiency of labeled glycans, suppress ions of other contaminants (e.g. peptides), and detect sialylated oligosaccharides. Using glycoprotein tryptic digests followed by enzymatic *N*-glycan liberation as a model case, we observed that a single type of analyte ion (labeled glycan) could suppress a large majority of peptide ions and other contaminants. In other words, the *N*-glycan profiling in an unpurified tryptic digest could be achieved without any purification steps. The detection of sialylated oligosaccharides in a complex mixture will also be addressed.

EXPERIMENTAL SECTION

Materials and Reagents. A complex-type *N*-linked oligosaccharide (Gal(β1−4)GlcNAc(β1−2)Man(α1−6)(Gal(β1−4)GlcNAc-(β1−2)Man(α1−3))Man(β1−4)GlcNAc(β1−4)GlcNAc, NA2) was purchased from Prozyme (San Leandro, CA). Girard's reagents T and P were acquired from Tokyo Kasei Kogyo (Tokyo, Japan). (3-(2-(Methoxycarbonyl)ethyl)-2-indolyl)trimethylammonium iodide and 3-Et-1-(2-(2-methoxycarbonylmethyl-1*H*-indol-3-yl)-ethyl)-pyridinium perchlorate were ordered from Sigma-Aldrich (St. Louis, MO). N^{α}-benzoyl-L-arginine methyl ester bicarbonate was purchased from Sigma Chemical (St. Louis, MO), L-arginine methyl ester dihydrochloride was acquired from Fluka (Milwaukee, WI), and (*Z*)-Phe-Arg methyl ester was acquired from Chem-Impex International (Wood Dale, IL). Ovalbumin (grade VI), asialofetuin (type II) from fetal calf serum, ribonuclease B from bovine pancreas, and human α_1-acid glycoprotein were purchased from Sigma Chemicals. 2,5-Dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (HCCA) were obtained from Bruker Daltonics (Billerica, MA). Recombinant PNGase F (E.C. 3.2.2.18) was procured from Roche Diagnostics (Penzberg, Germany), and sequencing grade-modified trypsin (EC 3.4.21.4) was purchased from Promega Corp (Madison, WI).

Synthesis of Reagents. *(3-(2-(Hydrazinocarbonyl)ethyl)-2-indolyl)trimethylammonium (HEITA).* (3-(2-(Methoxycarbonyl)-ethyl)-2-indolyl)trimethylammonium iodide (21.7 mg, 55.9 μmol) was dissolved in methanol (2.8 mL), and hydrazine monohydrate (0.5 mL, 10.3 mmol) was added. The reaction mixture was stirred for 12 h at room temperature. The titled compound was isolated by reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (80:20, vol) at a yield of ∼72%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.57−6.97 (m, 4H, Ph), 4.13 (m, 2H, $CONHNH_2$); positive ion mode MALDI-TOF *m/z* 261.3 (M^+).

3-Et-1-(2-(2-hydrazinocarbonylmethyl-1H-indol-3-yl)-ethyl)-pyridinium (EHIEP). 3-Et-1-(2-(2-methoxycarbonylmethyl-1*H*-indol-3-yl)-ethyl)-pyridinium perchlorate (11.1 mg, 26.2 μmol) was dissolved in methanol (0.99 mL), and hydrazine monohydrate (0.28 mL, 5.9 mmol) was added. The reaction mixture was stirred for 12 h at room temperature. The titled compound was isolated by reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (80:20, vol) at a yield of ∼74%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.76 (t, 2H, J = 6.5 Hz, CH_2N), 4.31 (bs, 2H, $CONHNH_2$); positive ion mode MALDI-TOF *m/z* 323.0 (M^+).

L-Arginine Hydrazide (Rh). L-Arginine methyl ester dihydrochloride (RO−Me) was prepared according to the previously described procedure with minor modifications.[22] RO−Me (5.0 mg, 19.1 μmol) was dissolved in methanol (0.96 mL), and hydrazine monohydrate (0.35 mL, 7.3 mmol) was added. The reaction mixture was stirred for 6 h at room temperature. The titled compound was isolated using reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (86:14, vol) at a yield of ∼76%; positive ion mode MALDI-TOF *m/z* 189.1 ($M + H)^+$.

N^{α}-Benzoyl-L-arginine Hydrazide (BRh). N^{α}-benzoyl-L-arginine methyl ester bicarbonate (4.5 mg, 12.7 μmol) was dissolved in methanol (0.63 mL), and hydrazine monohydrate (0.23 mL, 4.7 mmol) was added. The reaction mixture was stirred for 6 h at

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room temperature. The titled compound was isolated by reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (80:20, vol) at a yield of ~87%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.31−7.37 (m, 5H, Ph), δ 4.42 (m, 1H, α-CH), 4.24 (bs, 2H, CONHNH_2); positive ion mode MALDI-TOF m/z 293.2 $(M + H)^+$.

N^{α}-(Benzyloxycarbonyl)arginine Hydrazide (ZRh). $NaHCO_3$ (168 mg, 2 mmol) and *Z* chloride (313 μL, 1 mmol) was added to a solution of R−OMe (261 mg, 1.0 mmol) in H_2O (5 mL). The mixture was stirred for 12 h at room temperature. The reaction mixture was extracted with $CHCl_3$, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine. The extraction was concentrated, and the residue was purified by silica gel chromatography, eluting it with $CHCl_3$/MeOH (5:1) to give 0.3 g (31% yield) of (*Z*)-arginine methyl ester ((*Z*)R−OMe); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (m, 5H, Ph), 5.08 (d, 1H, J = 12.1 Hz, 1/2 × CH_2Ph), 5.03 (d, 1H, J = 12.1 Hz, 1/2 × CH_2Ph), 4.23 (bs, 1H, α-CH), 3.67 (s, 3H, $COOCH_3$). (*Z*)R−OMe (2.9 mg, 9.0μmol) was dissolved in methanol (0.45 mL), and hydrazine monohydrate (0.17 mL, 3.4 mmol) was added. The reaction mixture was stirred for 6 h at room temperature. The titled compound was isolated by reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (66:34, vol) at a yield of ~68%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37 (m, 5H, Ph), 5.01 (s, 2H, CH_2Ph), 4.22 (bs, 2H, CONHNH_2), 4.09 (bs, 1H, α-CH); positive ion mode MALDI-TOF m/z 323.2 $(M + H)^+$.

