Journal of Chromatography B, 869 (2008) 20–30

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous glycosylation analysis of human serum glycoproteins by high-performance liquid chromatography/tandem mass spectrometry

Akira Harazono[a,*], Nana Kawasaki[a,b], Satsuki Itoh[a], Noritaka Hashii[a], Yukari Matsuishi-Nakajima[a,b], Toru Kawanishi[a], Teruhide Yamaguchi[a]

[a] Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kami-yoga, Setagaya-Ku, Tokyo 158-8501, Japan
[b] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), Kawaguchi Center Building, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 19 December 2007
Accepted 5 May 2008
Available online 10 May 2008

Keywords:
Glycosylation analysis
Human serum
Glycopeptide
LC/MS
LC/MS/MS
Immunoglobulin G
Haptoglobin
Transferrin
Ceruloplasmin

ABSTRACT

Changes in the glycosylation of some serum proteins are associated with certain diseases. In this study, we performed simultaneous site-specific glycosylation analysis of abundant serum glycoproteins by LC/Qq-TOF MS of human serum tryptic digest, the albumin of which was depleted. The glycopeptide peaks on the chromatogram were basically assigned by database searching with modified peak-list text files of MS/MS spectra and then based on mass differences of glycan units from characterized glycopeptides. Glycopeptide of IgG, haptoglobin and ceruloplasmin were confirmed by means of a comparison of their retention times and *m/z* values with those obtained by LC/MS of commercially available glycoproteins. Mass spectrometric carbohydrate heterogeneity in the assigned glycopeptides was analyzed by an additional LC/MS. We successfully demonstrated site-specific glycosylation of 23 sites in abundant serum glycoproteins.

1. Introduction

Glycosylation of proteins is a common post-translational modification of proteins [1], and most proteins in serum are glycosylated [2]. Changes in the oligosaccharide moieties of certain serum glycoproteins are associated with human diseases. Oligosaccharides lacking galactose residues in immunoglobulin G (IgG) are increased in rheumatoid arthritis [3,4] and Crohn's syndrome [5]. Congenital disorders of glycosylation (CDG) are genetic disorders in the N-linked glycosylation processing pathway [6], and can be diagnosed by glycosylation analysis of serum glycoproteins [7], such as transferrin and haptoglobin. Significant increases in fucose levels and oligosaccharide branches in haptoglobin have been found to be associated with ovarian cancer [8,9], lung cancer [10–12], pancreatic cancer [13] and hepatocellular carcinoma [14]. Changes in glycosylation are also found in acute-phase proteins, such as alpha-1-acid glycoprotein and ceruloplasmin, in lung cancer [15]. These findings suggest the potential of the glycosylation analysis of serum glycoproteins in diagnosis of some diseases and an investigation of new biomarkers. At present the glycosylation of each protein is examined individually, therefore simultaneous analysis of serum glycoproteins has been required for rapid diagnosis with a limited amount of sample.

Mass spectrometry (MS) is known as a powerful tool for the glycosylation analysis of serum proteins. For the glycosylation analysis of serum glycoproteins, the enrichment of glycopeptides by lectin-affinity or hydrophilic chromatography is useful due to their low ionization efficiency, ionization suppression effects, and microheterogeneity [16–19]. There are still concerns about the loss of some glycopeptides during the preparation procedure, biased recoveries toward certain glycan structures, and low reproducibility of recovery. Liquid chromatography/mass spectrometry (LC/MS) is effective for the separation of glycopeptides and for the simultaneous glycosylation analysis.

Abbreviations: ESI, electrospray ionization; Fuc, fucose; GlcNAc, N-acetylglucosamine; Hex, hexose; HexNAc, N-acetylhexosamine; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NeuAc, N-acetylneuraminic acid; Qq-TOF, quadrupole–quadrupole time-of-flight mass spectrometry; TIC, total ion chromatogram; EIC, extracted ion chromatogram.

* Corresponding author. Tel.: +81 3 3700 9074; fax: +81 3 3700 9084.
E-mail addresses: harazono@nihs.go.jp (A. Harazono), nana@nihs.go.jp (N. Kawasaki).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.006

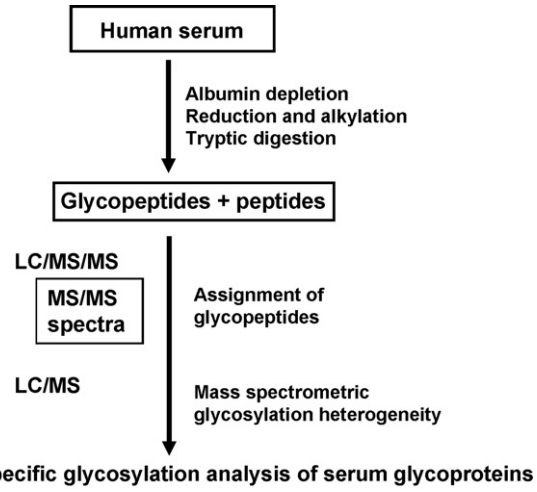

Fig. 1. Strategy for glycosylation analysis of abundant glycoproteins in serum. Human serum in which albumin was roughly removed was reduced and alkylated at cysteine residues. A mixture of peptides resulting from trypsin digestion was subjected to LC/MS/MS and LC/MS. Glycopeptides were assigned by elucidating MS/MS spectra (database searching). Mass spectrometric heterogeneity at each glycosylation site was analyzed by an additional LC/MS.

Recent progress in MS/MS and multiple-stage MS (MS^n) of glycopeptides allows for the characterization of both peptide and oligosaccharide moieties based on fragment ions [17,20–27]. Previously it was demonstrated that the Qq-TOF type mass spectrometer provides highly abundant carbohydrate-related ions at lower m/z values such as m/z 204 $[HexNAc+H]^+$ and 366 $[HexHexNAc+H]^+$, glycopeptide-related ions with sequentially lost saccharide units, including $[peptide+H]^+$ and $[peptide+GlcNAc+H]^+$ at higher m/z values, and b- and y-ions derived from peptide backbone [20,23,26,28]. These fragment ions could be used in database search to deduce peptide of glycopeptide.

In this study we demonstrated LC/MS(/MS) of human serum digest for the simultaneous glycosylation analysis of abundant serum proteins. Fig. 1 shows the strategy for the glycosylation analysis. Human serum, the albumin of which was depleted, was carboxymethylated and digested with trypsin. LC/MS/MS of the digest was performed by using the LC/Qq-TOF MS instrument. Glycopeptide ions were basically assigned by database searching with modified peak-list text files. Mass spectrometric heterogeneity at each glycosylation site was analyzed by an additional LC/MS, in which the acquisition of MS/MS was not allowed. By LC/MS of albumin-depleted human serum digest, we were successful in the site-specific glycosylation analysis of abundant serum glycoproteins.

2. Experimental

2.1. Materials

Pooled normal human serum was purchased from Sigma (St. Louis, MO, USA). Human haptoglobin and polyclonal immunoglobulin G, which were purified from normal human serum, were purchased from Calbiochem (San Diego, CA, USA). Modified trypsin was purchased from Promega (Madison, WI, USA). The water used was obtained from a Milli-Q water system (Millipore, Bedford, MA). All other reagents were of the highest quality available.

2.2. Sample preparation

Human serum (5 μl) was depleted of albumin using the Montage Albumin Depletion Kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. Lyophilized albumin-depleted sample and each of the glycoproteins (100 μg) were dissolved in 50 μl of 0.5 M Tris–HCl buffer (pH 8.5) that contained 7 M guanidine hydrochloride and 5 mM EDTA. After the addition of 2 μl of 1 M dithiothreitol, the mixture was incubated for 30 min at 56 °C. Then, 4.7 μl of 1 M sodium monoiodoacetate was added, and the resulting mixture was incubated for 30 min at room temperature in the dark. The reaction mixture was applied to a PD-10 column (GE Healthcare, Little Chalfont, UK) to remove the reagents, and a fraction of the carboxymethylated proteins was dried. The sample was redissolved in 50 μl of 50 mM Tris–HCl buffer (pH 8.0). An aliquot of 1 μl of modified trypsin prepared as 1 μg/μl was added, and then the mixtures were incubated for 12 h at 37 °C. The enzyme digestions were stopped by boiling for 10 min and stored at −20 °C before analysis.

