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LC–MS analysis of glycopeptides of recombinant monoclonal antibodies by a rapid digestion procedure

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ABSTRACT

N-glycan analysis of recombinant monoclonal antibodies (mAbs) usually requires the removal of oligosaccharides by PNGase F followed by 2-AB labeling, normal phase high performance liquid chromatography (NP-HPLC) separation and fluorescence detection. Alternatively antibodies can be completely digested by trypsin to generate glycopeptides for analysis by liquid chromatography–mass spectrometry (LC–MS). Here, we report the development of a rapid digestion procedure to generate glycopeptides for quantitative LC–MS analysis. Recombinant monoclonal antibodies were digested using a combination of Lys-C and trypsin at 37 °C for 15 min. The glycan profiles from this rapid digestion procedure are in good agreement with those from LC–MS analysis of glycopeptides from completely digested antibodies and those from NP-HPLC analysis of 2-AB labeled PNGase F released oligosaccharides. This rapid digestion procedure was applied to the comparison of oligosaccharides of two different antibodies. Glycopeptides from the two antibodies were differentially labeled with stable isotopes and analyzed simultaneously after a 1:1 mixing. The combination of the rapid digestion procedure and differential stable isotope labeling significantly reduced the turnaround time.

1. Introduction

The N-linked oligosaccharides on the conserved asparagine (Asn) residue in the Fc region have been thoroughly characterized and closely monitored during the development of recombinant monoclonal antibodies (mAbs) due to their importance to the structure, stability, and biological functions. The major glycoforms associated with recombinant mAbs from mammalian cell culture are core-fucosylated biantennary complex oligosaccharides with either zero (G0F), one (G1F) or two (G2F) terminal galactose residues. Complex oligosaccharides without core fucose (G0, G0-GlcNAc) and high mannose oligosaccharides are also observed.

Various methods have been used to determine the N-glycan profile of mAb. Released oligosaccharides have been directly analyzed using high pH anion exchange chromatography with pulsed amperometric detection [1,2]. More commonly, released oligosaccharides labeled with fluorescent tags have been analyzed by capillary electrophoresis with laser-induced fluorescence [3–8], normal phase high performance liquid chromatography (NP-HPLC) [9,10] or reversed-phase (RP) HPLC [3,11,12] with fluorescence detection. Mass spectrometry also plays an essential role in oligosaccharide analysis. For example, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has been used to determine oligosaccharide structures and their relative amounts [4,5,9]. Oligosaccharide structures that are assigned based on molecular weights can be confirmed by MS/MS fragmentation and sequential exoglycosidase digestion. Released oligosaccharides labeled with 2-aminobenzamide (2-AB) have also been analyzed by RP-HPLC with on-line MS detection [3,11,12]. Oligosaccharides that are still attached to the protein backbone have been analyzed by LC–MS. Analysis of the molecular weights of intact antibodies, their light chains and heavy chains after reduction, and proteolytic fragments such as Fab and Fc can provide a quick estimate of the relative abundance of the major glycoforms [3,10,13,14]. For more accurate molecular weight determination and structure confirmation, analysis is usually performed using glycopeptides obtained from digested IgG antibodies [10,12,13,15–18].

Several advantages of analyzing glycopeptides have been demonstrated [12,13,15,17,19–23]. First, glycopeptides can be readily retained by RP columns because of the hydrophobic nature of the peptide moieties, which allows an easy on-line mass spectrometry analysis. Second, the peptide moieties of glycopeptides most likely have different molecular weights and are the primary determinant of retention times from RP columns. Therefore, site-specific oligosaccharide structures and their relative abundance can be determined by glycopeptide analysis. Third, oligosaccharide structures assigned based on molecular weight can be readily confirmed by MS/MS fragmentation of glycopeptides because the

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major fragments are generated from breakage of the relatively weak glycosidic bonds under typical collision-induced dissociation (CID) conditions. Typical methods for LC–MS analysis of glycopeptide require the complete digestion of antibodies, which is tedious and time-consuming.

The first goal of our current study was to examine whether a rapid digestion procedure could be established for quick and accurate glycopeptide analysis. Recombinant monoclonal antibodies are composed of two light chains and two heavy chains and have a total of twelve domains including one variable domain (VL) and one constant domain (CL) from each light chain, and one variable domain (VH) and three constant domains (CH1, CH2 and CH3) from each heavy chain. The conserved Asn residue containing the N-linked oligosaccharides resides in the CH2 domain, which is the least stable domain when compared to the others [24–26]. Therefore, it was speculated that the CH2 domain may be more susceptible to protease digestion, and if so, glycopeptides could be generated even without complete digestion of the entire antibody.

