Quantitative Glycomics of Human Whole Serum Glycoproteins Based on the Standardized Protocol for Liberating N-Glycans*§

Yoko Kita‡§, Yoshiaki Miura‡, Jun-ichi Furukawa‡, Mika Nakano‡§, Yasuo Shinohara‡, Masahiro Ohno§, Akio Takimoto§, and Shin-Ichiro Nishimura‡¶

Global glycomics of human whole serum glycoproteins appears to be an innovative and comprehensive approach to identify surrogate non-invasive biomarkers for various diseases. Despite the fact that quantitative glycomics is premised on highly efficient and reproducible oligosaccharide liberation from human serum glycoproteins, it should be noted that there is no validated protocol for which deglycosylation efficiency is proven to be quantitative. To establish a standard procedure to evaluate N-glycan release from whole human serum glycoproteins by peptide-N-glycosidase F (PNGase F) treatment, we determined the efficiencies of major N-glycan liberation from serum glycoproteins in the presence of reducing agents, surfactants, protease treatment, or combinations of pretreatments prior to PNGase F digestion. We show that de-N-glycosylation efficiency differed significantly depending on the condition used, indicative of the importance of a standardized protocol for the accumulation and comparison of glycomics data. Maximal de-N-glycosylation was achieved when serum was subjected to reductive alkylation in the presence of 2-hydroxyl-3-sulfopropyl dodecanoate, a surfactant used for solubilizing proteins, or related analogues, followed by tryptic digestion prior to PNGase F treatment. An optimized de-N-glycosylation protocol permitted relative and absolute quantitation of up to 34 major N-glycans present in serum glycoproteins of normal subjects for the first time. Moreover PNGase F-catalyzed de-N-glycosylation of whole serum glycoproteins was characterized kinetically, allowing accurate simulation of PNGase F-catalyzed de-N-glycosylation required for clinical glycomics using human serum samples. The results of the current study may provide a firm basis to identify new diagnostic markers based on serum glycomics analysis. Molecular & Cellular Proteomics 6: 1437–1445, 2007.

Sequencing the human genome and that of various pathogens has opened the door for proteomics, which has dramatically facilitated the search for diagnostic biomarkers. Proteomics approaches have emerged as indispensable tools to identify new disease markers from clinical specimens (1, 2). In particular, human serum/plasma proteomes are considered the most informative proteomes from a medical/clinical point of view because they likely contain most human proteins as well as proteins derived from some pathogens (3).

An additional approach currently in development focuses on serum protein glycomics, the qualitative and quantitative characterization of the gross glycans present in serum (4). Indeed glycosylation is the most common posttranslational modification of cell surface and extracellular matrix proteins, and most plasma proteins are also thought to be heavily glycosylated. Changes in abundance and alterations in glycan profiles of serum and cell surface proteins have been shown to correlate with progression of cancer and other disease states (5–7). Recently Callewaert et al. (8) reported the glycomics analysis of 106 patients with chronic liver disorders at various stages of severity and revealed significant alterations in specific N-glycans depending on the presence of cirrhosis. Although prostate-specific antigen tests often suffer from lack of specificity in distinguishing benign prostate hyperplasia from prostate cancer, recent studies indicate that N-glycans of prostate-specific antigen found in prostate cancer differ significantly from those seen in benign prostate hyperplasia and therefore could be a potential indicator leading to improved sensitivity in diagnosing prostate cancer (9, 10).

For glycomics analysis, glycans are often released from protein backbones. As-linked type glycans can be cleaved enzymatically by peptide-N-glycosidase F (PNGase F)† (peptide-N\(^\text{+}\)-((aminooxy)acetyl)tryptophan-ylarginine methyl ester; HSD, 2-hydroxyl-3-sulfopropyl dodecanoate; IAA, iodoacetamide; MTT, 3-methyl-1-propanesulfonic acid, 2-hydroxy-3-lauroamido; PHM, 1-propanesulfonic acid, 2-hydroxy-3-myristamido; RA, rheumatoid arthritis.

