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Quantitative Matrix-assisted Laser Desorption/ionization Mass Spectrometry of Pyrene-derivatized Glycopeptides for Investigation of Mammalian Cell Glycomics

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Abstract

Glycan is one of the major information-rich biomolecules. Alterations in *N*-glycans can be associated with many diseases including cancer, and are often observed in the serum of affected patients. As an example, prostate-specific antigen (PSA) is a glycoprotein secreted by prostatic epithelial cells. The serum PSA assay is widely used for detection of prostate cancer (PCa). Detection of altered PSA glycan is regarded as a precise diagnosis. However, the limited amount of serum PSA available makes it difficult to determine detailed glycan structures, as the PSA level in serum is very low.

Mass spectrometry (MS) is a powerful tool for analyzing glycan structures. We have recently established a highly sensitive MS for both glycans and glycopeptides by pyrene derivatization. Matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight (MALDI-QIT-TOF) mass spectra of glycans derivatized with pyrene butanoic acid hydrazide (PBH) were compared with those of pyridylaminated (PA) glycans. Each derivatized glycan was prepared from the same amount of commercial PSA. PBH-labeled PSA glycans showed higher signal intensity with less fragmentation compared with PA-glycans, and gave spectra with high s/n ratio (in both positive- and negative-ion modes). For derivatization of the glycopeptides, pyrenyl diazomethane (PDAM) was used. The glycopeptide signals were greatly enhanced by this derivatization. PDAM-glycopeptides prepared from about 10 ng of PSA could be determined.

In this study, we demonstrated that MS analysis using pyrene derivatization provides higher sensitivity and stability for both glycans and glycopeptides, compared with HPLC and lectin affinity chromatography; furthermore comparable results were obtained to those seen with the other methods. We therefore conclude that our method is useful for investigation of mammalian cell glycomics.

Abbreviations: PSA: Prostate Specific Antigen; PBH: Pyrene Butanoic Acid Hydrazide; PDAM: Pyrenyl Diazomethane; PCa: Prostate Cancer; MALDI: Matrix-Assisted Laser Desorption/Ionization; QIT: Quadrupole Ion Trap; TOF: Time of Flight; MS: Mass Spectrometry; DHBA: 2,5-Dihydroxybenzoic Acid; CHCA: Alpha-cyano-4-Hydroxycinnamic Acid; PA: 2-Aminopyridine; HPLC: High-Performance Liquid Chromatography; ELISA: Enzyme-Linked Immunosorbent Assay; PNGase F: Peptide: N-Glycanase F; AAL: *Aleuria Aurantia* Lectin; WFA: *Wisteria Floribunda* lectin; TFA: Trifluoroacetic Acid

Introduction

Glycoform profiling by high-throughput sample preparation and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis is a new potential tool for the diagnosis of human diseases [1]. Using MALDI-MS, protein profiling has been applied to proteomic biomarker research. However, several aspects of MALDI-MS profiling need further evaluation and optimization before clinical use [2]. In this profiling, simple preparation steps to capture glycoproteins while removing lipids and salts are essential with biological fluids such as serum, urine, and tissue extracts. Because high-abundant proteins, salts, and lipids tend to mask the smaller amount of biomarkers present, profiling can be achieved only with highly abundant glycoproteins [3,4]. For example, prostate-specific antigen (PSA) is a glycoprotein synthesized and secreted by prostatic epithelial cells. The serum PSA assay is widely used for detection of prostate cancer (PCa). However, PSA tests still suffer from a lack of specificity in distinguishing benign prostatic hyperplasia (BPH) from PCa. Since alteration of glycans on many

glycoproteins have been reported during tumor progression, it is of great interest to study whether changes in glycosylation of PSA could be useful diagnostic indicators. As an initial effort, the PSA derived from a cell line (LNCaP) established from the lymph node of a patient with metastatic prostate carcinoma, was investigated [5]. Furthermore, it has been reported that PSA from prostate tumor tissue and from LNCaP cell contains *N*-glycans with more antennae than PSA from benign prostate hypertrophy tissues and seminal fluid [6,7]. Recently, Tajiri et al. [8] measured the MS spectra of PSA glycopeptides from PCa patient sera at high levels of PSA (1.3 and 1.8 μg / ml). On the other hand, Fukushita et al. [9] analyzed alteration of glycans on PSA from PCa patients' sera compared with those from BPH, using lectin affinity chromatography and enzyme-linked immuno-assay (ELISA). Although lectin is a powerful tool for glycan analysis, only partial information about structure is thereby obtained. Therefore, the limited amount of serum PSA available has made it difficult to determine detailed glycan structures to date. Sensitivity in clinical glycan profiling can be effectively improved by extensive pre-fractionation strategies, which still need critical evaluation before they can be used in high-throughput analysis.

