Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 513–520

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Glycosylation characterization of Human IgA1 with differential deglycosylation by UPLC–ESI TOF MS

Song C. Klapoetke*, Jian Zhang, Steven Becht

cGMP Lab, PPD Inc, 8551 Research Way, Suite 90, Middleton, WI 53562, United States

ARTICLE INFO

Article history:
Received 11 February 2011
Received in revised form 16 May 2011
Accepted 15 June 2011
Available online 22 June 2011

Keywords:
Glycosylation analysis
Human IgA1
Differential deglycosylation
LC-TOF MS
Glycan

ABSTRACT

Differential deglycosylation was introduced as an effective technique to characterize glycosylation in glycoprotein containing both N-linked and O-linked glycans at both protein and peptide levels. Human IgA1 was used as a model glycoprotein to demonstrate this technique. The glycans attached to Human IgA1 were removed from their attachment sites by an array of enzymes. After reduction by DTT, the resulting deglycoproteins were analyzed by UPLC–ESI TOF MS to estimate the numbers of N-glycan and O-glycan sites through differential masses. The deglycoproteins and unmodified glycoprotein were further digested to deglycopeptide through trypsin digestion. The glycopeptides and deglycopeptides were identified by UPLC–ESI TOF MS. Two N-glycan and four O-glycan sites were identified and confirmed at peptide levels. These results matched those from deglycoproteins. The N-glycosylation site and N-glycan sequence confirmation were also demonstrated in this study.

1. Introduction

Glycosylation represents the most pronounced and most complex post-translational modification of biopharmaceutical proteins. The functional activity of therapeutic glycoproteins frequently depends on their glycosylation. Therefore, glycosylation analysis is a vital part of the characterization of biopharmaceutical drug products.

In recent years, mass spectrometry (MS) has emerged as the most sensitive and specific technique in glycosylation analysis. Recent technical improvements resulted in a variety of successful analysis of glycosylated proteins such as α1-acid glycoprotein [1], recombinant IgG1 [2] and bacterial flagellin [3]. To characterized glycosylation, currently, the glycoprotein is first cleaved to glycopeptides by a protease (e.g., trypsin, chymotrypsin, Glu-C, or Lys-C). After subjecting the glycopeptides to enrichment and/or deglycosylation, the resulting glycopeptides, deglycopeptides and released glycans are analyzed by mass spectrometry for assignment of the glycosylation sites and glycan composition [4–9]. Numerous studies on glycosylation analysis have been reported with subtle difference using this strategy. For example, Segu and colleages mapped N-glycosylation sites by using a cocktail of endo- and exo-glycosidases (PNGase F, Endo-M, Endo-M/exoglycosidases) after trypsin digestion. LCMS analysis obtained a mass shift of 203 Da when endo-M was used at the N-glycosylation sites [10]. Wang et al. [7] identified the N-glycosylation sites by using PNGase F to remove N-glycans from the binding sites after trypsin digestion. GluC digestion was also incorporated to achieve additional cleavage for peptide containing two potential N-glycosylation sites. Tzur et al. introduced a two-dimensional array for both N- and O-glycans sequencing and site mapping [11]. The glycoprotein was digested to glycopeptides by trypsin then captured by lectin-array. These glycopeptides were also treated by a series of exoglycosidases then captured by lectin-array. The material trapped on lectin plates were analyzed by MALDI MS. By tracking how the target glycopeptides were retained on the lectin plate with their exoglycosidases treatment history, the glycan sequence information can be obtained.

In all these cases, the glycosylation characterization was only studied at the peptide level. With improved mass resolution, mass accuracy and sensitivity, it is possible to acquire accurate glycoprotein mass information by MS and characterize glycoprotein at the protein level. In this study, a new approach for glycosylation analysis is proposed by using a differential deglycosylation technique. The glycoprotein is firstly deglycosylated by an array of enzymes into deglycoproteins that can be analyzed by MS for mass information. The deglycoproteins are further digested into deglycopeptides by a protease and analyzed by MS. With this new strategy, the glycoprotein is studied at both protein and peptide levels for both N- and O-glycan sites and compositions. Data from both levels are orthogonal. To the best of our knowledge, this is the first application using this strategy on glycosylation analysis containing both N- and O-glycans.

* Corresponding author. Tel.: +1 608 345 2482; fax: +1 608 662 0395.
E-mail addresses: Song.Klapoetke@ppdi.com, Song.Klapoetke@gmail.com (S.C. Klapoetke).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.06.010

Human IgA1 was selected as a model glycoprotein to demonstrate this technique. Human IgA1 antibody is a well characterized glycoprotein with both N-linked and O-linked glycans [12–18]. Its primary structure and sequence are known and can be found in the RCSB protein data bank [19]. Briefly, Human IgA1 antibody is a tetrameric glycoprotien (approximately 160 kDa) composed of two identical heavy chains and two identical light chains linked to each other by disulfide bonds. The 12-domain structure of IgA1 is similar to that seen in IgG but with heavier glycosylation [12,18].

In this investigation, mass information was acquired from the desialylated, N-deglycosylated and O-deglycosylated forms of the protein. These data together were used to determine the number of N-linked and O-linked glycosylation sites. Furthermore, the glycosylation sites and glycan identification were confirmed through differential mass information generated from both deglycopeptide and intact glycopeptide by MS experiments. Removal of an N-glycan attached to asparagine leads to conversion of the asparagine to aspartic acid with a +1 mass shift. This mass shift was used to track N-glycosylation site by MS experiments. On the other hand, no mass change occurs when an O-glycan is removed from its attached amino acid (serine or threonine). The experimental design is summarized in Fig. 1.

MSMS experiment was performed to demonstrate the verification of N-glycan sequence using one dominant form of N-glycan as example. MSMS experiment was also used to confirm N-linked glycosylation sites.

To simplify the discussion, the heavy chains are designated as HC and the tryptic peptides of the heavy chains are designated as HT with fragment number. For example, HT19 represents fragment 19 of trypsin-cleaved peptide from the heavy chains. Deglycopeptide is used as a general term for a glycopeptide in which the glycans have been partially or completely removed. Other terms will be used in this study if specific treatment is involved. For example, desialylated peptide means glycopeptide with sialic acid removed. Similar terminology will be also used for glycoprotein.