2.3. LC/MS and LC/MS/MS

The tryptic digests corresponding to 0.01–0.3 μl of human serum or a tryptic digest of purified commercially available glycoprotein (0.1–1.0 μg) was loaded onto a nanotrap (AMR Inc., Tokyo, Japan). After a wash with 10 μl of 2% (v/v) acetonitrile containing 0.1% (v/v) TFA, the trapping column was switched into line with the column. HPLC was performed on a Paradigm MS 4 (Michrome BioResources, Auburn, CA, USA) equipped with a MonoCap High Resolution 750 column (0.2 mm × 750 mm, GL Sciences Inc., Tokyo, Japan) at a flow rate of about 2 μl/min. The eluents consisted of water containing 2% (v/v) acetonitrile and 0.1% (v/v) formic acid (pump A) and 90% acetonitrile and 0.1% formic acid (pump B). Samples were eluted with 5% of B for 2.5 or 5.0 min followed by a linear gradient from 5 to 90% of pump B in 85 min or by linear gradients from 5 to 25% for 80 min, 25–45% for the next 60 min, 45–65% for the next 40 min and 60–90% for the next 20 min (total 205 min).

Mass spectrometric analyses were performed using a QSTAR Pulsar i Qq-TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with a nano-electrospray ion source. The mass spectrometer was operated in positive ion mode. The nano-spray voltage was set at 1700 V. Mass spectra were acquired over m/z 1000–2000 for MS, and m/z 100–2000 for MS/MS. After every regular MS acquisition, MS/MS acquisitions were performed against the top two multiply charged ions by a data-dependent acquiring method. The precursor ions with the same m/z as previously acquired were excluded for 60 or 90 s. The collision energy was varied between 30 and 70 eV depending on the size and charge of the molecular ion. The accumulation time of the spectra was 1.0 s for MS, and 2.0 or 5.0 s for MS/MS. All signals were monoisotopically resolved.

2.4. Assignment of glycopeptide peaks by database search

Detection and assignment of glycopeptide ions from LC/ESI MS/MS data were performed by elucidating MS/MS spectra or database search. Briefly, glycopeptide ions were selected manually based on presence of oligosaccharide oxonium ions such as m/z 204 and 366 in their MS/MS spectra. The information of m/z values and charge states of peptides in the glycopeptides was deduced by sequential degradation pattern at N-glycan core structure in their MS/MS spectra. The MS/MS spectra of glycopeptides were converted to peak-list text files, and then oligosaccharide-related ions (m/z 168, 186, 204, 274, 292 and 366 or ions under m/z 370)

and the ions larger than peptide ion were deleted. Modified peaklist text files were submitted to against the nonredundant human Swiss–Prot protein database (version 48.2) using Mascot search engine with following parameters: a specified trypsin enzymatic cleavage with two possible missed cleavage, peptide tolerance of 1.2 Da, fragment ion tolerance of 0.8 Da, and variable modifications of cysteine (carboxymethylation) or cystein (carboxymethylation) and methionine (oxidation). Suggested peptides were validated by manual inspection of the spectra, and the presence of more than four consecutive fragments of amino acid sequence was used as criteria for peptide identification.

3. Results

3.1. Locating glycopeptides in the chromatogram

Mass spectrometric glycosylation analysis of human serum was performed by LC/Qq-TOF MS of tryptic digest using in a positive ion mode. In this method, all serum glycoproteins should be completely digested by trypsin. When the tryptic digest was subject to LC/MS/MS with the MS range m/z 400–2000, results of Mascot database search using 3 missed cleavage sites suggested that most peptides were completely digested (missed cleavage <1) and few incompletely digested peptides (missed cleavage 3) were present. Many missed cleavage sites were present at N- or C-terminal, or adjacent to two or more acidic amino acid residues (D, E or carboxymethylated C) (data not shown). Fig. 2A shows the total ion chromatogram (TIC) obtained by LC/MS/MS with MS range m/z 1000–2000 of tryptic digest (corresponding to approximately 0.3 μl of serum) using a reversed phase MonoCap High Resolution 750 column (0.2 mm × 750 mm) with a gradient of 5–90% of B in 205 min. In order to locate the glycopeptide peaks and determine m/z and charge state, the intensity of the oxonium $HexNAc^+$ (m/z 204.05–204.15) that arose by data-dependent MS/MS was depicted as the extracted ion chromatogram (Fig. 2B). We confirmed that most of these MS/MS spectra were of glycopeptides by the presence of abundant carbohydrate-derived ions, such as m/z 204 ($[HexNAc+H]^+$), 186 ($[HexNAc+H-H_2O]^+$), 292 ($[NeuAc+H]^+$), 274 ($[NeuAc+H-H_2O]^+$) and 366 ($[HexHexNAc+H]^+$).

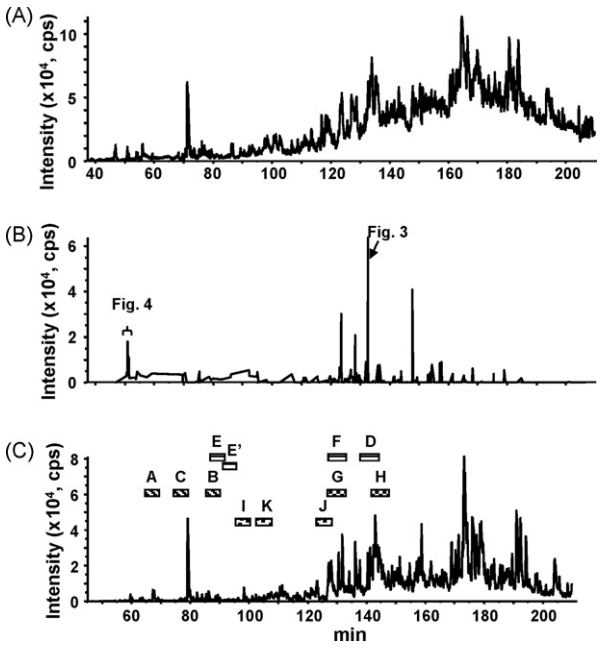

Fig. 2. LC/MS/MS and LC/MS of tryptic digest of human serum. (A) TIC (m/z 1000–2000) obtained by the LC/MS/MS. (B) EIC (m/z 204.05–204.15) obtained by the data-dependent MS/MS. (C) TIC obtained by the additional LC/MS in which data-dependent MS/MS was not allowed. Peak assignment: A, IgG1; B, IgG2; C, IgG3/IgG4; D–F, haptoglobin; G and H, transferrin; I–K, ceruloplasmin. Mass spectra of fractions A–K are shown in Fig. 7.

3.2. Assignment of glycopeptide peaks by a database search

Glycopeptides were assigned by manual database searching. As a representative example, the MS/MS spectrum acquired from $[M+4H]^{4+}$ (m/z 1221.8 (4+)) at 133 min is shown in Fig. 3. There are some abundant ions derived from carbohydrates, such as m/z 204, 186, 292, 274 and 366 in the lower m/z region. Degradation pattern and mass difference of 203 u between the fragment ions at m/z 1340.2 (2+) and those at 1441.7 (2+) in the higher m/z region suggests that the ions are $[peptide+2H]^{2+}$ and $[peptide+HexNAc+2H]^{2+}$, respectively. Based on these m/z values the molecular mass of

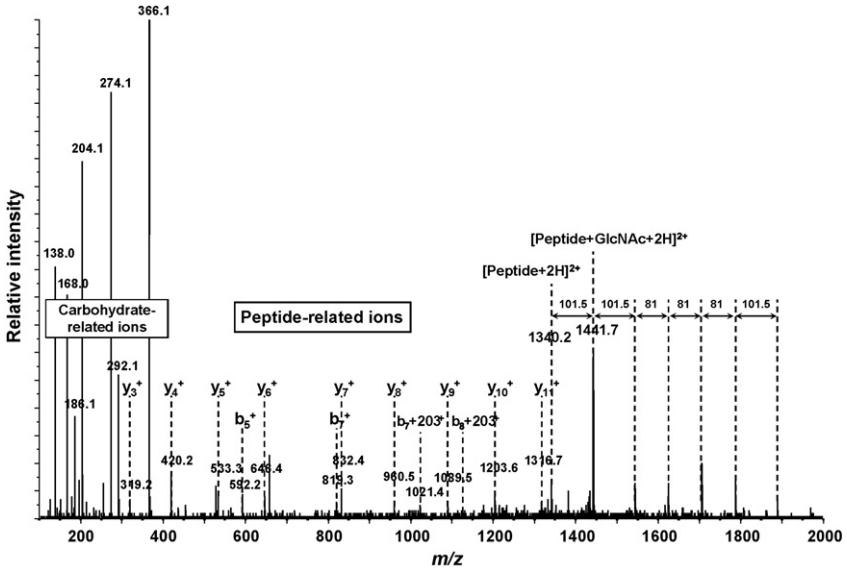

Fig. 3. MS/MS spectrum acquired from m/z 1221.8 (4+) by data-dependent LC/MS/MS of trypsin-digested human serum. Mascot database search using m/z 1340.2 (2+) of peptide and fragment ions (m/z 370–1300) suggested peptide sequence MVSHHN[184]LTTGATLINEQWLLTTAK in haptoglobin (P00738).