N^{α}-(Benzyloxycarbonylphenylalanylarginine Hydrazide (ZFRh). (*Z*)-Phe-Arg methyl ester, hydrochloride (2.7 mg, 5.3μmol) was dissolved in methanol (0.27 mL), and hydrazine monohydrate (0.1 mL, 2.1 mmol) was added. The reaction mixture was stirred for 6 h at room temperature. The titled compound was isolated using reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (66:34, vol) at a yield of ~77%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37 (m, 10H, Ph), 4.95 (d, 1H, J = 13.0 Hz, 1/2 × CH_2Ph), 4.91 (d, 1H, J = 12.6 Hz, 1/2 × CH_2Ph), 4.28 (m, 2H, Phe α- and Arg α-CH), 4.25 (bs, 2H, CONHNH_2); positive ion mode MALDI-TOF m/z 470.1 $(M + H)^+$.

N^{α}-(Benzyloxycarbonyl)tryptophanylarginine Hydrazide (ZWRh). To a solution of N^{α}-(benzyloxycarbonyl)tryptophan (846 mg, 2.5 mmol) in dry THF (6 mL), we added *N*-methylmorpholine (330 μL, 3.0 mmol) and isobutyl chloroformate (393μL, 3.0 mmol) at −20 °C. The mixture was heated to room temperature and stirred for 15 min, followed by the addition of a solution of the arginine methyl ester dihydrochloride (784 mg, 3.0 mmol) and $NaHCO_3$ (252 mg, 3.0 mmol) in H_2O (3 mL) at 0 °C. The reaction mixture was stirred for 1 h. Solvents were removed under reduced pressure. The residue was chromatographed on a column of silica gel using 5:1 $CHCl_3$/MeOH to give (*Z*)-Trp-Arg methyl ester (1.1 g, 87%); ^{1}H NMR (500 MHz, CD_3OD) δ 8.55−7.18 (m, 10H, Ph and indole), 4.93 (s, 2H, CH_2Ph), 4.33 (m, 2H, Trp α- and Arg α-CH), 3.63 (s, 3H, $COOCH_3$). (*Z*)-Trp-Arg methyl ester (5.4 mg, 10.6μmol) was dissolved in methanol (0.53 mL), and hydrazine monohydrate (0.2 mL, 4.0 mmol) was added. The reaction mixture was stirred for 6 h at room temperature. The titled compound was isolated using reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (66:34, vol) at a yield of ~76%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.13−6.95 (m, 10H, Ph and indole), 4.94 (s, 2H, CH_2Ph), 4.29 (m, 2H, Trp α- and Arg α-CH), 4.29 (m, 2H, CONHNH_2); positive ion mode MALDI-TOF m/z 509.2 $(M + H)^+$.

N^{α}-(Benzyloxycarbonyl)tyrosinylarginine Hydrazide (ZYRh). To a solution of N^{α}-(benzyloxycarbonyl)tyrosine (788 mg, 2.5 mmol) in dry THF (6 mL), we added *N*-methylmorpholine (330 μL, 3.0 mmol) and isobutyl chloroformate (393 μL, 3.0 mmol) at −20 °C. The mixture was heated to room temperature and stirred for 15 min and was followed by the addition of a solution of the arginine methyl ester dihydrochloride (784 mg, 3.0 mmol) and $NaHCO_3$ (252 mg, 3.0 mmol) in H_2O (3 mL) at 0 °C. The reaction mixture was stirred for 1 h. Solvents were removed under reduced pressure. The residue was chromatographed on a column of silica gel using 5:1 $CHCl_3$/MeOH to give (*Z*)-Tyr-Arg methyl ester (0.8 g, 67%); ^{1}H NMR (500 MHz, CD_3OD) δ 8.53−7.09 (m, 9H, Ph), 4.95 (d, 1H, J = 12.9 Hz, 1/2 × CH_2Ph), 4.92 (d, 1H, J = 12.9 Hz, 1/2 × CH_2Ph), 4.23 (m, 2H, Tyr α- and Arg α-CH), 3.62 (s, 3H, $COOCH_3$). (*Z*)-Tyr-Arg methyl ester hydrochloride (5.2 mg, 10.7μmol) was dissolved in methanol (0.54 mL), and hydrazine monohydrate (0.2 mL, 4.0 mmol) was added. The reaction mixture was stirred for 6 h at room temperature. The titled compound was isolated by reversed-phase chromatography with 50 mM ammonium bicarbonate/acetonitrile (66:34, vol) at a yield of ~66%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.35−6.63 (m, 9H, Ph), 4.97 (d, 1H, J = 13.4 Hz, 1/2 × CH_2Ph), 4.92 (d, 1H, J = 12.9 Hz, 1/2 × CH_2Ph), 4.37 (m, 2H, CONHNH_2), 4.20 (m, 2H, Tyr α- and Arg α-CH); positive ion mode MALDI-TOF m/z 486.2 $(M + H)^+$.

Digestion of Glycoproteins with Trypsin. Digestion of glycoproteins with trypsin was performed according to the procedure previously described.[23] Briefly, glycoproteins were solubilized with 0.1% (w/v) RapiGest SF (Waters, Milford, MA) solution buffered with 50 mM ammonium bicarbonate (pH of 7.8) at a concentration of 15 μM each. They were digested with 5 μg of modified trypsin at 37 °C for 1 h. Heating the reaction mixture at 100 °C for 3 min terminated the reaction.

Deglycosylation of Tryptic Digests by PNGase F. The tryptic digest of each glycoprotein was incubated by directly adding PNGase F (0.5 U) to the tryptic glycoprotein digest at 37 °C overnight. The reaction mixture was kept at −20 °C until further analysis.