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The other challenge in glycan profiling by MALDI-MS is establishment of the reproducibility of signal intensity. In biomarker research, the aim is to determine signal intensities that are different in case and control samples. However, poor reproducibility has been considered to be one of the major problems in glycan profiling with MALDI-TOF MS [10].

To resolve those problems, we established a highly sensitive MS detection method for glycans and glycopeptides by pyrene derivatization. The glycan derivatization method using pyrene butanoic acid hydrazide (PBH) has been developed for highly sensitive analysis of structures and functions, e.g., with high-performance liquid chromatography (HPLC), ELISA, or fluorescence polarity measurement. PBH-glycans have strong fluorescence with a long lifetime [11]. We found that PBH-glycans have advantages in negative-ion MALDI-QIT-TOF MS^n. Finally we have established a reliable method using this technique for the analysis of neutral oligosaccharides, such as fucosylated glycans containing blood group antigens H, Le^a, and Le^x. The key advantages of this method are not only high sensitivity, but also protection against loss of fucose. Furthermore, many fragment ions such as A-, D-, Y-, and C-type ions are observed, and determination of isomeric and branched structures is possible [12]. Using the method, we were able to obtain detailed structures of variously fucosylated oligosaccharides from human milk [13].

We also have established a glycopeptide derivatization method using pyrenyl diazomethane for highly sensitive MALDI-MS^n of glycopeptides in amounts of less than 100 fmol. This derivatization is unique, as the pyrene groups are easily released from glycopeptides during ionization when 2,5-dihydroxybenzoic acid (DHBA) is used as a matrix. As a result, most ions are observed as the underivatized form on the spectra. At the same time, pyrene derivatization dramatically reduces the ionization of peptides [14]. Furthermore, we show that on-plate pyrenyl diazomethane (PDAM) derivatization followed by negative-ion MALDI-MS^2 is a simple and robust method for the discrimination of α2,3- and α2,6-sialylation on glycopeptides [15].

In the present study, we prepared glycans or glycopeptides from the same samples and made measurements by various methods such as HPLC, lectin affinity and MS to determine whether MALDI-MS using pyrene-derivatized glycan or glycopeptides has advantages for investigation of mammalian cell glycomics.

Materials and Methods

Materials

Human PSA was purchased from Chemicon international (Temecula, CA, US). The pyrene reagents, PDAM and PBH were purchased from Molecular Probes, Inc (Eugene, OR). The solvents, methanol, ethanol, acetonitrile and butanol, were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Xylene and Cellulose fibrous medium were from Sigma-Aldrich (Steinheim, Germany). Highly purified MALDI matrix chemicals, DHBA and alpha-cyano-4-hydroxycinnamic acid (CHCA), were purchased from Shimadzu-Biotech (Kyoto, Japan). The agarose-conjugated lectins AAL (*Aleuria Aurantia*) and WFA (*Wisteria Floribunda*) were from J-Oil Mills Co (Tokyo, Japan) and Vector Laboratories, Inc (Burlingame, CA, US), respectively. Peptide: N-Glycanase F (PNGase F) from *Flavobacterium meningosepticum* was obtained from New England BioLabs (Hitchin, UK).