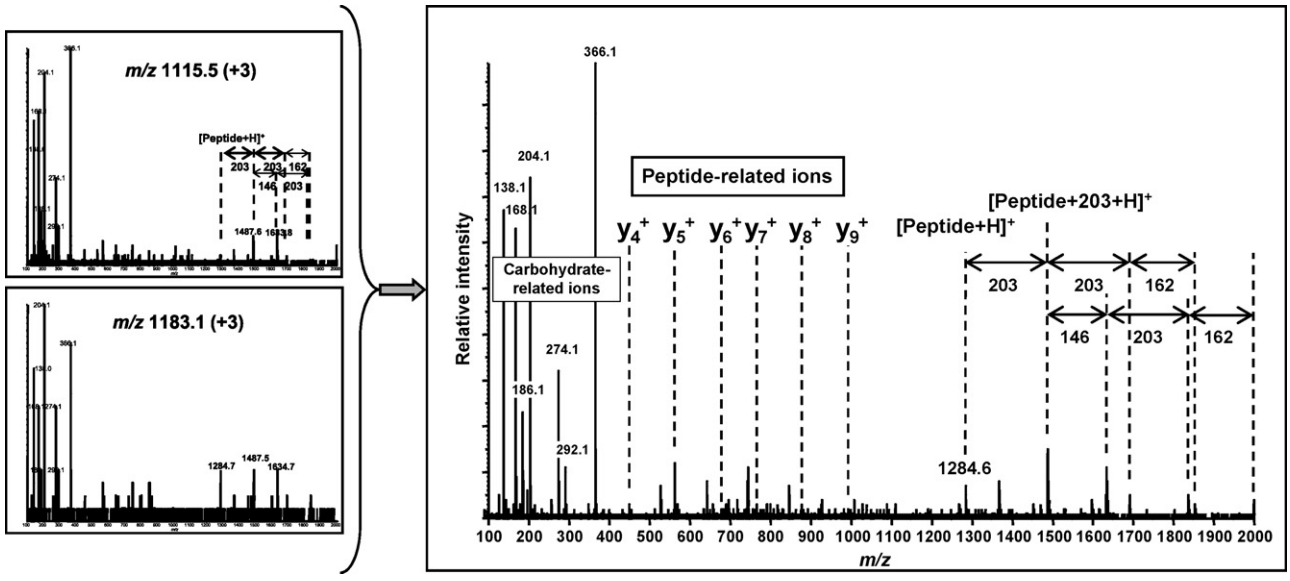

Fig. 4. Integrated MS/MS spectrum of *m*/*z* 1115.5 (3+) and 1183.1 (3+) at 52–53 min that show similar fragment patterns. Mascot database search using *m*/*z* 1284.5 (1+) of peptide and fragment ions (*m*/*z* 370–1280) suggested YKN[46]NSDISSTR in Ig mu chain C region (P01871).

the peptide was calculated to be 2678.4. For the peptide identification, a database search requires the *m*/*z* values and charge state of the peptide precursor ions and fragment ions but not of the carbohydrate- and glycopeptide-related ions. We deleted the carbohydrate-related ions in the lower *m*/*z* region (under *m*/*z* 370) and the glycopeptide-related ions in the higher *m*/*z* region (over *m*/*z* 1340) from the peak-list text files, and then submitted the modified peak-list text files for a Mascot database search of the human Swiss–Prot database with 1 missed cleavage, peptide tolerance of 1.2 Da, fragment ion tolerance of 0.8 Da and variable modifications of cysteine (carboxymethylation). The peptide suggested was MVSHHN[184]LTTGATLINEQWLLTTAK in human haptoglobin (P00738). As shown in Fig. 3, many ions were consistent with *b*- and *y*-series peptide fragment ions derived form MVSHHNLTTGATLINEQWLLTTAK. The molecular mass of the carbohydrate moiety was calculated to be 2204.7, which suggests the carbohydrate composition of $HexNAc_4Hex_5NeuAc_2$.

3.3. Assignment of glycopeptide peaks by a database search with integrated spectra

Glycopeptides that have the same peptide backbone show quite similar fragment patterns in the case of Qq-TOF MS. When glycopeptides showed insufficient peptide fragment ions in the CID-MS/MS spectra due to low peak intensity, we integrated the similar MS/MS spectra into one spectrum, and the integrated spectrum was submitted for a database search. As a representative example, the spectrum obtained by integrating two spectra of *m*/*z* 1115.5 (3+) and 1183.1 (3+) acquired around 60 min is shown in Fig. 4. Mascot database search using the information of *m*/*z* 1284.5 (1+) of peptide which was deduced by sequential degradation pattern at N-glycan core structure, and modified peak-list text files between *m*/*z* 370 and 1250 suggests that the peptide moiety is YKN[46]NSDISSTR in Ig mu chain C region (P01871).

By elucidating MS/MS spectra, 19 tryptic glycopeptides (20 N-glycosylation sites) in 14 glycoproteins were determined (Table 1). The ions, which were confirmed as glycopeptides by data-dependent MS/MS, were underlined. Other glycoforms, whose MS/MS spectra were not acquired, were assigned based on their mass difference of saccharide units from characterized glycopeptides. Since high intensity glycopeptide ions showed high quality of MS/MS spectra and were subjected to data-dependent MS/MS several times, many of them could be assigned. Low intensity glycopeptide ions showed poor MS/MS spectra for detection of peptide fragment ions, about 20% of MS/MS spectra of glycopeptides could not be assigned (data not shown).

3.4. Confirmation of glycopeptide peaks using commercially available glycoproteins

We conducted peptide mapping of commercially available polyclonal IgG and haptoglobin, and then *m*/*z* values and charge states of the glycopeptides were used for confirmation of assignment of glycopeptides and assignment of undetected glycopeptides. Glycosylation data of ceruloplasmin in previous report [28] was also utilized.

Tryptic digest (0.2 μg and 0.4 μg) of commercially available human polyclonal IgG was analyzed by LC/ESI MS/MS at *m*/*z* 400–2000 and 1000–2000 with a gradient of 5–90% of B in 85 min. The MS data were submitted for database searching against the human Swiss–Prot database using the computer program Mascot. Polypeptides of IgG heavy chain C region of IgG1 (P01857), IgG2 (P01859), IgG3 (P01860) and IgG4 (P01861) and light chain C region of Kappa (P01834) and Lambda (P01842) chain and other proteins were identified (data not shown). Fig. 5A and A′ show TIC of LC/MS/MS at *m*/*z* 1000–2000 of polyclonal IgG and EIC of data-dependent MS/MS at *m*/*z* 204.05–205.15, respectively. It was found that glycopeptide ions were eluted at 7–12 min (fraction A), 15–17 min (fraction C) and 18–21 min (fraction B) based on the presence of the oligosaccharide-related ions in their MS/MS spectra and mass differences of saccharide units. MS/MS spectra after 25 min were not of glycopeptides. The glycopeptide peaks from fraction A and fraction B were assigned as the glycopeptides containing Fc-glycosylation site in IgG1 (EEQYNSTYR) and IgG2 (EEQFNSTFR) based on data-dependent MS/MS spectra, respectively (data not shown). Data-dependent MS/MS spectra from fraction C suggested molecular mass of 1171.5 Da for the peptide, but could not suggest amino acid sequence due to low abundance of peptide fragment ions (data not shown). Based on the molecular mass of the peptide, the glycopeptide peaks from fraction C would be EEQYNSTFR from IgG3 (CAA67886) and/or EEQFNSTYR from IgG4 (P01861), which are attached to core-fucosylated agalacto-