Derivatization of Standard Oligosaccharides with Labeling Reagents. The derivatization reaction was modified from the procedure for hydrazone formation, as previously described.[24,25] For standard derivatization, 10 μL of each labeling reagent (200 μM) in 80% methanol and 1 μL of 10 μM oligosaccharide (NA2) were added to 10 μL of 50 mM ammonium bicarbonate. The mixture was then incubated at 90 °C for 1 h. After cooling the reaction tube on ice, the reaction mixture was accordingly mixed with DHB (10 g/L in 30% acetonitrile), and 0.5 μL of the resulting mixture was subjected to MALDI-TOF mass analysis without any purification.

Derivatization of Deglycosylated Tryptic Digests with Labeling Reagents. Typically, 1 μL of deglycosylated tryptic digest of ovalbumin, asialofetuin, ribonuclease B, or α_1-acid

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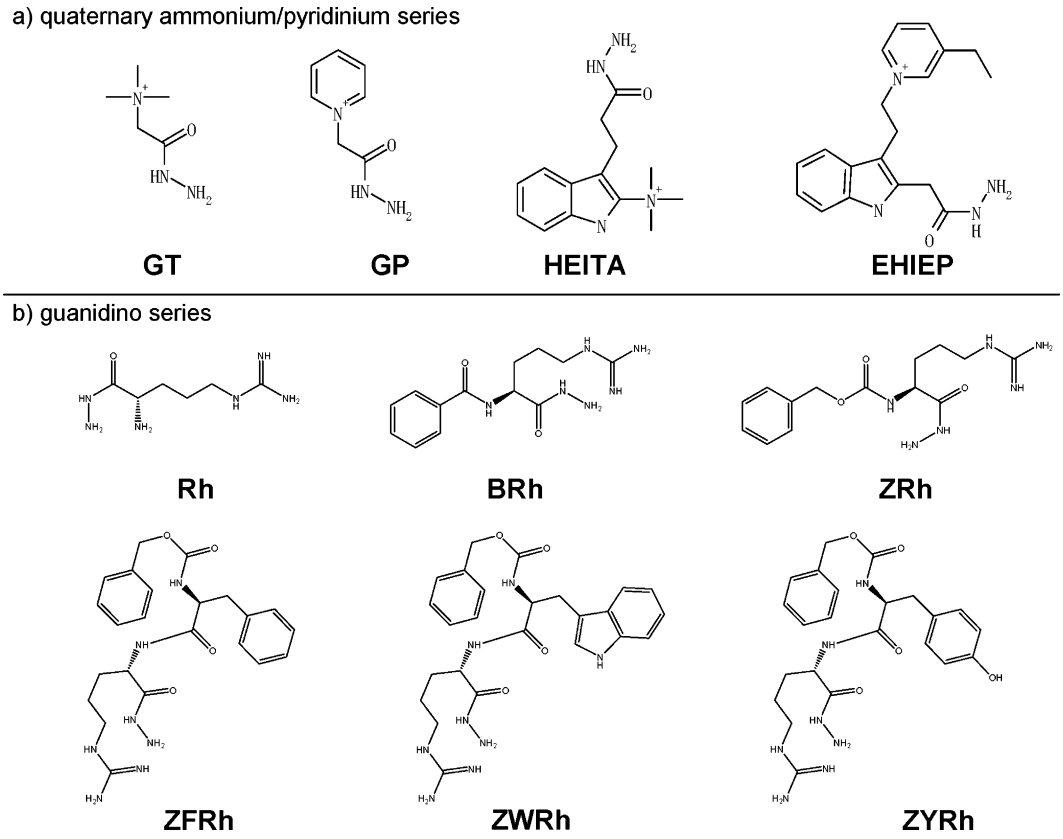

Figure 1. Structures of the reagents used in this study: (a) quaternary ammonium/pyridinium series and (b) guanidino series.

glycoprotein (15 μM) was mixed with 10 μL of each labeling reagent (200 μM) in 80% methanol and 9 μL of 50 mM ammonium bicarbonate. The mixture was incubated at 90 °C for 1 h. After cooling the reaction tube on ice, 1 μL of the reaction mixture was mixed with 9 μL of DHB (10 g/L in 30% acetonitrile), and 0.5 μL of the resulting mixture was subjected to MALDI-TOF mass analysis without any purification.

MALDI-TOF Mass Spectrometry. Mass measurements were carried out using an Ultraflex TOF mass spectrometer equipped with a pulsed ion extraction system (Bruker-Daltonik GmbH, Bremen, Germany). Ions were generated by a pulsed 337-nm nitrogen laser. The laser power was adjusted to a level slightly above the threshold for the formation of ions. All of the spectra were obtained using a reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. These were the results of the signal averaging of 100 laser shots. Typically, three spectra of one spot measured at different positions were averaged. Good spectrum quality of each derivatized glycan was typically obtained from the peripheral region of the DHB crystal. Acidic oligosaccharides were also analyzed at 20 kV without the reflectron in the positive ion mode.

The detection limits for the derivatized oligosaccharides were estimated by depositing each carbohydrate derivative on the target in decreasing amounts by serial dilution and analyzing them by MALDI-TOF MS, as described above.

RESULTS AND DISCUSSION

Basis of the Selection of Reagents Used. The structures of the reagents tested in this study are summarized in Figure 1. To achieve selective labeling of the oligosaccharides in the presence of other species, such as tryptic peptides, we employed the hydrazide functionality as the carbohydrate reactive center. The formation of hydrazone has advantages for the microscale analysis of oligosaccharides, since the substituted hydrazide couples to the oligosaccharide with high efficiency by simply heating the oligosaccharide with some fold of reagent molar excess.[24,25] Moreover, it does not necessarily require the purification step prior to MS analysis.[26] Since they are proven to be efficient in improving the sensitivity, we employed quaternary ammonium/pyridinium centers as ionization promoting functionalities (e.g., Girard's reagent T (GT),[26] trimethyl-(*p*-aminophenyl) ammonium (TMA-PA)[27]). In addition to two commercially available compounds, GT and Girard's reagent P (GP), two novel reagents, (3-(2-(hydrazinocarbonyl) ethyl)-2-indolyl)trimethylammonium (HEITA) and 3-Et-1-(2-(2-hydrazinocarbonylmethyl-1*H*-indol-3-yl)-ethyl)-pyridinium (EHIEP), were synthesized for this purpose (see the Experimental Section). The other class of ionization promoting functionality used in this study was the guanidino center, since it was also ionized with high efficiency.[28] Six reagents that possess both hydrazide and guanidino functionalities, Rh, BRh, ZRh, ZFRh, ZWRh, and ZYRh, were synthesized for this purpose (see the Experimental Section).