The second goal of our current study was to establish a method for comparative quantitation of various oligosaccharides from different antibodies. Methods for comparative quantitation have been reported in the literature by analyzing either the released stable isotope labeled oligosaccharides [27–36] or peptide moieties with stable isotope labels at the peptide termini and the glycosylation sites [37–39]. Here, the procedure of dimethyl labeling was employed to specifically label the peptide's N-terminal primary amine and the side chain of lysine (Lys) residue using reagents with stable isotopes and thus allowing detection and quantification of glycopeptides by mass spectrometry [40].

2. Materials and methods

2.1. Materials

The two recombinant monoclonal antibodies (referred to as mAb-A and mAb-B) were produced in transfected Chinese hamster ovary (CHO) cell lines and purified at Merck (Union, NJ). Guanidine hydrochloride, iodoacetic acid, dithiothreitol (DTT), formic acid and IgG1 from human myeloma plasma were obtained from Sigma (St. Louis, MO). Acetonitrile and trifluroacetic acid (TFA) were obtained from J.T. Baker (Phillipsburg, NJ). Trypsin was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Lys-C was purchased from Wako (Richmond, VA). PNGase F was purchased from Prozyme (San Leandro, CA). N-octylglucoside was purchased from Roche (Indianapolis, IN).

2.2. Complete digestion

mAb-A, mAb-B and IgG1 from human myeloma plasma were denatured and reduced at 37 °C for 30 min at final concentration of 1 mg/mL in a buffer of 6 M guanidine hydrochloride, 50 mM Tris, and 10 mM DTT, at a pH of approximately 8.0. The samples were then alkylated using 25 mM idodoacetic acid at 37 °C for another 30 min. Iodoacetic acid stock solution (1 M) was prepared in 1 M Tris, pH 8.0. The samples were then buffer exchanged into 20 mM Tris, pH 8.0, using NAP-5 column (GE healthcare, Piscataway, NJ). Trypsin digestion was carried out at 37 °C for 18 h using a trypsin to antibody ratio of 1:20 (w:w).

2.3. Rapid digestion

The rapid digestion procedure was established and optimized using mAb-A at a concentration of 3 mg/mL in PBS. mAb-A was pre-incubated at 55 °C for 5 min and then digested using trypsin at a 1:5 trypsin:antibody (w:w) ratio at the same temperature for 15 min. Optimization was attempted by including either 20 mM DTT or Lys-C at a final Lys-C: antibody ratio of 1:25 (w:w) in the sample preparations. After 5 min of incubation at 55 °C, the samples were then digested using trypsin at a 1:5 ratio at 55 °C for 15 min. The procedure of including Lys-C was repeated at temperatures of 37 °C, and 45 °C. The results demonstrated that the procedure of including Lys-C (1:25 ratio) during the 5 min pre-incubation and then digested by tryspin (1:5 trypsin:antibody ratio) at 37 °C for 15 min is optimal. mAb-B and IgG1 from human myeloma plasma were digested using the optimized procedure. After 15 min of digestion, the samples were incubated at 99 °C for 5 min and then centrifuged at 10,000 rpm for 5 min using an Eppendorf Minispin table top centrifuge. The supernatant from each sample was collected for LC–MS analysis.

2.4. Dimethyl labeling

The supernatants collected from digestion of 500 μg of either mAb-A or mAb-B following the optimized procedure were labeled with stable isotopes. Glycopeptides from mAb-A were labeled by the addition of 4 μL of 20% formaldehyde (CH_2O) and 12 μL of 50% sodium cyanoborohydride ($NaBH_3CN$). Glycopeptides from mAb-B were labeled by the addition of 4 μL of 20% formaldehyde (CD_2O) and 12 μL of 50% sodium cyanoborodeuteride ($NaBD_3CN$). The samples were incubated at room temperature for 60 min and then quenched by the addition of 8 μL of 8 M ammonium hydroxide (NH_4OH). The labeled samples were either analyzed separately or as a 1:1 combined mixture.