The abbreviations used are: PNGase F, peptide-N-glycosidase F; ALS, acid-labile surfactant; aoWR, N\(^\text{+}\)-((aminooxy)acetyl)tryptophanylarginine methyl ester; HSD, 2-hydroxyl-3-sulfopropyl dodecanoate; IAA, iodoacetamide; MTT, 3-methyl-1-propanesulfonic acid, 2-hydroxy-3-lauroamido; PHM, 1-propanesulfonic acid, 2-hydroxy-3-myristamido; RA, rheumatoid arthritis.
substrate carbohydrate moiety and a slightly modified protein in which Asn residues at the site of de-N-glycosylation are converted to Asp, whereas hydrazinolysis causes chemical modification including N-deacetylation of sialic acids and N-acetyl-D-hexosamines such as GlcNAc and GalNAc residues as well as extensive cleavage of polypeptide backbones. However, glycoproteins widely differ in susceptibility to enzymatic digestion because glycosylated sites are often obstructed by secondary and tertiary protein structure. To optimize efficiency of enzymatic release of N-glycans from individual/target glycoproteins, several conditions have been utilized using reducing agents, surfactants, protease treatment, or a combination of pretreatments prior to PNGase F digestion to make glycosylation sites more accessible. Although these procedures are often used to obtain qualitative information on N-glycan structures of specific glycoproteins, there are no standardized conditions allowing highly efficient and reproducible liberation of N-glycans from serum whole glycoprotein. It should be noted that quantitative glycomics is premised on non-biased, highly efficient, and reproducible oligosaccharide liberation from human serum glycoproteins. Therefore, our attention must be directed to establish a standardized procedure for liberating major N-glycans from human whole serum glycoproteins. Using an optimized protocol for quantitative glycomics, we revealed for the first time the absolute concentrations of major N-glycans occurring in human serum whole glycoproteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

1-Butanol, ammonium bicarbonate, and sodium phosphate buffer were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), 2-Hydroxy-3-sulfopropyl dodecanoate (HSD), 3-methyl-1-p-tolyltriazenes (MTT), and sodium cyanoborohydride were purchased from Aldrich. Trypsin was purchased from Sigma-Aldrich. PNGase F (recombinant) and Pronase were obtained from Hoffmann-La Roche and Calbiochem, respectively. Large scale preparation of PNGase F was carried out according to a previously reported method (14, 15). Briefly Flavobacterium meningosepticum (ATCC33958) was cultured, and the medium was centrifuged and filtered. The extract was concentrated by ultrafiltration, and ammonium sulfate was added to 90%. After centrifugation, the precipitate was resuspended in 0.1M sodium phosphate buffer pH 7.0 containing 1M ammonium sulfate and 1 mM EDTA and then centrifuged. The supernatant was applied to a TSK-3000 column (6.0-mm internal diameter × 75 cm) from Shimadzu Co. (Kyoto, Japan). 2,5-Dihydroxybenzoic acid, human angiotensin II, bombesin, and adrenocorticotropic hormone 18–39 were from Bruker Daltonics (Bremen, Germany). 2-Aminopyridine, acetonitrile (HPLC/MS grade), methanol (HPLC/MS grade), acetic acid, ammonium acetate, and other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Both forms of Asn residues at the site of de-N-deacetylation of sialic acids and N-acetyl-D-hexosamines such as GlcNAc and GalNAc residues as well as extensive cleavage of polypeptide backbones. However, glycoproteins widely differ in susceptibility to enzymatic digestion because glycosylated sites are often obstructed by secondary and tertiary protein structure. To optimize efficiency of enzymatic release of N-glycans from individual/target glycoproteins, several conditions have been utilized using reducing agents, surfactants, protease treatment, or a combination of pretreatments prior to PNGase F digestion to make glycosylation sites more accessible. Although these procedures are often used to obtain qualitative information on N-glycan structures of specific glycoproteins, there are no standardized conditions allowing highly efficient and reproducible liberation of N-glycans from serum whole glycoprotein. It should be noted that quantitative glycomics is premised on non-biased, highly efficient, and reproducible oligosaccharide liberation from human serum glycoproteins. Therefore, our attention must be directed to establish a standardized procedure for liberating major N-glycans from human whole serum glycoproteins. Using an optimized protocol for quantitative glycomics, we revealed for the first time the absolute concentrations of major N-glycans occurring in human serum whole glycoproteins.

