Comparison of Methods for Profiling O-Glycosylation

HUMAN PROTEOME ORGANISATION HUMAN DISEASE GLYCOMICS/PROTEOME INITIATIVE
MULTI-INSTITUTIONAL STUDY OF IgA1


Recently, the Human Proteome Organisation Human Disease Glycomics/Proteome Initiative (HGPI) coordinated an institutional study that evaluated methodologies that are widely used for defining the N-glycan content in glycoproteins. The study convincingly endorsed mass spectrometry as the technique of choice for glycomic profiling in the discovery phase of diagnostic research. The present study reports the extension of the Human Disease Glycomics/Proteome Initiative’s activities to an assessment of the methodologies currently used for O-glycan analysis. Three samples of IgA1 isolated from the serum of patients with multiple myeloma were distributed to 15 laboratories worldwide for O-glycomics analysis. A variety of mass spectrometric and chromatographic procedures representative of current methodologies were used. Similar to the previous N-glycan study, the results convincingly confirmed the pre-eminent performance of MS for O-glycan profiling. Two general strategies were found to give the most reliable data, namely direct MS analysis of mixtures of permethylated reduced glycans in the positive ion mode and analysis of native reduced glycans in the negative ion mode using LC-MS approaches. In addition, mass spectrometric methodologies to analyze O-glycopeptides were also successful. Molecular & Cellular Proteomics 9:719–727, 2010.

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The abbreviations used are: HGPI, Human Disease Glycomics/Proteome Initiative; Hex, hexose; HexNAc, N-acetylgalactosamine; N, HexNAc (GalNAc); H, Hex (Gal); NA, NeuAc (N-acetylneuraminic acid); ABHE, 2-(2-amino-2-deoxy-β-D-glucopyranosylamino)-2-hydrazinocarboxylethanamide; LTQ, linear trap quadrupole.
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evaluation of methodologies that are widely used for defining the N-glycan content in glycoproteins (1). Twenty laboratories around the world participated in the study in which the glycosylation of standard samples of transferrin and IgG was characterized by a variety of chromatographic and mass spectrometric techniques. Two clear messages emerged from this study. First, there was significant variance among the data sets from laboratories using chromatographic profiling that was likely due to incomplete derivatization with the fluorophores that had been used to “tag” the oligosaccharides to facilitate chromatography and provide a means of detection. Second, MS was shown to give consistent data in interlaboratory comparisons, and it was concluded that MS-based strategies provide the most effective means of both identification and quantitation of N-glycans in glycomics studies.

HGPI has now extended its comparison of analytical methodologies to encompass mucin-type O-glycosylation. In this type of glycosylation, O-glycans are attached by a GalNAc to the amino acids serine and threonine. They can occur as single O-glycans or clustered in mucin domains. Such domains are most abundant in the class of glycoproteins known as mucins, which typically have a great number of mucin domains arranged as tandem repeats, but are also found in many extracellular proteins like IgA, which is the subject of the current study. IgA1 represents one of two structurally and functionally distinct subclasses of IgA (2). The heavy chains of IgA1 molecules contain a hinge region segment between the first and second constant region domains. This segment, which has a high content of Pro, Ser, and Thr, is the site of attachment of usually up to six O-linked glycan chains (3–9). In circulatory IgA1, these O-glycans consist of GalNAc with a β1,3-linked Gal; both saccharides may be sialylated (3, 4). The carbohydrate composition of the O-linked glycans in the hinge region of normal human serum IgA1 is variable. The prevailing forms include Gal-GalNAc disaccharide and its mono- and disialylated forms (4, 5, 10). Gal-deficient variants with terminal GalNAc or sialylated GalNAc are rarely found in the O-glycans of normal serum IgA1 (5) but are much more common in IgA nephritis patients (11, 12) in whom IgG autoantibodies reactive to the hypogalactosylated IgA1 form immune complexes and lead to mesangioproliferative glomerulonephritis (13). These pathological features clearly demonstrate the importance of determining the profile of the total glycan pool as an initial but essential step in tackling the complex O-glycan structures. In the present study, IgA1 preparations from three patients with multiple myeloma were delivered by HGPI to 15 experienced academic laboratories, and the results of O-glycome analysis, especially the total glycoform profiles, obtained using different analytical methodologies were assessed. This study was designed to compare and evaluate various methods differing in sample preparation and analytical modes as well as to document levels of variance or consistency among the data.