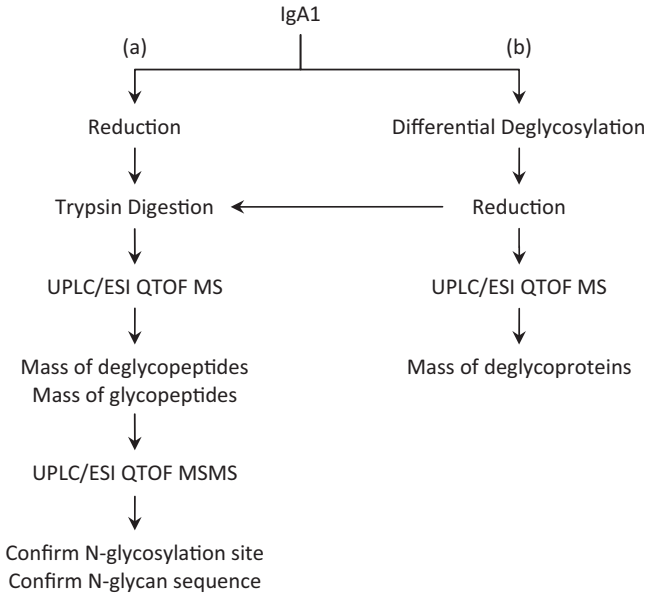

Fig. 1. Experimental design.

2. Materials and methods

2.1. Materials

All the reagents were of analytical reagent grade unless stated otherwise. Purified water was obtained from an in-house Milli-Q system. HPLC grade acetonitrile (ACN) was used for mobile phase preparation. PNGase F, sialidase and O-glycanase, were purchased from Prozyme (Hayward, CA). O-Glycanase can specifically remove the core structure of GalNAc-Gal intact form from serine or threonine after other monosacchlorides have been removed. Trypsin was purchased from Promega (Madison, WI). IgA1 was purchased from Antibody Research Corporation (St. Charles, MO).

2.2. Sample preparation

The N-glycan, sialic acid and O-glycan with disaccharides sequence as GalNAc-Gal were enzymatically removed from their respective glycosylation sites in IgA1 using an enzymatic deglycosylation kit (product code GK80115) from Prozyme (Hayward, CA). The vendor's procedure was used for the deglycosylation. Briefly, about 100 μg of IgA1 was reconstituted in 10 μL buffer provided in the kit (0.25 M sodium phosphate, pH 7.0), followed by the addition of enzyme before diluted to 50 μL with water. The enzymes were added as following: (a) 1 μL of sialidase (to remove sialic acids); (b) 1 μL PNGase F (to remove N-linked glycans); (c) 1 μL of sialidase and 1 μL of PNGase F (to remove sialic acid from the O-glycans and N-glycans); (d) 1 μL of sialidase, 1 μL of PNGase F and 1 μL of O-glycanase (to remove both N-linked and O-linked glycans). Therefore four types of solutions were prepared. Each solution was incubated for at least 40 h at 37 °C. The samples were then reduced with 40 mM DTT in 50 mM ammonium bicarbonate at 37 °C for 1 h (sample: DTT = 1:1, v/v). This resulted in a 1 mg/mL deglycosylated protein solution that was ready for analysis.

To confirm the glycosylation sites and glycan sequence, 20 μg of each of the above samples were digested with 1 μg trypsin (enzyme:protein = 1:20, w/w) in 50 μL 50 mM ammonium bicarbonate, diluted to final concentration of 0.2 mg/mL with water. The samples were incubated overnight at 37 °C. A reduced IgA1 sample was also digested without deglycosylation. This yielded deglycosylated/glycosylated peptides that were ready for analysis.

2.3. UPLC instrument and conditions

The deglycosylated proteins were analyzed on a Waters (Milford, MA) Acquity UPLC with UV detector. Liquid chromatographic separations for light chains and heavy chains were achieved with a gradient program by a Waters Acquity BEH C4 column (50 mm × 2.1 mm, 1.7 μm, part number 186004495) at 40 °C. The starting eluent was a 98:2 mixture of water with 0.1% formic acid (Mobile Phase A) and ACN with 0.1% formic acid (Mobile Phase B). The proportion of Mobile Phase B was increased linearly to 90% over 23 min after initial 2 min of holding. The column was washed with Mobile Phase B for 7 min before the eluent was returned to its initial composition. The column was allowed to re-equilibrate for 3 min prior to starting the next analysis. The flow rate is 0.25 mL/min with an injection volume of 5 μL.

The deglycosylated peptides prepared by trypsin digestion were analyzed on a Waters Acquity UPLC with UV detector. Liquid chromatographic separations for trypsin digestion products were achieved with a gradient program by a Waters Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm, product number 186003686) at 40 °C. The starting eluent was 98:2 mixture of water with 0.1% formic acid (Mobile Phase A) and ACN with 0.1% formic acid (Mobile Phase B). The proportion of Mobile Phase B was increased linearly to 50% over 90 min after initial 2 min of holding. The proportion of Mobile Phase B was then increased linearly to 98% over 2 min and held for 2 min. The eluent was returned to its initial composition in 2 min. The column was allowed to re-equilibrate for 2 min prior to

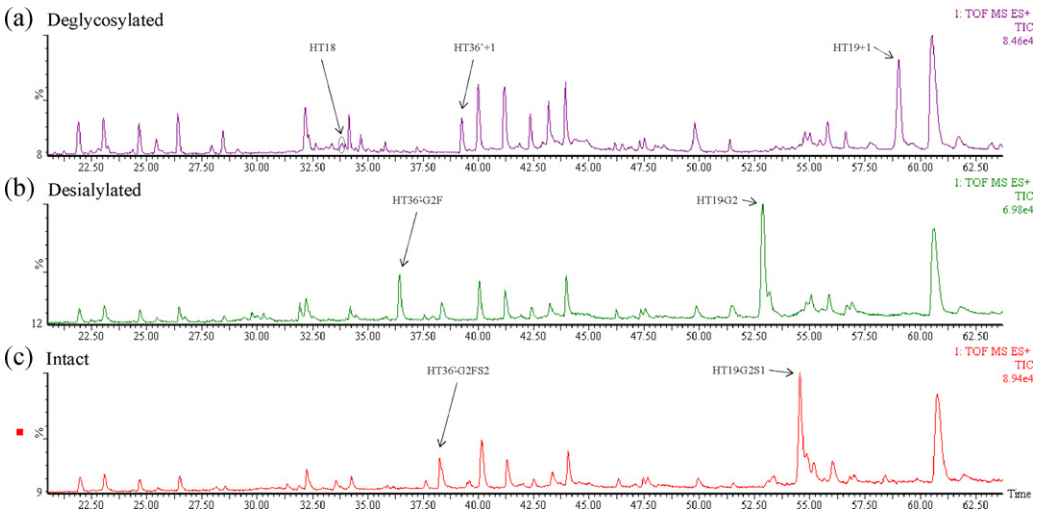

Fig. 2. UPLC/ESI TIC IgA1 tryptic peptide profiles from before and after differential deglycosylation. Panel (a): sample with both O-linked and N-linked glycans removed; panel (b): sample with sialic acids removed; panel (c) intact sample. All samples were reduced before trypsin digestion.

starting the next analysis. The flow rate was 0.20 mL/min with an injection volume of 5 μL.