**Release of N-Glycans by PNGase F**

Before PNGase F digestion, human serum (20 μl) was pretreated using nine different conditions as follows. In Condition A, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8) and 26 μl of H2O. In Condition B, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8), 21 μl of H2O, and 5 μl of trypsin (400 units) followed by incubation at 37 °C for 1 h. Then trypsin was heat denatured at 80 °C for 15 min. In Condition C, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8), 8 μl of H2O, and 8 μl of 50 mM DTT followed by incubation at 60 °C for 30 min. 5 μl of 135 mM iodoacetamide (IAA) in H2O was added, and the mixture was allowed to stand at room temperature for 1 h. Then 5 μl of trypsin (400 units) was added and incubated at 37 °C for 1 h followed by heat denaturation at 80 °C for 15 min. In Condition D, serum was diluted with an equal volume of 50 mM Tris/HCl buffer (pH 7.8) containing 2% (w/v) SDS and 2% 2-mercaptoethanol and heated to 95 °C for 5 min. Then an equal volume of buffer solution containing 8% (v/v) Triton X-100 was added. In Condition E, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8), 8 μl of 0.5% acid-labile surfactant (ALS; Waters, Milford, MA) in H2O, 8 μl of H2O, and 8 μl of 50 mM DTT followed by incubation at 55 °C for 45 min. Finally 5 μl of H2O was added and the mixture was allowed to stand at room temperature for 45 min. Finally 5 μl of H2O was added for PNGase F digestion. In Condition F, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8), 8 μl of 0.5% HSD in H2O, 8 μl of H2O, and 8 μl of 50 mM DTT followed by incubation at 55 °C for 45 min. 5 μl of 135 mM IAA in H2O was added, and the mixture was allowed to stand at room temperature for 45 min. Finally 5 μl of H2O was added for PNGase F digestion. In Conditions G, H, and I, serum was mixed with 4 μl of 100 mM ammonium bicarbonate (pH 7.8), 16 μl of 0.5% HSD (Condition G), 0.05% PHL (Condition H), or 0.005% PHM (Condition I) in H2O, and 8 μl of 50 mM DTT followed by incubation at 55 °C for 45 min. Finally 5 μl of 135 mM IAA in H2O was added, and the mixture was allowed to stand at room temperature for 45 min. Then 5 μl of trypsin (400 units) was added and incubated at 37 °C for 1 h followed by heat denaturation at 80 °C for 15 min. Subsequently...
all the pretreated serum samples were treated with PNGase F (2 units) at 37 °C for 24 h followed by heat denaturation at 90 °C for 15 min. The final volume of all samples was adjusted to 200 μl with 100 mm ammonium bicarbonate. All sample preparations were performed in triplicate except for Condition D, which was in duplicate.

Preparation of 2-Aminopyridine (PA)-oligosaccharides from Human Serum Glycoproteins

Following enzymatic release of serum N-glycans under the digestion conditions described above, an aliquot of each sample (50 μl) was digested with 20 μg of Pronase, and the mixture was purified by Bio-Gel P-4 column chromatography. Oligosaccharides obtained were reductively aminated with 1.7 M PA and 2.0 M sodium cyanoborohydride at 90 °C for 1 h and then purified on a Sephadex G-15 column using 10 mm ammonium bicarbonate as eluant (17). After removing the solvent, the sample was dissolved in 500 μl of water, and a 5-μl aliquot was injected into the reversed-phase HPLC system.

Preparation of Standard PA-oligosaccharide

Disialylated biantennary oligosaccharide (A2; NeuAcα2→6Galβ1→4GlcNAcβ1→2Man(1→6)2)[Man(1→3)]Man(1→4)GlcNAcβ1→4GlcNac was prepared by PNGase F digestion of sialylglycopeptide, which was purified from hen egg yolk (18). Briefly fresh egg yolk was treated with phenol, and the supernatant was purified by gel filtration (Sephadex G-50 column and Sephadex G-25 column) and chromatographed on an anion exchange column (DEAE-Toyopearl 650 M) and then a cation exchange column (CM-Sephadex C-25). Purified sialylglycopeptide was digested with PNGase F, and then a standard PA-oligosaccharide was prepared with the released sialyloligosaccharide by the procedure noted above.

Analysis of PA-oligosaccharides Obtained from Human Serum by Reversed-phase HPLC

PA-oligosaccharides were applied to an octadecylsilica silica (ODS, 6 × 150-mm; Shimadzu, Kyoto, Japan) HPLC column. A linear gradient elution was applied at a flow rate of 1.0 ml/min at 55 °C using 10 mm sodium phosphate buffer (pH 3.8) (solvent A) and solvent B containing 0.5% 1-butanol (solvent B) (A/B = 80:20 (0 min), 45:55 (70 min)). Fluorescence was monitored at 400 nm with excitation at 320 nm.

Methyl Esterification of Sialic Acid Residues and Labeling with aoWR

Following enzymatic release of serum N-glycans under Condition G, an aliquot of the sample (equivalent to 2 μl of serum) was digested with 2 μg of Pronase, and the mixture was subjected to purification on a Bio-Gel P-4 column. Whole N-glycans obtained were subjected to methyl esterification by treatment with MTT in DMSO-acetonitrile according to previously reported conditions with slight modification (19). Briefly lypsyHilized material (N-glycans) was dissolved in 20 μl of 100 mm HCl, and 480 μl of acetonitrile was added. The sample was then applied onto ~20 mg of labrotubes silica gel (latrom Laboratories, Inc., Tokyo, Japan) packed in a disposable filter column, Mobicol polypropylene column (1 ml, MobiTec, Göttingen, Germany), which had been preequilibrated with 1 m acetic acid and acetonitrile. The column was washed with acetonitrile by centrifugation. With the bottom cap in place, 100 μl of 100 mm MTT in a 1:1 mixture of acetonitrile and DMSO was added, and the column was incubated for 1 h at 60 °C. With the bottom cap still in place, 500 μl of acetonitrile was added to the column and briefly mixed. Next the bottom cap was removed. The column was washed with acetonitrile, 2% acetic acid in acetonitrile, and 96% acetonitrile in water, successively. The methyl esterified free oligosaccharides were eluted from the silica gel by 50% aqueous acetonitrile. The recovered oligosaccharides were labeled with aoWR according to a method described previously (16). Briefly an aliquot (50 μl) of the eluate (200 μl) was directly mixed with 5 μl of 2 mm aoWR and 50 μl of 2% acetic acid in acetonitrile, and the mixture was heated to 60 °C until the solvent evaporated (~1 h).