**RESULTS AND DISCUSSION**

Human IgA1 has both N- and O-glycosylation with the latter being the focus of this study. The O-glycans are located on the hinge region of the heavy chain, and five sites of glycosylation have been identified previously (Thr-225, Thr-228, Ser-230, Ser-232, and Thr-236), two of which (225 and 236) have been reported to be partially occupied (3–6). Tryptic cleavage yields the 38-amino acid hinge region O-glycopeptide: HYTNPSQDVTVCPCPVPST225PPT228PS230PS232TPPT236PSPSCCHPR. The mass of core peptide containing carbamidomethylated cysteine residues is 4135.88 (monoisotopic) or 4138.56 (average).

The 15 laboratories participating in the study analyzed tryptic glycopeptides, released oligosaccharides, or both. General work flows are shown in Schemes 1 and 2, and methodologies used by each laboratory are summarized in Table I. IgA1 derived from three patients with multiple myeloma (coded NUD, Sap-II, and VDS) (14, 15) were prepared in Japan and dispatched to the participating laboratories in 250-μg aliquots. Several of the participating laboratories additionally analyzed a sample of IgA pooled from healthy individuals.
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Glycopeptide Analysis—MALDI and/or ESI mass fingerprinting of cysteine-alkylated tryptic hinge O-glycopeptides was carried out by six laboratories. Data of remarkable consistency were obtained from most of these laboratories despite the use of a variety of sample handling procedures and mass spectrometric instrumentation (see Table I and supplemental methods). Exemplar MALDI and ESI mass spectra from O-glycopeptides isolated from solution digests using hydrophilic affinity extraction (16) by two laboratories (labs 1 and 4) are shown in Fig. 1 and supplemental Fig. S1, respectively. The enriched glycopeptide fraction was directly analyzed by MALDI-MS in linear TOF mode (lab 1) or subjected to on-line LC-ESI-MS (lab 4). Profiles obtained from on-line LC-ESI-MS of solution digests and by in-gel digestion followed by LC-ESI-MS are shown in Fig. 2 and supplemental Figs. S2 and S3 (labs 2, 5, and 6, respectively). Some laboratories additionally analyzed a pooled sample of IgA1 purified from healthy individuals. An exemplar mass fingerprint for the hinge glycopeptide of normal IgA1 is shown in supplemental Fig. S2.

Additional analyses to decrease the complexity of the MS profiles were carried out by lab 1 (supplemental Figs. S6 and S7). Desialylation removed the negative effect of sialic acid on the ionization efficiency in the MALDI and, the small signals corresponding to highly glycosylated glycopeptides with six or seven HexNAc residues could be observed (supplemental Fig. S6). The number of O-glycans attached to the glycopeptides was clearly demonstrated by the mass spectra after eliminating peripheral glycans attached to the Ser/Thr-GalNAc units by incubation with trifluoromethanesulfonic acid (supplemental Fig. S7). However, the current protocol of reactions did not guarantee good reproducibility, and some removal of GalNAc residues was observed. Deglycosylation by glycosidase (galactosidase) would be an alternative to this chemical treatment, but the removal of galactose residues was incomplete in a preliminary experiment carried out by lab 1 (data not shown).

The major advantages of glycopeptide analysis are clearly illustrated in this pilot study as follows. First, the average or maximum/minimum number of attached sites can be illustrated in this pilot study as follows. First, the average or maximum/minimum number of attached sites can be counted. Second, relative abundances of different glycoform

2 Y. Wada and M. Tajiri, unpublished observations.
compositions can be readily established at high sensitivity. Third, MS of glycopeptides does not miss the GalNAc-O-Ser/Thr (Tn epitope), which is characterized by components having a higher number of HexNAc than Hex such as 5N4H or 5N3H.