2.5. LC–MS analysis

An Agilent Infinity 1290 UHPLC (Santa Clara, CA) coupled with an Agilent 6538 UHD Q-TOF mass spectrometer was used to analyze the glycopeptides. Twenty μL of each sample was loaded into a Proto C18 column (250 × 1 mm i.d., 5 μm particle size, Higgins Analytical Inc., Mountain view, CA) with 98% mobile phase A (0.02% TFA and 0.08% formic acid in water) and 2% mobile phase B (0.02% TFA and 0.08% formic acid in acetonitrile) at a flow-rate of 50 μL/min. The peptides were separated and eluted using a gradient from 5% to 20% mobile phase B over 15 min. The column was then washed using 98% mobile phase B and re-equilibrated using 2% mobile phase B for 5 min. The column was heated at 60 °C. The MS was operated in positive ion mode with a full mass scan from m/z 200 to 2000. Source temperature and fragmentor voltage were optimized for quantitation.

2.6. 2-AB labeling and NP-UPLC analysis

mAb-A and mAb-B were diluted to 1 mg/mL using phosphate buffered saline (PBS), pH 7.4 and N-octylglucoside at a final concentration of 1% (w/v). The antibodies were digested using PNGase F at a ratio of 1 μL PNGase F to 100 μg antibody at 37 °C overnight. Proteins were precipitated by heating at 99 °C for 5 min and then centrifuged at 10,000 rpm for 5 min. The supernatant from each sample was collected and dried by speed-vacuum. The dried samples were labeled by reductive amidation using the 2-AB labeling kit from Prozyme (San Leandro, CA) following the manufacture's instruction. Extra labeling reagent was removed using the GlycoClean S cartridges (Prozyme). The labeled oligosaccharides were then dried and reconstituted in 200 μL of 72% acetonitrile in water and analyzed by UPLC system (Waters, Milford, MA) with fluorescence detection. Approximately 1 μL of each sample was injected onto an Acquity UPLC BEH glycan column (1.7 μm, 2.1 × 150 mm, Waters) using 72% mobile phase A (acetonitrile) and 28% mobile phase B (100 mM ammonium formate, pH 4.4) and then the oligosaccharides were eluted off the column using a linear gradient from the initial condition to 62% mobile phase A and 38% mobile

phase B within 30 min at a flow-rate of 0.4 mL/min. The column was then washed and re-equilibrated using the initial condition before the next injection. The column temperature was set at 60 °C. The excitation wavelength was set at 330 nm and the emission wavelength was set at 420 nm. The oligosaccharide structures were assigned based on their molecular weights from LC–MS measurement and the commonly reported oligosaccharides of recombinant monoclonal antibodies analyzed by 2-AB labeling and NP-UPLC analysis.

3. Results and discussion

3.1. Principle of the method

The N-linked oligosaccharides are attached to the conserved Asn residue of the CH2 domain, which is the least stable domain when compared to the others. Therefore, the CH2 domain may be preferentially digested to generate glycopeptides for LC–MS analysis. The proposed rapid digestion procedure is outlined in Supplementary Figure 1. mAb-A was digested for 15 min and then the sample was heated to precipitate any large antibody fragments that were not fully digested as well as proteolytic enzymes used for digestion. After centrifugation, the supernatant was collected for LC–MS analysis. The rapid digestion conditions were optimized based on comparison to the well-established methods including NP-UPLC analysis of 2-AB labeled oligosaccharides and LC–MS analysis of glycopeptides from complete digestion.

Dimethyl labeling of glycopeptides from mAb-A and mAb-B was carried out as outlined in Supplementary Figure 1B. Glycopeptides from mAb-A were modified by the "light" reagents of formaldehyde (CH_2O) and cyanoborohydride ($NaBH_3CN$). Glycopeptides from mAb-B were modified by the "heavy" reagents of formaldehyde (CD_2O) and cyanoborodeuteride ($NaBD_3CN$). In addition to peptide N-terminal primary amine, glycopeptides generated from the rapid digestion procedure contain an internal Lys residue, which was also labeled. Therefore, the molecular weight increases after labeling are 56 Da for glycopeptides from mAb-A and 68 Da for glycopeptides from mAb-B. The larger molecular weight difference is advantageous to avoid overlapping of isotope patterns.