MALDI-TOF Mass Spectrometry

Methyl-protected and aoWR-labeled N-glycans were dissolved in 10 μl of water, the mixture was directly mixed with 2,5-dihydroxybenzoic acid (10 mg/ml in 30% acetonitrile) at a 1:10 dilution, and an aliquot (1 μl) was deposited on a stainless steel target plate. MALDI-TOF data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a LIFT-TOF/TOF facility controlled by FlexControl 2.0 software according to the general procedure reported previously (20, 21). All spectra were obtained using a reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. These spectra were the sum of 1,000 laser shots. All peaks were picked by FlexAnalysis 2.0 using the Sophisticated Numerical Annotation Procedure (SNAP) algorithm that fits isotopic patterns to the matching experimental data. The algorithm provides the monoisotopic mass, the intensity and area under the envelope of the isotopic cluster, and the resolution of the peaks in the cluster. Estimation of N-linked type oligosaccharide structures was obtained by input of peak masses into the GlycoMod Tool (Swiss Institute of Bioinformatics) and GlycoSuite (Proteome Systems).

Enzyme Kinetics Study

Kinetics analysis of PNGase F was carried out at 37 °C in 100 mm ammonium bicarbonate (200 μl), and the reaction was terminated by heating in boiling water. Each velocity was determined at a 30–150 μM N-glycan concentration using serum denatured under Condition G. The amount of released oligosaccharides was determined by reductive amination with PA and HPLC analysis as described above. Initial rates were defined as the amount of product formed after incubation for 60 min, and K_m and V_max values were determined by Sigma Plot, Enzyme Kinetics Module (SYSTAT Software Inc., Chicago, IL). Deglycosylation using PNGase F was characterized by Michaelis-Menten kinetics.

RESULTS AND DISCUSSION

Effect of PNGase F Digestion Conditions on Efficiency of Deglycosylation of Serum Whole Glycoproteins—Glycoproteins differ widely in susceptibility to PNGase F deglycosylation such that they often require denaturation prior to enzymatic treatment. Glycoproteins are typically denatured by heating in an appropriate detergent (e.g. SDS (22, 23) or ALS (24)) or by protease (e.g. trypsin or chymotrypsin) pretreatment (17, 25, 26) with or without reductive alkylation. ALS, sodium-[2-methyl-2-undecyl-1,3-dioxolan-4-yl] methoxyl]-1-propanesulphonate, is designed to degrade at low pH condition to eliminate surfactant-caused interference with analysis (24). However, one of the decomposition products contains a ketone group; hence it can seriously interfere with labeling of oligosaccharides required to improve detection sensitivity via reductive amination or hydrazone/oxime formation toward the hemiacetal-reducing terminus. To overcome this difficulty, we...
sought alternatives with chemical properties similar to ALS and chose to evaluate HSD as a designated surfactant (Fig. 1). A quantitative comparison among different digestion conditions was evaluated by an established reversed-phase HPLC method following pyridylation of released oligosaccharides. The areas of 14 major peaks in Fig. 2 (major oligosaccharide(s) present in each peak) were used for quantitative analysis.

As shown in Fig. 3a, the total amount of deglycosylated glycans differs significantly depending on the conditions used. When enzyme digestion was performed without denaturation pretreatment (Condition A), the releasing efficiency was significantly low, supporting previous findings that denaturation of substrate before deglycosylation is indispensable for high efficiency release of glycans. Tryptic digestion prior to PNGase F digestion (Condition B) and in combination with reductive alkylation (Condition C) improved efficiencies by ~88 and ~127%, respectively, over Condition A. Likewise deglycosylation efficiency was improved following treatment with solubilizing agents combined with reductive alkylation, although improvement differed depending on which surfactants were used. SDS, ALS, or HSD improved efficiency by ~63, ~75, or ~104%, respectively. Without reductive alkylation, improvement by addition of ALS and HSD remained ~19 and ~52%, respectively (data not shown), indicating that using reductive alkylation is quite effective in improving deglycosylation efficiency not only for protease-assisted but also for surfactant-assisted deglycosylation. Combining HSD, reductive alkylation, and trypsin digestion (Condition G) improved deglycosylation efficiency by ~134% compared with Condition A, an improvement nearly the same as that of Condition C. However, considering that only 22 of the most abundant human serum proteins constitute 99% of the total protein mass (28) and that low abundance glycoproteins highly resistant to PNGase F may occur in the remaining 1%,

the most rigorous condition (Condition G) would be recommended to ensure maximal deglycosylation efficiency. Note that HSD was proven useful as a protein solubilizer for the first time, although its efficacy as a cleaning agent has been reported (29). Because we confirmed the utility of HSD for PNGase F-catalyzed deglycosylation, we synthesized HSD analogues (PHL and PHM; Fig. 1) and evaluated their activities as protein solubilizer(s). When serum was reductively alkylated in the presence of PHL (Condition H) or PHM (Condition I) rather than HSD (Condition G) followed by trypsin treatment prior to PNGase F digestion, we observed that deglycosylation efficiencies were almost identical to that achieved by Condition G (Fig. 3b).