Release of glycans from PSA

All samples used in this study were prepared from a commercially available PSA product. Cleavage of the *N*-glycans from PSA by PNGase F was carried out according to the manufacturer's instructions with some modification. Briefly, each glycoprotein (10 μg for 2-aminopyridine (PA) or PBH derivatization) was dissolved in water (50 μl) and denatured with a denaturing solution (5 μl) at 100°C for 10 min. To the cooled solution were added the following: reaction buffer (6 μl), detergent solution (6 μl), and PNGase F solution (1 μl ; 500 NEB units). Samples were incubated at 37°C for 12 h, and then heat-shocked at 100°C for 5 min before drying with speed vac. The de-sialylation reaction was performed in 0.8% trifluoroacetic acid (TFA) at 80°C for 40 min. Released *N*-glycans were purified with cellulose fibrous medium using a hydrophilic affinity method as described previously [15]. In brief, the sample was dissolved in a butanol/ ethanol/ water (4: 1: 1) solution and poured onto the disposable column containing 150 mg of cellulose medium equilibrated with the same solution. Captured glycans were eluted with 50% ethanol. Obtained glycan samples were dried with speed vac. and derivatized.

Preparation of PA derivatized PSA glycans

Pyridylaminated-glycans were prepared using a pyridylamination manual kit (Takara, Kyoto, Japan) according to the manufactures' protocol [16]. Briefly, 20 μl of 2-aminopyridine in acetic acid was added to the tube containing dried PSA glycans and heated at 80°C for 90 min. A volume of 20 μl of borane dimethylamine–acetic acid was then added, and the mixture was heated at 80°C for 30 min. Excess reagents were removed using a cellulose cartridge Glycan preparation kit (Takara, Japan) according to the procedures recommended by the supplier. Finally, PA-glycans were dissolved in 100 μl of 10% methanol solution and subjected to MS.

Preparation of PBH derivatized PSA glycans

PBH-glycans were prepared as previously described [11,12]. PBH in methanol (500nmol / 20μl) was mixed with the dried glycan sample and heated at 80°C for 90 min. Then 1.7 M NaBH$_4$ solution (30 μl) was added to the labeling mix and incubated at 40°C for 20 min. The addition of water and chloroform (1:1, V/V) allowed labeled oligosaccharides with or without reduction to be extracted into the aqueous phase. PBH-glycans were further purified using a Sep-Pac C18 cartridge (Waters Corp., Milford, MA) and dried with speed vac. Finally, PBH-glycans were dissolved in 100 μl of 10% methanol solution and subjected to MS.

Preparation of glycopeptides from PSA

To obtain glycopeptide sample, 10 μg of PSA was digested with thermolysin. The digestion was performed in 50 μl of 50 mM ammonium bicarbonate solution with 50 units of thermolysin at 56°C for 12 h. The sample was then dried with speed vac and de-sialylated with 100 μl of 0.8% TFA at 80°C for 40 min. The sample was desalted by passing through a 50 mg-carbon cartridge (GL science, Japan). The glycopeptides were dissolved in binding solution (5% acetonitrile and 0.1% TFA) and poured onto the carbon cartridge. Bound glycopeptides were eluted with the solution (80% acetonitrile containing 0.1% of TFA) and dried with speed vac. Finally, glycopeptide sample was further purified with cellulose according to the procedure described in the "*Release of glycans from PSA*" section.

On plate PDAM derivatization of glycopeptides

Sample solution was spotted on a mirror-polished stainless steel MALDI target and dried. PDAM solution was prepared with dimethyl sulfoxide at a 10nmol / μl concentration, and 0.25 μl of the solution was spotted onto the dried sample. The PDAM reaction was performed at 80°C for 5 min. In order to remove excess PDAM, the plate was rinsed with xylene and briefly dried under vacuum.

Mass spectrometry

One µl of matrix solution (10mg/ ml in 60% acetonitrile) was spotted onto the dried sample. MALDI-TOF mass spectra were acquired using an AXIMA-QIT instrument consisting of a quadrupole ion trap and reflector time-of flight analyzer (Shimadzu Biotech, Manchester, UK). MALDI-TOF MS was also performed in the linear mode using a Shimadzu Biotech AXIMA-TOF² instrument. For linear MS, 1µl of CHCA solution (the compound in 60% acetonitrile at a concentration of 5mg/ml) was spotted onto the sample as the matrix solution.

