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Comparison of the methods for profiling glycoprotein glycans—HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study

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Mass spectrometry (MS) of glycoproteins is an emerging field in proteomics, poised to meet the technical demand for elucidation of the structural complexity and functions of the oligosaccharide components of molecules. Considering the divergence of the mass spectrometric methods employed for oligosaccharide analysis in recent publications, it is necessary to establish technical standards and demonstrate capabilities. In the present study of the Human Proteome Organisation (HUPO) Human Disease Glycomics/Proteome Initiative (HGPI), the same samples of transferrin and immunoglobulin-G were analyzed for *N*-linked oligosaccharides and their relative abundances in 20 laboratories, and the chromatographic and mass spectrometric analysis results were evaluated. In general, matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS of permethylated oligosaccharide mixtures carried out in six laboratories yielded good quantitation, and the results can be correlated to those of chromatography of reductive amination derivatives. For underivatized oligosaccharide alditols, graphitized carbon-liquid chromatography (LC)/electrospray ionization (ESI) MS detecting deprotonated molecules in the negative ion mode provided acceptable quantitation. The variance of the results among these three methods was small. Detailed analyses of tryptic glycopeptides employing either nano LC/ESI MS/MS or MALDI MS demonstrated excellent capability to determine site-specific or subclass-specific glycan profiles in these samples. Taking into account the variety of MS technologies and options for distinct protocols used in this study, the results of this multi-institutional study indicate that MS-based analysis appears as the efficient method for identification and quantitation of oligosaccharides in glycomic studies and endorse the power of MS for glycopeptide characterization with high sensitivity in proteomic programs.

Key words: N-linked/transferrin/immunoglobulin/mass spectrometry/glycopeptide

Glycosylation is a common posttranslational modification, providing a highly diverse structure variation to more than half of all secretory and cellular proteins (Apweiler et al. 1999). Several lines of evidence have indicated that attachment of a specific monosaccharide to core glycans or branches changes glycoprotein function, and the resulting transformation of cellular phenotypes is suggested to be involved in various biological or pathological processes such as cancer, infection, and reproduction (Taniguchi et al. 2001; Hakomori 2002; Helenius and Aebi 2004). From a pharmacological point of view, glycosylation profoundly affects biological activity, function, clearance from the circulation, and crucially the antigenicity of recombinant proteins (Brooks 2004; Jefferis 2005). Increasing knowledge of the biological significance of glycosylation has resulted from the development of analytical

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methods, among which mass spectrometry (MS) is an essential tool as it allows rapid and high sensitivity profiling and detailed characterization of heterogeneous glycan structures. In fact, in recent years, mass spectrometric analysis by soft ionization techniques, electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), has been employed in most studies of oligosaccharide structure (Harvey 1999; Dell and Morris 2001; Mechref and Novotny 2002; Zaia 2004).

The capability of MS and data interpretation is based on various instrumental factors. For example, the physicochemical properties such as charge and internal energy of generated ions are largely dependent on the ionization methods employed. Different types of mass analyzers have specific time frames for detection and this affects the fragmentation patterns observed in the mass spectrum. Moreover, there are still other factors to consider with oligosaccharides such as derivatization, ion polarity and the nature of ion species derived from protonation and deprotonation, alkali-metal cations, and different anion adduction. Although all of these factors affect, to varying extents, the relative intensities of the molecular ions for different oligosaccharide structures, it is also true that good quantitation can be expected with appropriately designed measurements, as already reported (Viseux et al. 2001). While detailed reviews of current mass spectrometric techniques and analysis workflow are available, the emerging need for rapid and sensitive analysis of glycoprotein glycans makes it desirable to compare the performance of standard method(s). To this end, the Human Proteome Organisation (HUPO) Human Disease Glycomics/Proteome Initiative (HGPI) has formed a consortium with expertise in glycobiology and has distributed *N*-glycosylated protein samples, transferrin and immunoglobulin-G (IgG) from healthy individuals to participating members for analysis. The released oligosaccharides were analyzed by MS or conventional chromatography, and in some cases, glycopeptides obtained by enzymatic proteolysis were also analyzed by MS. This report describes these analyses of glycoprotein glycans, focusing mainly on relative quantitative profiling of structural heterogeneity.

Results

The laboratories enrolled in this study analyzed released oligosaccharides or glycopeptides, or both. The methods are summarized in Table I (The laboratory numbers designated in Table I and the succeeding figures are not the same as those given to the authors in the title.).

Human transferrin contains two *N*-linked complex type oligosaccharides at Asn-432 and Asn-630 (Spik et al. 1975). Human IgG has a conserved *N*-linked glycosylation site, Asn-297, in the heavy chain of the fragment crystallizable (Fc) region, with variable glycosylation in the fragment antigen binding (Fab) depending on the presence or absence of the glycosylation motif in the variable region. The oligosaccharides attached within the Fc region are of a complex type (Takahashi et al. 1987), showing higher heterogeneity than transferrin, and were the subject of this study.

For transferrin, the evaluation was primarily focused on the *N*-acetylneuraminic (sialic) acid levels. Sialic acids are major residues giving negative charge to the molecule and play a key role in various biological processes such as infection and cellular communication. The rather labile glycosidic linkage of sialic acid to the oligosaccharide chain can be cleaved during sample preparation or analysis, thus making quantitation difficult. For IgG, galactosylation levels were evaluated because of the possible alterations implicated in pathological conditions (Axford 1999). In these respects, detection of minor glycans, specifically, the fucosylation and triantennary branching of the transferrin glycans and the bisecting *N*-acetylglucosamination of IgG glycans, was evaluated.

In addition, in the glycopeptide analysis, different oligosaccharide profiles at two glycosylation sites of transferrin and those of different IgG subclass molecules were identified.

Chromatographic analysis

Chromatography of reductively aminated oligosaccharides is generally accepted as a standard method of quantitation, in which the fluorescence correlates with the amounts of individual components. In the present study, five laboratories carried out this type of analysis utilizing 2-aminopyridine, 2-aminobenzamide, or 2-aminobenzoate, as the labeling agent (Table I), and the results were compared with those obtained by MS.

The chromatographic measurements of the oligosaccharides from sample A transferrin are shown in Figure 1A (labs 1–4), in which the relative abundances of differently sialylated biantennary chains and trisialo-triantennary and fucosylated disialo-biantennary chains in the total oligosaccharides are presented. One laboratory (lab 1) discriminated the monosialo-binatennary isomers bearing a sialic acid at either antenna, and the values were summed for data presentation. In labs 1–4, the levels of monosialo- and disialo-biantennary, fucosylated disialo-biantennary, and trisialo-triantennary chains were 7.6 ± 7.0, 70.8 ± 12.3, 5.1 ± 2.9, and 9.7 ± 7.5 [mean $\pm$ standard deviation (SD), %] respectively, apparently showing a considerable variance among reports. No laboratories detected significant amounts of asialo-biantennary chain.