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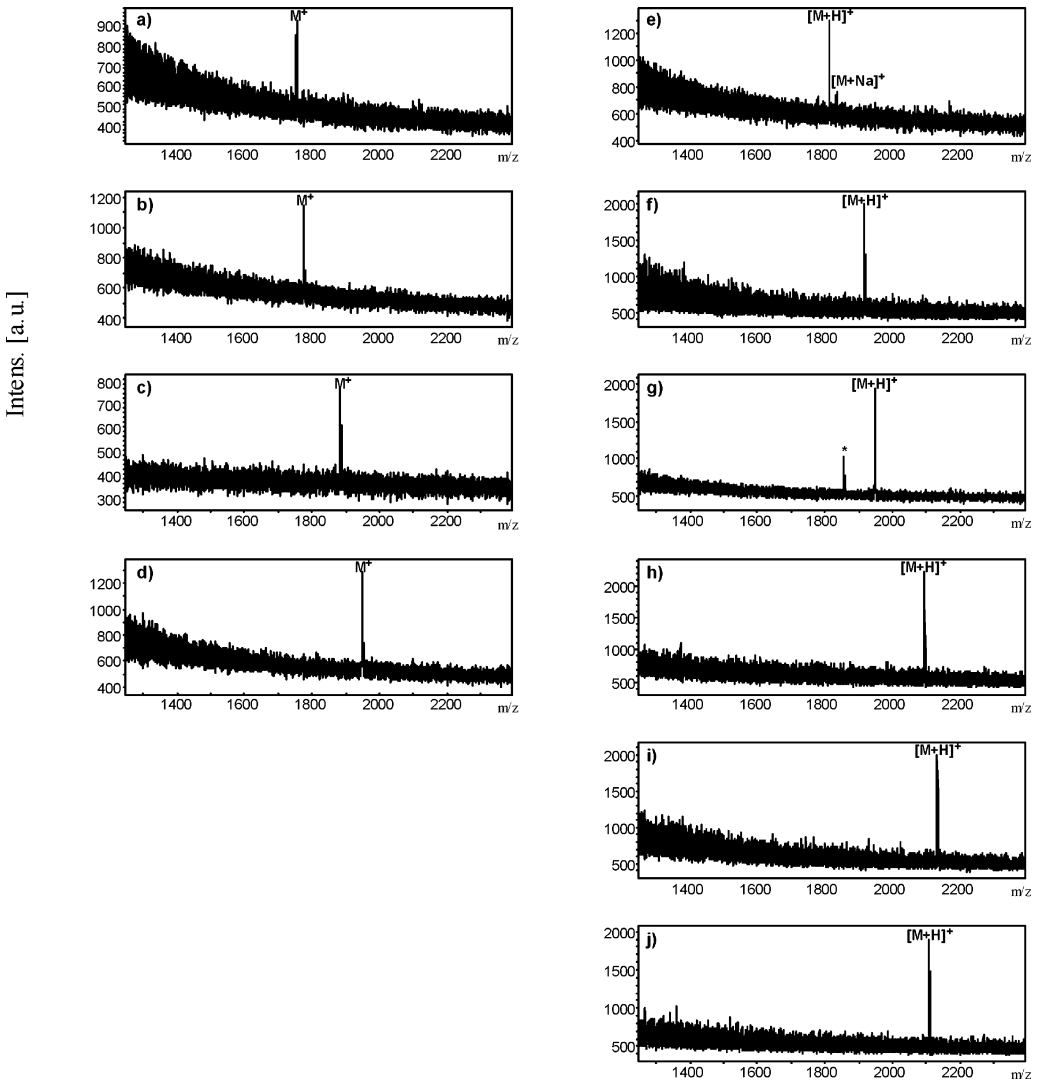

Figure 2. MALDI-TOF spectra of the derivatized asialobiantennary *N*-glycan (NA2) by (a) GT, (b) GP, (c) HEITA, (d) EHIEP, (e) Rh, (f) BRh, (g) ZRh, (h) ZFRh, (i) ZWRh, and (j) ZYRh. The sample amounts loaded on the target were 50 fmol for GT, GP, HEITA, Rh, and ZRh and 4.5 fmol for EHIEP, BRh, ZFRh, ZWRh and ZYRh. The asterisk shown in (g) indicates the byproduct. All spectra were acquired with DHB as matrix.

Evaluation of Enhanced Detection Sensitivity upon Derivatization. All of the reagents properly reacted with a model oligosaccharide, asialobiantennary *N*-glycan (NA2), to give expected hydrazones under the conditions described in the Experimental Section. As shown in Figure 2a−d, derivatives of GT, GP, HEITA, and EHIEP were detected solely as M^+ at m/z 1754.9, 1774.8, 1883.8, and 1945.9, respectively, on positive reflectron mode MALDI-TOF analysis using DHB as a matrix. M^+ ions were the only ion species detected that were consistent with the previous observations for those derivatives of other reagents having a quaternary ammonium center,[26,27] and this may be explained by the presence of a permanent positive charge. On the other hand, derivatives of Rh, BRh, ZRh, ZFRh, ZWRh, and ZYRh were detected as $[M + H]^+$ at m/z 1811.8, 1915.8, 1945.9, 2092.8, 2131.8, and 2108.8, respectively (Figure 2e−j).