Acquisition and analysis of UPLC-UV data were performed by using Waters MassLynx (version 4.1).

2.4. ESI-QTOF instrument and conditions

The mass spectrometer (Waters QTOF Premier), equipped with an electrospray source and lockspray, was run in positive mode (ES+). The sample eluted from the UPLC column was directed to the QTOF after UV detector. Mass spectrometric data was acquired in MS scan mode. Data acquisition and analysis were performed using Waters MassLynx (version 4.1). Mass spectrometer settings for MS analysis were as follows: capillary voltage 3.0 kV, cone voltage 25 V, source temperature 110 °C, desolvation temperature 350 °C, collision energy 5.0 V, scan range 500–3000 for deglycoprotein and 50–3000 for tryptic digestion samples. The MSMS experiments were performed in MSE mode and a collision energy ramp from 20 to 50 V was applied to precursors.

3. Results and discussion

Due to the high degree of heterogeneity in the molecule, the protein charge state patterns for intact IgA1 and reduced IgA1 samples were either too weak to be deconvoluted or the deconvolution results were not reliable. Reducing this heterogeneity through deglycosylation is a necessary step for obtaining reliable mass information. Because all of the IgA1 glycosylations are located in the constant regions of the heavy chains, this investigation focuses on the mass information obtained from the heavy chains.

3.1. Deglycoprotein MS results

The molecular weights of the desialylated, N-deglycosylated, N-deglycosylated and desialylated and fully deglycosylated forms of the heavy chains are presented in Table 1. The molecular weight of a main component was obtained for each sample, as well as several minor components due to heterogeneity in the attached glycans. For the samples with sialic acid removed (HC-SA, HC-N-SA and HC-N-SA-O in Table 1), the mass differences among these com-

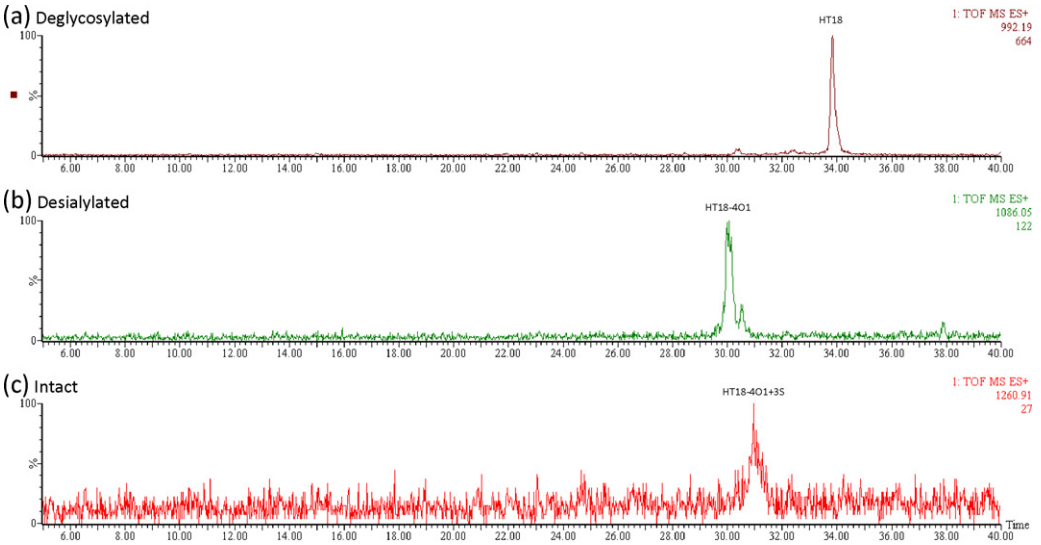

Fig. 3. UPLC/ESI XIC of HT18 peptide from before and after differential deglycosylation. Panel (a): extracted ion at $m/z = 992.19^{4+}$ as expected ion for HT18 with O-glycans removed; panel (b): extracted ion at $m/z = 1086.05^{5+}$ as expected ion for glycosylated HT18 with sialic acids removed; panel (c): extracted ion at $m/z = 1260.91^{5+}$ as expected ion for main glycoform of glycosylated HT18 with four O-glycans (each with O1 as core structure) with total of three sialic acids.