A majority of IgG oligosaccharides are of the fucosylated biantennary type, and are partially galactosylated. The contents of each differently galactosylated species among total fucosylated biantennary oligosaccharides from sample B IgG were calculated and are shown in Figure 2A (labs 1–5). Two laboratories (labs 1 and 5) discriminated the isomers with a galactose at either antenna, and the values were combined and represented by the monogalactosyl species. The most abundant structure was the monogalactosyl form, in three reports, whereas it was either agalactosyl or digalactosyl species in other laboratories. The number of galactose residues in a fucosylated biantennary oligosaccharide from this sample ranged from 0.82 (lab 3) to 1.59 (lab 4), and had a mean of 1.16 ± 0.28 for the five laboratories (Table II). The relative abundance of the monogalactosylated/fucosylated species with versus without bisecting *N*-acetylglucosamine (GlcNAc) ranged from 2.2 to 3.4% in three laboratories (labs 1, 4, and 5), whereas others overestimated abundance or did not present the data due to insufficient separation of the oligosaccharides with or without bisecting GlcNAc.

MS of oligosaccharides

MS of oligosaccharides was carried out with MALDI or liquid chromatography (LC)/ESI MS (Table I). The former was

Table I. Methods of glycoprotein glycan analysis

Lab[a]	Analyte/ derivatization	Methods of separation and quantitation	Ion polarity, TOF mode, MALDI matrix	Supportive or supplemental measurements and others
Lab 1	Os/PA	AE/RP/NP-LC/FL		
Lab 2	Os/PA	NP-LC/FL		Structure verification by LC/ESI MS (− ion)
Lab 3	Os/AA	NP-LC/FL		Structure verification by MALDI linear tof ms (− ion, DHB)
Lab 4	Os/AB	NP-LC/FL		Structure verification by MALDI linear TOF MS (+ ion, DHB)
	Gp	MALDI TOF MS	+ ion, linear, CHCA	Qualitative data only
Lab 5	Os/AB	NP-LC/FL		
	Os/none	MALDI TOF MS	+ ion, DHB	Exoglycosidase digestion followed by ESI MS/MS (− ion) for linkage analysis. Transferrin not examined
Lab 6	Os/perMe	MALDI TOF MS	+ ion, ref, DHB	Glycosyl composition by GC/MS
Lab 7	Os/perMe	MALDI TOF MS	+ ion, ref, DHB	ESI TOF MS
Lab 8	Os/perMe	MALDI TOF MS	+ ion, ref, DHB	Detailed structures by ESI MS/MS (+ ion)
	Gp	RP-LC/ESI MS/MS	+ ion	Structural analysis and mostly qualitative data
Lab 9	Os/perMe	MALDI TOF MS	+ ion, ref, DHB	Detailed structures by MALDI MS/MS (+ ion)
	Gp	RP-LC/ESI MS/MS	+ ion	Data submission of each single sample for transferrin and IgG
Lab 10	Os/PH	MALDI TOF MS	+/− ion, ref, DHB	Data submission of each single sample for transferrin and IgG
Lab 11	Os/perMe	MALDI TOF MS	+ ion, ref, DHB	Structure verification by esi ms (+/− ion) and detailed structures by ESI MS^n (+ ion) for underivatized Os
Lab 12	Os/perMe	MALDI TOF MS	+ ion/ref/DHB	
	Gp	RP-LC/ESI MS/MS	+ ion	Qualitative data only
Lab 13	Os/PA	MALDI TOF MS	+ ion, DHB	Detailed structures by MALDI MS^n (+ ion)
Lab 14	Os/none	MALDI TOF MS	+/− ion, ATT/DHB	− ion for sialylated Os. Transferrin not examined
Lab 15	Os/$NaBH_4$	Gr-LC/ESI MS/MS	− ion (Tf), + ion (IgG)	
	Gp	RP-LC/ESI MS/MS	+ ion	
Lab 16	Os/$NaBH_4$	Gr-LC/ESI MS/MS	− ion	
Lab 17	Gp	MALDI TOF MS	+ ion, linear, DHB	
Lab 18	Gp	RP-LC/ESI MS/MS	+ ion	Structural analysis and mostly qualitative data
Lab 19	Gp	RP-LC/ESI MS/MS	+ ion	Structural analysis and mostly qualitative data
Lab 20	Gp	MALDI TOF MS	+ ion	Qualitative data only

[a]The laboratory numbers are not the same as those given to the authors.
AA, aminobenzoic acid; AB, aminobenzamide; AE, anion-exchange; ATT, 6-aza-2-thiothymine; DHB, 2,5-dihydroxybenzoic acid; FL, fluorescence; Gp, glycopeptides, Gr, graphitized carbon; NP, normal phase; Os, released oligosaccharides; PA, pyridylamination; perM, permethylation; PH, phenylhydrazine; RP, reversed phase; ref, reflectron; Tf, transferrin; +/− ion, positive/negative ion.

employed by many laboratories, most of which derivatized by permethylation prior to analysis. Permethylation stabilizes the sialic acid residues by converting them to methyl esters, thus preventing sialic acid loss while also improving the efficiency of positive ion formation.

Transferrin

The results of sample A transferrin with attention to the sialylation of biantennary oligosaccharides and the minor glycans with triantennary branching or fucosylation are summarized in Figure 1A (labs 6–13, 15, and 16), and a typical MALDI time-of-flight (TOF) mass spectrum of permethylated oligosaccharides is shown in Figure 3. SDs of the data provided by two laboratories, lab 12 ($n = 7$) and lab 16 ($n = 2$), were small especially for the combination of permethylation and MALDI MS (lab 12). The abundance of monosialylated species was 11.0 ± 5.1% (mean ± SD) in seven laboratories using a combination of permethylation and MALDI MS, a little higher than the results obtained with chromatography (Figure 1B). Four laboratories had levels below 15% for the monosialylated chain, whereas the higher levels obtained by others (labs 10–12) were probably due to sialic acid loss during sample preparation. The (fully sialylated) triantennary oligosaccharide level ranged from 0 to 5.6%, apparently lower than the chromatographic data, and two laboratories (labs 6 and 10) failed to detect this oligosaccharide. The fucosylated oligosaccharide in seven laboratories (labs 6–12) ranged from 0 to 9.2%, comparable to the chromatographic results. One laboratory (lab 12) repeated the measurement for the same sample and reported good intra-assay coefficients