3.2. LC–MS analysis of glycopeptides

Glycopeptides generated from complete trypsin digestion and from rapid digestion were first analyzed to identify the major glycoforms. The initial rapid digestion was performed at 55 °C for 15 min with a trypsin: antibody ratio of 1:5 (w:w). The MS spectra of glycopeptides from mAb-A using either rapid or complete trypsin digestion are shown in Figs. 1 and 2 respectively. The major glycoforms are identified based on the common N-linked oligosaccharide structures of recombinant monoclonal antibodies and the accurate molecular weights of glycopeptides. Rapid digestion generated a peptide (TKPREEQYNSTYR) that has four more amino acid than the peptide (EEQYNSTYR) from complete digestion, suggesting that the antibody was not fully digested. Complex oligosaccharides with a core fucose and either zero (G0F), one (G1F) or two (G2F) galactose residues were observed. In addition, G0F with the loss of one GlcNAc (G0F-GlcNAc), G0, and high mannose with five (Man 5) or six (Man 6) mannose residues were also observed. The assigned structure can be readily confirmed by CID fragmentation of the glycopeptides. As an example, the MS/MS spectrum of the glycopeptide containing G0F from the complete digestion is shown in Supplementary Figure 2.

Table 1
The percentage of various glycoforms of mAb-A obtained from 2-AB labeling, LC–MS analysis of glycopeptides from complete digestion. The percentage was calculated by dividing the peak area of each individual glycoform by the total peak area of all glycoforms and then multiplied by 100. The data shows the mean ± standard deviation value calculated from triplicate experiments.

Glycoforms	2-AB	LC–MS
G0F	69.41 ± 0.11	70.41 ± 0.27
G0F-GlcNAc	1.02 ± 0.02	0.71 ± 0.06
G0	4.68 ± 0.02	5.08 ± 0.13
G1F	20.56 ± 0.15	21.19 ± 0.32
G2F	2.56 ± 0.01	2.32 ± 0.06
M5	1.42 ± 0.01	0.46 ± 0.05
M6	0.35 ± 0.0	0.10 ± 0.00

3.3. Optimization of mass spectrometer parameters

Because of the labile nature of the glycosidic bonds, the mass spectrometer parameters (source temperature and fragmentor voltage) were optimized to reduce in-source fragmentation, which will significantly affect quantitation results. Oligosaccharide G0F-GlcNAc is present naturally at very low level. It can also be generated by in-source fragmentation of G0F through the loss of a GlcNAc. Therefore the percentage of the extracted ion chromatograms (EIC) peak area of G0F-GlcNAc over the sum of the EIC peak areas of G0F and G0F-GlcNAc was used to monitor in-source fragmentation. Lower percentage indicates lower in-source fragmentation and vice versa.

There are several observations during the optimization of the mass spectrometer parameters (Supplementary Tables 1, 2, 3 and 4). First, the source temperature and fragmentor voltage has a significant impact on in-source fragmentation of doubly charged ions, while the impact on triply charged ions is minimal. This is true for both the shorter peptide from complete digestion and the longer peptide from rapid digestion. To illustrate the difference in in-source fragmentation, a MS spectrum acquired using glycopeptides from rapid digestion is shown in Fig. 3. Clearly, much higher amount of the G0F-GlcNAc is observed for the doubly charged ions compared to the triply charged ions. A mass spectrum from complete digestion is shown in Supplementary Figure 3, as another example. Second, for the doubly charged ions, lower source temperature results in lower level of fragmentation, while higher source temperature resulted in higher level of in-source fragmentation. The same relationship was also observed between the level of in-source fragmentation and fragmentor voltage. In addition, source temperature and fragmentor voltage seem to have synergic effect. Much higher impact was observed when both parameters were set up higher. Third, lower source temperature and lower fragmentor voltage result in lower peak areas of the glycopeptides. A source temperature of 200 °C and fragmentor voltage of 125 V was chosen for all further experiments to minimize in-source fragmentation. In addition, triply charged ions were used for relative quantitation because they are less impacted by source temperature and fragmentor voltage.

3.4. Optimization of the rapid digestion procedure

As discussed earlier, the rapid digestion procedure was optimized based on comparison of data from 2-AB labeling followed by NP-UPLC and LC–MS analysis of glycopeptides from complete digestion. Overall, there is a good agreement between 2-AB labeling and LC–MS analysis of the glycopeptides from complete digestion except Man 5 and Man 6 (Table 1). However, the percentage of various glycoforms from the initial rapid digestion procedure is significantly different from that of 2-AB labeling and LC–MS analysis