The approximate detection limits for each derivatized oligosaccharide was determined by acquiring the spectra of the derivatized NA2 in decreasing amount using serial dilution. The derivatized NA2 by GT, GP, HEITA, and EHIEP could be detected down to 20, 10, 7.5, and 1 fmol, respectively, loaded on the target. Despite the previous observations that derivatives incorporating a constitutive cationic charge by reacting with Girard's reagent T, TMAPA, and the dimethylation of the reductive amination product of sugars with benzylamine[29] equally increased the sensitivity about an order of magnitude, the detection limits of the derivatives of GT, GP, HEITA, and EHIEP differed considerably while they all carried constitutive cationic charge. Likewise, the detection limits of derivatized NA2 by the guanidino series reagents differed ~10-fold: the derivatized NA2 by Rh, BRh, ZRh, ZFRh, ZYRh, and ZWRh could be detected down to 10, 1, 5, 1, 1, and 1 fmol, respectively, loaded on the target. Derivatization of NA2 using ZRh tended to give a relatively intense signal of the byproduct at 90 mu below the molecular ion $[MH]^+$, which may be attributable to the cleavage of the benzyl group. Krause et al. demonstrated that signals from arginine-containing peptides are generally stronger than lysine-containing peptides in a tryptic digest,[28] so a recent flurry of research has been directed toward increasing mass spectral signal intensities from lysine-containing peptides,[17,18] for example, modifying lysine residues with *O*-methylisourea to form more basic homoarginine residues. The current study has revealed

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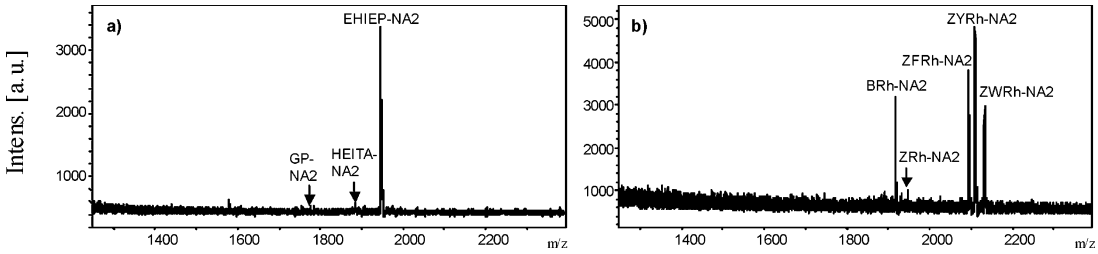

Figure 3. MALDI-TOF spectra obtained for the mixed reaction mixture of derivatized asialobiantennary *N*-glycan (N2) by (a) GT, GP, HEITA, and EHIEP and (b) Rh, BRh, ZRh, ZFRh, ZWRh, and ZYRh. The sample amounts loaded onto the target were (a) 114 fmol and 10 fmol, each. All spectra were acquired with DHB as matrix.

that the incorporation of hydrophobic residue(s), such as phenyl, indole, and benzoyl groups together with the guanidino group, further increase detection sensitivity. This observation is partly supported by the recent publication of Baumgart et al. that the signal-enhancement impact of peptides depends on the amino acid compositions as well as on the identity of adjacent amino acids to arginine.[30]

To further compare the relative sensitivities of the different reagent derivatives, each derivatization reaction mixture by GT, GP, HEITA, and EHIEP was mixed in equal quantities and analyzed using MALDI-TOF to compare the relative intensity of each derivative under exactly the same analytical conditions. Derivative of EHIEP was predominantly detected with slight signal intensities of the derivatives of GP and HEITA. GT derivative was not observed (Figure 3a). Note that the derivatives of GT, GP, and HEITA were clearly detectable at this concentration when they were measured independently. Likewise, the reaction mixtures by Rh, BRh, ZRh, ZFRh, ZWRh, and ZYRh were mixed and analyzed by MALDI-TOF. Derivative ions of BRh, ZFRh, ZWRh, and ZYRh were dominantly observed at comparable intensities with a lesser signal intensity of the ZRh adduct (Figure 3b). Rh derivative was not even visible, while it should be detectable at this concentration when it is measured independently. This may be explained by ion suppression, in which glycan derivatives having higher sensitivities could suppress those having lesser sensitivities.

Evaluation of the Peptides' Ion-Suppressing Ability of Each Reagent. Ovalbumin tryptic digest was used as one of the model studies. Following the tryptic digestion of ovalbumin, it was further digested with PNGase F in the same vial to remove the *N*-glycans and was subjected to MALDI-TOF analysis using DHB as matrix. Since glycoproteins widely differ in their susceptibility to enzymatic deglycosylation,[31] tryptic digestion prior to PNGase F digestion has been commonly used to ensure complete *N*-glycan liberation.

As shown in Figure 4a, tryptic digest gave reasonable peptide mass fingerprints in the MALDI-TOF analysis, whereas a better quality peptide mass fingerprint (PMF) result was obtained with CHCA (data not shown). This is often the first choice matrix for PMF due to the predominantly higher mass peak intensities when compared to DHB.[32] Additional PNGase F digestion of the tryptic digest gave plural new signals while retaining most of the peptides' signals at comparable intensities. Six of the new signals were identical to the molecular ions ($[M + Na]^+$) of the previously reported most abundant *N*-glycans of ovalbumin[33,34] (see Figure 4b).

When an aliquot of deglycosylated tryptic ovalbumin digest was directly derivatized with a 70-fold excess of GT, GP, HEITA, or EHIEP and subjected to MALDI-TOF analysis by simply mixing the reaction mixture with DHB, the resulting mass spectra differed significantly, depending on the reagent used (Figure 4c–f). The result obtained from the EHIEP derivatives gave the most remarkable result. Almost all of the peptides' ions disappeared, and all of the m/z values of the newly appeared ions upon EHIEP derivatization were identical to those of previously reported *N*-glycans (mainly high-mannose and hybrid structures). For the derivatives of GT, GP, and HEITA, peptide ion suppression was modest. Many of the peptides' ions were still visible, and only a limited number of the derivatized glycans were detectable. Upon EHIEP derivatization, the detection of major ovalbumin oligosaccharides could be achieved at an amount equivalent to 10 fmol of glycoprotein by serial dilution (data not shown), which is consistent with the estimated detection limit of the EHIEP-derivatized glycan.