Table 1
Summary of analysis of serum glycoproteome with higher ion intensities

Glycopeptide					Oligosaccharide	Protein (Protein ID)	Theoretical MW
Retention time (min)	m/z[a]	Charge	Observed MW	Relative peak intensity[b]	Observed MW	Glycopeptide Peptide sequence Deduced oligosaccharide composition[c]	Peptide Oligosaccharide
						Ig gamma-1 chain C region (P01857)	1[d]
						EEQYNSTYR[A]	1188.50
67.3	1479.6	2+	2957.1	13.1	1768.6	[HexNAc]4[Hex]5[Fuc]1	1768.64
67.3	1297.0	2+	2592.0	3.0	1403.5	[HexNAc]3[Hex]4[Fuc]1	1403.51
67.4, 67.6	1398.5	2+	2795.1	33.1	1606.5	[HexNAc]4[Hex]4[Fuc]1	1606.59
67.4, 67.7	1216.0	2+	2429.9	4.3	1241.4	[HexNAc]3[Hex]3[Fuc]1	1241.45
67.7	1317.5	2+	2633.0	27.8	1444.5	[HexNAc]4[Hex]3[Fuc]1	1444.53
67.9	1500.1	2+	2998.1	2.3	1809.6	[HexNAc]5[Hex]4[Fuc]1	1809.67
	1000.4	3+	2998.0				
67.9	1581.1	2+	3160.2	0.2	1971.7	[HexNAc]5[Hex]5[Fuc]1	1971.72
68.0	1406.6	2+	2811.1	0.9	1622.6	[HexNAc]4[Hex]5	1622.58
68.2, 68.4	1325.5	2+	2649.0	2.8	1460.5	[HexNAc]4[Hex]4	1460.53
68.2	1419.1	2+	2836.1	2.9	1647.6	[HexNAc]5[Hex]3[Fuc]1	1647.61
68.5	1244.5	2+	2486.9	1.4	1298.4	[HexNAc]4[Hex]3	1298.48
69.1	1625.1	2+	3248.1	2.2	2059.6	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
	1083.7	3+	3248.1				
69.6	1544.1	2+	3086.2	0.5	1897.6	[HexNAc]4[Hex]4[NeuAc]1[Fuc]1	1897.68
						EEQYNSTYRVVSVLTVLHQDWLNGK[e]	2977.49
161.9, 162.2	1147.0	4+	4584.1		1606.6	[HexNAc]4[Hex]4[Fuc]1	1606.59
162.4	1106.5	4+	4422.1		1444.6	[HexNAc]4[Hex]3[Fuc]1	1444.53
						EEQYNSTYRVVSVLTVLHQDWLNGKEYK[e]	3397.69
156.4	1034.3	5+	5166.4		1768.7	[HexNAc]4[Hex]5[Fuc]1	1768.64
156.6, 157.1	1001.8	5+	5004.0		1606.3	[HexNAc]4[Hex]4[Fuc]1	1606.59
						Ig gamma-2 chain C region (P01859)	1[d]
						EEQFNSTFR[B]	1156.51
85.4	1463.6	2+	2925.1	6.3	1768.6	[HexNAc]4[Hex]5[Fuc]1	1768.64
85.5	1281.0	2+	2560.0	1.4	1403.5	[HexNAc]3[Hex]4[Fuc]1	1403.51
85.7, 86.3	1382.5	2+	2763.1	19.4	1606.5	[HexNAc]4[Hex]4[Fuc]1	1606.59
85.7, 86.4	1200.0	2+	2397.9	2.8	1241.4	[HexNAc]3[Hex]3[Fuc]1	1241.45
85.7	1565.1	2+	3128.2	0.0	1971.7	[HexNAc]5[Hex]5[Fuc]1	1971.72
86.0	1484.1	2+	2966.2	1.0	1809.6	[HexNAc]5[Hex]4[Fuc]1	1809.67
86.5	1301.5	2+	2601.0	21.8	1444.5	[HexNAc]4[Hex]3[Fuc]1	1444.53
86.5	1390.5	2+	2779.0	0.0	1622.5	[HexNAc]4[Hex]5	1622.58
86.9	1403.0	2+	2804.1	1.8	1647.5	[HexNAc]5[Hex]3[Fuc]1	1647.61
87.0, 87.5	1309.5	2+	2617.0	0.1	1460.5	[HexNAc]4[Hex]4	1460.53
87.6	1228.5	2+	2454.9	0.2	1298.4	[HexNAc]4[Hex]3	1298.48
89.4	1609.1	2+	3216.2	1.6	2059.7	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
	1073.1	3+	3216.2				
90.0	1528.1	2+	3054.2	1.5	1897.6	[HexNAc]4[Hex]4[NeuAc]1[Fuc]1	1897.68
	1019.1	3+	3054.1				
						Gamma 3 immunoglobulin constant heavy chain (CAA67886)	2[d]
						EEQYNSTFR[C]	1172.51
						Ig gamma-4 chain C region (P01861)	1[d]
						EEQFNSTYR[C]	1172.51
76.4	1471.6	2+	2941.1	1.0	1768.6	[HexNAc]4[Hex]5[Fuc]1	1768.64
76.5	1289.0	2+	2576.0	0.2	1403.5	[HexNAc]3[Hex]4[Fuc]1	1403.51
76.6, 76.8	1390.6	2+	2779.1	3.4	1606.6	[HexNAc]4[Hex]4[Fuc]1	1606.59
76.5, 76.8	1208.0	2+	2413.9	0.4	1241.4	[HexNAc]3[Hex]3[Fuc]1	1241.45
76.7	1492.1	2+	2982.1	0.2	1809.6	[HexNAc]5[Hex]4[Fuc]1	1809.67
76.9	1309.5	2+	2617.0	3.6	1444.5	[HexNAc]4[Hex]3[Fuc]1	1444.53
77.0	1398.6	2+	2795.1	0.1	1622.6	[HexNAc]4[Hex]5	1622.58
76.9	1317.5	2+	2633.0	0.1	1460.5	[HexNAc]4[Hex]4	1460.53
77.0	1411.1	2+	2820.1	0.3	1647.6	[HexNAc]5[Hex]3[Fuc]1	1647.61
77.4	1236.4	2+	2470.8	0.1	1298.3	[HexNAc]4[Hex]3	1298.48
78.5	1617.1	2+	3232.1	0.3	2059.6	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
79.1	1536.1	2+	3070.1	0.1	1897.6	[HexNAc]4[Hex]4[NeuAc]1[Fuc]1	1897.68
						Haptoglobin (P00738)	4[d]
						MVSHHNLTTGATLINEQWLLTTAK[D]	2678.39
137.9	1531.7	3+	4592.1		1913.7	[HexNAc]4[Hex]5[NeuAc]1	1913.68
	1149.0	4+	4592.0	30.1	1913.6		

Table 1 (*Continued*)