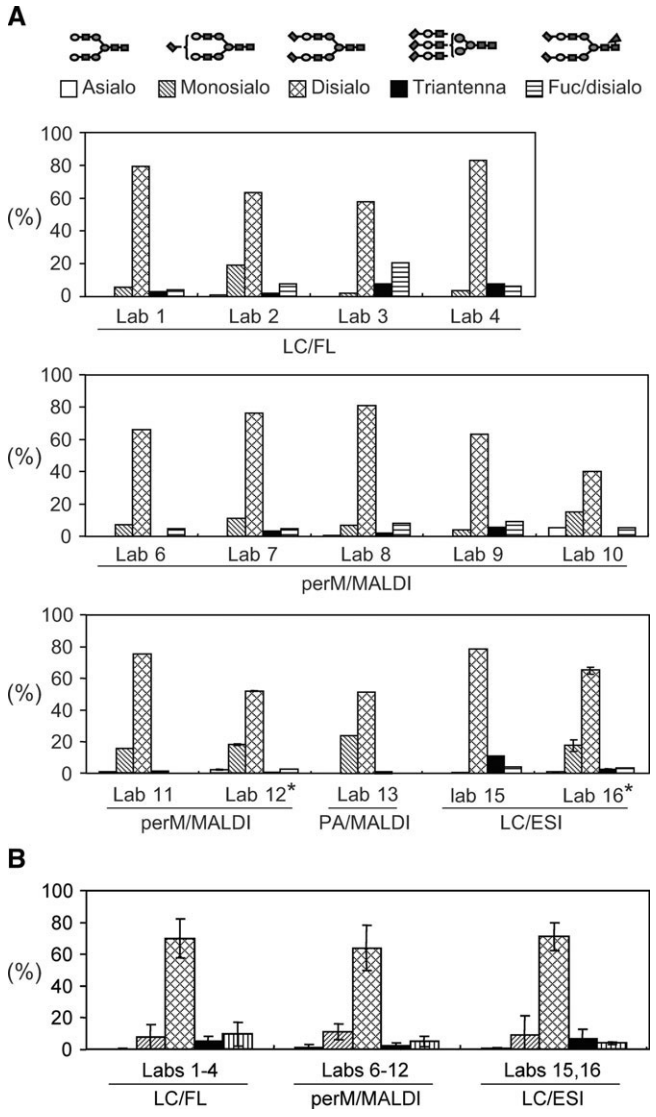


Fig. 1. Relative quantities of transferrin oligosaccharides from sample A.The amounts of all glycoforms in total oligosaccharides are presented. Abbreviations are those presented in Table 1. (**A**) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing SD from repeated measurements by lab 12 ($n = 7$) and lab 16 ($n = 2$) show intra-assay variance. The SDs of lab 12 were quite small. (**B**) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography ($n = 4$), a combination of permethylation and MALDI MS ($n = 7$), and LC/ESI MS ($n = 2$). Lab 5 was excluded from the calculation (see text). The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

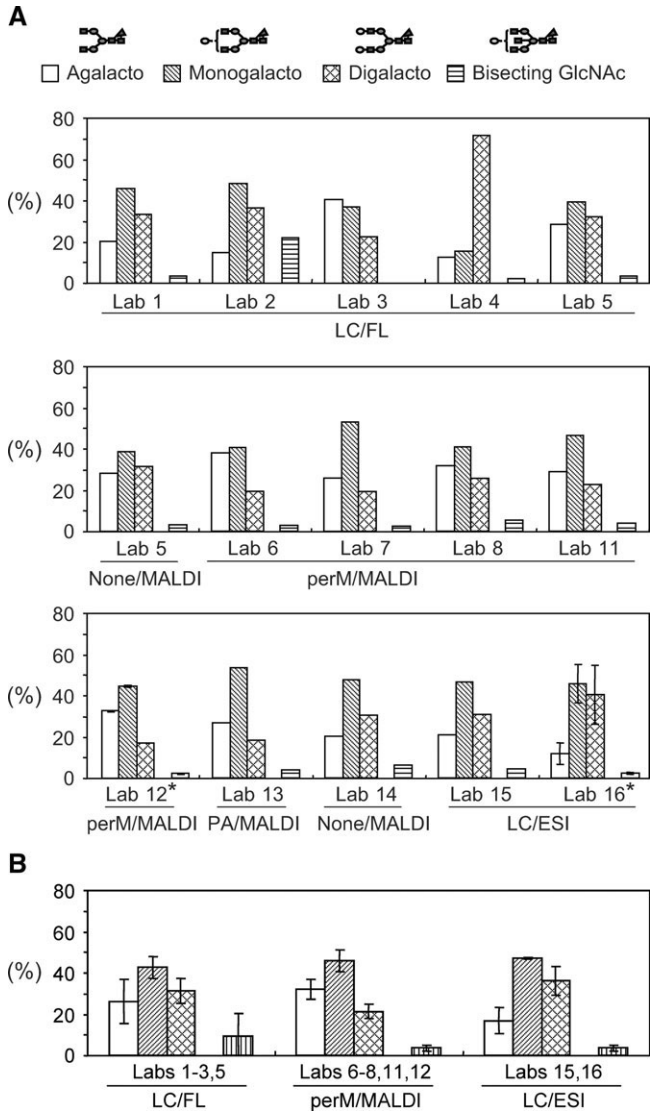


Fig. 2. Relative quantities of IgG oligosaccharides from sample B.Three bars on the left, for each laboratory, show the relative abundance of differently galactosylated species as a percentage of the total. That for monogalactosylated biantennary oligosaccharide bearing bisecting GlcNAc represents the content of total oligosaccharides identified. (**A**) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing SD from repeated measurements by lab 12 ($n = 7$) and lab 16 ($n = 2$) show intra-assay variance. The SDs of lab 12 were quite small. (**B**) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography ($n = 4$), a combination of permethylation and MALDI MS ($n = 5$), and LC/ESI MS ($n = 2$). Lab 4 was excluded from the calculation. The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

of variation (CVs) less than 5% for either oligosaccharide. One laboratory applied pyridylaminated oligosaccharides to MALDI TOF MS, which showed significant sialic acid loss. LC/ESI MS was employed by two laboratories (labs 15 and 16), and the results were a little different from each other with respect to the levels of monosialo and triantennary species. In most measurements employing MALDI MS or LC/ESI MS, the fucosylated species was more abundant than the triantennary branching species, consistent with the chromatographic data. In summary, the results from three different methods, which were carried out in different laboratories, were similar to each other as shown in Figure 1B.

IgG

The results for sample B IgG are presented in Figure 2A (labs 5–8, 11–16), with attention to the galactosylation levels as well as the minor component, monogalactosylated biantennary oligosaccharide bearing bisecting GlcNAc. Galactosylation in this sample was evaluated according to the relative abundance

Table II. Galactosylation levels of different IgG samples (mol)

		A	B	C
Lab 1	Chromatography	0.91	1.13	1.14
Lab 2	Chromatography	1.15	1.21	1.31
Lab 3	Chromatography	0.74	0.82	0.94
Lab 4	Chromatography	1.54	1.59	1.69
Lab 5	Chromatography	0.95	1.03	1.10
Lab 5	MALDI	0.71	0.83	0.87
Lab 6	MALDI	0.77	0.81	0.95
Lab 7	MALDI	0.73	0.93	1.05
Lab 8	MALDI	0.86	0.94	1.04
Lab 11	MALDI	0.97	0.94	1.07
Lab 12	MALDI	0.76	0.84	0.96
Lab 13	MALDI	0.89	0.91	0.96
Lab 14	MALDI	0.95	1.10	0.96
Lab 15	LC/ESI MS	1.10	1.10	1.25
Lab 16	LC/ESI MS	1.20	1.29	1.32
	Mean	0.95	1.03	1.11
	SD	0.22	0.21	0.21

