RESEARCH ARTICLE

Detection and Quantitation of Afucosylated N-Linked Oligosaccharides in Recombinant Monoclonal Antibodies Using Enzymatic Digestion and LC-MS

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Abstract

The presence of N-linked oligosaccharides in the CH2 domain has a significant impact on the structure, stability, and biological functions of recombinant monoclonal antibodies. The impact is also highly dependent on the specific oligosaccharide structures. The absence of core-fucose has been demonstrated to result in increased binding affinity to Fcγ receptors and, thus, enhanced antibody-dependent cellular cytotoxicity (ADCC). Therefore, a method that can specifically determine the level of oligosaccharides without the core-fucose (afucosylation) is highly desired. In the current study, recombinant monoclonal antibodies and tryptic peptides from the antibodies were digested using endoglycosidases F2 and H, which cleaves the glycosidic bond between the two primary GlcNAc residues. As a result, various oligosaccharides of either complex type or high mannose type that are commonly observed for recombinant monoclonal antibodies are converted to either GlcNAc residue only or GlcNAc with the core-fucose. The level of GlcNAc represents the sum of all afucosylated oligosaccharides, whereas the level of GlcNAc with the core-fucose represents the sum of all fucosylated oligosaccharides. LC-MS analysis of the enzymatically digested antibodies after reduction provided a quick estimate of the levels of afucosylation. An accurate determination of the level of afucosylation was obtained by LC-MS analysis of glycopeptides after trypsin digestion.

Key words: LC-MS, Recombinant monoclonal antibody, Glycosylation, Afucosylation

Introduction

N-linked oligosaccharides in the CH2 domain are important for the structure, stability, and Fc receptor mediated biological functions of antibodies [1–4] and play a critical role in the development of recombinant monoclonal antibody (mAb) therapeutics [2]. The impact on structure,

stability, and biological functions is also highly dependent on the presence of various specific oligosaccharide structures [1]. For example, it has been found that absence of the core-fucose can significantly enhance the FcγRIIIa binding affinity of IgG1, resulting in increased antibody-dependent cellular cytotoxicity (ADCC) [5–7]. Higher level of afucosylation is desired if ADCC is the mechanism of action of the recombinant mAbs. On the other hand, lower level of afucosylation is required for the development of recombinant monoclonal antibodies if ADCC is not part of the mechanism of action. Therefore, it is critical to accurately monitor the level of afucosylation for the development of recombinant mAbs.

Electronic supplementary material The online version of this article (doi:10.1007/s13361-012-0397-8) contains supplementary material, which is available to authorized users.

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Received: 13 March 2012
Revised: 9 April 2012
Accepted: 18 April 2012
Published online: 9 May 2012

Oligosaccharides are routinely analyzed after being released from glycoprotein, either directly or after derivatization. Released oligosaccharides can be analyzed by high performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD) [8], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) [9–11], and a graphitized carbon column with mass spectrometry [12, 13]. A variety of chemistry has been employed to derivatise released oligosaccharides either to introduce a chromophore or fluorophore for detection by absorption or fluorescence [14–23], or to introduce a positive charged group to enhance mass spectrometry response [20, 24–26]. Fluorescence labeling using 2-amino-benzamide (2-AB) or less frequently 2-aminobenzoic acid (2-AA) followed by normal-phase high performance liquid chromatography (NP-HPLC) separation has become the most commonly used method because of the high sensitivity, high resolution, and good reproducibility [21, 27–29]. In addition, many of the derivatization reagents add hydrophobicity to the otherwise hydrophilic oligosaccharides and allow them to be analyzed by reversed-phase HPLC (RP-HPLC) [14–18].

Oligosaccharides have also been analyzed while they are still attached to glycoproteins or glycopeptides. For example, the major oligosaccharides of recombinant monoclonal antibodies have been determined by LC-MS analysis of the molecular weights of intact antibodies, Fab and Fc fragments or light chain and heavy chain [30]. More commonly, glycopeptides from enzymatic digestion are analyzed to obtain additional site-specific oligosaccharide structure distribution and site-occupancy information, which are not available from the analysis of released oligosaccharides. The other advantage of analyzing glycopeptides is the use of RP-HPLC because of the hydrophobic nature of the amino acid moiety. RP-HPLC with either off-line or on-line mass spectrometry has been routinely used for the analysis of glycopeptides [13, 30–35] because not only glycopeptides can be retained by RP-HPLC columns, but they also can be separated mainly based on difference in the amino acid sequences.