Chromatographic Analysis of Tagged O-Glycans—Chromatography of reductively aminated oligosaccharides carrying fluorescent “tags” at their reducing ends is a well-established procedure for quantitating mixtures of N-glycans. It remains a popular method despite issues of variance between laboratories (1) because it does not require highly sophisticated and expensive instrumentation. Exploitation of this type of methodology in the O-glycan field is far less common because free reducing sugars are required for the tagging reaction, and no tools are available that are capable of liberating O-glycans efficiently and cleanly from glycopeptides or glycoproteins while retaining their reducing sugars. Unfortunately, no broad spectrum O-glycanase has yet been discovered. Therefore, the release of O-glycans requires base-catalyzed chemical elimination, and it has been known for over 40 years that the core 1 arm of O-glycans is readily “peeled” from the 3-position of the reducing GalNAc residue unless the latter is reduced. Hence, reductive elimination is the only way of obtaining an artifact-free O-glycan population. Nevertheless, chromatographic/tagging methodologies can play a useful role in O-glycan analysis, for example in the analysis of complex mucins (17), provided peeling products and other chemical artifacts are taken into account in data.
analysis. In the present study, three laboratories (labs 2, 7, and 8) analyzed base-eliminated fluorescently tagged glycans, and their data nicely encapsulate the issues that need to be considered when such methods are used. O-Glycans were released manually with anhydrous hydrazine (labs 2 and 7) or automatically with lithium hydroxide (lab 8) or were tagged with phenylhydrazine, 2-(2-aminobenzoylamino)-2-hydrazino-carbonyl-ethanethiol (ABHE), or 2-aminobenzoic acid, respectively. The ABHE tagging procedure involved a nanoparticle immobilization/derivatization step and subsequent release of the tagged O-glycans (see supplemental methods). Work flows and protocols are given in Scheme 2 and supplementary methods, and chromatographic profiles are given in supplemental Figs. S8–S10. Comparative quantitative data are shown in Table II. The stark discrepancies between the data sets highlight the artifact problems mentioned earlier. Nevertheless, where replicate experiments were carried out, reasonable consistency was observed, so it can be concluded that comparative chromatographic profiling is a valid technique under carefully controlled conditions.

Mass Spectrometric Analysis of Reduced O-Glycans—Nine labs analyzed reductively eliminated O-glycans using three general MS strategies: (i) positive ion mode MALDI-MS fingerprinting supplemented by MALDI and/or ESI-MS/MS sequencing of mixtures of permethylated glycans (labs 1 and 9–12), (ii) negative ion mode ESI-MS fingerprinting and ESI-MS/MS sequencing of native glycans that were purified and separated by graphitized carbon on-line LC (labs 5, 13, and 14), and (iii) negative and positive ion mode MALDI-FT-ICR-MS of mixtures of native glycans without on-line LC purification (lab 15).

Work flows and protocols are given in Scheme 2 and supplemental methods, respectively, and typical data are shown in supplemental Figs. S11 and S12. Quantitative information was extracted from MALDI fingerprints and LC-MS profiles by measuring peak heights and peak areas, respectively. With one exception (lab 13; see supplemental methods), no corrections were made for differences in the response factors of the various glycans. Exemplar quantitative data are shown in Table III for permethylated and native samples, respectively, and data from all eight labs are collated in supplemental Figs. S8–S14. Collectively, these data are broadly in line with the conclusions of the glycopeptide profiling experiments (see above), namely that most of the glycans are core 1 type with monosialylated core 1 dominating in Sap-II and non-sialylated core 1 being the major glycan in VDS and NUD. For technical reasons, the neutral glycans were missed in some of the on-line experiments. On-line graphitized carbon LC will not detect the Tn antigen as single monosaccharides are not retained. Minor core 2 glycans were most reliably observed in the analyses of permethylated samples (supplemental Figs. S11 and S13 for confirmation via tandem MS). A weakness of both methodologies is that monosaccharides such as Tn are difficult
to analyze reliably because of masking by matrix peaks in MALDI data and early elution together with impurities in on-line LC-MS.