Lectin fractionation of PSA

AAL lectin-agarose was equilibrated with PBS and 100 µl of the resin was removed into a 1.5ml tube. One hundred µg PSA in 100 µl of solution was dissolved in 300 µl PBS and reacted with the prepared lectin at 4°C for 90 min. The flow through was divided into two fractions (200 µl / tube). The lectin was then washed 3 times with 200 µl PBS. Bound PSA was eluted (three times) with 200 µl PBS containing 50 mM L-fucose. Twenty µl of each fraction was applied to a Pierce BCA protein assay kit (Thermo scientific). The flow through and eluted fractions were combined respectively, and dried with speed vac. Each fraction was dissolved in 400 µl PBS and reacted with 100 µl WFA lectin–agarose (equilibrated with PBS) at 4°C for 90 min. The following procedures were performed as with the AAL-lectin assay, except that 50 mM *N*-acetylgalactosamine (GalNAc) in PBS was used as the eluent.

HPLC analysis of PA-glycans

PA-glycans were separated by HPLC using a chromatography system (LC20-AD) equipped with a fluorescence detector (Shimadzu, RF-10A XL). Reverse-phase HPLC was performed with an Inertsil GL WP300 C18 column (2.1 × 250 mm) equilibrated with 10 mM acetic acid / triethanolamine (pH 4.0). The analysis was performed at a flow rate of 0.3 ml / min at 40°C by linearly increasing the concentration of 1-butanol from 0% to 0.25% over 50 min. PA-glycans were detected by fluorescence using excitation and emission wavelengths of 320 nm and 400 nm, respectively. One twentieth part of the prepared PA-glycan solution (5 µl of total 100 µl) was injected to the HPLC equipment.
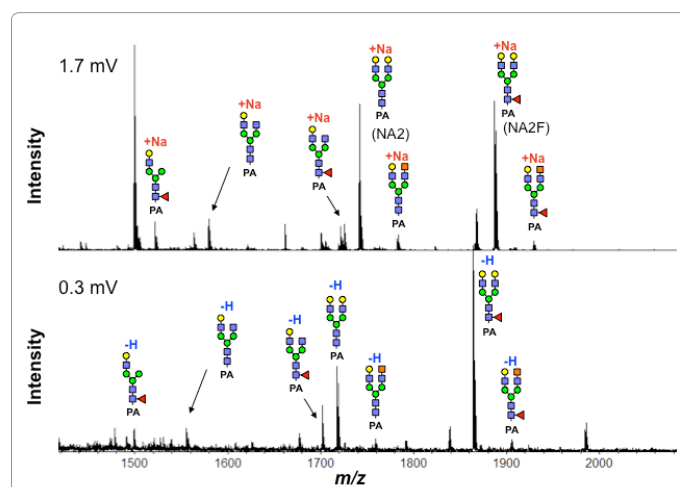

Figure 1: MS spectra of PA-glycans obtained by MALDI-QIT-TOF MS. Glycans labeled with PA were detected as the [M+Na]⁺ or [M-H]⁻ ions by MALDI-QIT-TOF MS. The MS spectra in positive (upper) and negative (lower) ions are shown. The schematic glycan structures are denoted for each signal in the chart. *N*-acetylglucosamine, Mannose, Fuc, Galactose and GalNAc are indicated as blue squares, green circles, red triangles yellow squares and orange squares, respectively.
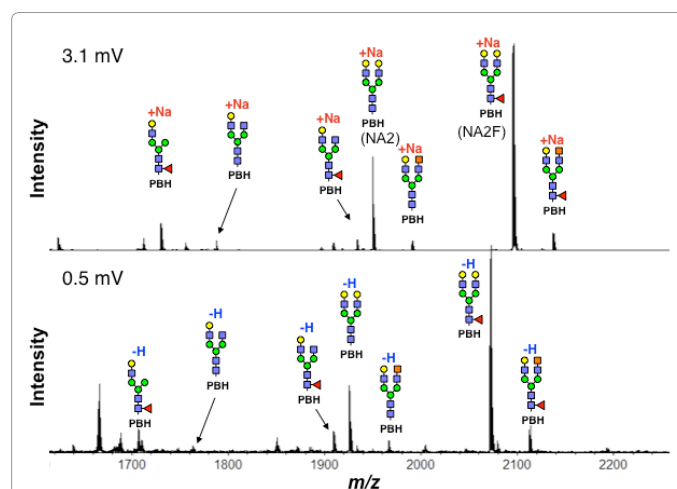