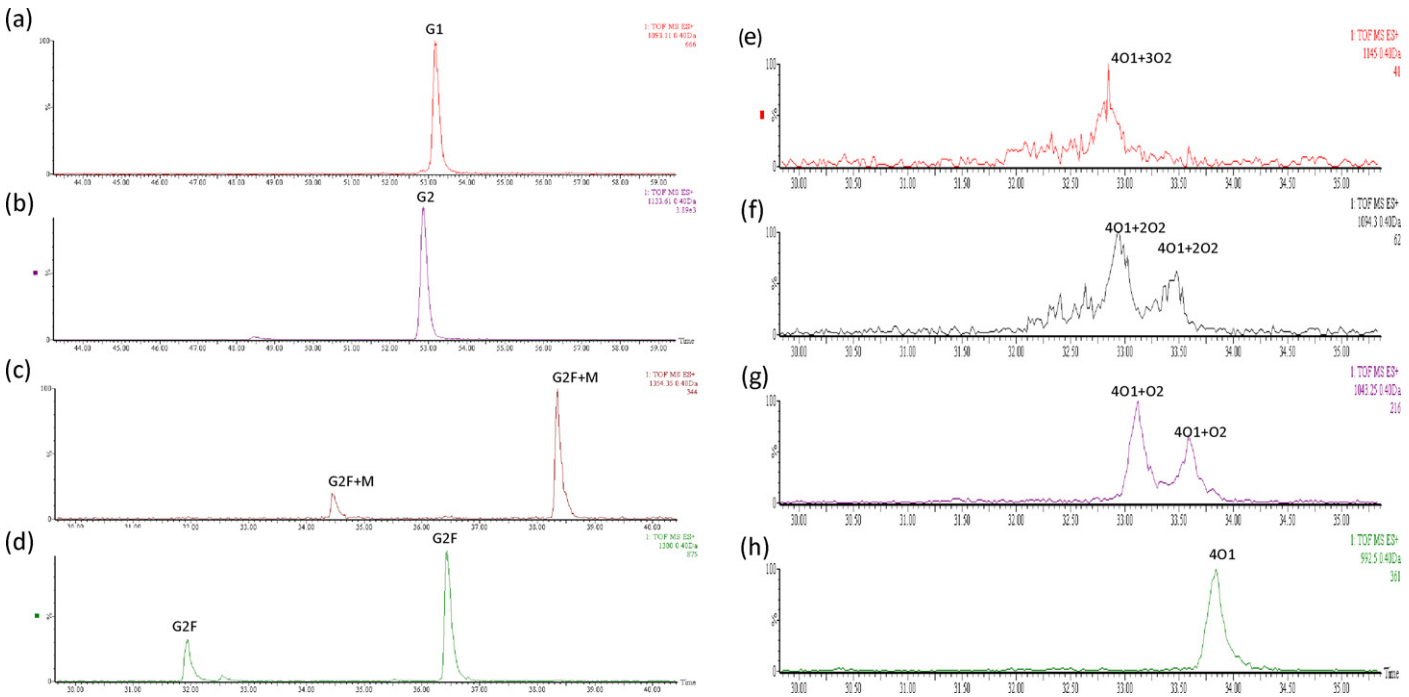

Fig. 4. UPLC/ESI XIC of detectable glycoforms from desialylated glycopeptides. (a) Extracted ion at $m/z = 1093.11^{4+}$ as expected ion for G1 with intensity of 666 from HT19; (b) extracted ion at $m/z = 1133.61^{4+}$ as expected ion for G2 with intensity of 3890 from HT19; (c) extracted ion at $m/z = 1354.35^{3+}$ as expected ion for G2F + M with intensity of 162 as the more intense peak from HT36′; (d) extracted ion at $m/z = 1300.40^{3+}$ as expected ion for G2F with intensity of 875 as the more intense peak from HT36′; (e) extracted ion at $m/z = 1145.04^{4+}$ as expected ion for 4O1 + 3O2 with intensity of 41 as the more intense peak from HT18; (f) extracted ion at $m/z = 1094.30^{4+}$ as expected ion for 4O1 + 2O2 with intensity of 62 as the more intense peak from HT18; (g) extracted ion at $m/z = 1043.25^{4+}$ as expected ion for 4O1 + O2 with intensity of 216 as the more intense peak from HT18; (h) extracted ion at $m/z = 992.50^{4+}$ as expected ion for. 4O1 with intensity of 361 from HT18. All glycoforms were extracted from the most intense isotopic peak mass and not the monoisotopic peak mass.

ponents were either 162 Da or 203 Da (within ±5 Da or 0.01% mass accuracy). In some cases, multiple series of these components were observed in one sample. With samples containing sialic acids (HC-N in Table 1) additional 291 Da mass differences were also observed.

Using the differential mass from each main component, the masses of the desialylated N-glycan, desialylated O-glycan, intact O-glycan and total sialic acids attached to O-glycans were calculated and reported in Table 2. According to Arnold et al.'s review, the N-glycans of human immunoglobulins are predominantly complex glycans with some high mannose glycans ranging in mass from 1216.42 (Man-5) to 1971.72 (G2FB) for the desialylated forms [20] (also see their glycan composition and residue mass in Table 3). Therefore, in the Human IgA1 sample, the number of N-glycan sites is most likely two since the total desialylated glycan mass of 3391 Da is too heavy for one glycan and too light for three glycans. For the desialylated O-glycans, there are four potential O-glycan sites with a core structure of GalNAc-Gal for a total glycan mass of 1460 Da (365 each, $1460 = 4 \times 365$) as reported in Table 2 (the actual mass was 1464 Da within ±5 Da mass accuracy). Intact O-glycans mass was also calculated as 2335 Da, so three sialic acids were present among all O-glycan sites. Due to microheterogeneity, these results only represent the main glycoforms in Human IgA1.

The molecular weight of light chains was 23,405 Da from all four deglycosylated samples, which confirmed that glycosylation is restricted to the heavy chains.

3.2. Glycopeptides and deglycopeptide MS results

Glycopeptide and deglycopeptide MS results are presented in Table 4. Molecular weights of the three types of peptides were acquired as intact, desialylated and deglycosylated forms. At the peptide level, obvious retention time shifts were observed within the three types of samples as presented in Table 4 and Fig. 2. Removal of the terminal sialic acids reduces the retention time of the peptide fragments. On the other hand, removal of entire glycans increases the retention time. With the known

Table 1
Human IgA1 heavy chain molecular weight after an array of deglycosylations.

Glycoprotein	HC-SA (m_1)	HC-N (m_2)	HC-N-SA (m_3)	HC-N-SA-O (m_4)
Main component mass (Da)	55,606	53,086	52,215	50,751
Minor components mass (Da)	55,407	52,216	52,255	50,913
	55,807	51,418	52,376	50,953
	55,968	53,505	52,416	51,115
	56,176	52,662	52,577	51,318
		52,708		
		52,793		
		52,869		
		52,997		

HC-SA: sample treated with sialidase; HC-N: sample treated with PNGase F; HC-N-SA: sample treated with both sialidase and PNGase F; HC-N-SA-O: sample treated with sialidase, O-glycanases and PNGase F. All samples were reduced after deglycosylation; m_1 = mass of desialylated heavy chain; m_2 = mass of N-deglycosylated heavy chain; m_3 = mass of desialylated and N-deglycosylated heavy chain; m_4 = mass of both N- and O-deglycosylated heavy chain.