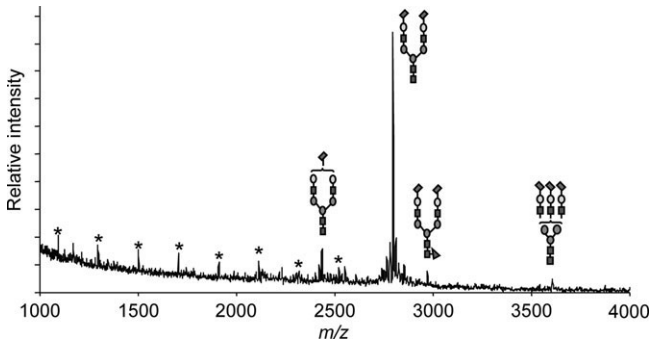


Fig. 3. MALDI mass spectrum of permethylated oligosaccharides from sample A transferrin.Oligosaccharides released by PNGase F were permethylated, and analyzed by MALDI TOF MS in positive ion and reflectron mode. The signals from polyhexose as an internal calibrant are indicated by asterisks. The mass spectrum was provided by lab 7.

of three major species as a percentage of the total. Repeated measurements were performed in labs 12 and 16. The CVs in lab 12 with permethylation and MALDI MS were quite small. SDs of the measurement by lab 16 were exceptionally large for this specific sample, but the CVs for these species were less than 10% for samples A and C in the same laboratory. With either MALDI or LC/ESI MS, the most abundant *N*-linked oligosaccharide from sample B IgG was monogalactosylated species by both MALDI and LC/ESI MS. The galactosylation levels calculated from the MALDI MS measurements ranged from 0.80 to 1.10 (0.93 ± 0.10) mol per fucosylated biantennary oligosaccharide chain, but those calculated from LC/ESI MS measurements (1.10 and 1.29 mol) were slightly higher (Table II). The relative levels of the monogalactosylated and fucosylated glycan bearing bisecting GlcNAc calculated by mass spectrometric measurements were within the range of 2.2–14.0% of total oligosaccharides, which was comparable to that obtained by chromatography. Similarly to the transferrin study described above, the results on IgG oligosaccharides from three different methods were comparable with each other (Figure 2B). Typical mass spectra of permethylated oligosaccharides are shown in Figure 4.

Reproducibility of MALDI MS quantitation of permethylated oligosaccharides

Reproducibility of MALDI MS quantitation was evaluated by a supplementary experiment carried out in one laboratory (lab 17), where sample B (0.3 mg) was divided into three portions and each sample was separately subjected to permethylation followed by MS.

The CVs of the five repeated measurements, or intra-assay CV, were less than 10% (1.3–8.8% for three major oligosaccharide species and 12–34% for the minor one (Table III). The inter-assay CV was 0.1–4.2% for major species and 13% for the minor one. In this experiment, the sensitivity for small components was approximately 0.4% of their proportion among total oligosaccharides, when defined by the detection of ions with a signal-to-noise ratio of more than 2.

MS of glycopeptides

Nine laboratories performed MS of glycopeptides, and seven presented qualitative data or quantitative results for a single sample. Two laboratories presented sufficient, as well as remarkable, data with LC/ESI MS/MS (lab 15) or MALDI TOF MS (lab 17).

First, the difference between the oligosaccharide profiles at the two glycosylation sites of transferrin was revealed as shown in Figure 5A, where the levels of fucosylation and triantennary branching of transferrin were higher for the oligosaccharide attached to Asn-630 than for that attached to Asn-432, and this site-specific oligosaccharide profile difference was quite consistent between the results from these two laboratories. As shown by the MALDI TOF mass spectra in Figures 5B and C, the heterogeneity of oligosaccharide structures was more obvious at Asn-630, whereas a very low level of fucosylation could be identified in the Asn-432 oligosaccharides by tandem MS of the ion observed at m/z 3830 (data not shown). In addition, the level of fucosylated biantennary oligosaccharide exceeded that of the triantennary oligosaccharide, consistent with the chromatographic or mass spectrometric analyses of the released oligosaccharides. On the other hand, sialic acid loss was observed in the glycopeptide ions generated by MALDI. Good reproducibility was reported by lab 17 (Figure 5A).

Serum IgG is polyclonal and is thus a mixture composed of different primary protein structures. The amino acid sequence of the tryptic peptide involving the *N*-glycosylation site Asn-297 is heterogeneous; and EEQYNSTYR and EEQFNSTFR representing two subclass molecules, IgG1 and IgG2, respectively, are abundant. In this study, three IgG samples (A, B, and C) from different individuals were analyzed. The relative abundances of subclass molecules were estimated as 1/3, 1/4, and 2/1 (IgG1/IgG2), for samples A, B, and C, respectively, on the basis of the total intensities of the corresponding groups of ions in the MALDI linear TOF mass spectra of enriched glycopeptides (Supplementary Figure 1). The

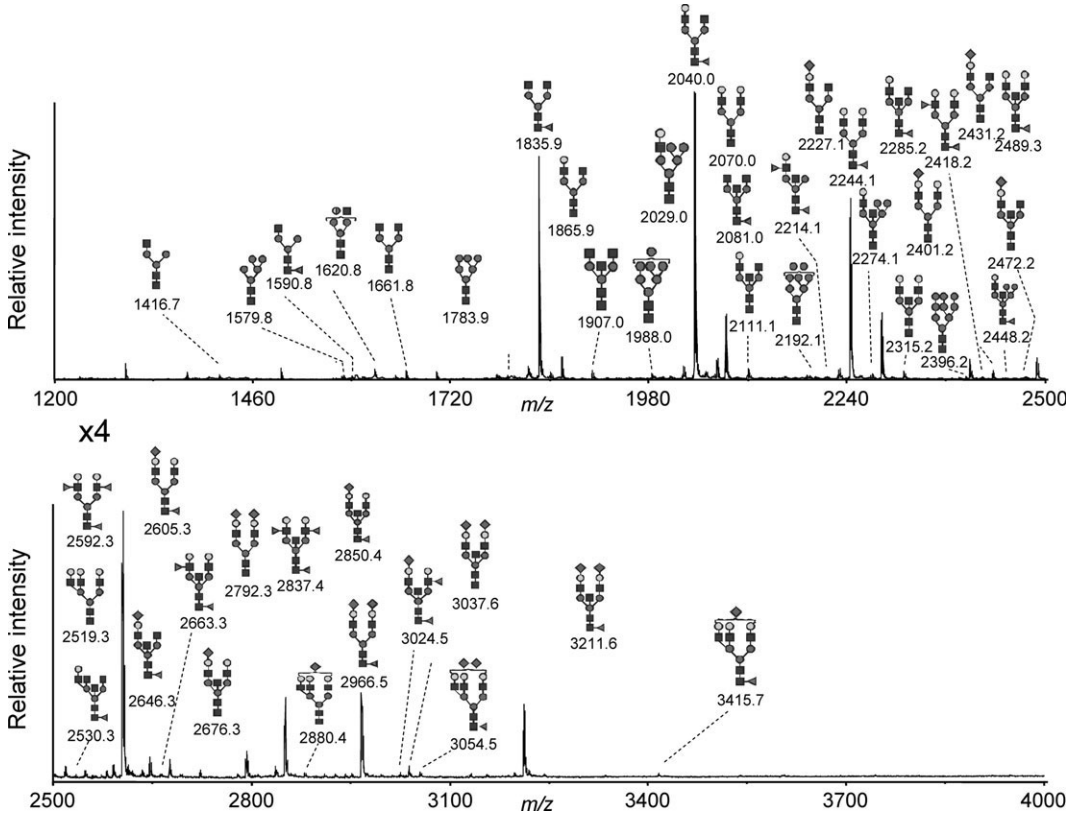
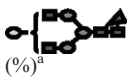
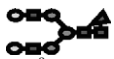
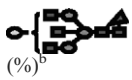


Fig. 4. MALDI mass spectra of permethylated oligosaccharides from sample B IgG. The mass spectrum was provided by lab 8.