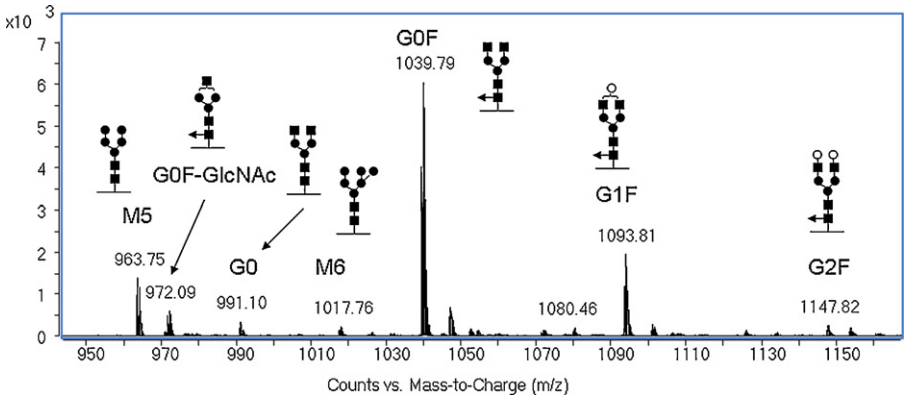

Fig. 1. MS spectrum of the glycopeptides obtained from rapid digestion at 55 °C for 15 min of mAb-A. Peaks of triply charged ions were labeled. Peak assignment was based on the commonly observed N-linked oligosaccharides of recombinant monoclonal antibodies and the measured molecular weights. Solid square represents GlcNAc, solid triangle represents Fuc, solid circle represents Man, and empty circle represents Gal.

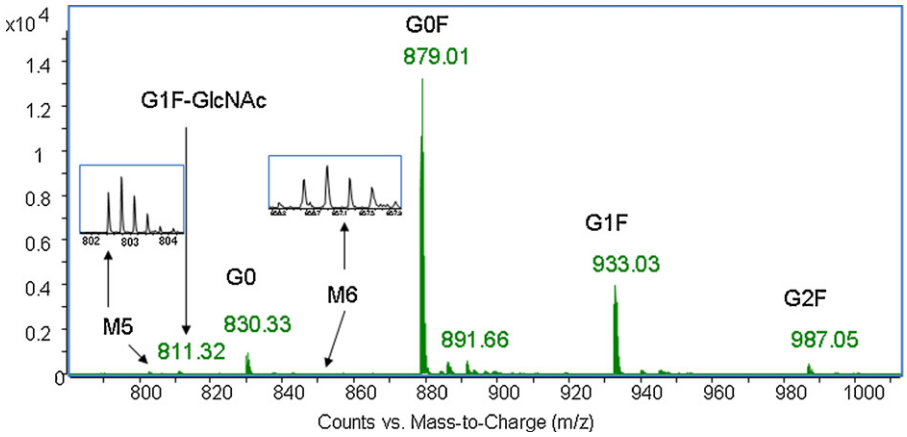

Fig. 2. MS spectrum of the glycopeptides obtained from complete digestion of mAb-A. Peaks of triply charged ions were labeled. Peak assignment was based on the commonly observed N-linked oligosaccharides of recombinant monoclonal antibodies and the measured molecular weights.

of glycopeptides from complete digestion (Table 2, column 2), indicating the necessity to optimize the digestion procedure.

One hypothesis to explain the difference is the preferential digestion of mAb-A with certain types of oligosaccharides. For example, higher level of G0F-GlcNAc and high mannose oligosaccharides could be the result of preferential digestion of mAb-A with such types of oligosaccharides. It was reasoned that further unfolding of the CH2 domain may allow similar accessibility of the cleavage sites for antibodies with different oligosaccharides. To test this hypothesis, DTT was included in the sample preparation to determine whether the reduction of disulfide bonds could increase the unfolding and digestion efficiency. However, no significant improvement in the percentage of various oligosaccharides in mAb-A was observed (Table 2, column 3). Attempt was also made to use Lys-C, which can selectively cleave the hinge region [41], to remove Fab and thus increases the accessibility of the CH2 domain. The percentage of various glycoforms from digestion including Lys-C was much closer to that obtained from the well-established methods of 2-AB labeling and LC–MS analysis of glycopeptides from complete digestion (Table 2, column 4). The procedure of including Lys-C was further optimized by digestion at relative lower temperatures of 37 °C and 45 °C. As expected, lower total peak areas of glycopeptides were obtained from digestion at lower temperature, suggesting less efficient proteolysis. Interestingly, the percentage of various oligosaccharides obtained from digestion at 37 °C (Table 2, column 6) is in good agreement with 2-AB labeling

Table 2
The percentage of various glycoforms of mAb-A obtained from LC–MS analysis of glycopeptides from rapid digestion at 55 °C for 15 min (trypsin, 55 °C), 55 °C for 15 min with DTT (trypsin DTT, 55 °C), and from rapid digestion using a combination of Lys-C and trypsin at 55 °C (55 °C), 45 °C (45 °C) and 37 °C (37 °C). The percentage was calculated by dividing the EIC peak area of each individual glycoform by the total peak area of all glycoforms and then multiplied by 100. The data represent mean ± standard deviation values from triplicate experiments.