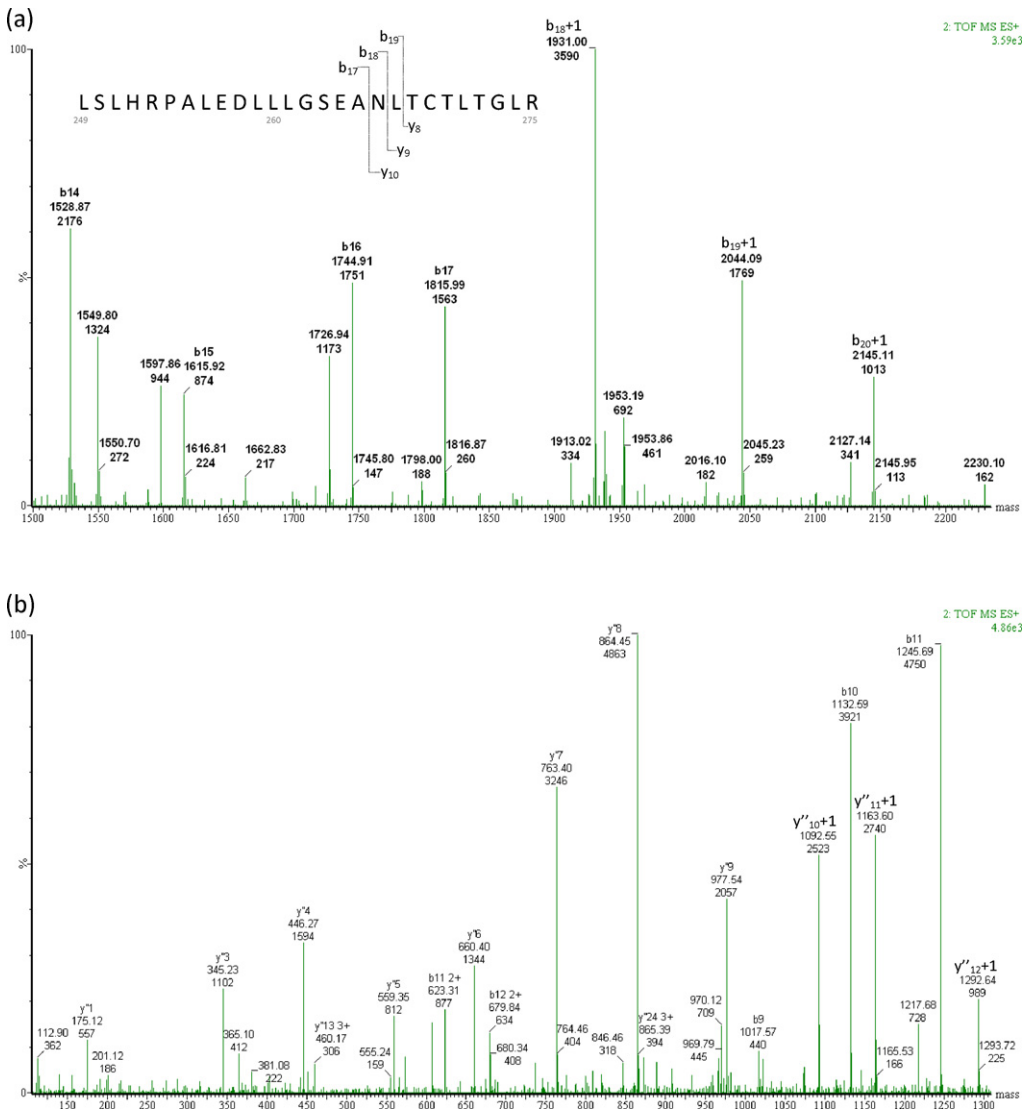

Fig. 5. ESI + MSMS spectra of HT19 around the Asn266 site at retention time of 59.1 min. (a) b ions, the expected b18 ion as $m/z = 1930.00^+$ was not found. Instead a +1 Da ion ($m/z = 1931.00^+$) was found. Similar shifts were observed for b19 and b20 (labeled as b19 + 1 and b20 + 1); (b) y ions, the expected y10 ion ($m/z = 1091.55^+$) was not found. Instead a +1 Da ion ($m/z = 1092.55^+$) was found. Similar shifts were observed for y11 and y12 (labeled as y11 + 1 and y12 + 1).

amino acid sequence and specificity of tryptic digestions [19], the deglycosylated peptide mass results can be tracked. For the deglycosylated samples in Table 4 and Fig. 2, the peak at about 59.2 min is the N-deglycopeptide HT19 with +1 mass shift from the theoretical mass while the peak at about 39.4 min is the other N-deglycopeptide HT36 with +1 mass shift and C-terminal tyrosine truncated (referred to as HT36′ in the following section). The +1 mass shift is due to the conversion of the asparagines to aspartic acid mediated by deglycosylation (the mass accuracy for the data is <±0.1 Da). The peak at about 34.2 min is the O-deglycopeptide HT18 without any mass shift from the theoretical mass. Due to poor ionization efficiency from multiple glycosylation sites on HT18, the intact peptides and desialylated peptides were not able to be visually tracked as were the N-glycopeptides HT19 and HT36′ seen in Fig. 2. Their existence can be tracked on extracted ion chromatogram instead (see in Fig. 3).

Using the differential masses from the three types of samples, the masses of attached main glycan forms from each glycopep-

Table 2
Differential mass of main component after an array of deglycosylations.

Glycoprotein	Differential mass	Results (Da)	Estimated main glycoforms
Desialylated O-glycans	$\Delta M_1 = m_3 - m_4$	1464	4 O-glycans
Desialylated N-glycans	$\Delta M_2 = m_1 - m_3$	3391	2 N-glycans
Intact O-glycans	$\Delta M_3 = m_2 - m_4$	2335	4 O-glycanswith3SA
Total sialic acids from O-glycan	$\Delta M_4 = m_2 - m_3$	871	3SA

SA: sialic acid. The estimation was based on the main component masses and therefore the glycoforms represent the main components. m_1 = mass of desialylated heavy chain; m_2 = mass of N-deglycosylated heavy chain; m_3 = mass of desialylated and N-deglycosylated heavy chain; m_4 = mass of both N- and O-deglycosylated heavy chain; ΔM_1 = residue mass of total O-glycans in desialylated form; ΔM_2 = residue mass of total N-glycans in desialylated form; ΔM_3 = residue mass of total O-glycans; ΔM_4 = residue mass of total sialic acid attached to O-glycans.