Table III. Reproducibility of quantitation by MALDI MS of permethylated oligosaccharides

IgG sample B	 (%)[a]	 (%)[a]	 (%)[a]	 (%)[b]
1 ($n = 5$)	38.9 ± 2.5	44.4 ± 1.4	16.7 ± 1.5	0.91 ± 0.32
2 ($n = 5$)	37.7 ± 1.1	44.5 ± 0.7	17.8 ± 0.9	0.84 ± 0.14
3 ($n = 5$)	37.3 ± 0.5	44.5 ± 0.8	18.1 ± 0.4	1.08 ± 0.13

[a]Relative abundance as a percentage of the total of these major species.
[b]Proportion among total oligosaccharides.

amino acid sequences of these glycopeptide ions were verified by tandem MS in a separate experiment (data not shown). The galactosylation levels of IgG1 or IgG2 for each sample were calculated from the corresponding signals observed in the LC/ESI or MALDI mass spectrum (Supplementary Figure 2A) and summarized in Table IV. The results from these independent analyses were consistent, i.e., the agalacto species was more abundant than the digalactosylated species in the IgG2 from samples A and B, but not in the IgG1 from sample C. The oligosaccharides on the major subclass molecules, IgG2 for samples A or B, and IgG1 for sample C, should contribute more to the global glycan profiles of total IgG in each sample. The results from the MS of IgG glycopeptides were quite consistent with the chromatographic or mass spectrometric measurements of the corresponding released oligosaccharides (Supplementary Figure 2B). Hypogalactosylation of IgG2 relative to IgG1 is not a common finding among different individuals (unpublished observation by YW).

The levels of minor glycans with bisecting GlcNAc in the major IgG subclass molecules were comparable between LC/ESI MS and MALDI MS (data not shown). The numbers of oligosaccharide structures identified as constituting more than 1% of the total oligosaccharides from sample B IgG were compared by different methods. Fifteen structures were seen by lab 1 using chromatography, 9 and 14 by lab 7 and lab 8, respectively, analyzing permethylated oligosaccharides with MALDI MS, 15 by LC/ESI MS of glycopeptides and 10 by MALDI TOF MS of glycopeptides (Supplementary Figure 3), indicating glycopeptide analysis to be sufficiently sensitive to detect minor glycans on IgG. No significant amounts of glycopeptides derived from other regions of IgG were found in these samples.

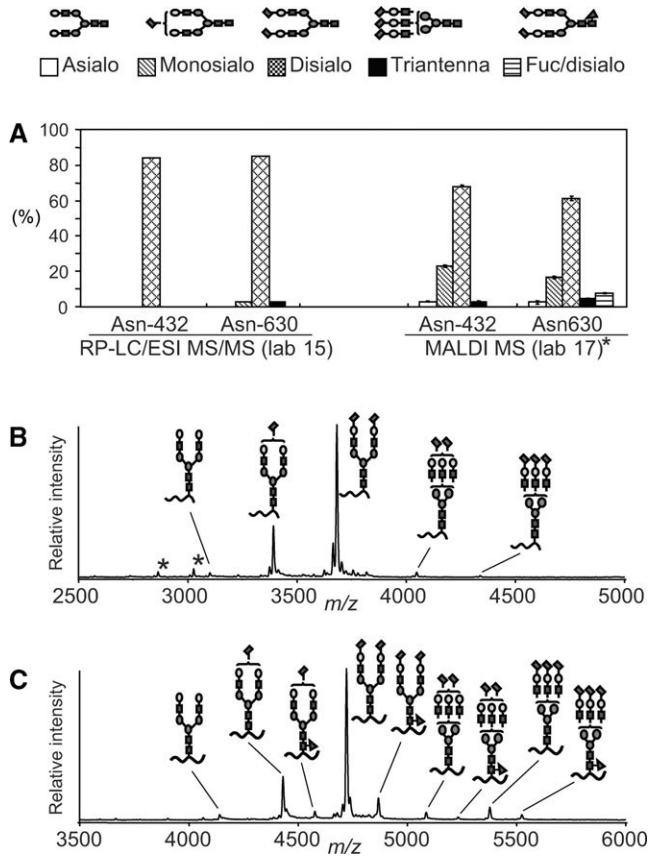


Fig. 5. Mass spectrometric analysis of glycopeptides for site-specific glycan profiling of sample A transferrin.(**A**) Relative abundances of the oligosaccharides at each *N*-glycosylation site measured by RP-LC/ESI MS/MS (lab 15) or MALDI linear TOF MS of tryptic glycopeptides (lab 17). (*) The error bars representing SD from repeated measurements by lab 17 ($n = 5$) show intra-assay variance. (**B, C**) MALDI mass spectra of tryptic glycopeptides. The glycopeptides containing Asn-432 (B) or Asn-630 (C) were isolated by reversed phase chromatography and analyzed by MALDI linear TOF MS (lab 17). The ions indicated by asterisks are derived from the glycosidic cleavage during measurements.

Discussion

Oligosaccharides

The chromatographic quantitation of the reductively aminated oligosaccharides showed a significant variance among laboratories, despite the fact that the method has been accepted as established. Whether or not labeling efficiency is uniform for different glycan structures and/or different fluorescent aromatic amines remains unclear, but the variance seems to be attributable to the use of different reaction protocols, which can result in incomplete derivatization. In addition, the insufficient separation of specific glycans, e.g., those with or without bisecting GlcNAc, resulted in a considerable variation in the quantitation (Figure 2B), while the chromatographic method discriminates isomers, e.g., monogalacto species bearing a galactose at either antenna in IgG.

It is generally accepted that MS does not allow real quantitation for oligosaccharides unless stable isotope-labeled analogs are incorporated as internal standards. In this multi-institutional study, analyzing the same transferrin and IgG glycoprotein samples, however, MS has yielded quite comparable results with chromatography in the (relative) quantitation of oligosaccharides (Figures 1B and 2B). For MALDI, especially, the in-source and postsource decay of glycans are so well-known that one suspects it would be too difficult to detect their intact structures. Parameters such as laser wavelength and power, extraction voltages, and "hot" or "cool" matrices directly influence the internal energy of generated ions and the resulting fragmentation (Harvey 1999). Furthermore, the distribution of molecular species in complex glycan mixtures within the matrix spot on the target depending on sample preparation is also a critical point and sufficient "averaging" of laser shots is essential. However, there are publications describing good quantitation of oligosaccharides based on the signal intensities observed in the MALDI mass spectrum (Harvey 1993; Garozzo et al. 1994). Indeed, a comparative study of the signal intensities generated from a mixture of equimolar amounts of various *N*-linked glycans did not show any significant difference for compounds with molecular masses over about 1 kDa (Naven and Harvey 1996). In the present study, although different MALDI TOF instruments were used, the analytical parameters, such as the type of laser (nitrogen that emits at 337 nm), the choice of matrix (2,5-dihydroxybenzoic acid, DHB), the type of ion species for permethylated oligosaccharide detection (sodium-adduct ions $[M+Na]^+$), and the mode of TOF analysis (reflectron) were the same among the laboratories and yielded consistent results. In MALDI MS, the ions generated by hundreds of laser shots, each of which produces one mass spectrum, are accumulated from different points of laser irradiation. The procedure makes the reproducibility excellent in the repeated measurements by lab 12 and in the supplemental experiment, and the inter-assay reproducibility in the same laboratory was also quite good (Table III).