Figure 2: MS spectra of PBH-glycans obtained by MALDI-QIT-TOF MS. MS spectra of PBH-glycans were obtained by MALDI-QIT-TOF MS. The signals corresponding to glycan derivatives were detected as the [M+Na]⁺ ions (upper) and as the [M-H]⁻ ions (lower).
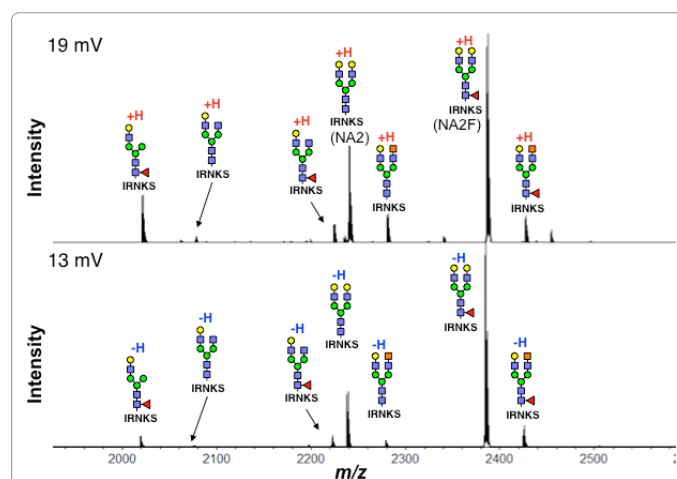

Figure 3: MS spectra of PDAM-glycopeptides by MALDI-QIT-TOF MS. MS spectra of PDAM-derived glycopeptides were obtained by MALDI-QIT-TOF MS. The signals corresponding to glycopeptides were detected as the [M+H]⁺ ion (upper) and the [M-H]⁻ ion (lower).

Results and Discussion

MALDI-TOF MS

Prepared glycan derivatives and derivatized glycopeptides were analyzed by MALDI-QIT TOF MS using both positive and negative ion modes. One hundredth part of each sample was measured with raster (2300 µm × 2300 µm area, 50 µm spacing, 20 shots each for 2209 points). PA-glycans showed sufficient signal intensity with high s/n ratio (1.7mV for positive ion mode and 0.3mV for negative ion mode) (Figure 1). The signal intensity of negative ion from PA-glycans was weaker than that in positive ion mode. The main glycan structures in PSA were NA2F and NA2 as reported by several researchers [17,9]. However, a quite high NA2-PA signal was observed in positive ion mode. It seems that considerable metastable fragmentation of fucose (Fuc) was observed during ion trapping event with post-source decay using the AXIMA-QIT instrument.

MS spectra of the same amounts of PBH-glycans are shown in

(Figure 2). PBH-glycans showed relatively stronger signal intensity with high s/n ratio (3.1 mV for positive ion mode and 0.5 mV for negative ion mode) compared with those of PA-glycans. Generally, it is known that negative-ion formation from neutral glycan is difficult. We have found that glycans derivatized with pyrene produced an abundance of ions compared with other derivatizations such as with PA [11]. In this study, we reconfirmed that PBH-glycans give higher MS intensity with less fragmentation, and are more suitable for MS detection than PA-glycans.

The MS spectra of PDAM-derivatized PSA glycopeptides are shown in (Figure 3). The signal patterns for the PSA glycopeptides and the two types of glycan derivatives were almost identical. PDAM-glycopeptides showed quite strong signals and high s/n ratios in both positive and negative ions (19 mV for positive ion mode and 13 mV for negative ion mode). Furthermore, the sample generated sufficient MS signals when one thousandth part of the total volume was measured (data not shown). The signal intensity was greatly increased with PDAM derivatization, but almost no peaks were observed at the expected *m/z* values corresponding to PDAM-attached glycopeptides. We have previously demonstrated using different matrices that release of a pyrene moiety is due to the in-source decay reaction, rather than post-source decay [15].

For calibration, relative quantification assay was performed with synthesized glycopeptides, NA2-IRNKS and [^{13}C] NA2-IRNKS. The signal intensity patterns of all derivatives are shown in Figure 4. The column heights in the Figure are indicated relative to the NA2F-IRNKS signals as 100%. The measurement errors were confirmed for all MS signals with duplicate analysis from two independent experiments, and calculated to be less than 5% for each signal in all MS spectra (data not shown). These results indicated that except for PA-glycans in positive-MALDI-QIT MS, relative glycoform patterns were almost the same.