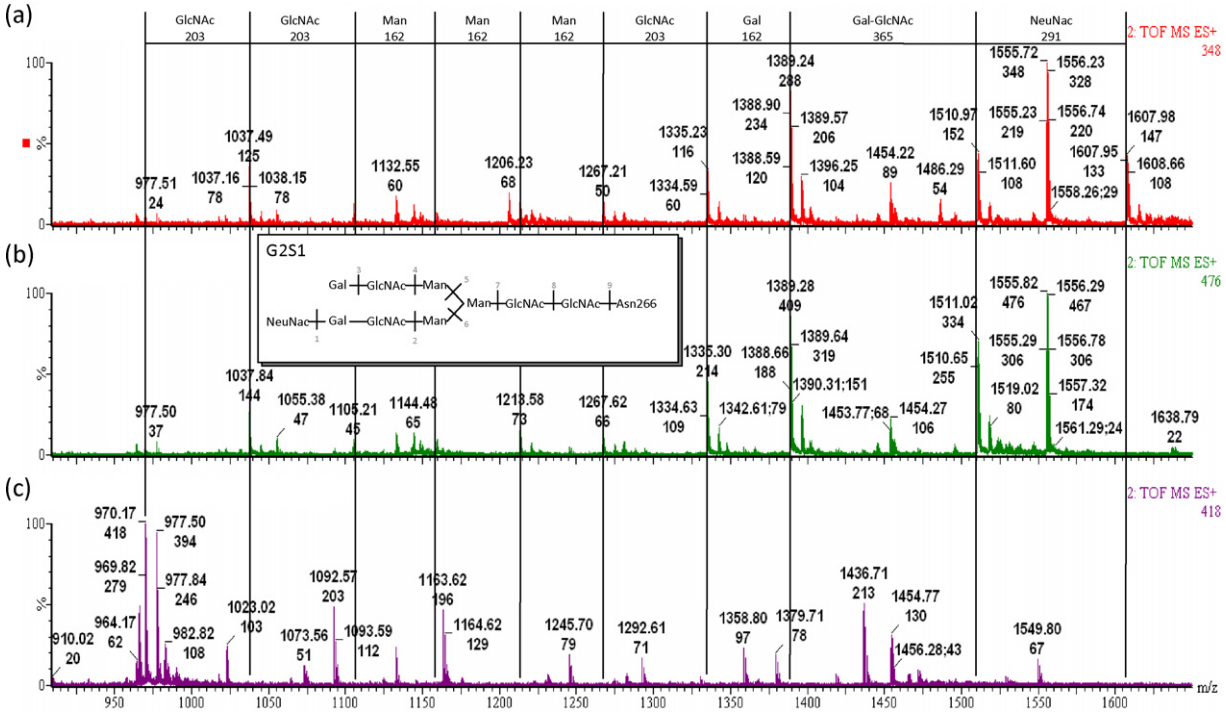

Fig. 6. ESI + MSMS spectra of HT19 glycopeptide before and after differential deglycosylation. (a) Intact at 54.5 min; (b) desialylated at 52.9 min; and (c) deglycosylated at 59.1 min. The figure shows the sequential cleavage of glycosidic bonds to confirm glycan G2S1's sequence (from $m/z = 1607.98^{3+}$ to 970.17^{3+}). Peak with m/z of 1555.72^{2+} has same mass as the peak with m/x of 1037.49^{3+}. See Fig. 7 for expanded scale.

Table 3
Glycan naming convention and theoretical mass.

Glycan	Monosaccharide composition	Residue mass	Code
1	$Man_5GlcNAc_2$	1216.42	Man-5
2	$Man_3GlcNAc_4$	1298.48	G0
3	$Gal_1Man_3GlcNAc_4$	1460.53	G1
4	$Gal_2Man_3GlcNAc_4$	1622.58	G2
5	$NeuNac_1Gal_2Man_3GlcNAc_4$	1913.68	G2S1
6	$Gal_2Man_3GlcNAc_4Fuc_1$	1768.64	G2F
7	$Gal_2Man_4GlcNAc_4Fuc_1$	1930.69	G2F + M
8	$NeuNac_2Gal_2Man_3GlcNAc_4Fuc_1$	2350.83	G2FS2
9	$Gal_1GalNAc_1$	365.13	O1
10	$GalNAc_1$	203.08	O2
11	$Man_9GlcNAc_2$	1864.63	Man-9
12	$Gal_2Man_3GlcNAc_5Fuc_1$	1971.72	G2FB

Note: The residue mass is calculated from respective monosaccharide composition. The monosaccharide composition assignment is based on previous study on human immunoglobulins and Human IgA1 [15–18,20].

Table 4
Mass of main glycopeptide after an array of deglycosylation and tryptic digestion.

Glycopeptide	HT18	HT19	HT36′
Theoretical			
(m/z)	992.21^{4+}	969.53^{3+}	1064.03^{2+}
Intact			
RT (min)	31.36	54.55	38.24
m_5 (m/z)	1260.91^{5+}	1205.79^{4+}	1493.73^{3+}
Desialylated			
RT (min)	30.28	52.96	36.48
m_6 (m/z)	1086.05^{5+}	1133.01^{4+}	1299.73^{3+}
Deglycosylated			
RT (min)	34.17	59.20	39.41
m_7 (m/z)	992.19^{4+}	969.84^{3+}	1064.52^{2+}
$\Delta M_5 = m_7 -$ theoretical (Da)	0	1	1

HT18: HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCHPR (residues 211–248); HT19: LSLHRPALEDLLLGSEANLTCTLTGLR (residues 249–275); HT36′: LAGKPTHVNVSVVMAEVDGTC (residues 454–474, residue 475 = Y); m_5 = mass of intact glycopeptide; m_6 = mass of glycopeptide in desialylated form; m_7 = mass of deglycopeptide; ΔM_5 = mass difference between deglycopeptide and theoretical.

tide were calculated and reported in Table 5. For peptide HT19, the main glycan is an N-glycan with mass of 1622.52 Da, which represents the desialylated form as G2. One attached sialic acid (mass of 291 Da) is inferred from the mass difference between the intact HT19 and the desialylated HT19. Therefore, the tentative glycan assignment is G2S1 (see Table 3 for the glycan monsaccharides composition and residue mass). Similarly, for peptide HT36′, the main glycan is an N-glycan with mass of 1768.83 Da, which represents the desialylated form as G2F. Two attached sialic acids (mass of 2×291 Da) are inferred from the mass difference between the intact HT36′ and the desialylated HT36′. The tentative glycan assignment is G2FS2. The total glycan mass of 3391.35 Da (1622.52 Da + 1768.83 Da) as the N-glycan main glycoform is consistent with the results from deglycoprotein in Table 2. For peptide HT18, the main glycans were four O-linked glycans with a mass of 365 Da and disaccharide sequence of GalNAc-Gal as the core desialylated form (referred to as O1 core in the following sections) and an additional three sialic acids in the intact form. These results are also consistent with those from the deglycoprotein analysis in Table 2. According to Tarelli et al.'s study [16], an O-glycan with GalNAc only as the core structure was also observed in Human IgA1 (referred to as O2 core in the following sections). The O-glycanase used in this study can only cleave disaccharides sequence as GalNAc-Gal from the attached amino acids; therefore, the main glycoform was four O-glycans. Each of them contained an O1 core structure.