Regarding the determination of sialylation levels, the glycosidic linkage of sialic acid is susceptible to prompt (in-source) decay in the MALDI process, and the sialic acid-substituted oligosaccharides are likely to decompose in the flight tube in the TOF instrument as well. In addition, the number of sialic acids affects the relative ionization efficiencies (Sutton et al. 1994). Consequently, the signal intensity of underivatized sialylated oligosaccharides in the MALDI mass spectrum cannot be directly related to concentration as shown by the results obtained by lab 13 for oligosaccharides only derivatized at the reducing end by aminopyridine (Figure 1). Permethylation, which has been used for more than 40 years in order to modify hydroxyl groups of sugars (Hakomori 1964; Ciucanu and Kerek 1984; Ciucanu and Cotello 2003) methylates the carboxylic acid group of the sialic acid to produce a neutral sugar, whereupon esterified sialic acid loss is avoided and the ionization efficiency becomes equivalent to that of natively neutral oligosaccharides. In addition, this type

Table IV. Galactosylation levels of different IgG subclass molecules (mol)

		A	B	C
Lab 15	IgG1	0.85	0.97	1.07
	IgG2	0.89	0.79	0.99
Lab 17	IgG1	Not determined	Not determined	1.16
	IgG2	0.90	0.91	Not determined

of derivatization prevents salt formation, which complicates the mass spectrum and impairs the signal-to-noise ratio for the individual molecular ion species. Furthermore, the lack of hydroxyl groups also prevents the cleavage of other glycosidic bonds (Lemoine et al. 1996), making the permethylated oligosaccharides resistant to in-source fragmentation. In fact, the sialylation levels determined by MALDI MS after permethylation were acceptable, although a variance exists among laboratories, as was the case for chromatography. A special setup of derivatization required a smaller amount of samples (5 μg glycoprotein) to meet the high sensitivity of MALDI MS for permethylated oligosaccharides (Kang et al. 2005), whereas routine analysis used approximately 100 μg in this study.

The levels of triantennary oligosaccharides determined by MALDI MS were lower than those from chromatography, though not markedly. It is not possible to decide which value is the correct one, but the decreased triantennary structures seen by MS may be due to the increased mass of 810.4 Da of an additional antenna, making the detector response for the triantennary oligosaccharide ions at m/z 3602.8 weaker than that for the biantennary ions at m/z 2792.4, or there may be increased collision-induced dissociation (CID) of an enlarged cross-section of structure. The peak broadening effect due to isotopes is only 17% for this mass increase and thus does not produce a substantial error even when the quantitation is based on the peak height in the MALDI mass spectrum. On the other hand, core fucosylation, which occurs at the sixth position of the reducing terminal GlcNAc in the transferrin oligosaccharides, does not affect quantitation by MALDI MS (Naven and Harvey 1996). It is worth noticing that the level of the triantennary oligosaccharide was lower than that of the fucosylated species in measurements with either MALDI MS or chromatography, indicating that MALDI MS is similar in its ability to detect minor glycans. Similarly, good relative quantitation by MALDI MS was confirmed by IgG analysis, as the monogalactosylated species was the most abundant and the calculated level of galactosylation was comparable to that obtained by chromatography.

ESI MS was employed by two laboratories, where oligosaccharide alditols were separated by graphitized carbon chromatography and introduced on-line to an ESI mass spectrometer operated in the negative ion mode (Karlsson et al. 2004). The reproducibility of LC/ESI MS was acceptable from the data of lab 16. Regarding sialylated oligosaccharides, ESI is soft enough to detect the intact oligosaccharides with sialylation unless high nozzle-skimmer potential is applied, even when sialic acids are not derivatized. However, the charge of sialic acids affects the efficiency of negative ion formation, and thus the distribution of multiple-charge ions in the mass spectrum is different from that for neutral oligosaccharides, disturbing straightforward quantitation of sialic acid-substituted oligosaccharides in the mass spectrum or ion chromatogram at the same time as neutral glycans. Nevertheless, LC/ESI MS results were comparable to those from MALDI MS or chromatography as shown in Figures 1B and 2B, except that an increased level of monosialylated oligosaccharides from the transferrin sample was reported by one laboratory, possibly due to sialic acid loss during sample preparation. A brief summary of the methods for oligosaccharide analysis including capillary electrophoresis-MS (Zamfir et al. 2000) is presented in Table V.

In this study, some laboratories also presented MS/MS spectra of permethylated or reductively aminated oligosaccharides. For example, the product ion mass spectra provided branching and linkage information (Supplementary Figure 4). This method is used for structure verification, qualitatively, and is not primarily directed toward quantitation. However, along with the establishment of the strategies for *de novo* carbohydrate sequencing by an algorithm incorporating the fragmentation database and informatics (Ashline et al. 2005; Tang et al. 2005), quantitation of oligosaccharide isomers will be realized in the near future.

Glycopeptides

Glycopeptide analysis allows the identification of site-specific oligosaccharide structures, which are essential to understand the role of local oligosaccharide structures in protein folding and functions. However, it is conceivable that the analysis of glycopeptides is difficult as compared to that of unglycosylated peptides or oligosaccharides, since chemical properties differ between the glycan and peptide components. Moreover, each glycopeptide is often a minor constituent in the peptide mixtures after enzymatic digestion of glycoproteins because of the large number of glycoforms expressed by many proteins at each site. These problems may be overcome by employing LC/MS and selecting the glycopeptides in the chromatogram by detection of the CID-generated glycan-specific oxonium ions (Huddleston et al. 1993). Alternatively, glycopeptide fraction can be enriched by lectin or other extraction tools, and the fraction can be directly analyzed by MS, especially when the glycoprotein has a small number of glycosylation sites, as is the case for transferrin and IgG (Wada et al. 2004). The glycan and peptide structures of glycoproteins can then be elucidated by MS/MS of glycopeptide ions (Liu et al. 1993; Krokhin et al. 2004).