It is still challenging to accurately determine the level of afucosylation using current methods. Peak overlapping and lack of detection of low abundant oligosaccharides are the two major challenges for the commonly used NP-HPLC method or MS-based methods. It has been reported that core-fucosylated complex oligosaccharide without galactose residues and lacking one GlcNAc residue (G0F-GlcNAc) overlaps with complex oligosaccharides without galactose and lacking the core-fucose (G0) when analyzing by NP-HPLC [36]. In addition, oligosaccharides with six, seven, or even higher number of mannose residues can be hardly detected because of their low abundances [36]. Although MS-based methods can differentiate oligosaccharides based on their unique molecular weights (except isobaric forms), they usually suffer from lower sensitivity because of micro heterogeneity of oligosaccharides [30].

The goal of the current study is to establish a method that can accurately determine the level of afucosylation in recombinant mAbs based on the combination of enzymatic digestion and LC-MS analysis. Endo F2 and Endo H digestion converts the otherwise complicated population of oligosaccharides into either monosaccharide or disaccharide depending on the presence or absence of the core-fucose residue. Oligosaccharides without the core-fucose will be converted to monosaccharide with GlcNAc only. Oligosaccharides with the core-fucose will be converted to disaccharide with GlcNAc and Fuc. Low abundant oligosaccharides, though they may not be detectable as an individual species before enzymatic digestion, will be collapsed to either GlcNAc or GlcNAc with Fuc, which should be readily detectable by LC-MS analysis. Therefore, LC-MS analysis after enzymatic digestion provides a quantitative approach to accurately determine the level of afucosylation.

Experimental

Materials

Three recombinant monoclonal antibodies (referred to as mAb-A, mAb-B, and mAb-C) were produced by transfected Chinese hamster ovary (CHO) cell lines and purified at Merck (Union, NJ). *N*-octylglucoside was purchased from Roche (Indianapolis, IN, USA). Endoglycosidase H and α -(1–2,3,4,6) fucosidase were purchased from Prozyme (San Leandro, CA, USA). Endoglycosidase F2, iodoacetic acid, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, trifluoroacetic acid (TFA), and guanidine hydrochloride were purchased from J. T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from EMD (Gibbstown, NJ, USA). Trypsin was purchased from Worthington (Lakewood, NJ, USA).

Trypsin Digestion

The antibodies at 1 mg/mL (500 μ L of each) were denatured and reduced in the presence of 6 M guanidine hydrochloride in 20 mM ammonium bicarbonate with 10 mM DTT at 37 °C for 30 min, which was followed by alkylation using 25 mM iodoacetic acid at 37 °C for 30 min. The samples were buffer-exchanged into 20 mM ammonium bicarbonate using NAP-10 column (GE Healthcare, Piscataway, NJ, USA). Digestion was performed by the addition of trypsin to a final trypsin to antibody ratio of 1:20 (w:w) at 37 °C for 18 hours.

Endo H and Endo F2 Digestion

Endo F2 and Endo H digestion was performed while the recombinant mAbs were either still intact or after trypsin digestion. Digestion conditions for Endo F2 and Endo H were optimized for complete digestion. For digestion of oligosaccharides attached to intact antibodies, each antibody

(50 µg) was dissolved at 1 mg/mL in 50 mM sodium acetate with a final concentration of 1 % *N*-octylglucoside at pH 4.5, and was incubated with a mixture of 5 µL of Endo F2 and 5 µL of Endo H at 37 °C for 18 h. After the digestion, 2 µL of 8 M ammonium solution was added to each sample to adjust pH to 8.0 and reduced by 10 mM DTT for 30 min at 37 °C. For digestion of oligosaccharides attached to peptides, 100 µL of peptide mixtures from each antibody was dried using a speed-vacuum and then reconstituted in 100 µL of 50 mM sodium acetate, pH 4.5. Then, 5 µL of Endo F2 and 5 µL of Endo H were added to each reconstituted sample followed by incubation at 37 °C for 18 h. The reduced protein samples and the digested peptide samples were then analyzed by LC-MS.

Fucosidase Digestion

Glycopeptides after Endo H and Endo F2 digestion were further digested using fucosidase, which specifically removes the core-fucose. To do this, 5 µL of fucosidase was added to 50 µL of glycopeptides from each antibody and incubated at 37 °C for overnight (16–18 h). The samples were then analyzed by LC-MS.