Among the guanidino series reagents, derivatization with BRh, ZFRh, ZWRh, and ZYRh allowed for oligosaccharide-specific ion embossment under the presence of tryptic peptides of ovalbumin (Figure 4h, j, k, and l). Derivatization with ZRh also allowed for the detection of the glycan ions, though the ability to suppress the peptides' ions was behind those of EHIEP, BRh, ZFRh, ZWRh, and ZYRh (Figure 4i). Rh derivatization barely allowed the detection of a few major glycans among the peptides' signals (Figure 4g). Thus, the specific glycan ion embossment ability in a complex mixture appeared to correlate well with the relative sensitivity that the derivatization by each reagent could achieve.

To elucidate whether the observed peptides' ion suppression phenomenon is attributable to the presence of excess reagent or the presence of the labeled glycan species, the deglycosylated tryptic digest of ovalbumin was mixed with a 100-fold excess of EHIEP, and an aliquot was analyzed by MALDI-TOF. It was confirmed that oligosaccharides do not react with EHIEP unless the reaction mixture is heated. Almost all of the peptides' ions disappeared on the spectrum, indicating that the observed pep-

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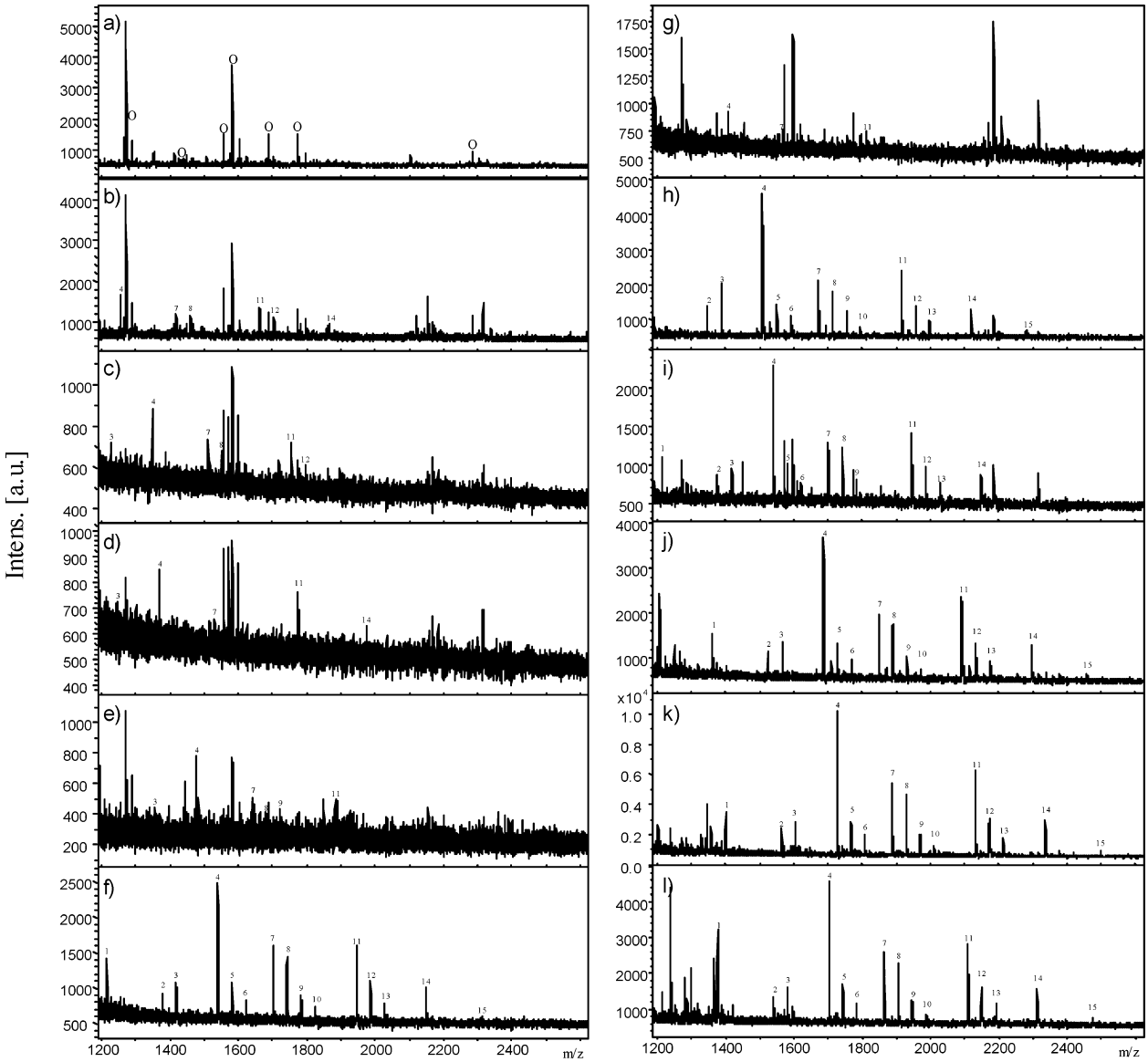