The relative quantitative profiles of released N-glycans were compared among the three most efficient digestion conditions (Conditions C, F, and G). As shown in Fig. 3c, the profile obtained under Condition F differed somewhat from those obtained under Conditions C and G possibly due to the presence of glycosylation sites that are less susceptible to PNGase F in the absence of trypsin digestion. The observation that additional trypsic digestion substantially improved deglycosylation efficiency appeared to be reasonable because it was indicated earlier that hydrolysis rates of PNGase F may be primarily determined by peptide length (30). However, trypsin digestion further complicates biological material and therefore makes the glycomics analysis even more challenging. The reproducibility of deglycosylation efficiency was fairly good (coefficient of variation <8%) throughout Conditions A–G when sample preparations from serum and HPLC analyses were performed in triplicate (except for Condition D, which was performed in duplicate). The relative quantity of each peak, calculated by the relative peak area ratios, was also reproducible (i.e. coefficient of variation of relative quantity was less than 14% for those peaks whose relative quantities are more than 1%) throughout Conditions A–G. In this regard, trypsin digestion may be omitted to simplify the analysis depending on the purpose of the study. Our finding, however, indicates that the quantitative serum glycomic profile could be severely affected by the digestion condition used, and therefore care should be taken when handling the quantitative glycomics data. As an efficient tool to rapidly purify oligosaccharides from highly complicated biological matrices, we recently developed a chemoselective glycoblotting platform utilizing synthetic polymers displaying aminooxy functionality (31–33). The combination of the described standardized sugar-liberating protocol and glycoblotting technique for high throughput, large scale disease-related serum glycomics is currently in progress in our laboratory.

Relative and Absolute Quantification of Major N-Glycans Present in Human Serum—Gross human serum N-glycan profiles have been analyzed by three-dimensional HPLC (17), LC-sonic spray ionization-MS (27), high throughput capillary electrophoresis (8), and MALDI-TOF (34). Following our successful optimization of PNGase F digestion conditions suitable for hu-
man serum proteins, the major N-glycans present in human serum were quantified. Oligosaccharides of normal serum A released under Condition G were methyl esterified followed by labeling with aoWR(H) and subjected to MALDI-TOF analysis. It has been communicated that oligosaccharides with masses greater than about 1000 Da exhibited similar signal strengths, irrespective of structure, when examined on the MALDI-TOF MS system (35). In addition, methyl esterified N-glycans are proven to exhibit signal strength in positive ion MALDI-TOF MS comparable to neutral oligosaccharides (36); thus a positive ion MALDI mass spectrum of mixtures of neutral and methyl esterified sialic acid containing oligosaccharides should reflect the relative proportions of those glycans.

As shown in Fig. 4, the analysis allowed detection of signals of up to 34 glycans. We confirmed that all these signals are aoWR derivatives by comparing m/z differences when label-

**TABLE I**

Major oligosaccharides in each peak Fig. 2

| Peak | Oligosaccharide Structure |
|------|---------------------------|
| #1   | Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3 |
| #2   | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Manβ1-4GlcNAcβ1-4GlcNAc |
| #3   | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Manβ1-4GlcNAcβ1-4GlcNAc |
| #4   | Neu5Acα2-3Galβ1-4GlcNAcβ1-4Manα1-3  |
|      | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Manβ1-4GlcNAcβ1-4GlcNAc |
| #5   | GlcNAcβ1-2Manα1-6  |
|      | Fucα1-6 |
| #6   | Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Fucα1-6 |
| #7   | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6  |
|      | Fucα1-6 |
| #8   | GlcNAcβ1-2Manα1-6  |
|      | Fucα1-6 |
|      | Galβ1-4GlcNAcβ1-2Manα1-3 |

**Fig. 2.** Reversed-phase chromatogram showing separation of PA-derivatized oligosaccharides of PNGase F-treated human serum. The numbers correspond to the structures in Table I. AU, arbitrary units.

**Molecular & Cellular Proteomics 6.8**

1441
N-glycans were the two most abundant analog of aoWR(H) (16). Mono- and disialylated biantennary glycans were detected for many high abundance glycoproteins (tri- and tetra-antennary) were detected at relatively low rates.