Glycopeptide					Oligosaccharide	Protein (Protein ID)	Theoretical MW
Retention time (min)	m/z[a]	Charge	Observed MW	Relative peak intensity[b]	Observed MW	Glycopeptide Peptide sequence Deduced oligosaccharide composition[c]	Peptide Oligosaccharide
141.4	1221.8	4+	4883.1	88.7	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						M(O)VSHHNLTTGATLINEQWLLTTAK	2694.38
137.3	1153.0	4+	4608.1	28.9	1913.7	[HexNAc]4[Hex]5[NeuAc]1	1913.68
140.5, 141.1	1225.8	4+	4899.1	64.3	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
	1634.1	3+	4899.1		2204.8		
						NLFLNHSENATAK[E]	1457.73
86.3	1395.0	4+	5576.0	0.1	4118.3	[HexNAc]8[Hex]10[NeuAc]3	4118.45
87.0	1650.3	4+	6597.3	0.3	5139.6	[HexNAc]10[Hex]12[NeuAc]4	5139.81
87.6	1595.6	4+	6378.3	0.9	4920.6	[HexNAc]9[Hex]11[NeuAc]4[Fuc]1	4920.73
87.9	1559.1	4+	6232.3	2.7	4774.5	[HexNAc]9[Hex]11[NeuAc]4	4774.68
88.6	1504.3	4+	6013.0	0.1	4555.3	[HexNAc]8[Hex]10[NeuAc]4[Fuc]1	4555.60
88.9	1467.8	4+	5867.1	4.1	4409.4	[HexNAc]8[Hex]10[NeuAc]4	4409.54
90.4	1759.6	4+	7034.5	0.2	5576.8	[HexNAc]10[Hex]12[NeuAc]5[Fuc]1	5576.96
90.7	1723.1	4+	6888.5	0.5	5430.8	[HexNAc]10[Hex]12[NeuAc]5	5430.90
91.5	1668.3	4+	6669.4	0.3	5211.6	[HexNAc]9[Hex]11[NeuAc]5[Fuc]1	5211.83
91.7	1631.8	4+	6523.3	0.4	5065.6	[HexNAc]9[Hex]11[NeuAc]5	5065.77
87.8	1124.7	3+	3371.2	–	1913.5	[HexNAc]4[Hex]5[NeuAc]1	1913.68
91.6	1221.7	3+	3662.2	–	2204.5	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						VVLHPNYSQVDIGLIK[F]	1794.00
127	1358.6	3+	4072.8	2.2	2278.8	[HexNAc]5[Hex]6[NeuAc]1	2278.81
128.2	1236.9	3+	3707.7	3.1	1913.7	[HexNAc]4[Hex]5[NeuAc]1	1913.68
131.4	1455.6	3+	4363.8	4.8	2569.8	[HexNAc]5[Hex]6[NeuAc]2	2569.90
	1092.0	4+	4363.8				
131.8	1333.9	3+	3998.7	89.2	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
	1000.7	4+	3998.7		2204.7		
134.1	1552.7	3+	4655.0	7.5	2860.9	[HexNAc]5[Hex]6[NeuAc]3	2861.00
134.1	1164.7	4+	4654.9		2860.9		
						Transferrin (P02787)	2[d]
						CGLVPVLAENYNK[G]	1476.73
126.1	1252.8	3+	3755.5	0.9	2278.8	[HexNAc]5[Hex]6[NeuAc]1	2278.81
127.0	1131.1	3+	3390.4	1.7	1913.7	[HexNAc]4[Hex]5[NeuAc]1	1913.68
129.8	1349.9	3+	4046.7	1.6	2569.9	[HexNAc]5[Hex]6[NeuAc]2	2569.90
130.6	1228.2	3+	3681.5	46.8	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
133.1	1446.9	3+	4337.8	0.8	2861.0	[HexNAc]5[Hex]6[NeuAc]3	2861.00
						QQQHLFGSNVTDCSGNFCLFR[H]	2516.08
143.8	1623.3	3+	4866.9	4.1	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
143.9	1181.2	4+	4720.9		2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
	1574.6	3+	4720.9	50.5	2204.8		
146.0	1842.0	3+	5523.0	0.9	3007.0	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1	3007.06
146.2	1793.4	3+	5377.3	1.6	2861.2	[HexNAc]5[Hex]6[NeuAc]3	2861.00
						Ceruloplasmin (P00450)	4[d]
						EHEGAIYPDNTTDFQR[I]	1891.83
95.6	1415.2	3+	4242.7	0.1	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
	1061.7	4+	4242.6		2350.8		
96.3	1366.5	3+	4096.6	6.0	2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
	1025.2	4+	4096.7		2204.9		
98.1, 98.5	1633.9	3+	4898.7	0.4	3006.9	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1	3007.06
	1226.0	4+	4899.9		3008.0		
98.8	1585.2	3+	4752.7	0.4	2860.9	[HexNAc]5[Hex]6[NeuAc]3	2861.00
	1189.2	4+	4752.6		2860.8		
						ENLTAPGSDSAVFFEQGTTR[J]	2125.99
127.0	1493.2	3+	4476.6	0.0	2350.6	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
127.4	1444.5	3+	4330.6	2.8	2204.6	[HexNAc]4[Hex]5[NeuAc]2	2204.77
129.3	1663.3	3+	4987.0	0.2	2861.0	[HexNAc]5[Hex]6[NeuAc]3	2861.00
						ELHHLQEQNVSNAFLDK[K]	2021.00
104.3	1093.9	4+	4371.7	1.4	2350.7	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
	1458.3	3+	4371.8	0.2	2350.8		
105.2	1057.4	4+	4225.7	4.5	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
	1409.6	3+	4225.6	1.2	2204.6		

Table 1 (*Continued*)

Glycopeptide					Oligosaccharide	Protein (Protein ID)	Theoretical MW
Retention time (min)	m/z[a]	Charge	Observed MW	Relative peak intensity[b]	Observed MW	Glycopeptide Peptide sequence Deduced oligosaccharide composition[c]	Peptide Oligosaccharide
106.6	1294.6	4+	5174.2	1.2	3153.2	[HexNAc]5[Hex]6[NeuAc]3[Fuc]2	3153.12
106.8, 107.4	1258.0	4+	5027.9	2.0	3006.9	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1	3007.06
107.7	1221.5	4+	4881.8	1.9	2860.8	[HexNAc]5[Hex]6[NeuAc]3	2861.00
						Alpha-1-antitrypsin (P01009)	3[d]
						YLGNATAIFFLPDEGK	1754.89
154.6	1369.6	3+	4105.7	2.2	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
154.8	1320.9	3+	3959.7	140.6	2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						Alpha-2-HS-glycoprotein (P02765)	2[d]
						VCQDCPLLAPLNDTR	1772.81
136.9	1326.9	3+	3977.7		2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						Alpha-2-macroglobulin (P01023)	8[d]
						VSNQTLSLFFTVLQDVPVR	2162.17
187.9, 188.8	1505.3	3+	4512.7	5.1	2350.6	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
188.3	1456.7	3+	4367.0	22.5	2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						Beta-2-glycoprotein 1 (P02749)	4[d]
						VYKPSAGNNSLYR	1467.75
83.5	1273.8	3+	3818.5	1.5	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
83.6	1225.2	3+	3672.5	6.9	2204.8	[HexNAc]4[Hex]5[NeuAc]2	2204.77
85.2	1492.6	3+	4474.6	0.3	3006.9	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1	3007.06
85.4	1443.9	3+	4328.6	0.5	2860.8	[HexNAc]5[Hex]6[NeuAc]3	2861.00
						LGNWSAMPSCK	1250.54
109.7	1152.8	3+	3455.3		2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						Complement C3 (P01024)	3[d]
						TVLTPATNHMGNVTFTIPANR	2254.15
121.0	1265.9	3+	3794.6	5.4	1540.4	[HexNAc]2[Hex]7	1540.53
121.2	1211.8	3+	3632.5	47.8	1378.4	[HexNAc]2[Hex]6	1378.48
121.6	1157.8	3+	3470.4	10.2	1216.3	[HexNAc]2[Hex]5	1216.42
						Hemopexin (P02790)	5[d]
						SWPAVGNCSSALR	1404.65
115.3	1252.8	3+	3755.5	0.7	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
115.8	1204.1	3+	3609.4	10.3	2204.7	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						ALPQPQNVTSLLGCTH	1735.86
115.8	1314.5	3+	3940.4	10.3	2204.5	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						Ig alpha-1 chain C region (P01876)	2[d]
						Ig alpha-2 chain C region (P01877)	4[d]
						LSLHRPALEDLLLGSEANLTCTLTGLR	2963.58
165.2, 165.7	1157.8	4+	4627.2	8.0	1663.6	[HexNAc]5[Hex]4	1663.61
165.8	1117.3	4+	4465.0	15.4	1501.4	[HexNAc]5[Hex]3	1501.56
165.9	1046.0	4+	4180.0	5.6	1216.4	[HexNAc]2[Hex]5	1216.42
169.2	1220.3	4+	4877.2	48.8	1913.6	[HexNAc]4[Hex]5[NeuAc]1	1913.68
168.8, 169.4	1256.9	4+	5023.5	1.3	2059.9	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
169.9	1179.8	4+	4715.3	4.0	1751.7	[HexNAc]4[Hex]4[NeuAc]1	1751.62
170.0	1169.6	4+	4674.2	5.1[f]	1710.6	[HexNAc]3[Hex]5[NeuAc]1	1710.60
169.0	1271.2	4+	5080.8	21.0[f]	2117.2	[HexNAc]5[Hex]5[NeuAc]1	2116.76
	1017.2	5+	5081.0		2117.4		
169.9	1230.4	4+	4917.6	11.8	1954.0	[HexNAc]5[Hex]4[NeuAc]1	1954.70
173.1	1293.2	4+	5168.8	9.0	2205.2	[HexNAc]4[Hex]5[NeuAc]2	2204.77
						PALEDLLLGSEANLTCTLTGLR[e]	2357.21
174.4	1287.2	3+	3858.6	14.3	1501.4	[HexNAc]5[Hex]3	1501.56
176.7	1424.6	3+	4270.9	65.8	1913.7	[HexNAc]4[Hex]5[NeuAc]1	1913.68
176.4	1492.5	3+	4474.5	20.5	2117.3	[HexNAc]5[Hex]5[NeuAc]1	2116.76
						Ig alpha-2 chain C region (P01877)	
						TPLTANITK	957.55
84.1	1006.8	3+	3017.2	3.7	2059.7	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
	1509.6	2+	3017.2		2059.6		
84.1	1074.4	3+	3220.3	4.8	2262.8	[HexNAc]5[Hex]5[NeuAc]1[Fuc]1	2262.81
	1611.2	2+	3220.3		2262.8		
87.2	1103.8	3+	3308.3	1.3	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1	2350.83
						Ig mu chain C region (P01871)	5[d]
						YKNNSDISSTR[e]	1283.61

Table 1 (*Continued*)

Glycopeptide					Oligosaccharide	Protein (Protein ID)	Theoretical MW
Retention time (min)	*m/z*[a]	Charge	Observed MW	Relative peak intensity[b]	Observed MW	Glycopeptide Peptide sequence Deduced oligosaccharide composition[c]	Peptide Oligosaccharide
59.7	1115.5	3+	3343.4	40.1	2059.7	[HexNAc]4[Hex]5[NeuAc]1[Fuc]1	2059.73
60.3	1183.1	3+	3546.4	16.4	2262.8	[HexNAc]5[Hex]5[NeuAc]1[Fuc]1	2262.81

[a] Underlines indicated that these ions were assigned by elucidating data-dependent MS/MS of LC/ESI MS/MS of human serum digest.
[b] Centroid peak intensity (count per sec) in integrated MS spectra during glycopeptide eluting period.
[c] Oligosaccharide compositions were deduced from molecular weights.
[d] Number of potential N-glycosylation sites.
[e] Missed cleavage or unexpected digestion.
[f] Other ions with same *m/z* overlapped.
[A–K] Mass spectra were shown in Fig. 7A–K.
All masses are monoisotopic. Cysteine residue was carboxymethylated. Potential N-glycosylation sites were underlined. M(O), oxidized methionine; Fuc, fucose; Hex, hexose; HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid

or mono/diagalacto-biantennary complex-type glycan. Integrated mass spectra of fraction A, B and C were shown in Fig. 5B–D.