LC-MS Analysis

An Agilent Infinity 1290 UHPLC (Santa Clara, CA, USA) coupled with an Agilent 6538 UHD Q-TOF mass spectrometer was used to analyze the reduced protein and the digested peptide samples. For reduced protein samples, 10 µL of each sample was loaded onto a Poros R2 column (2.1×30 mm; Applied Biosystems, Foster City, CA, USA) held at 70 °C. A gradient from 85 % mobile phase A (0.1 % formic acid in water) and 15 % mobile phase B (0.1 % formic acid in acetonitrile) to 40 % mobile phase B in 10 min was used to separate antibody light chain and heavy chain at a flow-rate of 1 mL/min. Ion spray voltage was set at 5500 V and the gas temperature was set at 200 °C. Fragmentor was set at 125 V.

For digested peptide samples, 10 µL of each sample was loaded onto a Halo Peptide ES-C18 column (2.1×150 mm, 2.7 µm; Advanced Materials Technology, Wilmington, DE, USA) held at 60 °C. Peptides were eluted off the column using a short gradient of 98 % mobile phase A (0.1 % formic acid in water) and 2 % mobile phase B (0.1 % formic acid in acetonitrile) to 35 % mobile phase B in 11 min at a flow-rate of 0.25 mL/min. The column was washed using 98 % mobile phase B for 5 min and then equilibrated using 2 % mobile phase B for 5 min before the next injection. Ion spray voltage was set at 4500 V and the gas temperature was set at 200 °C.

Results and Discussion

Principle of the Method

Recombinant mAb contains two major types of oligosaccharides, complex and high mannose. Complex oligosac-

charides account for the majority of the glycans, whereas high mannose oligosaccharides are present at relatively low abundance. Complex oligosaccharides are present mainly with the core-fucose residue. A very low percentage of complex oligosaccharides are present without core-fucose residue. On the other hand, high mannose oligosaccharides lack the core-fucose residue. Although only two major types of oligosaccharides are associated with recombinant mAbs, a complicated population of oligosaccharides is commonly observed because of microheterogeneity. For example, complex oligosaccharides can exist with the core-fucose with either zero (G0F), one (G1F), or two (G2F) galactose. Complex oligosaccharides without the core-fucose can also exist with either zero (G0), one (G1) or two terminal galactose (G2). Different number of mannose (Man) residue is the major reason for microheterogeneity of high mannose oligosaccharides. Microheterogeneity not only results in complicated mass spectra with many peaks, but also leads to lower sensitivity for MS detection. As a result, low abundant oligosaccharides are difficult to detect. Therefore, it is challenging to determine the level of afucosylation by direct analysis of intact antibodies, reduced antibodies, and the glycopeptides.

The proposed strategy is outlined in Figure 1. Oligosaccharides that are still attached to recombinant mAb or peptides after trypsin digestion were digested with Endo F2 and Endo H. Endo F2 cleaves the glycosidic bond between the two primary GlcNAc residues of mainly complex oligosaccharides, to a lesser extent of high mannose oligosaccharides. Endo H cleaves the glycosidic bond between the two primary GlcNAc residues of high mannose type oligosaccharides. A combination of Endo F2 and Endo H will reduce the complexity of the total oligosaccharides to either monosaccharide with GlcNAc, or disaccharides with GlcNAc and the core-fucose residue, which represents afucosylated species and fucosylated species, respectively. Antibodies and glycopeptides after Endo F2 and Endo H digestion were then analyzed by LC-MS. The presence and absence of fucose is not expected to affect the ionization efficiency significantly and the level of afucosylation can thus be calculated.

LC-MS Analysis of the Reduced Antibodies

The recombinant mAbs were reduced and then analyzed by LC-MS either before or after Endo F2 and Endo H digestion. Mass spectra of the heavy chain from the three mAbs with the N-linked oligosaccharides are shown in Figure 2. Multiple peaks were observed corresponding to the molecular weights of the respective heavy chains with the commonly known oligosaccharides, G0, G0F, G1F, and G2F. High mannose oligosaccharides were not detected although they were detected when glycopeptides were analyzed, as will be discussed later. After Endo F2 and Endo H digestion, mass spectra with fewer peaks were observed. The main peaks observed correspond to the

Figure 1. Complex and high mannose oligosaccharides are commonly observed in recombinant monoclonal antibodies. Digestion using a combination of Endo H and Endo F2 reduces the complexity of the total oligosaccharides to either monosaccharide with GlcNAc, or disaccharide with GlcNAc and the core-fucose residue

respective heavy chain with monosaccharide, GlcNAc, or disaccharide, GlcNAc with the core-fucose residue. Enzy-

matic digestion using Endo F2 and Endo H followed by LC-MS analysis thus allowed a direct observation of the