Lectin fractionation and MS

In order to verify the reproducibility of signal intensity of pyrene derivatives, relative ratios of glycans obtained by MS were compared
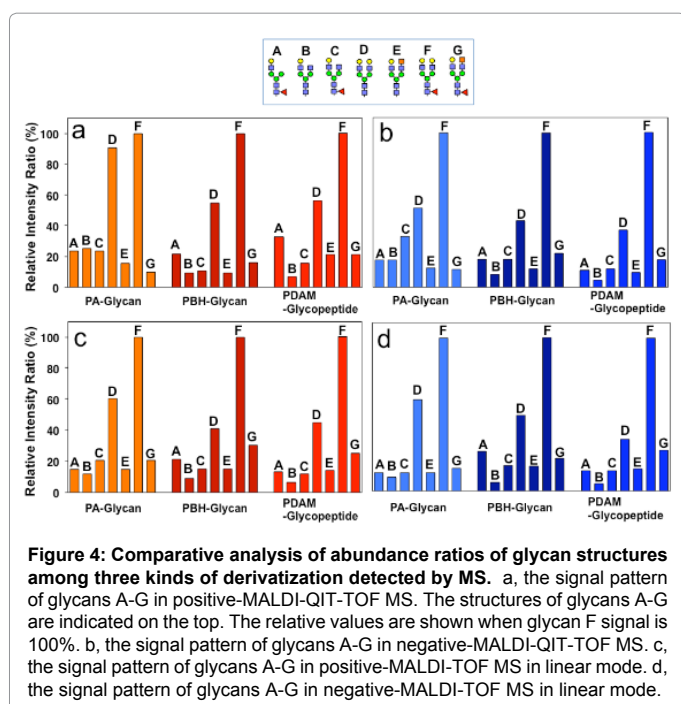

Figure 4: Comparative analysis of abundance ratios of glycan structures among three kinds of derivatization detected by MS. a, the signal pattern of glycans A-G in positive-MALDI-QIT-TOF MS. The structures of glycans A-G are indicated on the top. The relative values are shown when glycan F signal is 100%. b, the signal pattern of glycans A-G in negative-MALDI-QIT-TOF MS. c, the signal pattern of glycans A-G in positive-MALDI-TOF MS in linear mode. d, the signal pattern of glycans A-G in negative-MALDI-TOF MS in linear mode.
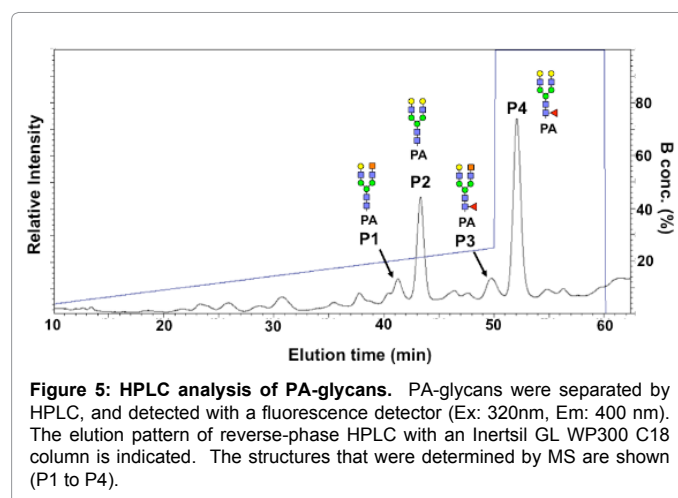

Figure 5: HPLC analysis of PA-glycans. PA-glycans were separated by HPLC, and detected with a fluorescence detector (Ex: 320nm, Em: 400 nm). The elution pattern of reverse-phase HPLC with an Inertsil GL WP300 C18 column is indicated. The structures that were determined by MS are shown (P1 to P4).