For each glycosylation site, there is more than one peak corresponding to the respective glycopetides as presented in Fig. 4 and Table 5. Two, four and six glycoforms were found at the Asn_{266} site, Asn_{462} site and HT18 O-glycopeptide, respectively, as desialylated form. This illustrates the heterogeneity on N-glycosylation sites and up to two additional O2 core O-glycans sites. Desialylated glycopeptides were used to identify potential glycoforms for each glycosylation site. At the intact glycopeptide level, glycoforms at each glycosylation site could be further modified by sialylation. The

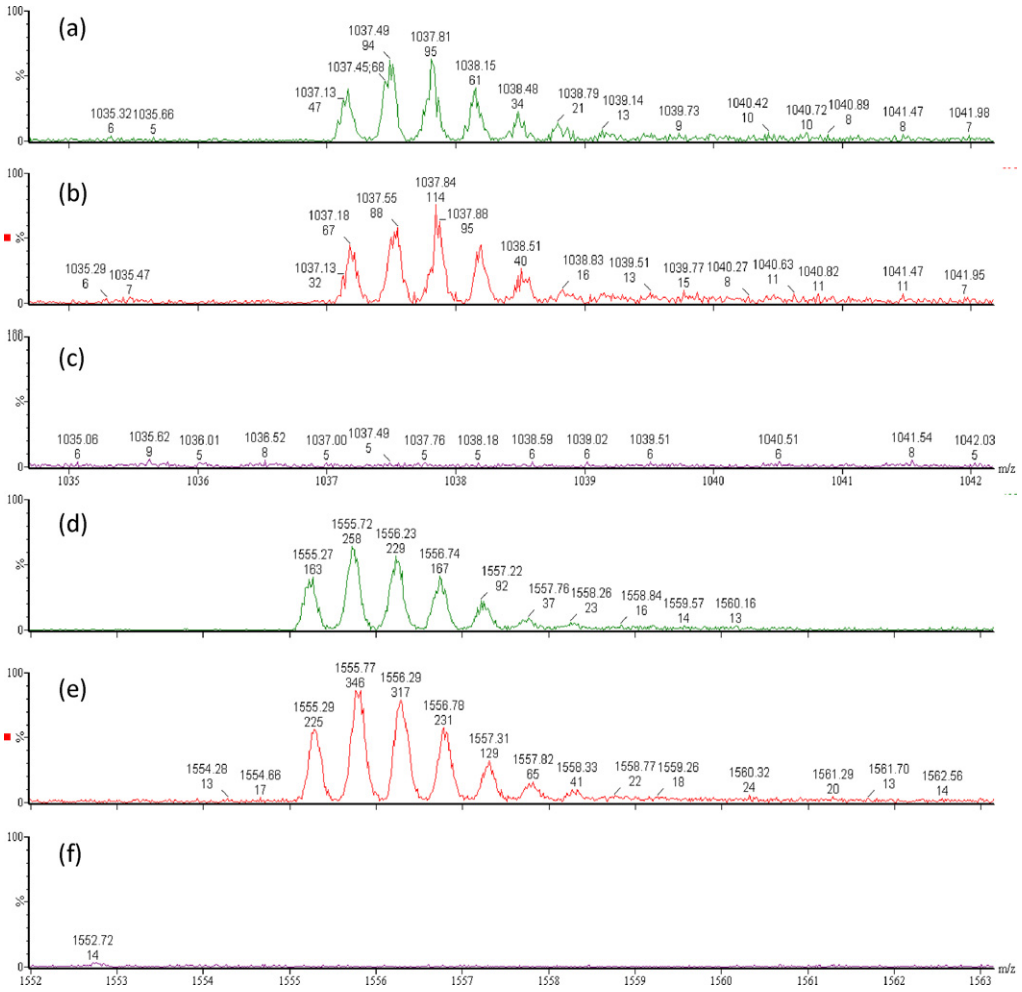

Fig. 7. Expanded scale of Fig. 6. Panels (a–c) represent m/z at 1037 region and panels (d–f) represent m/z at 1555 region. Three charge states were observed for peak at m/z around 1037 while only two charge states were confirmed for the peak at m/z around 1555.

intact glycoforms from O-glycan cannot be obtained due to low ion intensity.

3.3. Glycopeptides and deglycopeptide MSMS results

The purpose of MSMS experiment was to confirm N-glycosylation site and N-glycan sequence. Specifically, G2S1 attached to HT19 was selected to demonstrate the glycan sequence verification. The sequence verification of G2FS2 attached to HT36′ was also performed (data not shown).

The N-linked glycosylation sites can be confirmed by MSMS analysis of the N-deglycosylated sample. For the peak at about 59.1 min (HT19), the b ions increased in mass by 1 Da starting from b18 and the y ions increased in mass by 1 Da starting from y10 as shown in Fig. 5. This indicates that Asn_{266} was deamidated into Asp_{266} after deglycosylation. Therefore, the N-glycan was attached to Asn_{266} of HT19. Similarly, the peak at 39.3 min (HT36′) contained Asn_{462} deamidated HT36′.

The N-glycan monosaccharide sequence confirmation was also performed by MSMS experiment. For the N-glycan attached to HT19, the MSMS mass spectra of intact, desialylated and deglycosylated peptides are presented in Figs. 6 and 7. It was observed that the same MSMS mass spectrum of intact HT19 (panel (a) in Fig. 5) and desialylated HT19 (panel (b) in Fig. 4) was obtained except for the ion of 1608.2^{2+} which represents one additional sialic acid with mass difference of 291 Da. It was also obvious that the major fragments from the intact and desialylated samples were from the cleavage of glycosidic bonds while the major fragments from the deglycosylated sample were from the cleavage of peptide bonds as seen in Fig. 6. Therefore, a totally different MSMS

Table 5
Differential mass of main glycopeptide after an array of deglycosylations.

Glycopeptide	$\Delta M_6 = m_5 - m_6$(Da)	$\Delta M_7 = (m_6 - m_7) + \Delta M_5$ (Da)	Main glycoform	Desialylated glycoforms
HT18	3 × 291	1460.49	4O1 + 3SA	6
HT19	1 × 291	1622.52	G2S1	2
HT36′	2 × 291	1768.83	G2FS2	4
HT19+HT36′	3 × 291	3391.35	G2S1/G2FS2	NA

N-linked glycan monosaccharide compositions were confirmed by an MSMS experiment as G2S1 attached to Asn_{266} of HT19 and G2FS2 attached to Asn_{462} of HT36′ with C-terminal Y truncated. Desialylated glycoforms were glycans observed in desialylated tryptic digested sample. m_5 = mass of intact glycopeptide; m_6 = mass of glycopeptide in desialylated form; m_7 = mass of deglycopeptide; ΔM_5 = mass difference between deglycopeptide and theoretical; ΔM_6 = residue mass of total sialic acid attached to the glycopeptide; ΔM_7 = residue mass of total glycan in desialylated form.

mass spectrum for deglycosylated HT19 was observed compared to the other two samples. The sequence specific pattern of G2S1 was confirmed by this experiment as shown in Figs. 6 and 7. Similarly, the N-glycan attached to HT36′ was also confirmed to be G2FS2.