Figure 4. MALDI-TOF spectra of (a) tryptic digest of ovalbumin, (b) tryptic and PNGase F digest of ovalbumin, and direct derivatives of deglycosylated tryptic digest of ovalbumin by (c) GT, (d) GP, (e) HEITA, (f) EHIEP, (g) Rh, (h) BRh, (i) ZRh, (j) ZFRh, (k) ZWRh, and (l) ZYRh. The sample amounts loaded on the target were 750 fmol equiv to glycoprotein for (a) and (b), and 170 fmol equiv to glycoprotein for c−l. All spectra were acquired with DHB as matrix. Ions labeled with O in (a) are ovalbumin peptides. 1, $(Hex)_3(HexNAc)_2$; 2, $(Hex)_4(HexNAc)_2$; 3, $(Hex)_3(HexNAc)_3$; 4, $(Hex)_5(HexNAc)_2$; 5, $(Hex)_4(HexNAc)_3$; 6, $(Hex)_3(HexNAc)_4$; 7, $(Hex)_6(HexNAc)_2$; 8, $(Hex)_5(HexNAc)_3$; 9, $(Hex)_4(HexNAc)_4$; 10, $(Hex)_3(HexNAc)_5$; 11, $(Hex)_5(HexNAc)_4$; 12, $(Hex)_4(HexNAc)_5$; 13, $(Hex)_7(HexNAc)_2$; 14, $(Hex)_5(HexNAc)_5$; and 15, $(Hex)_6(HexNAc)_5$.

tides' ion suppression is attributable to the presence of excess reagent. On the other hand, when the reaction mixture was analyzed after the removal of unreacted excess EHIEP, most of the peptides' ions were observed together with labeled glycans (data not shown). These results indicated that efficient peptides' ion suppression could be realized only when excess reagents were present in the sample.

The detection of EHIEP-labeled oligosaccharide was found to be quite tolerant of the large excess presence of EHIEP. The presence of a large EHIEP excess did not give any detection sensitivity reduction of EHIEP-labeled NA2, even when a 2000-fold excess of EHIEP was present (data not shown). This was also true for BRh, ZFRh, ZWR, and ZYRh. We thus concluded that a higher reagent/sample molar ratio was advantageous for an efficient oligosaccharide-specific ion embossment on the MALDI-TOF analysis. To the authors' knowledge, this is the first successful example demonstrating the direct *N*-glycan profiling in an unpurified glycoprotein tryptic digest without any purification steps.

It has been considered that the high sensitivity on MALDI is due to the high efficiencies during both the solid−gas phase transition and ionization processes. Despite the fact that it is widely used in many fields, the theories and models of MALDI are still crude.[35] The detailed mechanism of the observed highly efficient ion suppressing ability needs to be further elucidated.

Applicability to Other Glycoproteins, Including Those That Carry Sialylated Oligosaccharides. To further evaluate selective oligosaccharide detection under the presence of the tryptic peptides, this protocol was applied to other glycoproteins, asialofetuin, ribonuclease B, and α_1-acid glycoprotein. The de-

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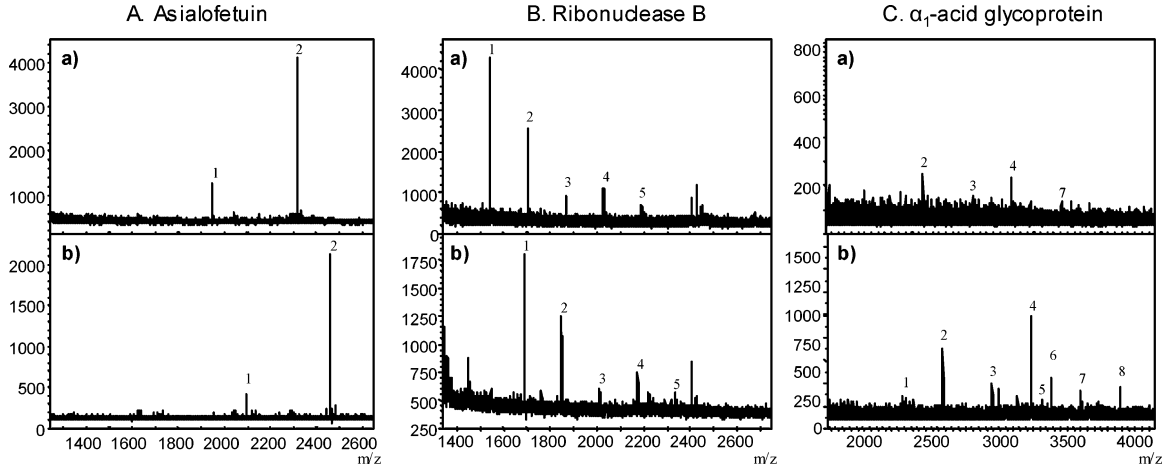

Figure 5. MALDI-TOF spectra of derivatized tryptic and PNGase F digest of (A) asialofetuin, (B) ribonuclease B, and (C) α_1-acid glycoprotein by EHIEP (a) and ZFRh (b). The sample amounts loaded onto the target were 170 fmol equiv to glycoprotein. All spectra were acquired with DHB as matrix. Ions with numbers are identical to (A) 1, $(Hex)_2(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$; 2, $(Hex)_3(HexNAc)_3$ + $(Man)_3(GlcNAc)_2$; (B) 1, $(Hex)_2$ + $(Man)_3(GlcNAc)_2$; 2, $(Hex)_3$ + $(Man)_3(GlcNAc)_2$; 3, $(Hex)_4$ + $(Man)_3(GlcNAc)_2$; 4, $(Hex)_5$ + $(Man)_3(GlcNAc)_2$; 5, $(Hex)_6$ + $(Man)_3$-$(GlcNAc)_2$; (C) 1, $(Hex)_2(HexNAc)_2(Neu5Ac)_1$ + $(Man)_3(GlcNAc)_2$; 2, $(Hex)_2(HexNAc)_2(Neu5Ac)_2$ + $(Man)_3(GlcNAc)_2$; 3, $(Hex)_3(HexNAc)_3(Neu5Ac)_2$ + $(Man)_3(GlcNAc)_2$; 4, $(Hex)_3(HexNAc)_3(Neu5Ac)_3$ + $(Man)_3(GlcNAc)_2$; 5, $(Hex)_4(HexNAc)_4(Neu5Ac)_2$ + $(Man)_3(GlcNAc)_2$; 6, $(Hex)_3(HexNAc)_3$-$(dHex)_1(Neu5Ac)_3$ + $(Man)_3(GlcNAc)_2$; 7, $(Hex)_4(HexNAc)_4(Neu5Ac)_3$ + $(Man)_3(GlcNAc)_2$; 8, $(Hex)_4(HexNAc)_4(Neu5Ac)_4$ + $(Man)_3(GlcNAc)_2$.