Figure 2. Mass spectra of the heavy chain from three mAbs before and after Endo F2 and Endo H digestion. Multiple peaks correspond to the respective heavy chain with G0, G0F, G1F, or G2F [upper panels of (a), (b), and (c)]. Only two peaks were observed after Endo F2 and Endo H digestion, which correspond to the respective heavy chain with either GlcNAc or GlcNAc and the core fucose [lower panels of (a), (b), and (c)]. Other low abundant peaks are due to either Non-covalent adducts or chemical modification such as oxidation

afucosylated heavy chain and comparison between different antibodies. The level of afucosylation can be estimated by the relative intensity of the peak corresponding to heavy chain with GlcNAc and the peak corresponding to heavy chain with GlcNAc and fucose for each antibody. However, more accurate comparison can only be obtained from analyzing of glycopeptides, as will be discussed later.

LC-MS Analysis of the Glycopeptides from Trypsin Digestion

The level of afucosylation was also determined by analyzing glycopeptides as outlined in Figure 1 in the Supplement Section. Glycopeptides were first analyzed directly by LC-MS. The level of afucosylation was calculated by dividing the amount of glycopeptides without the core-fucose by the sum of all glycopeptides using extracted ion chromatogram (EIC). Glycopeptides after digestion using Endo F2 and Endo H were analyzed again by LC-MS. The level of afucosylation was calculated by dividing the amount of peptide with GlcNAc by the sum of the peptides with either GlcNAc or GlcNAc with core-fucose using EIC peak areas.

The mass spectrometer parameters were optimized for the analysis of glycopeptides. In-source fragmentation

was minimized to avoid the breakdown of the labile glycosidic bonds, which otherwise will affect quantitation of afucosylation significantly. For Agilent 6538 Q-TOF MS used in this study, two source parameters control the in-source fragmentation, the fragmentor voltage and the gas temperature. Minimal level of fragmentation and stable signals for all glycopeptides were achieved when the gas temperature was set at 200 °C and fragmentor was set at 125 V. For example, there was minimal difference in the ratio of G0/G0F when the fragmentor voltage was set at either 125 or 175 V with various source temperatures (Supplement Table 1). This set of settings was used for all the experiments to analyze glycopeptides.

The various glycoforms of the glycopeptides from the three mAbs elute between 6.0 to 6.5 min. An average full scan MS spectrum was used to identify the glycans presented on this glycopeptides (Figure 3). Peaks were assigned to the commonly observed oligosaccharides based on the molecular weights. The high resolution and high mass accuracy of Q-TOF MS allows confident identification of 9 glycoforms (± 10 ppm). The glycan identity was further confirmed by MS/MS experiment. As an example, an MS/MS spectrum obtained from G0F glycopeptide is shown in Figure 4. A series of oligosaccharides breakage fragment ions were observed, which confirms the oligosaccharide

Figure 3. Full scan mass spectrum obtained from the analysis of the glycopeptides from mAb-A tryptic digestion. The spectrum was acquired across the retention time window where the glycopeptides with different oligosaccharide structures eluted. Square represents GlcNAc, circle represents Man, triangle represents Fuc, and diamond represents Gal

Figure 4. CID MS/MS spectrum obtained from the glycopeptide with G0F. The major fragment ions correspond to fragmentation of glycosidic bonds. Doubly charged ions are labeled, while unlabeled peaks are singly charged. A series of fragment ions confirmed the oligosaccharide structure. Square represents GlcNAc, circle represents Man, and triangle represents Fuc

structure determined by full scan MS. The amino acid sequence information of the glycopeptide was not available from direct fragmentation of the glycopeptides because the spectrum is dominated by the fragment ions from the breakage of the glycosidic bonds.

The level of afucosylation was then determined either by direct calculation using glycopeptides detected by LC-MS or by calculation using glycopeptides detected by LC-MS after Endo F2 and Endo H digestion. First, the afucosylation level was calculated by dividing the EIC peak areas of glycopeptides without the core-fucose by the sum of the EIC peak area of all glycopeptides detected and then multiplied by 100. As shown in Table 1 (direct analysis), slightly different levels of afucosylation are associated with the three recombinant monoclonal antibodies. Secondly, tryptic peptides from the three mAbs were further digested using Endo F2 and Endo H as described above. An example of full scan MS was shown in Figure 5a. The peak with an *m/z* of 696.81 corresponds to the glycopeptide with GlcNAc only. The peak with an *m/z* of 769.84 corresponds to the glycopeptide with GlcNAc and the core-fucose. The identities of the two