We observed that the concentration of one agalactosylated glycans (oligosaccharides 3, 6, and 11 in Table II) is derived primarily from IgG, which has two N-glycosylation sites per molecule, the calculated total concentration of those oligosaccharides (50–75 μM) is well within the range of serum IgG concentration (50–100 μM). To detect changes in glycan expression profiles of less abundant glycans, further study to effectively pre-fractionate minor components of serum is in progress in our laboratory.

We also analyzed the serum N-glycomic profile of a patient with RA (Supplemental Fig. S1 and Supplemental Table S1). We observed that the concentration of one agalactosylated glycans (oligosaccharide 3 in Fig. 4 and Table II) was about 7–12 times higher in a patient with RA than those of normal subjects, most likely reflecting the well documented hypogalactosylation of serum IgG in RA (6, 42). It was also

Fig. 3. Comparison of PNGase F digestion conditions on N-glycan release efficiencies (a and b) and N-glycan profiles of human serum glycoproteins (c). Mean ± S.D. (n = 3), f mean ± range (n = 2). Only reduction with 2-mercaptoethanol was performed. Optimized concentrations employed for HSD, PHL, and PHM were 0.2, 0.02, and 0.002%, respectively; thus PHL and PHM appeared to be more effective than HSD in improving PNGase F-catalyzed deglycosylation. Red. Alkyl., reductive alklyation. The error bars represent the S.D. value.

Fig. 4. MALDI-TOF MS spectra showing the gross N-glycan profiles of human whole serum from a normal subject. N-Glycans were methyl esterified and labeled with aoWR prior to analysis. The numbers correspond to the structures in Table II.

and then analysis of the relative signal strength of each glycan obtained from MALDI-TOF MS analysis. Absolute quantitation of the major N-glycan was performed by an absolute calibration method following injection of known concentrations of the same oligosaccharide onto the HPLC system. The estimated absolute concentration of each major N-glycan present in normal sera A–C is summarized in Table II. To assess the reproducibility of the quantitation methods used, which include methyl esterification, aoWR derivatization, and quantitation by MALDI-TOF analysis, these procedures were performed in triplicate for the analysis of normal serum C, and mean values are shown with S.D. The reproducibility was found to be reasonably good because S.E. values were mostly within 20% for those signals whose absolute concentrations were higher than 6 μM (which corresponds to ~1% of total N-glycan concentration). The total concentration of N-glycans was calculated to be 700–850 μM. To the best of our knowledge, the absolute concentration of N-glycans in human whole serum was determined for the first time with estimation of variability associated with several healthy controls. Given that a large proportion of core fucosylated neutral biantennary glycans (oligosaccharides 3, 6, and 11 in Table II) is derived from IgG, which has two N-glycosylation sites per molecule, the calculated total concentration of those oligosaccharides (50–75 μM) is well within the range of serum IgG concentration (50–100 μM). To detect changes in glycan expression profiles of less abundant glycans, further study to effectively pre-fractionate minor components of serum is in progress in our laboratory.

We also analyzed the serum N-glycomic profile of a patient with RA (Supplemental Fig. S1 and Supplemental Table S1). We observed that the concentration of one agalactosylated glycans (oligosaccharide 3 in Fig. 4 and Table II) was about 7–12 times higher in a patient with RA than those of normal subjects, most likely reflecting the well documented hypogalactosylation of serum IgG in RA (6, 42). It was also
observed that the calculated total concentration of core fucosylated neutral biantennary glycans (oligosaccharides 3, 6, and 11 in Table II), three of major N-glycans of IgG, was ~270 μM, which is 3–6 times higher than that in normal subjects. This may be attributable to the previous findings that the mean IgG level was raised above normal values (43) and that the increase reflects unknown autoimmune reactions in the early stage of RA (44). Thus, the described absolute quantitation may lead to improved sensitivity in diagnosing RA because it allows estimating the concentration of oligosaccharides that properly reflects the relative abundance of glycoprotein(s) in a disease state.

Kinetics Analysis of PNGase F Using Human Serum as Substrate—Speed is required to apply glycomics analysis to clinical applications such as analysis of disease diagnosis and prognosis. To shorten the time required for deglycosylation of human serum glycoproteins, enzyme kinetics analysis was performed using whole serum glycoproteins as substrate following denaturation in Condition G. Although deglycosylation by PNGase F is known to follow Michaelis-Menten kinetics, the enzyme kinetics has not been elucidated when gross human serum glycoproteins were used as substrates. Based on the concentration (~0.8 mM) of N-glycosylation sites present in whole serum glycoproteins estimated above, $K_{\text{m}}$ and $V_{\text{max}}$ values were determined to be $2.40 \times 10^1 \mu \text{M}$ and $7.12 \times 10^{-2} \mu \text{M/min}$, respectively (Fig. 5a). These values are in good agreement with the range previously reported for several glycopeptides (30).