In summary, the study has shown that both strategies i and ii are reliable strategies for semiquantitative O-glycan analysis except for the analysis of Tn. On the other hand, strategy iii in

Fig. 2. ESI mass spectra of IgA O-glycopeptides of Sap-II, VDS, and NUD samples (lab 2). a, deconvoluted spectra of three samples. Monoisotopic masses of [M + H]⁺ ions are indicated in the mass spectrum of Sap-II. b, real ESI mass spectrum of Sap-II before deconvolution. The charge state of each peptide ion signal is given in parentheses. Samples were analyzed by LC-MS in positive ion mode using a Paradigm MS4 HPLC system coupled to a Thermo FT-ICR mass spectrometer. Mass accuracy was ±0.1 Da.
which native samples were analyzed as mixtures by MS without permethylation or on-line LC purification gave equivocal data. Notably, spectra were characterized by dominant matrix and artifact peaks, and only one molecular ion species was assignable to an authentic IgA \(\text{O}\)-glycan (see supplemental Fig. S14). It was also observed in some experiments that small quantities of glycopeptides (with two or more amino acids) are recovered from reductive elimination reactions, and their presence may need to be taken into account when data are analyzed.

**Conclusions**—In this study, the \(\text{O}\)-glycan content of three IgA1 samples was analyzed by 15 participating laboratories.

Six of these laboratories used either MALDI or ESI to obtain mass fingerprints of the hinge glycopeptide (which has up to five occupied \(\text{O}\)-glycosylation sites), three laboratories eliminated the \(\text{O}\)-glycans without reduction and then tagged the free reducing ends prior to chromatographic analysis, and nine laboratories used reductive elimination to release the glycans, which were then analyzed by MS after permethylation (five laboratories) or as native glycans by LC-MS (three laboratories) or MS alone (one laboratory). The main conclusions that can be drawn from the study are summarized below.

**Tryptic glycopeptides that were purified using comparable chromatographic procedures gave broadly similar mass fingerprints.**

Fig. 3. Comparison of glycan profiles obtained by laboratories having used MS of glycopeptides as analytical tool. Relative abundance of glycan structures was calculated from the signal intensities in the mass spectra of Sap-II (Figs. 1 and 2 and supplemental Figs. S1 and S2). Error bars, 2 S.D. Protonated ions were analyzed. MS mode was MALDI-linear TOF in lab 1 and ESI LTQ-FT-ICR, ESI Orbitrap, and ESI Q-TOF in labs 2, 4, and 5, respectively.

**TABLE II**
Comparative data obtained by labs 2, 7 and 8 using chromatography analyses

Keys to symbols - light square: \(\text{N}\)-acetylgalactosamine, dark square: \(\text{N}\)-acetylglucosamine, circle: galactose, diamond: \(\text{N}\)-acetylneuraminic acid.

<table>
<thead>
<tr>
<th>(\text{O})-linked glycan structure</th>
<th>Lab 2</th>
<th>Lab 7</th>
<th>Lab 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>NUD</td>
<td>VDS</td>
<td>Sap II</td>
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<tr>
<td>N-acetylglactosamine</td>
<td>39.0</td>
<td>40.4</td>
<td>8.0</td>
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<tr>
<td>N-acetylglucosamine</td>
<td>36.5</td>
<td>37.1</td>
<td>58.4</td>
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<tr>
<td>Galactose</td>
<td>13.3</td>
<td>8.1</td>
<td>21.2</td>
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<tr>
<td>Neuraminic acid</td>
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<tr>
<td>total</td>
<td>112</td>
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</tbody>
</table>

**TABLE III**
\(\text{O}\)-glycans comparative results obtained by lab 9 (MALDI-TOF MS) and 13 (LC-ESI MS)

*Combination of the two isomers; \([\text{M}^+\text{Na}]^-\) corresponds to the mass of a deuterated permethylated glycan analyzed in positive mode (see Supplementary Methods for lab 9), \([\text{M}-\text{H}]^-\) corresponds to the mass of a native glycan analyzed in negative mode. For keys see Table II legend.