peaks were confirmed by fucosidase digestion of the glycopeptides containing fraction. After fucosidase digestion, only the peak with *m/z* 696.8 was observed (Figure 5b). The percentage of afucosylation was calculated by dividing the EIC peak area of the glycopeptides with GlcNAc only by the sum of the EIC peak areas of the glycopeptides with GlcNAc or with GlcNAc and core-fucose and then multiplied by 100. The results are shown in Table 1 (Endo H and F2). The difference in the afucosylation levels of the three antibodies are in agreement with the difference calculated from direct analysis. However, the afucosylation level obtained after Endo F2 and Endo H digestion is higher compared with that obtained from direct glycopeptides analysis. Higher level of afucosylation is not due to the loss of fucose during mass spectrometry analysis. Using the ratio of G0/G0F as an indicator, slightly higher level of G0 was observed when the fragmentor voltage was set at 250 V compared with a setting of 125 V (Supplement Table 1), indicating a small loss of fucose. However, there was no difference between fragmentor voltage of 175 and 125 V. In addition, the same instrument parameters were used for direct analysis of glycopeptides and peptides after Endo F2 and Endo H digestion. Higher level of afucosylation after Endo F2 and Endo H digestion was expected and further highlighted that oligosaccharide microheterogeneity causing lower detection sensitivity could be a serious problem for the accurate determination of afucosylation. Endo F2 and Endo H digestion converted those low abundant oligosaccharides that may not be detectable as individual species

Table 1. A Fucosylation % Obtained from the Three MABs. The Data Represent an Average ± Standard Deviation from Three Experiments

Samples	Direct analysis	Endo H and F2
mAb-A	9.67±0.33	13.7±0.32
mAb-B	7.15±0.45	11.5±0.34
mAb-C	8.27±0.32	11.9±0.10

Figure 5. Mass spectra of the glycopeptides after various digestions. **(a)** Full scan mass spectrum obtained from the analysis of glycopeptides after Endo H and Endo F2 digestion; **(b)** full scan mass spectrum obtained from the analysis of the glycopeptides after further digestion using fucosidase

Figure 6. CID MS/MS spectrum obtained from the glycopeptide with GlcNAc with core-fucose residue. The major fragment ions correspond to fragmentation of peptide bonds, suggesting peptide sequence can be obtained by CID fragmentation of glycopeptide with small oligosaccharide

into either monosaccharide with GlcNAc or disaccharide with GlcNAc with the core-fucose, which are readily detectable and, therefore, allowed an accurate determination of the level of afucosylation.

The other advantage of LC-MS analysis after Endo F2 and Endo H digestion is determination of the glycosylation site and amino acid sequence around the glycosylation site experimentally by CID fragmentation. Unlike fragmentation by MALDI MS/MS [37] or electron transfer disassociation (ETD)-MS/MS [38], experimental determination of the glycosylation site is impossible by ESI-CID fragmentation of glycopeptides with larger oligosaccharides because the spectrum is dominant by fragment ions from the breakage of the glycosidic bonds (Figure 4). Although this may not be critical for the analysis of glycopeptides from recombinant monoclonal antibodies because of the presence of only one conserved glycosylation site for the majority of antibodies, it may help to determine site-specific glycosylation for proteins with more than one glycosylation site. The glycopeptides containing N-linked GlcNAc with core-fucose residue was fragmented by CID. The collision energy was optimized, and 40 V was used. Under this CID condition, fragment ions corresponding to the peptide with GlcNAc and the core-fucose can hardly be detected, however, fragment ions corresponding to the peptide with GlcNAc alone can be readily detected (Figure 6). The observed Y_5 , Y_6 , and Y_7 ion masses are in agreement with those calculated from the peptide fragments with N-linked GlcNAc. The site of glycosylation was localized to the Asn residue as predicted from the sequence.

Conclusion

An LC-MS-based method that specifically quantified the level of afucosylation in recombinant mAbs was developed. The method used a combination of Endo F2 and Endo H digestion to reduce the otherwise complicated oligosaccharide population to either monosaccharide of GlcNAc residue or disaccharides of GlcNAc with the core-fucose residue. Furthermore, it also collapsed low abundant species that are typically undetectable into either GlcNAc or GlcNAc with the core fucose. The level of afucosylation could, therefore, be easily determined for each mAb and compared between different mAbs. Analysis of reduced mAbs after Endo F2 and Endo H provided a quick estimation and comparison of the level of afucosylation. An accurate determination can only be obtained by analysis of the glycopeptides. In addition to achieve an accurate quantitation of afucosylated oligosaccharides, the method also allowed a direct determination of the site of glycosylation by CID fragmentation because the larger sizes of oligosaccharides were reduced to smaller size oligosaccharides with either monosaccharide or disaccharide.

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