Haptoglobin has four potential N-glycosylation sites. We performed peptide mapping using a tryptic digest of haptoglobin under a chromatographic condition similar to that of human serum. Fig. 6A and A′ show TIC obtained by LC/MS/MS with mass range *m/z* 1000–2000 and EIC of data-dependent MS/MS at *m/z* 204.05–205.15, respectively. Glycopeptides for four potential glycosylation sites were assigned by elucidating MS/MS spectra (spectra were not shown). Glycopeptides of NLFLN^{207}HSEN^{211}ATAK containing two N-glycosylation sites were eluted in fraction E as two glycosylated forms (Fig. 6B) and fraction E′ as one glycosylated forms (Fig. 6C). The former glycosylated form was more abundant than the later form. These glycosylation sites could not be characterized separately by trypsin digestion. Glycopeptides of VVLHPN^{241}YSQVDIGLIK and MVSHHN^{184}LTTGATLINEQWLLTTAK were eluted in fractions F and D, respectively (Fig. 6D and E). From the molecular masses of oligosaccharides we inferred that a majority of oligosaccharides in haptoglobin are di-, tri-, and tetra-antennary forms and that some oligosaccharides were not fully saturated with NeuAc, and few glycans were fucosylated.

Using the data of relative retention times, accurate *m/z* values and charge states obtained by peptide mapping of commercially available glycoproteins, we confirmed already assigned glycopeptides and further assigned undetected glycopeptides (IgG3/IgG4 and two sites of ceruloplasmin), with the exceptions of one of the glycopeptides from ceruloplasmin, intensity of which was only noise levels.

3.5. Site-specific glycosylation analysis

To analyze the heterogeneity of glycosylation at each site, we performed an additional LC/MS in which switching to MS/MS was not allowed (Fig. 2C). Utilizing the information of retention time, accurate *m/z* and charge state of assigned glycopeptides by LC/MS/MS, corresponding glycopeptides were assigned in LC/MS data by mass chromatogram. When two or more glycoforms were detected, mass spectrometric heterogeneity was calculated using

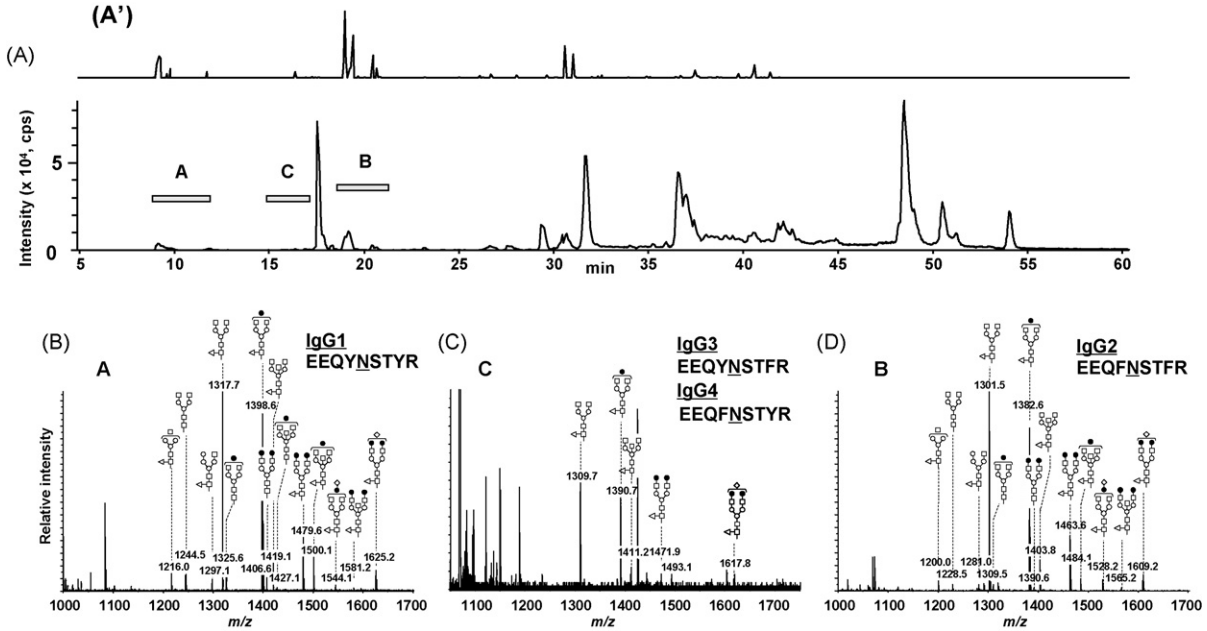

Fig. 5. Peptide map of commercially available human polyclonal IgG. (A) TIC (*m/z* 1000–2000) obtained by LC/MS/MS of trypsin-digested IgG. (A′) EIC (*m/z* 204.05–204.15) obtained by data-dependent MS/MS. (B) Mass spectrum of peak A, which was assigned as glycopeptides of EEQYNSTYR of IgG.1 (P01857). (C) Mass spectrum of peak C, which would be glycopeptides of EEQYNSTFR of IgG3 (CAA67886) and/or EEQFNSTYR of IgG4 (P01861). (D) Mass spectrum of peak B, which was assigned as glycopeptides of EEQFNSTFR of IgG2 (P01859).