MS of glycopeptides is usually performed in the positive ion mode to detect $[M+H]^+$ ions. In this case, protonation occurs on the peptide portion, and thus the signal intensity of the glycopeptide is largely dependent on the proton affinity of the peptide component of the molecule, thus rationalizing the quantitation of different glycoforms of a peptide according to their signal intensities. However, there are still a few concerns. First, glycopeptides are larger than oligosaccharides and thus are accompanied by an increased risk of CID. Since the glycan moiety is more labile than the peptide backbone, the resulting cleavage of the glycosidic bonds may result in overestimation of the glycoforms with a smaller number of building saccharides. This effect will be evident in the MALDI produced ions, in which internal energies are higher than those of ESI ions. In previous reports, however, neutral oligosaccharides had minimal affect upon the ionization efficiencies of glycopeptides, and consequently, the integration of MALDI TOF signals of several desialylated glycopeptides yielded excellent quantitative correlations with published data obtained by established high-performance liquid chromatography (HPLC) techniques (Sutton et al. 1994; Harmon et al. 1996). Second, the sialic acids of glycopeptides cannot be appropriately derivatized without deleterious modifications of the peptide component. In MALDI MS, the sialic acid loss occurs both in-source and postsource as described above, and occurs over a significant time frame in which a hydrogen transfers between suitably positioned functional groups (Harvey

Table V. Methods for structural analysis and quantitation of oligosaccharides

	Advantages	Disadvantages
Reductive amination and fluorescence-labeling/chromatography	Standard method for quantitation (if appropriately prepared); possible isomer discrimination; Independence of detector response on glycan size or structure	Various conditions for derivatization
Permethylation / +MALDI-TOF MS	Invariant conditions for derivatization available; protection of weak glycosidic linkages (e.g. sialic acid)	Loss of alkali-labile substituents
		Discrimination of isobaric species unable without MS/MS; instruments available
		Inverse correlation of detector response to molecular mass
No derivatization/ + MALDI-TOF MS	Easy and quick	Less sensitive
		Glycosidic bond cleavage, if not the matrix choice approapriate
Reducing end derivatization / +MALDI-TOF MS	Possible sharing of samples with chromatography when fluorescence-labeled	Glycosidic bond cleavage, if not the matrix choice appropriate
Direct inlet nanoESI MS and MS/MS	No loss of sample due to derivatization; detailed sequence analysis; superior sensitivity by sprayer chips	
No derivatization/LC/-ESI MS(/MS)	Easy MS/MS acquisition for structure elucidation; data-dependent analysis after run	Different charge distribution between neutral and acidic glycans; requirement of nanospray for good sensitivity
CE-MS and MS/MS	No loss of sample due to derivatization; detailed sequence analysis; high flexibility of protocols	

1999). Consequently, the sialic acid loss increases with the longer delay time before extraction (Naven et al. 1997); and is prominent in the MALDI ion-trap type of mass separators. Most studies of sialylated glycopeptides with MALDI TOF MS are conducted in the linear mode so that the postsource decay ions are not separated. On the other hand, the sialic acid loss is minimal in ESI, but there are charge-dependent effects on the ionization efficiency and on the distribution of multiple-charge ions of the oligosaccharides.

Despite these problems, the site-specific analysis of glycoprotein oligosaccharides by MS is becoming popular. In the present study, the results from two laboratories on glycopeptides, one with MALDI MS and another with LC/MS, were consistent with each other, and were informative in terms of the oligosaccharide profiles specific to the protein sequence. The galactosylation levels at different polymorphic sequences of IgG explained well the global glycan profiles determined by the analyses of released oligosaccharides, and the oligosaccharide profiles of two glycosylation sites of transferrin were clearly shown to be different from each other. Although these findings could be obtained by isolation of each glycopeptide and subsequent analysis of released oligosaccharides, the direct analysis of glycopeptides performed herein is by far easier and more rapid as well as being sufficiently sensitive for minor glycans. The glycopeptide analysis requires a fairly small amount of samples (less than 10 μg glycoprotein in this study), and thus will contribute a great deal to the emerging field of glycoproteomics.

In conclusion, MALDI MS of permethylated oligosaccharides is as reliable as chromatographic methods for elucidating glycan profiles based on mass mapping of the compositional analysis. The publicly available murine and human MALDI MS glycomics data, which is being acquired by the Consortium for Functional Glycomics (www.functionalglycomics.org), for systems biology research are based on this approach. Good relative quantitation data can be achieved without neutralization of sialic acid residues by LC/ESI MS utilizing porous graphitized carbon as a separation medium for oligosaccharide alditols. Quantitative and relative quantitative datasets can be obtained by peptide sequencing and determination of glycosylation sites by LC-MS/MS of native glycopeptides. Analysis of glycopeptides is not yet widely implemented outside specialist glycobiology laboratories, but it is clear from both the present study and the increasing volume of publication in this area that glycoproteomic strategies are now sufficiently mature to allow practical site-specific characterization of the oligosaccharide profiles of even highly heterogeneous glycoproteins (Harazono et al. 2005; Imre et al. 2005; Tajiri et al. 2005; Chalabi et al. 2006). Our present results justify the use of MS for relative quantitation of oligosaccharides, and highlight that glycopeptide MS will be a key interfacing technique in proteomics and functional glycomics in the future.

Methods

Glycoprotein samples

The blood samples were obtained from three healthy Japanese donors (A, B, and C) with the permission from the Medical Ethics Committee of Osaka University Graduate School of Medicine. Transferrin and IgG were purified from individual serum by immunoaffinity with rabbit polyclonal antibody against human transferrin and by protein G affinity chromatography, respectively, and then lyophilized. The purity of distributed samples was validated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. Each 1 mg sample from six specimens (A, B, and C for transferrin and IgG) in total was delivered to 26 laboratories at ambient temperature. Twenty of these laboratories

submitted results. The stability of these materials during transport was guaranteed by a test incubation, during which neither degradation nor modification of protein and glycan moieties was observed after one week of storage at 37 °C. In most laboratories, a 100 μg sample was used for each analysis.

Release of oligosaccharides

A majority of laboratories employed the method of oligosaccharide release from enzymatic digests of glycoproteins, typically as follows. Proteins (0.5 mg of transferrin or IgG) were dissolved in 0.5 mL of 6 M guanidium hydrochloride, 0.25 M Tris–HCl, pH 8.5 and reduced with 5 mg of dithiothreitol under N_2 at room temperature for 3 h. Then, 9 mg of iodoacetamide were added to the solution, followed by incubation in the dark at room temperature for 30 min for carbamidomethylation. The reagents were removed by a gel fitration column, NAP-5 (GE Healthcare, Piscataway, NJ), equilibrated with 0.05 N HCl, and the recovered proteins were lyophilized. The alkylated proteins were dissolved in 50 mM ammonium hydrogen carbonate, pH 8.0, and digested with 10 μg of trypsin at 37°C for 3 h. Subsequently, 20 U of Peptide: *N*-Glycosidase F (PNGase F) (*N*-glycanase F) (Roche, Mannheim, Germany) were added to the solution, followed by incubation at 37°C for 12 h to release *N*-linked oligosaccharides from glycopeptides. The solution was then passed through a solid phase extraction Sep-Pak Light C18 cartridge (Waters, Millford, MA), and the oligosaccharides in the pass-through fraction were recovered and lyophilized.

When oligosaccharides were released directly from glycoproteins, the in-solution or in-gel release method was used. For in-solution release, the 0.5 mg protein samples were dissolved in 400 μL of water and 40 μL of 10× denaturing solution (5% SDS and 10% β-mercaptoethanol). The sample was then denatured at 100°C for 5 min. After cooling, a 40 μL of reaction buffer (0.5 M sodium phosphate, pH 7.5) containing 10% Nonidet P-40 were added. The sample was mixed and incubated with PNGase F at 37 °C for 12 h. The digested sample solution was run through a C18 cartridge to remove the detergent, and then lyophilized (Sheeley and Reinhold 1998). The sample was desalted on a graphitized carbon column or by the normal phase extraction method (Wada et al. 2004).