to those obtained from lectin fractionation. It is reported that AAL has a strong affinity to fucosylated glycans, with a strong affinity for core-fucose (1, 6 linkage moiety) [18]. We used AAL lectin-agarose to divide PSA glycans into two fractions containing core-fucosylated or unfucosylated moieties. From calculations using the BCA protein assay, the relative amounts of fucosylated and unfucosylated PSA were estimated to be 70% and 29.4%, respectively (Table 1). To determine the relative amount of LacdiNAc structure, fucosylated and unfucosylated fraction were subsequently applied to WFA, which has binding ability to the beta-glycosidic linkage of GalNAc (Table 1) [19]. Recently, WFA lectin has also been used to detect *N,N'*-diacetyllactosediamine (GalNAc beta 1,4 GlcNAc, LacdiNAc) [20,21]. Relative amounts of the four fractions thus obtained, Fuc(+) / LacdiNAc(+), Fuc(+) / LacdiNAc(-), Fuc(-) / LacdiNAc(+) and Fuc(-) / LacdiNAc(-) were estimated to be 4.2%, 66.4%, 21.7% and 7.7%, respectively. Results obtained from lectin fractionation were compared with MS results and are summarized in (Table 1). The pyrene derivatives detected on MS gave similar values to those seen with the lectin assay when compared to the MS signals of PA-glycans. Some fractions observed by lectin assay contained several glycan structures as a single group. In such cases, the percentages of the relative structures on MS were calculated as the sum of relative signal intensities.

HPLC analysis

Further investigation of MS data reproducibility was verified by comparison with results of HPLC analysis. PA-glycans were separated by HPLC, and detected with a fluorescence detector. The results of reverse-phase HPLC with an Inertsil GL WP300 C18 column were indicated in (Figure 5). In order to elucidate the glycan structures contained in each peak, each fraction was collected and subjected to MALDI-MS. Glycan structures in four major peaks (P1 to P4) were determinable, although contaminating compounds in the H0PLC buffer may inhibit MS detection of lower amounts of other PA-glycans. The abundance ratios of four types of structure determined by HPLC were compared with the same structures observed by MALDI-QIT MS (Table 2). Relative ratios were calculated based on the total peak area or signal intensity of the selected four types of structures designated as 100%. In the case of PA-glycans, the HPLC and MS abundance ratios differed substantially since defucosylation on PA-glycans occurs in MS. On the contrary, as expected, the relative value of PDAM-glycopeptides detected by MS was consistent with that obtained by HPLC. This means that PDAM-derivatized glycopeptides are relatively stable in MS and have higher reproducibility than PA-glycans. In conclusion, pyrene-

Glycan Types			Fuc(-) LacdiNAc(-)		Fuc(-) LacdiNAc(+)		Fuc(+) LacdiNAc(-)		Fuc(+) LacdiNAc(+)	
Lectin (AAL / WFA)			21.7		7.7		66.4		4.2	
	MS Types		QIT	Linear	QIT	Linear	QIT	Linear	QIT	Linear
TOF-MS	PA-Glycans	Pos	40.2	29.8	5.4	6.1	51.0	55.8	3.4	8.5
		Neg	28.3	32.9	5.1	5.7	61.9	59.3	4.7	7.1
	PBH-Glycans	Pos	28.9	21.6	4.2	6.5	59.7	58.8	7.2	13.1
		Neg	23.2	23.4	5.4	6.8	61.5	61.1	9.9	8.9
	PDAM-Glyco-Peptides	Pos	24.8	23.8	8.3	6.6	58.9	57.9	8.3	11.7
		Neg	21.6	18.7	4.9	7.0	64.4	61.3	9.2	12.9

Table 1: PSA glycan or glycopeptide abundance ratios observed by MS and lectin assay (%).

Glycan Types (Peak no.)		NA2 LacdiNAc (P1)	NA2 (P2)	NA2F LacdiNAc (P3)	NA2F (P4)
HPLC (PA-Glycans)		7.2	31.0	10.1	51.6
QIT Positive ion mode	PA- Glycans	7.2	41.9	4.5	46.3
	PDAM- Glyco -Peptides	10.6	28.3	10.6	50.4

Table 2: PSA glycan or glycopeptide abundance ratios observed by MS and HPLC (%).

labeled glycans or glycopeptides are useful for the study of glycomics from viewpoints of high sensitivity and reliability.

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