4. Conclusion

Differential deglycosylation is an effective technique for characterizing glycosylation of glycoproteins containing both N-linked and O-linked glycans. In Human IgA1, two N-linked glycan sites and at least four O-glycan sites with GalNAc-Gal as the core structure were estimated by mass information from deglycoproteins and confirmed by mass information from deglycopeptides and glycopeptides. N-Linked glycosylation can be associated with a specific asparagine due to +1 mass shifts that occur after deglycosylation. Since removal of O-glycans does not result in a mass change, the sites of O-linked glycosylation can be linked only to a specific peptide fragment. These results are consistent with previous study [12,13,15–18]. In Novak et al.'s study [15], IgA1 proteases were used to further cleave HT18 peptide to three pieces. We did not verify this approach due to the availability of the special proteases.

In this study, the number of both N-linked and O-linked glycosylation sites can be determined at the glycoprotein and glycopeptide levels. These two methods are complementary. With both methods, this approach provides one more dimension of results compared to the current bottom up only approach. Since Human IgA1 contains both N-linked and O-linked glycans, the methodology can be easily adapted to other glycoproteins with both N-linked and O-linked glycans. Site specific N-glycan information can also be determined with this methodology when only one glycosylation site is involved for each glycopeptide.

Due to the lack of a universal enzyme for O-linked glycan cleavage, the choice of enzyme for O-glycan can be tuned to the specific O-glycans studied. For example, β (1–4) Galactosidase and β-N-acetylglucosaminidase can be added for additional cleavage specificity [21]. If the sequence is known, the deglycoprotein samples can be used to verify the main amino acid sequence for quality control. The deglycoprotein samples can also be used to check if the deglycosylation process is complete before protease treatment.

Acknowledgements

The authors wish to thank Dr. Hui Cai for her helpful discussion about primary structure of human immunoglobulins and to Joel Galang for his assistance with proofreading and preparing the tables and figures.

References

[1] M.M. Ivancic, H.S. Gadgil, H.B. Halsall, M.J. Treuheit, LC/MS analysis of complex multiglycosylated human α1-acid glycoprotein as a model for developing identification and quantitation methods for intact glycopeptide analysis, Anal. Biochem. 400 (2010) 25–32.
[2] S. Sinha, G. Pipes, E.M. Topp, P.V. Bondarenko, M.J. Treuheit, H.S. Gadgil, Comparison of LC and LC/MS methods for quantifying N-glycosylation in recombinant IgGs, J. Am. Soc. Mass Spectrom. 19 (2008) 1643–1654.
[3] M. Schirm, I.C. Schoenhofen, S.M. Logan, K.C. Waldron, P. Thibault, Identification of unusual bacterial glycosylation by tandem mass spectrometry analyses of intact protein, Anal. Chem. 77 (2005) 7774–7782.
[4] H. Geyer, R. Geyer, Strategies for analysis of glycoprotein glycosylation, Biochim. Biophys. Acta 1764 (2006) 1853–1869.
[5] R.R. Drake, E.E. Schwegler, G. Malik, J. Diaz, T. Block, A. Mehta, O.J. Semmes, Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers, Mol. Cell. Proteomics 5 (2006) 1957–1967.
[6] B.A. Budnik, R.S. Lee, J.A.J. Steen, Global methods for protein glycosylation analysis by mass spectrometry, Biochim. Biophys. Acta 1764 (2006) 1870–1880.
[7] F. Wang, A. Nakouzi, R.H. Angeletti, A. Casadevall, Site-specific characterization of the N-linked oligosaccharides of a murine immunoglobulin M by high-performance liquid chromatography/electrospray mass spectrometry, Anal. Biochem. 314 (2003) 266–280.
[8] S.J. North, P.G. Hitchen, S.M. Haslam, A. Dell, Mass spectrometry in the analysis of N-linked and O-linked glycans, Curr. Opin. Struct. Biol. 19 (2009) 498–506.
[9] H.J. An, J.W. Froehlich, C.B. Lebrilla, Determination of glycosylation sites and site-specific heterogeneity in glycoprotein, Curr. Opin. Chem. Biol. 13 (2009) 421–426.
[10] Z.M. Segu, A. Hussein, M.V. Novotny, Y. Mechref, Assigning N-Glycosylation sites of glycoproteins using LC/MSMS in conjunction with endo-M/exoglycosidase mixture, J. Proteome Res. 9 (2010) 3598–3607.
[11] Y. Tzur, A. Markovich, R.G. Lichtenstein, A two-dimentional array for simultaneous sequencing of N- and O-glycans and their glycoforms on specific glycosylation sites, J. Proteome Res. 7 (2008) 1188–1198.
[12] M.K. Boehm, J.M. Woof, M.A. Kerr, S.J. Perkins, The Fab and Fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering an homology modeling, J. Mol. Biol. 286 (1999) 1421–1447.
[13] T.S. Mattu, R.J. Pleass, A.C. Willis, M. Kilian, M.R. Wormakd, A.C. Lellouch, P.M. Rudd, J.M. Woof, R.A. Dwek, The glycosylation and structure of human serum IgA1, Fab and Fc regions and the role of N-glycosylation on Fca receptor interactions, J. Biol. Chem. 273 (1998) 2260–2272.
[14] A.J. Infante, F.W. Putnam, Primary structure of a human IgA1 immunoglobulin, J. Biol. Chem. 254 (1979) 9006–9016.
[15] J. Novak, M. Tomana, M. Kilian, L. Coward, R. Kulhavy, S. Barnes, J. Mestecky, Heterogeneity of O-glycosylation in the hinge region of human IgA1, Mol. Immunol. 37 (2000) 1047–1056.
[16] E. Tarelli, A.C. Smith, B.M. Hendry, S.J. Challacombe, S. Pouria, Human serum IgA1 is substituted with up to six O-glycans as shown by matrix assisted laser desorption ionization time-of-flight mass spectrometery, Carbohydr. Res. 339 (2004) 2329–2335.
[17] K. Takahashi, Y. Hiki, H. Odani, S. Shimozato, H. Iwase, S. Sugiyama, N. Usuda, Structural analyses of O-glycan sugar chains on IgA1 hinge region using SELDI-TOFMS with various lectins, Biochem. Biophys. Res. Commun. 350 (2006) 580–587.
[18] M.A. Kerr, The structure and function of human IgA, Biochem. J. 271 (1990) 285–296.
[19] M.K. Boehm, J.M. Woof, M.A. Kerr, S.J. Perkins, The Fab and Fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering an homologh modeling, J. Mol. Biol. 286 (1999) 1421–1447, PDB ID: 1IGA.
[20] J.N. Arnold, M.R. Wormald, R.B. Sim, P.M. Rudd, R.A. Dwek, The impact of glycosylation on the biological function and structure of human immunoglobulins, Annu. Rev. Immunol. 25 (2007) 21–50.
[21] PrO-LINK Extender™ Kit for Complex O-linked Glycans, Product Code: GK80115, Prozyme, Hayward, CA.