For in-gel release (Royle et al. 2006), 80 μg samples were reduced with 0.5 M dithiothreitol for 10 min at 70 °C, alkylated with 100 mM iodacetamide for 30 min at room temperature, then run over three lanes on 10% SDS–PAGE gels and visualized with Coomassie blue. Protein bands were excised, cut into approximately 1 mm^3, frozen for 2 h at −20 °C, then washed with alternating 1 mL acetonitrile and 1 mL 20 mM $NaHCO_3$ pH 7 (five washes, 30 min each) and the gel pieces lyophilized. *N*-linked glycans were released *in situ* with 100 U/mL PNGase F with overnight incubation. Glycans were extracted by washing with 3× 0.2 mL water, 1× 0.2 mL acetonitrile, 1× 0.2 mL water, 1× 0.2 mL acetonitrile (30 min each). Samples were lyophilized ready for MS or fluorescent labeling.

Fluorescence labeling and chromatography of oligosaccharides

For fluorescence detection, the oligosaccharides were subjected to reductive amination at the reducing end with 2-aminopyridine (Natsuka and Hase 1998), 2-aminobenzamide (Bigge et al. 1995) or 2-aminobenzoate (Anumula and Dhume 1998). The typical procedure using 2-aminobenzoate are as follows. To the lyophilized oligosaccharides, a solution (200 μL) of 2-aminobenzoate and sodium cyanoborohydride, freshly prepared by dissolution of both reagents (30 mg each) in methanol (1 mL) containing sodium acetate and 2% boric acid was added. The mixture was kept at 80 °C for 1 h. After cooling, the solution was applied to a column of Sephadex LH-20 (1 × 30 cm) equilibrated with 50% methanol. Earlier eluted fractions showing fluorescence at 410 nm with 335 nm-wavelength irradiation were collected and evaporated to dryness. The residue was dissolved in water (100 μL), and a portion (10 μL) was analyzed by HPLC. Separation was done at 50°C with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo; 4.6 × 250 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min, at which point solvent B was increased to 95% over 80 min and kept at this composition for a further 100 min. The flow rate was 1.0 mL/min throughout the analysis. Detection was performed by fluorometry with $\lambda_{ex} = 350$ nm and $\lambda_{em} = 425$ nm.

The oligosaccharides derivatized with 2-aminopyridine were analyzed by multidimensional chromatography (Takahashi 1996). In some laboratories, the separated oligosaccharide were analyzed by MALDI TOF MS for structure verification.

MALDI MS of permethylated oligosaccharides

Permethylation was performed by the solid sodium hydroxide technique (Dell et al. 1993; Lemoine et al. 1996; Ciucanu and Costello 2003). Briefly, five pellets (approximately 1 g) of NaOH were ground in a dry mortar to obtain a fine powder. This should be done as quickly as possible to minimize absorption of moisture from the atmosphere. The NaOH powder was mixed with 4 mL of anhydrous dimethyl sulfoxide. The oligosaccharide sample released from glycoproteins was dried in a glass tube. Approximately 1 mL of the slurry was added to the sample followed by 0.5 mL of iodomethane. The sample was agitated at room temperature for 10 min. The reaction was then terminated by addition of 2 reaction volumes of water. Subsequently, 1 mL of chloroform was added, and the mixture was vortexed for 30 s and centrifuged at 3000*g* to facilitate partitioning. The top aqueous layer was removed and the chloroform layer was then washed three additional times with 4 mL of water. The chloroform was evaporated to obtain a dried permethylated sample. One laboratory used the capillary permethylation method as described previously (Kang et al. 2005).

For MALDI MS, the dried permethylated sample was resuspended in 10 μL of pure methanol. The sample was mixed with an equal volume of DHB matrix solution at 20 mg/mL in 80% methanol and then spotted onto the MALDI plate. To attain good ion statistics the spectra presented were generated from several sub-spectra of 100 laser shots. The peak height of the $[M+Na]^+$ monoisotopic ions or the integrated peak area for their entire isotopic cluster was measured for relative quantitation.

Reproducibility of the quantitation was examined in one laboratory (lab 17) as follows. Sample B IgG (0.3 mg) was divided into three portions, and each sample was separately subjected to permethylation according to the procedures described above. The MALDI spectrum was acquired with a Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, CA) in reflectron mode. The signals from a total of 500 shots at 10 different laser irradiation spots were averaged, and the measurement was repeated five times.

The oligosaccharides derivatized at the reducing end or those with intact nonreducing hydroxyls were analyzed by MALDI MS in a few laboratories, among which a simple on-target derivatization with phenylhydrazine was carried out in lab 10 (Lattová et al. 2006).

Analysis of oligosaccharides by LC/ESI MS or LC/ESI MS/MS

The alditol forms of oligosaccharides were analyzed by LC/ESI MS (Karlsson et al. 2004). Typically, the enzymatically released oligosaccharides were converted into alditols by incubation in 20 μL of 0.5 M sodium borohydride/20 mM potassium hydroxide solution at 50 °C for 2 h. The resulting solutions were neutralized by addition of 1 mL of glacial acetic acid, desalted and dried. Borate was removed by repeated addition and evaporation of 50 μL of 1% acetic acid in methanol. Oligosaccharide samples were dissolved in water and subjected to negative ion LC/MS employing a graphitized carbon column (Hypercarb, Thermo Electron; 0.2 × 150 mm), using a linear gradient formed by 5 mM ammonium acetate/2% acetonitrile (solvent A) and 5 mM ammonium acetate/80% acetonitrile (solvent B) (Kawasaki et al. 1999). The deprotonated $[M-H]^-$ ions were measured, and the peak areas of the multiple-charge ions corresponding to one specific component were summed up manually for relative quantitation data.

MS of glycopeptides

Glycoproteins were reduced and alkylated, and then digested with trypsin as described above. Resulting peptide/glycopeptide mixtures were analyzed with LC/MS(/MS) or MALDI MS (Huddleston et al. 1993; Wada et al. 2004; Harazono et al. 2005). In either case, protonated peptides were monitored for detection. In LC/MS, glycopeptide profiles can be inferred from a low CID energy MS survey, while the molecular weight contribution of the core peptide can usually be inferred from the MS/MS data. Relative quantitation was carried out in the same way as LC/MS of oligosaccharides. For MALDI MS, glycopeptides were enriched from an enzymatic digest and the resulting glycopeptide mixture was mixed with DHB matrix solution at 10 mg/mL in 0.1% trifluoroacetic acid/50% acetonitrile and analyzed in linear mode (Wada et al. 2004). Relative quantitation was based on the intensities (heights) of the signals.

Data presentation

The relative abundances of the glycoforms identified were reported by each participating laboratory.

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Supplementary data

Supplementary data are available at Glycobiology online (http://glycob.oxfordjournals.org).

Conflict of interest statement

None declared.

Abbreviations

CID, Collision-induced dissociation; DHB, 2,5-dihydroxybenzoic acid; LC, liquid chromatography; Fab, fragment antigen binding; Fc, fragment crystallizable; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; HGPI, Human Disease Glycomics/Proteome Initiative; HUPO, Human Proteome Organisation; IgG, immunoglobulin-G; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PNGase F, Peptide: *N*-Glycosidase F; SD, standard deviation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TOF, time-of-flight.

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