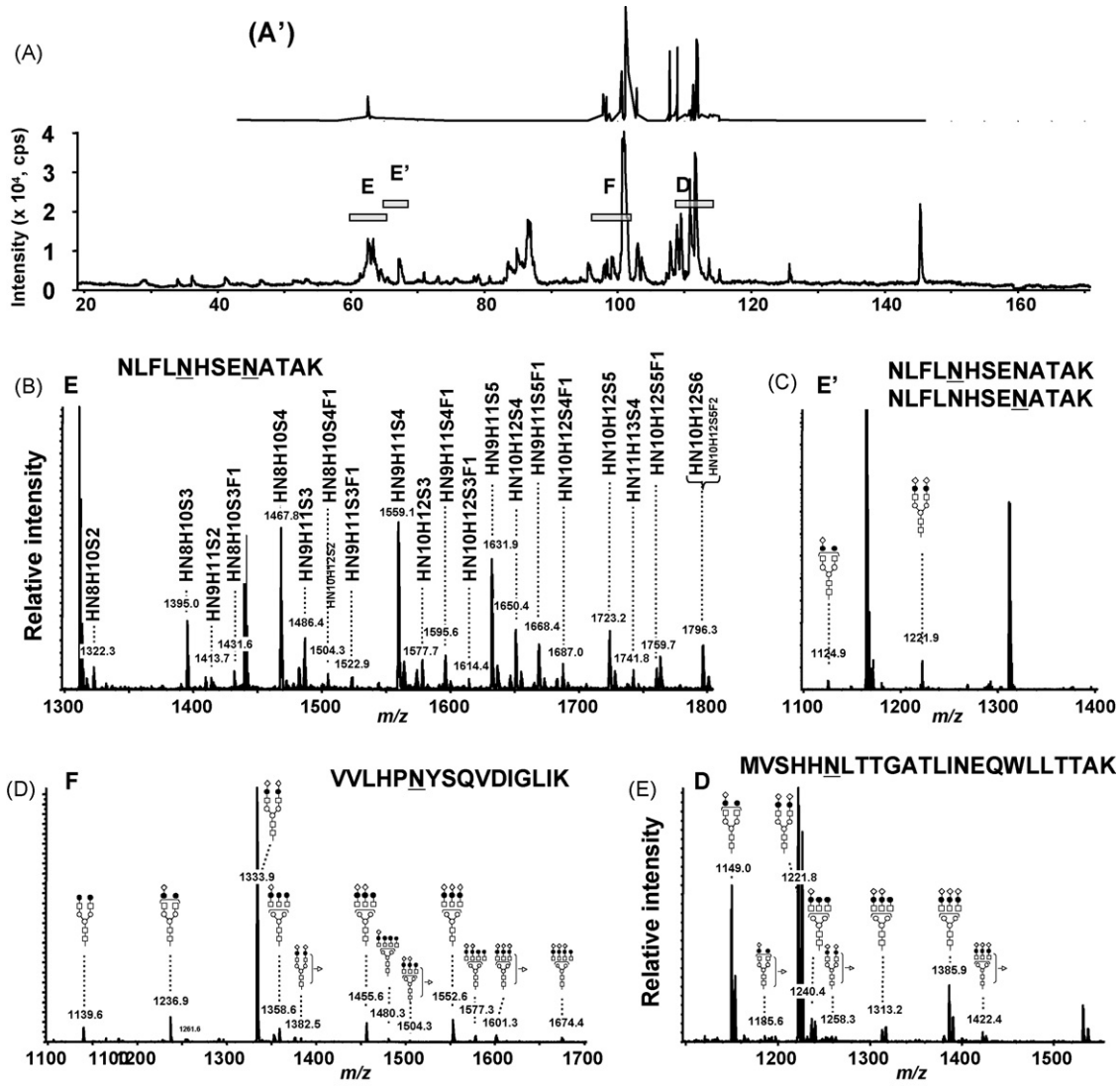

Fig. 6. Peptide map of commercially available human haptoglobin. (A) TIC (*m*/*z* 1000–2000) obtained by LC/MS/MS of trypsin-digested haptoglobin. (A′) EIC (*m*/*z* 204.05–204.15) obtained by data-dependent MS/MS. (B) Mass spectrum of peak E, which was assigned as glycopeptides of NLFLN^{207}HSEN^{211}ATAK attached to two N-glycan. (C) Mass spectrum of peak E', which was assigned as glycopeptides of NLFLN^{207}HSEN^{211}ATAK attached to one N-glycan. (D) Mass spectrum of peak F, which was identified as VVLHPN^{241}YSQVDIGLIK. (E) Mass spectrum of peak D, which was identified as MVSHHN^{184}LTTGATLINEQWLLTTAK. H, hexose; HN, *N*-acetylhexosamine; S, *N*-acetylneuraminic acid; F, fucose.

integrated mass spectra during the periods eluting the glycopeptides with same peptide. In Fig. 7, we show integrated mass spectra of fraction A–K (Fig. 2C) as the mass spectrometric heterogeneity of glycosylation in IgG1 (Fig. 7A), IgG2 (Fig. 7B), IgG3/IgG4 (Fig. 7C), haptoglobin (Fig. 7D–F), transferrin (Fig. 7G and H) and ceruloplasmin (Fig. 7I–K). Centroid ion intensity (count/sec) of each glycopeptide at the most intense isotope distribution was used as relative peak intensity. The mass spectrometric heterogeneity of the Fc-glycosylation sites of IgG1 (Fig. 7A) and IgG2 (Fig. 7B) was consistent with those of the commercially available polyclonal IgG (Fig. 5B and D) and previous reports [29]. The glycosylation pattern of haptoglobin at each site was similar to that of the commercially available haptoglobin except that peak intensities of minor glycoforms were noise level (Figs. 6B–E and 7D–F). The glycosylation of transferrin (Fig. 7G and H) at each site was consistent with previous reports [29]. Three glycopeptides of the four expected ones derived from ceruloplasmin could be assigned on the chromatogram of the serum sample (Fig. 7I–K), and their glycosylation patterns were in agreement with those in our previous reports [28]. Table 1 summarized LC retention time, *m*/*z* and charge, relative peak intensities of assigned glycopeptides in LC/MS. No O-glycosylated peptides were detected in this study. It would be due to low amount of O-glycosylation in serum and huge sample complexity.

4. Discussion

Alteration of glycans in several serum glycoproteins is a potential marker for several diseases. Several glycomic approaches to the diagnosis using mass spectrometric techniques have been proposed. The most common procedure involves analyzing the liberated glycans by MALDI-TOF MS or LC/ESI-MS, but this method provides no information on the glycosylation sites or protein sources. Another approach involves mass spectrometric analysis of glycopeptides resulting from proteolytic digestion. The enrichment of glycopeptides is useful due to their low ionization efficiency, but loss of glycopeptides cannot be avoidable. In the present study, we

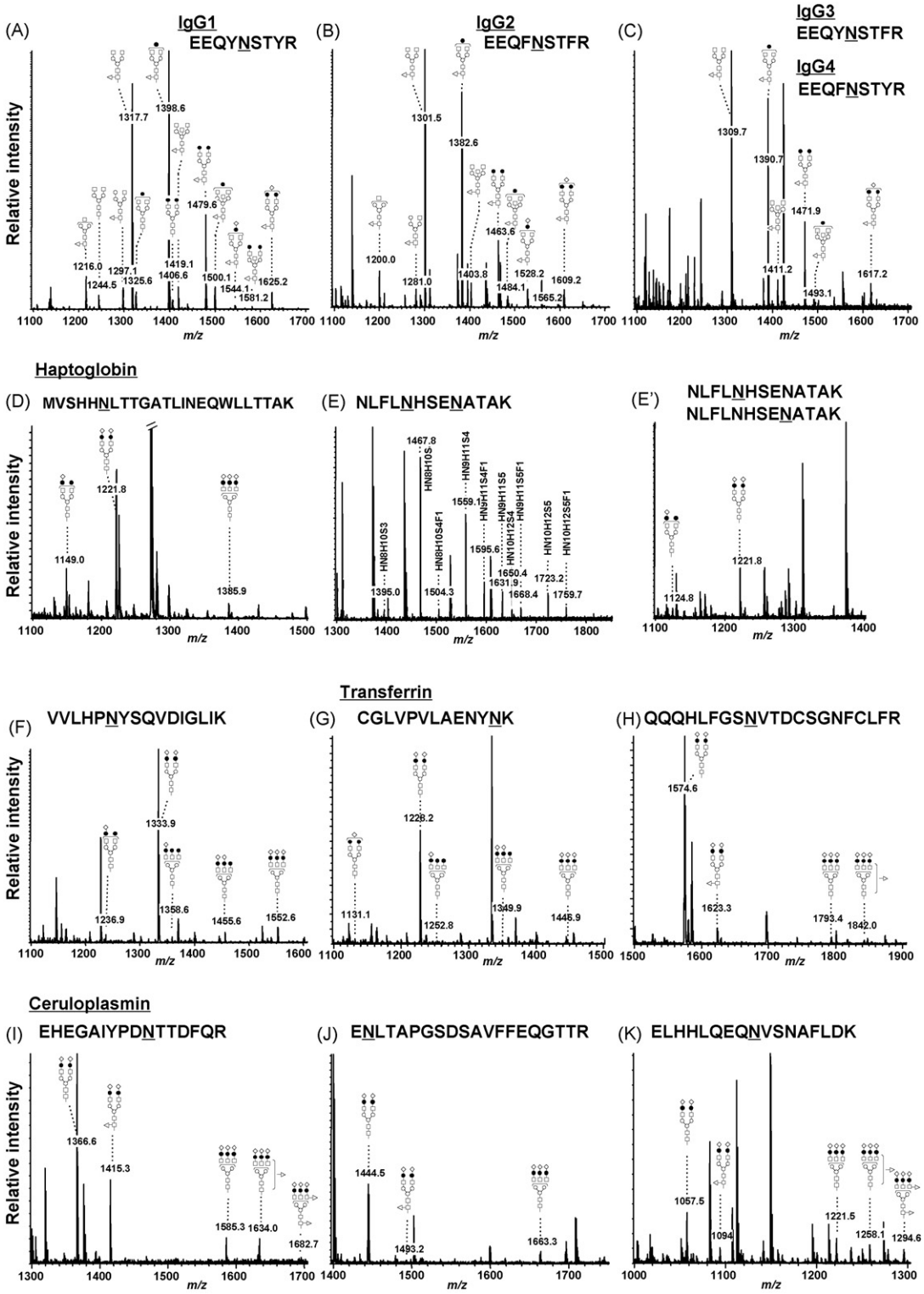

Fig. 7. Mass spectrometric heterogeneity of glycosylation in abundant serum glycoproteins. Integrated mass spectra obtained by the additional LC/MS of human serum digest. (A) IgG1; (B) IgG2; (C) IgG3/IgG4; (D–F) haptoglobin ((E) diglycosylated; (E′), monoglycosylated); (G and H) transferrin; (I–K) ceruloplasmin.

performed LC/MS(/MS) with high resolution separation to obtain mass spectrometric glycosylation profiles at each glycosylation site of abundant glycoproteins in human serum.