As shown in Fig. 5b, the reaction time course simulated from the estimated kinetics parameter showed fairly good agreement with experimental data. Both simulated and experimental data indicate that the amount of recovered N-glycans reached a plateau between 12 and 24 h under the conditions used. Fig. 5c shows the time course of recovery of each major individual N-glycan. Deglycosylation rates differ substantially among these glycoforms: those of sialylated glycans tended to be slower than those of neutral glycans. However, considering that it is understood that the structure of glycoforms has
Quantitative Glycomics of Human Serum Glycoproteins

Fig. 5. Kinetics analysis of PNGase F in human whole serum glycoproteins. a, Lineweaver-Burk plot of PNGase F-catalyzed N-deglycosylation of whole human serum glycoproteins. b and c, time course of PNGase F-catalyzed N-deglycosylation of whole serum human glycoproteins where 20 µl of serum was treated with 2 units of PNGase F under Condition G. Solid lines and symbols represent the simulation results and experimental data, respectively. Recovery of total N-glycans is shown in b, and that of each individual major N-glycan is shown in c. The numbers shown in c correspond to those shown in Table I. Data shown are mean ± S.D. (n = 3). The error bars represent the S.D. value.

little effect on PNGase F activity (30), the different deglycosylation rates likely reflect varying susceptibility of serum glycoproteins to PNGase F. This observation again promotes awareness that the quantitative serum glycomic profile can be significantly affected by digestion conditions. Based on kinetics parameters, enzymatic activity was simulated under various conditions (i.e. the amount of serum and enzyme or reaction time). Accordingly we experimentally confirmed that PNGase F-catalyzed deglycosylation times could be shortened substantially while maintaining recovery efficiency identical to that achieved by Condition G followed by 24 h of PNGase F digestion (data not shown). Under the optimized protocol, 5 µl of human serum is pretreated according to Condition G (<3 h) followed by digestion with 10 units of PNGase F only for 2 h. We believe that the present protocol ensures maximal deglycosylation of general glycoproteins and should meet requirements of high throughput clinical glycomics.

In the present report, we optimized conditions of PNGase F-catalyzed deglycosylation suitable for high throughput human serum glycomics based on quantitative comparisons of typical digestion conditions and a detailed kinetics study. We show that deglycosylation efficiency can differ significantly depending on conditions used, thus indicating the importance of a standardized protocol for the accumulation and comparison of glycomics data. Furthermore relative and absolute quantitation of human whole serum glycomics was achieved for the first time, providing a firm basis to explore the clinical glycomics research. Glycomics and proteomics are two of the few options available for identifying serum biomarkers, because DNA- or RNA-based diagnostics are not applicable to serum in which no corresponding genome or transcriptome exists. In this regard, it is worthy to mention that serum glycomics analysis offers more than just a means to explore disease-related glycan markers because the discovery of a unique glycan expression profile promptly provides a valuable strategy in developing a particular glycoform-focused reverse genomics.

Acknowledgments—We are grateful to Prof. A. Minami and Dr. N. Iwasaki for the provision of serum samples of rheumatoid arthritis patients. We appreciate Dr. H. Nakagawa and Dr. K. Deguchi for valuable suggestions and discussion on HPLC and mass analysis of N-glycan profiling. We thank Satomi Kudo for her expert technical assistance.

* This work was supported in part by a “Development of System and Technology for Advanced Measurement and Analysis (SENTANI)” from the Japan Science and Technology Agency (JST). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Petricoin, E. F., Ardekani, A. M., Witt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., and Liotta, L. A. (2002) Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359, 572–577
2. Veenstra, T. D., Conrads, T. P., Hood, B. L., Avellino, A. M., Ellenbogen, R. G., and Morrison, R. S. (2005) Biomarkers: mining the biofluid proteome. Mol. Cell. Proteomics 4, 409–418
3. Anderson, N. L., Polanski, M., Pieper, R., Gatlin, T., Timrali, R. S., Conrads, T. P., Veenstra, T. D., Adkins, J. N., Pounds, J. G., Fagan, R., and Lobley, A. (2004) The human plasma proteome: a nondendritic list developed by combination of four separate sources. Mol. Cell. Proteomics 3, 311–326
4. Dube, D. H., and Bertozzi, C. R. (2005) Glycans in cancer and inflammation—potential for therapeutics and diagnostics. Nat. Rev. Drug Discov. 4, 477–488
5. Kim, Y. S., Hwang, S. Y., Oh, S., Sohn, H., Kang, H. Y., Lee, J. H., Cho, E. W., Kim, J. Y., Yoo, J. S., Kim, N. S., Kim, C. H., Miyoshi, E., Taniguchi, N., and Ko, J. H. (2004) Identification of target proteins of N-acetylglucosaminyl-transferase V and fucosyltransferase VIII in human gastric tissues by glycomic approach. Proteomics 4, 3353–3358
6. Parekh, R. B., Dwark, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, M., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T., and Kobata, A. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature 316, 452–457
7. Meezan, E., Wu, H. C., Black, P. H., and Robbins, P. W. (1969) Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. II. Separation of glycoproteins and glycopeptides by Sephadex chromatography. Biochemistry 8, 2518–2524
8. Callaway, N., Van Vlierberghen, H., Van Hecke, A., Laroy, W., Delanghe, J.,...
and Contreras, R. (2004) Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. Nat. Med. 10, 429–434