Glycoforms	Trypsin 55 °C	Trypsin + DTT 55 °C	Lys-C + trypsin		
			55 °C	45 °C	37 °C
G0F	56.45 ± 0.54	57.75 ± 0.48	68.77 ± 0.08	68.78 ± 0.26	69.65 ± 0.37
G0F-GlcNAc	6.46 ± 0.15	3.71 ± 0.10	0.93 ± 0.03	0.80 ± 0.07	0.82 ± 0.04
G0	3.99 ± 0.14	6.12 ± 0.40	5.94 ± 0.05	5.70 ± 0.08	5.98 ± 0.11
G1F	16.90 ± 0.30	18.52 ± 0.08	17.23 ± 0.07	17.89 ± 0.11	17.96 ± 0.20
G2F	1.93 ± 0.13	2.16 ± 0.14	1.84 ± 0.03	1.88 ± 0.09	1.88 ± 0.17
M5	12.32 ± 0.21	10.10 ± 0.17	4.21 ± 0.06	3.95 ± 0.04	2.96 ± 0.13
M6	3.99 ± 0.14	1.63 ± 0.07	1.07 ± 0.03	1.00 ± 0.02	0.75 ± 0.05

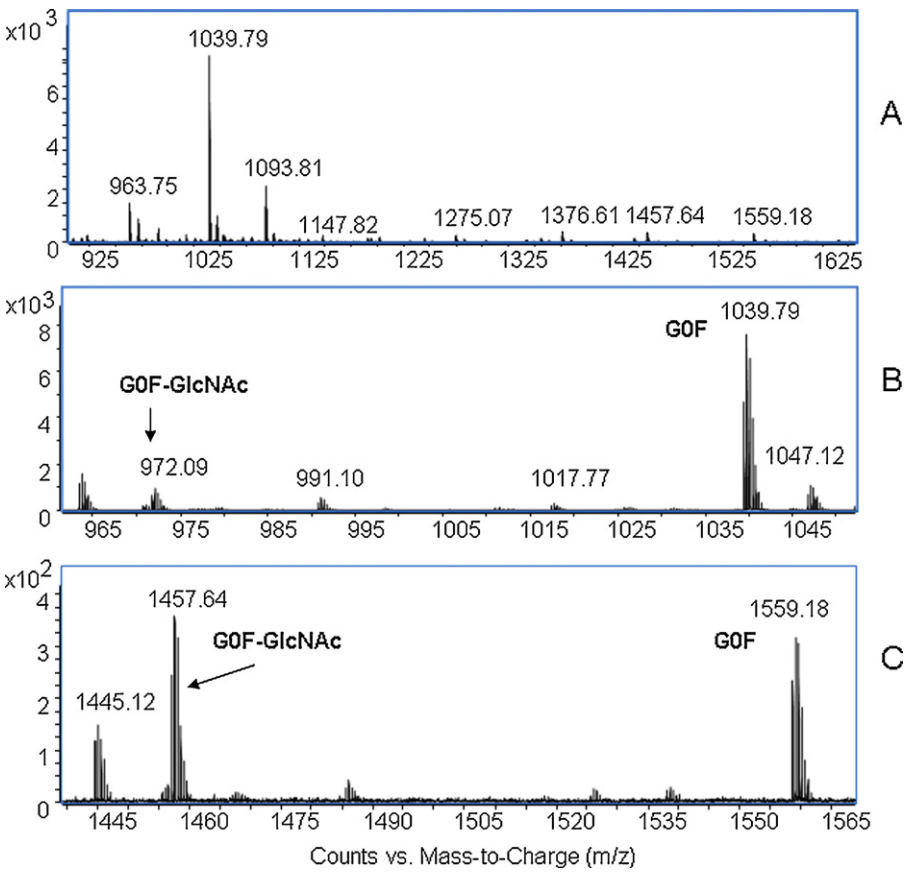

Fig. 3. Mass spectra of the glycopeptides from rapid digestion at 55 °C for 15 min of mAb-A. (A) Mass spectrum of the glycopeptides acquired using a source temperature of 200 °C and in-source fragmentor voltage of 250 V; (B) mass spectrum from (A) with the triply charged G0F and G0F-GlcNAc enlarged; (C) mass spectrum from (A) with the doubly charged G0F and G0F-GlcNAc enlarged. The relative peak height ratio of G0F-GlcNAc to G0F is much higher when the doubly charged peaks were compared. This is in agreement with data shown in Supplementary Tables 1 and 2, where the EIC peak areas were used to determine the ratio of G0F-GlcNAc to G0F.