MS/MS spectra are useful for detection and assignment of glycopeptide ions. Because MS/MS spectra of glycopeptide precursor ions have abundant carbohydrate B-ions, such as m/z 204 ($[HexNAc+H]^+$), and 366 ($[HexHexNAc+H]^+$), presence of these ions is a useful indication of the selection of glycopeptide precursor ions. MS/MS spectra of glycopeptide also contain ions of peptide and peptide plus glycans and several *b*- and *y*-series fragment ions of peptide backbone when using Qq-TOF mass spectrometer. These allow us to differentiate the glycopeptide ions with differ-

ent peptide backbone and further to deduce peptide containing N-linked glycosylation sites by database search. When MS/MS spectra of the glycopeptides obtained in a data-dependent manner were poor for detection of peptide fragment ions, improvement of MS/MS spectra quality by integrating several similar MS/MS spectra into one spectrum was effective. Composition of attached glycan can be deduced from molecular weight of glycan. Utilizing the data of site-specific glycosylation analysis of commercial glycoproteins (IgG, haptoglobin and ceruloplasmin) allowed us assign the corresponding glycopeptides in complex LC/MS(/MS) chromatogram.

We preliminary performed LC/MS/MS of serum tryptic digest to locate glycopeptides and assign by data-dependent MS/MS. Using LC retention time, accurate *m*/*z* and charge state of assigned glycopeptides, we successfully determined mass spectrometric heterogeneity of 23 glycosylation sites in 15 glycoproteins by LC/MS analyze using digest corresponding 0.3 μl of serum. Although there have been many reports on the analysis of human serum digest to show the glycosylation sites of abundant serum glycoproteins [30–33], less has been reported on their glycosylation. Glycopeptides detected in this study were those derived from glycoproteins which are present at about 0.2–5 mg/ml in human serum, and only glycopeptides with higher ionization efficiency were detected. Thus, it was suggested that detection limit of our method without sample enrichment procedure would be >0.2 mg/ml. It was thought that sample comlexicity, ionization suppression of low abundant glycopeptides and necessity of high quality of MS/MS spectrum for database searching reduced sensitivity. In order to characterize more glycosylation sites, combination of glycopeptide enrichment and depletion of abundant serum proteins is needed.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare, a Grant-in-Aid from the Ministry of Education, Culture, Sports and Technology, and Core Research for the Evolutional Science and Technology Program (CREST), Japan Science and Technology Corp (JST).

References

[1] A. Varki, Glycobiology 3 (1993) 97.
[2] Z. Yang, W.S. Hancock, J. Chromatogr. A 1053 (2004) 79.
[3] R.B. Parekh, R.A. Dwek, B.J. Sutton, D.L. Fernandes, A. Leung, D. Stanworth, T.W. Rademacher, T. Mizuochi, T. Taniguchi, K. Matsuta, et al., Nature 316 (1985) 452.
[4] L.A. Omtvedt, L. Royle, G. Husby, K. Sletten, C.M. Radcliffe, D.J. Harvey, R.A. Dwek, P.M. Rudd, Arthr. Rheum 54 (2006) 3433.
[5] R. Dube, G.A. Rook, J. Steele, R. Brealey, R. Dwek, T. Rademacher, J. Lennard-Jones, Gut 31 (1990) 431.
[6] H.H. Freeze, Glycobiology 11 (2001) 129R.
[7] M. Ferens-Sieczkowska, K. Zwierz, A. Midro, I. Katnik-Prastowska, Arch. Immunol. Ther. Exp. (Warsz) 50 (2002) 67.
[8] S. Thompson, E. Dargan, G.A. Turner, Cancer Lett. 66 (1992) 43.
[9] R. Saldova, L. Royle, C.M. Radcliffe, U.M. Abd Hamid, R. Evans, J.N. Arnold, R.E. Banks, R. Hutson, D.J. Harvey, R. Antrobus, S.M. Petrescu, R.A. Dwek, P.M. Rudd, Glycobiology 17 (2007) 1344.
[10] Y. Otake, I. Fujimoto, F. Tanaka, T. Nakagawa, T. Ikeda, K.K. Menon, S. Hase, H. Wada, K. Ikenaka, J. Biochem. (Tokyo) 129 (2001) 537.
[11] B. Kossowska, M. Ferens-Sieczkowska, R. Gancarz, E. Passowicz-Muszynska, R. Jankowska, Clin. Chem. Lab. Med. 43 (2005) 361.
[12] G.A. Turner, Adv. Exp. Med. Biol. 376 (1995) 231.
[13] N. Okuyama, Y. Ide, M. Nakano, T. Nakagawa, K. Yamanaka, K. Moriwaki, K. Murata, H. Ohigashi, S. Yokoyama, H. Eguchi, O. Ishikawa, T. Ito, M. Kato, A. Kasahara, S. Kawano, J. Gu, N. Taniguchi, E. Miyoshi, Int. J. Cancer 118 (2006) 2803.
[14] M.A. Comunale, M. Lowman, R.E. Long, J. Krakover, R. Philip, S. Seeholzer, A.A. Evans, H.W. Hann, T.M. Block, A.S. Mehta, J. Proteome Res. 5 (2006) 308.
[15] J.E. Hansen, J. Iversen, A. Lihme, T.C. Bog-Hansen, Cancer 60 (1987) 1630.
[16] R.R. Drake, E.E. Schwegler, G. Malik, J. Diaz, T. Block, A. Mehta, O.J. Semmes, Mol. Cell Proteomics 5 (2006) 1957.
[17] Y. Wada, M. Tajiri, S. Yoshida, Anal. Chem. 76 (2004) 6560.
[18] M. Tajiri, S. Yoshida, Y. Wada, Glycobiology 15 (2005) 1332.
[19] P. Hagglund, J. Bunkenborg, F. Elortza, O.N. Jensen, P. Roepstorff, J. Proteome Res. 3 (2004) 556.
[20] J.F. Nemeth, G.P. Hochgesang Jr., L.J. Marnett, R.M. Caprioli, Biochemistry 40 (2001) 3109.
[21] J.P. Hui, T.C. White, P. Thibault, Glycobiology 12 (2002) 837.
[22] K. Hakansson, H.J. Cooper, M.R. Emmett, C.E. Costello, A.G. Marshall, C.L. Nilsson, Anal. Chem. 73 (2001) 4530.
[23] O. Krokhin, W. Ens, K.G. Standing, J. Wilkins, H. Perreault, Rapid Commun. Mass Spectrom. 18 (2004) 2020.
[24] U.M. Demelbauer, M. Zehl, A. Plematl, G. Allmaier, A. Rizzi, Rapid Commun. Mass Spectrom. 18 (2004) 1575.
[25] M. Wuhrer, C.H. Hokke, A.M. Deelder, Rapid Commun. Mass Spectrom. 18 (2004) 1741.
[26] A. Harazono, N. Kawasaki, T. Kawanishi, T. Hayakawa, Glycobiology 15 (2005) 447.
[27] S. Itoh, N. Kawasaki, A. Harazono, N. Hashii, Y. Matsuishi, T. Kawanishi, T. Hayakawa, J. Chromatogr. A 1094 (2005) 105.
[28] A. Harazono, N. Kawasaki, S. Itoh, N. Hashii, A. Ishii-Watabe, T. Kawanishi, T. Hayakawa, Anal. Biochem. 348 (2006) 259.
[29] Y. Wada, P. Azadi, C.E. Costello, A. Dell, R.A. Dwek, H. Geyer, R. Geyer, K. Kakehi, N.G. Karlsson, K. Kato, N. Kawasaki, K.H. Khoo, S. Kim, A. Kondo, E. Lattova, Y. Mechref, E. Miyoshi, K. Nakamura, H. Narimatsu, M.V. Novotny, N.H. Packer, H. Perreault, J. Peter-Katalinic, G. Pohlentz, V.N. Reinhold, P.M. Rudd, A. Suzuki, N. Taniguchi, Glycobiology 17 (2007) 411.
[30] J. Bunkenborg, B.J. Pilch, A.V. Podtelejnikov, J.R. Wisniewski, Proteomics 4 (2004) 454.
[31] T. Liu, W.J. Qian, M.A. Gritsenko, D.G. Camp 2nd, M.E. Monroe, R.J. Moore, R.D. Smith, J. Proteome Res. 4 (2005) 2070.
[32] Y. Wang, S.L. Wu, W.S. Hancock, Glycobiology 16 (2006) 514.
[33] P. Hagglund, R. Matthiesen, F. Elortza, P. Hojrup, P. Roepstorff, O.N. Jensen, J. Bunkenborg, J. Proteome Res. 6 (2007) 3021.