glycosylated tryptic digest of each glycoprotein was derivatized with 130-fold excesses of EHIEP and ZFRh, and the reaction mixture was directly subjected to MALDI-TOF analysis using DHB as the matrix (Figure 5). EHIEP derivatization allowed for the detection of all of the previously reported oligosaccharides of asialofetuin[36] and ribonuclease B,[37] and the peptides' signal were largely suppressed, although a few relatively intense contaminant signals were observed around m/z 2400 for ribonuclease B. However, regarding α_1-acid glycoprotein, EHIEP barely allowed for the detection of a few derivatized glycan ions, whereas the peptides' ion suppression was fairly successful. Unlike asialofetuin and ribonuclease B, almost all of the oligosaccharides of α_1-acid glycoprotein are reported to be sialylated.[38] Although labeling reagents having constitutive cationic charge have been reported to successfully improve the detection sensitivities of oligosaccharides, to the authors' knowledge, their application had been limited to neutral oligosaccharides. Considering that oligosaccharide derivatives of reagents having constitutive cationic charge (e.g., Girard's reagents T and P, HEITA, EHIEP) are detected solely as M^+ on the MALDI-TOF analysis, the reduction of the net positive charge due to the incorporation of negative charge(s) from sialic acid may drastically decrease desorption/ionization efficiency.

In contrast, ZFRh derivatization allowed for the detection of oligosaccharides for all of the model proteins used, regardless of the presence or absence of sialic acid. Sialylated oligosaccharides, detected solely as $[M + H]^+$, were detected for neutral oligosaccharides. The detection of the major sialylated oligosaccharides of α_1-acid glycoprotein could be achieved at an amount equivalent to 10 fmol of glycoprotein loaded on the target by serial dilution, suggesting that the detection sensitivity of sialylated oligosaccharide is comparable to that of a neutral oligosaccharide. The mass spectrum obtained for α_1-acid glycoprotein was both qualitatively and quantitatively consistent with that of the previous report, which was obtained after converting sialic acid into methyl ester by its reaction with methyl iodide.[38] However, the relative signal intensity of each sialylated oligosaccharide could somewhat vary among the spectra acquired from firing the laser at different locations on the target. Careful use of threshold laser irradiance was required to detect ions that correspond to intact sialylated oligosaccharides in a positive reflectron mode. On the other hand, in a positive linear mode, the signal detection of the ZFRh derivatives of intact sialylated oligosaccharides was much less sensitive to the laser irradiance power. The relative signal intensity of each sialylated oligosaccharide was fairly stable among spectra acquired from firing the laser at different locations on the target as far as peripheral regions of the crystal were chosen (data not shown). Despite the previous observation that sialylated oligosaccharides analyzed with DHB in either the linear or reflectron positive mode often lose sialic acid, and these problems are further aggravated as the number of sialic acid residues incorporated into the oligosaccharide increases,[39] ZFR derivatives appeared to be quite stable through ionization under linear mode and may even be detectable under reflector mode. This may be explained by the highly improved ionization/desorption efficiency upon derivatization that allowed labeled glycans to be detected with a much lower laser power, and hence, the internal energy supplied by the laser may be reduced. This superior nature observed for ZFRh is also true for BRh, ZWRh, and ZYRh.

Securing a series of labeling reagents could provide some practically useful applications. For instance, when more than one reagent is used for a single sample, one can be assured that the detected signals originate from oligosaccharides by comparing the m/z differences of the detected signals of each spectrum with those of the molecular weights of the employed labeling reagents. This is especially true for glycans that are present at lower

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concentrations and with molecular weights that fall intermediate to those of the more abundant glycans.

The current protocol did not allow for the entire suppression of the ions of other species (e.g., a few intense signals observed for ribonuclease B and several weak signals observed for asialofetuin). These ions would complicate the *N*-glycan profiling of the real sample in which the glycosylation characteristics are unknown; however, the concept to selectively enhance the ionization/desorption efficiency of glycans in a crude mixture upon glycan-selective derivatization and, hence, to minimize the sample preparation steps have been proven. To integrate the described protocol with the current proteome analysis workflow, further study is currently in progress in our laboratory.

CONCLUSION

In this article, we proposed and demonstrated a novel concept that glycan ions could be highly selectively embossed on MALDI-TOF, even without the need for a sample cleanup under the condition that the ionization efficiency of glycan is selectively and substantially enhanced upon glycan-specific derivatization (hydrazone formation). We prepared a series of novel derivatization reagents that possess either quaternary ammonium/pyridinium or guanidino functionalities as ionization-promoting groups. The ability to enhance the signal is dependent not only on the presence of these functionalities, but also on the other part of the reagent structure. The incorporation of hydrophobic residue, such as the phenyl, indole, and benzoyl groups, further increased the sensitivity and was necessary to achieve significant ion suppression of other contaminants, such as peptides. Moreover, the derivatization of oligosaccharides with ZFRh and related compounds could allow for the sensitive detection of oligosaccharides, regardless of the presence or absence of sialic acid.

Mass spectrometry has in the past decade become accepted as a key analytical technique among biology and biochemistry laboratories. Due to its inherent limitation in the direct analysis of complex mixtures, mass spectrometry has often been adopted to combine sophisticated separation technologies, that is, LC, GC, gel electrophoresis, and capillary electrophoresis, prior to analysis. Though the central role of such a separation prior to the detection and evaluation of analytes will be indisputable, the concept to regulating ion enhancement and suppression upon derivatization may provide a new way to help reduce the number of sample preparation steps.

ACKNOWLEDGMENT

This work was supported in part by the National Project on Functional Glycoconjugate Research Aimed at Developing for New Industry from the Ministry of Education, Science, Sports and Culture of Japan.

Received for review May 18, 2004. Accepted August 27, 2004.

AC0492766