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<tr>
<td>Structure</td>
<td>(m/z)</td>
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<tr>
<td>N-acetylglactosamine</td>
<td>535</td>
<td>70.0</td>
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<tr>
<td>N-acetylglucosamine</td>
<td>784</td>
<td>26.3</td>
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<tr>
<td>Galactose</td>
<td>675</td>
<td>23.0</td>
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<tr>
<td>Neuraminic acid</td>
<td>894</td>
<td>2.3</td>
</tr>
<tr>
<td>unidentified component</td>
<td>749</td>
<td>0.7</td>
</tr>
<tr>
<td>total</td>
<td>1257</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**TABLE IV**
Comparative data obtained by labs 2, 7 and 8 using MALDI-TOF MS and LC-ESI MS.

*Combination of the two isomers; \([\text{M}^+\text{Na}]^-\) corresponds to the mass of a deuterated permethylated glycan analyzed in positive mode (see Supplementary Methods for lab 9), \([\text{M}-\text{H}]^-\) corresponds to the mass of a native glycan analyzed in negative mode. For keys see Table II legend.

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<tr>
<td>total</td>
<td>1257</td>
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gerprints irrespective of whether MALDI or ESI was used. It was noted, however, that the more highly sialylated glycoforms were somewhat less abundant in the MALDI fingerprints, suggesting some loss of sialic acid during the analysis. Nevertheless, the fact that most of the sialic acid was preserved demonstrates that MALDI is a suitable tool for profiling sialylated glycoforms provided the linear ion mode is used in the TOF analysis. Semiquantitative assignment of total glycan compositions deduced from ion abundances in the mass fingerprints enabled intersample comparisons with respect to heterogeneity and overall levels of sialylation. The likely presence of the Tn epitope in some of the glycoforms was revealed by compositions rich in HexNAc. The compositional data give clues to site occupancy: for example, the number of sites occupied cannot be greater than the number of HexNAc residues in the glycoform composition, whereas detailed profiling by MS of glycopeptides requires prior knowledge of the glycan structures obtained by other methods such as chromatography of tagged glycans. Electron capture dissociation or electron transfer dissociation MS/MS instrumentation is capable of defining precise glycosylation sites as well as the size or suggested structures of glycans at specific sites (6, 7, 18). Indeed, FT-MS equipped with electron capture dissociation has also been used for structure determination of O-glycans at specific sites (6, 7, 18). This makes analysis of the glycopeptides after proteolytic degradation ideal. However, in the mucins with more complex and longer oligosaccharides, this approach will be less useful. The O-glycans then have to be alkaline-released. Permethylation followed by direct MALDI-MS and separation by liquid chromatography coupled to ESI-MS analysis are the two methods of choice for the structural characterization of O-glycans in complex mixtures. The permethylation methods used in this study are suitable for neutral and sialic acid-containing compounds but need to be modified for sulfated oligosaccharides (20).

The IgA from patients with multiple myeloma is derived from a B-cell clonal population that tends to be almost monoclonal with the progression of the disease. Although the glycosylation of myeloma proteins will thus be more homogenous than is observed in polyclonal IgA from healthy individuals, the O-glycans exhibit a degree of heterogeneity specific for each myeloma protein (7, 10). The differences observed here probably reflect varying regulation of O-glycosylation in the different B-cell clones. The distribution of O-glycan heterogeneity in NUD, VDS, and SAP-II is comparable to a published analysis of myeloma IgA1 from other sources (7).

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