and LC–MS analysis of the glycopeptides from complete digestion. The results from the combination of Lys-C and trypsin digestion indicated that cleavage of the hinge region might be sufficient to expose the CH2 domain to digestion with no preference over specific glycoforms. On the other hand, higher levels of glycopeptides with G0F-GlcNAc and high mannose residues from digestion at 45 °C and 55 °C suggested antibodies with those oligosaccharides were more susceptible to digestion at elevated temperatures. Digestion using trypsin alone at 37 °C was also tested. The total peak area of the glycopeptides was approximately 10 times lower than that from digestion using the combination of Lys-C and trypsin. Therefore, quantitation using trypsin alone was not attempted.

Although recombinant monoclonal IgG1s share almost identical amino acid sequence in the Fc regions, a second recombinant antibody (mAb-B) and an IgG1 from human myeloma plasma were also digested using the rapid digestion procedure. The data for the recombinant mAb-B is summarized in Supplementary Table 5, which demonstrate a good agreement among the three different methods except the level of mannose oligosaccharides. The data for the IgG1 from human myeloma plasma is summarized in Supplementary Table 6. Glycoforms of G0F, G1F and G2F that were present in the recombinant IgG1 antibodies were also detected in the human IgG1. In addition, glycoforms with bisecting GlcNAc or with sialic acid were also detected in the human IgG1, but not in the recombinant IgG1 antibodies. Nevertheless, a good agreement was observed between the rapid digestion and the complete digestion. Taken together, those results suggest that the rapid digestion procedure is applicable to determine the percentage of various glycoforms of recombinant antibodies as well as human IgG1 antibodies.

3.5. Comparative quantitation

Glycopeptides obtained from rapid digestion of mAb-A and mAb-B were labeled with reagents containing different stable isotopes and then analyzed by LC–MS after 1:1 mixing. A typical mass spectrum is shown in Fig. 4. A representative mass spectrum containing peaks corresponding to differentially labeled G0F glycopeptides is shown in Fig. 5. The m/z difference between the two triply charged peak series is 4, which corresponds to labeling at two sites including the undigested Lys and the peptide N-terminal primary amine. There is no overlapping between the two peak series, which facilitates data analysis for comparative quantitation.

The ratios of various glycoforms were calculated by dividing the EIC peak areas of glycopeptides from mAb-A by those of the glycopeptides containing the respective oligosaccharides from mAb-B. For comparison, the ratios of various glycoforms from mAb-A and mAb-B were also calculated using their relative percentage from analysis of glycopeptides without dimethyl labeling. As shown in Table 3, a good agreement was observed among the three data sets. The major difference observed is the ratio of Man 5 between the two antibodies. Similar levels of Man 5 in mAb-A and mAb-B were determined by 2-AB labeling, and LC–MS analysis of glycopeptides from complete and rapid digestion, while, lower level of Man5 was observed in mAb-A than in mAb-B by dimethyl labeling. Although, more experiments are warranted, dimethyl labeling

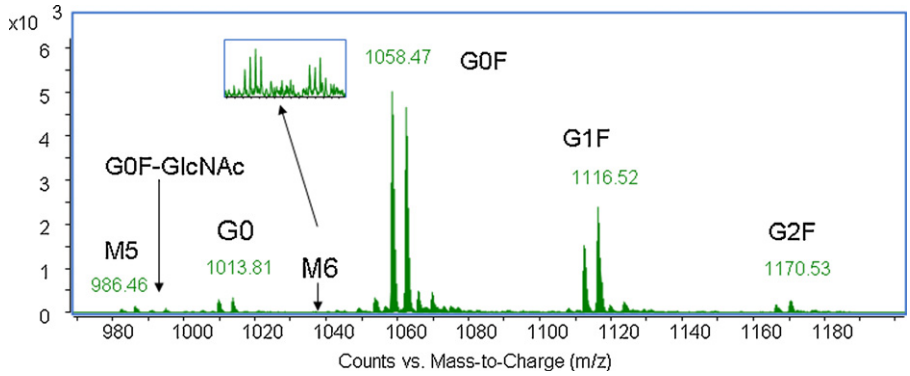

Fig. 4. Mass spectrum of the dimethyl labeled glycopeptides from mAb-A and mAb-B from rapid digestion. The same types of oligosaccharides were detected and labeled as Figs. 1 and 2. The double peaks represent glycopeptides with either "light" or "heavy" labels.