9. Ohyama, C., Hosono, M., Nitta, K., Oh-edaa, M., Yoshikawa, K., Habuchi, T., Aral, Y., and Fukuda, M. (2004) Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. Glycobiochemistry 14, 671–679

10. Peracaula, R., Tabares, G., Royle, L., Harvey, D. J., Dwek, R. A., Rudd, P. M., and de Llorens, R. (2003) Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. Glycobiology 13, 457–470

11. Yosizawa, Z., Sato, T., and Schmid, K. (1966) Hydrazinolysis of α-1 carbohydrate glycopolypeptide. Biochim. Biophys. Acta 121, 417–419

12. Takasaki, S., Mizuochi, T., and Kobata, A. (1982) Hydrazinolysis of asparagine-linked sugar chains to produce free oligosaccharides. Methods Enzymol. 83, 263–268

13. Takahashi, N. (1977) Demonstration of a new amidease acting on glycopeptides. Biochem. Biophys. Res. Commun. 76, 1194–1201

14. Plummer, T. H., Jr., and Tarentino, A. L. (1991) Purification of the oligosaccharide-removing enzymes of Flavobacterium meningosepticum. Glycobiology 1, 257–263

15. Tarentino, A. L., Gomez, C. M., and Plummer, T. H., Jr. (1985) Deglycosylation of asparagine-linked glycan by peptide-N-glycosidase F. Biochemistry 24, 4665–4671

16. Uematsu, R., Furukawa, J., Nakagawa, H., Shinohara, Y., Deguchi, K., Monde, K., and Nishimura, S.-I. (2005) High throughput quantitative glycomics and glycomega-focused proteomics of murine dermis and epidermis. Mol. Cell. Proteomics 4, 1977–1989

17. Nakagawa, H., Kawamura, Y., Kato, K., Shimada, I., Arata, Y., and Takahashi, N. (1995) Identification of neutral and sialyl-N-linked oligosaccharide chains from human serum glycoproteins using three kinds of high-performance liquid chromatography. Anal. Biochem. 226, 130–138

18. Seko, A., Koketsu, M., Nishizono, M., Enoki, Y., Ibrahim, H. R., Juneja, L. R., Kim, M., and Yamamoto, T. (1997) Occurrence of a sialylglycopeptide and free sialylglycans in hen’s egg yolk. Biochim. Biophys. Acta 1335, 23–32

19. Miura, Y., Shinohara, Y., Furukawa, J., Nagahori, N., and Nishimura, S.-I. (2007) Rapid and simple solid-phase esterification of sialic acid residues for quantitative glycomics by mass spectrometry. Chem. Eur. J. 13, 4797–4804

20. Kurogoci, M., and Nishimura, S.-I. (2004) Structural characterization of N-glycopeptides by matrix-dependent selective fragmentation of MALDI-TOF/TOF tandem mass spectrometry. Anal. Chem. 76, 6097–6101

21. Hato, M., Nakagawa, H., Kurogoci, M., Akama, T. O., Marth, J. D., Fukuda, M. N., and Nishimura, S.-I. (2006) Unusual N-glycan structures in α-mannosidase II/IIx double null embryos identified by a systematic glycomics approach based on two-dimensional LC mapping and matrix-dependent selective fragmentation method in MALDI-TOF/TOF mass spectrometry. Mol. Cell. Proteomics 5, 2148–2157

22. Goodarzi, M. T., and Turner, G. A. (1998) Reproducible and sensitive determination of charged oligosaccharides from haptoglobin by PNGase F digestion and HPAEC/PAD analysis: glycan composition varies with disease. Glycoconjug. J. 15, 469–475

23. Mann, A. C., Self, C. H., and Turner, G. A. (1994) A general method for the complete deglycosylation of a wide variety of serum glycoproteins using peptide-N-glycosidase F. Glycobiology 4, 253–261

24. Yu, Y. Q., Gilar, M., Lee, P. J., Bouvier, E. S., and Gbler, J. C. (2003) Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. Anal. Chem. 75, 6023–6028

25. Yu, Y. Q., Gilar, M., Kaska, J., and Gbler, J. C. (2005) A rapid sample preparation method for mass spectrometric characterization of N-linked glycans. Rapid Commun. Mass Spectrom. 19, 2331–2336

26. Nakano, M., Kakehi, K., and Lee, Y. C. (2003) Sample clean-up method for analysis of complex-type N-glycans released from glycopeptides. J. Chromatogr. A 1005, 13–21