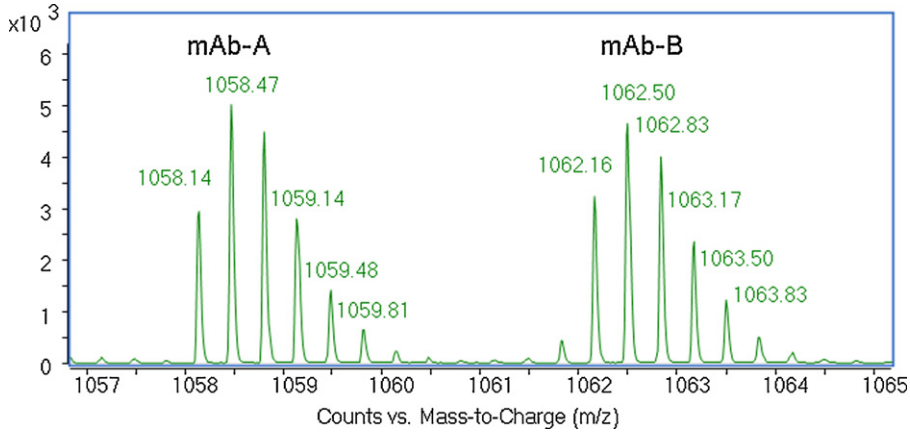

Fig. 5. A portion of the mass spectrum from Fig. 4 was enlarged to show glycopeptides with G0F from either mAb-A or mAb-B. The peak series with lower m/z corresponds to the glycopeptide from mAb-A and the peak series with higher m/z corresponds to the glycopeptide from mAb-B.

has the advantage of analyzing the two samples simultaneously and thus eliminated sample preparation variation and instrument fluctuation. In addition, when the samples were analyzed by 2-AB and LC–MS without dimethyl labeling, the relative percentage of each glycoform from each antibody was calculated first, which was then used to calculate the ratios. Therefore, the ratios of various glycoforms are interdependent, inaccurate quantitation of one glycoform also affecting others. On the other hand, when the samples were analyzed after dimethyl labeling, the ratio of each glycoform between mAb-A and mAb-B was calculated independent of other glycoforms. Inaccurate determination of the ratio of one glycoform will not impact the accuracy of the ratios of other glycoforms.

Table 3
The ratios of various glycoforms from mAb-A and mAb-B. The ratios were calculated using the average relative percentage of various glycoforms that were determined by 2-AB, and LC–MS analysis of glycopeptides from complete and rapid digestion. For glycopeptides with stable isotope labeling, the ratios of various glycoforms were calculated from three experiments and the data represent average ± standard deviation.

Glycoforms	2-AB	Complete	Rapid	Rapid + labeling
G0F	1.21	1.21	1.19	1.18 ± 0.03
G0F-GlcNAc	0.57	0.65	0.75	0.64 ± 0.08
G0	0.95	1.03	1.13	1.11 ± 0.02
G1F	0.68	0.68	0.63	0.75 ± 0.03
G2F	0.61	0.63	0.61	0.72 ± 0.07
M5	0.92	0.96	1.07	0.69 ± 0.03
M6	2.92	1.43	1.36	1.40 ± 0.05

4. Conclusion

A rapid digestion procedure followed by LC–MS analysis was established for relative quantitation of glycans from recombinant monoclonal antibodies. Digestion of antibodies using a combination of Lys-C and trypsin at 37 °C for 15 min resulted in the generation of glycopeptides that are suitable for quantitative LC–MS analysis. Although, the antibody may not be fully digested, the glycopeptides generated are representative of the oligosaccharide profiles of the recombinant monoclonal antibodies. Glycopeptides generated from two antibodies were further analyzed after differential dimethyl labeling, which allowed simultaneous analysis of two samples for binary comparison. This rapid digestion procedure alone or followed by dimethyl labeling will be a valuable tool for comparing the relative abundance of the oligosaccharide structures among different antibodies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.09.004.

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