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Characterization of the glycosylation state of a recombinant monoclonal antibody using weak cation exchange chromatography and mass spectrometry

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Abstract

Recombinant monoclonal antibody heterogeneity is inherent due to various enzymatic and non-enzymatic modifications. In this study, a recombinant humanized monoclonal IgG1 antibody with different states of glycosylation on the conserved asparagine residue in the CH_2 domain was analyzed by weak cation exchange chromatography. Two major peaks were observed and were further characterized by enzymatic digestion and mass spectrometry. It was found that this recombinant monoclonal antibody contained three glycosylation states of antibody with zero, one or two glycosylated heavy chains. The peak that eluted earlier on the cation exchange column contained antibodies with two glycosylated heavy chains containing fucosylated biantennary complex oligosaccharides with zero, one or two terminal galactose residues. The peak that eluted later from the column contained antibodies with either zero, one or two glycosylated heavy chains. The oligosaccharide on the antibodies eluted in the later peak was composed of only two GlcNAc residues. These results indicate that conformational changes in large proteins such as monoclonal antibodies, caused by different types of neutral oligosaccharides as well as the absence of oligosaccharides, can be differentiated by cation exchange column chromatography.

Keywords: Recombinant monoclonal antibody; Oligosaccharides; WCX-10; Mass spectrometry

1. Introduction

A recombinant monoclonal antibody is synthesized based on specific gene sequences for both light and heavy chains, which should result in the production of a homogeneous population of molecules with the same protein sequence. However, enzymatic and non-enzymatic modifications introduced during protein synthesis and assembly, cell culture, purification, formulation, storage and incubation under various accelerated stability conditions often convert the homogeneous population into a heterogeneous one. Thus antibody products do demonstrate heterogeneity in characteristics such as molecular weight, charge, and conformation.

Ion exchange chromatography is a method commonly used to analyze antibody charge heterogeneity. When analyzed by ion-exchange chromatography, monoclonal antibodies usually elute in multiple peaks. Multiple peaks of different retention times have been determined to be due to factors such as the absence or presence of a C-terminal lysine (Lys) residue [1–8], cyclyzation of the N-terminal glutamine to pyroglutamate [2,7], deamidation [5,7,9–12], the presence of sialic acid [3,7], isomerization [11], amidation [8], expression of a leader or intron sequence [6,7], or oxidation of methionine residues [13].

The effect of various modifications on the elution of antibodies from ion exchange columns can be classified into three categories including direct contribution to charge difference, positional effect and conformational effect. Firstly, modifications can affect the elution of antibodies from ion exchange chromatography columns by contribution to charge directly. For example, a Lys residue introduces a positive charge. Therefore, on cation exchange columns antibodies with two C-terminal Lys will elute later than antibodies with one C-terminal Lys, followed by antibodies containing no C-terminal Lys. Treatment with carboxypeptidase B, which removes C-terminal Lys, changes this profile of three peaks with different Lys residues into a single peak containing antibodies with no C-terminal

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Lys residues [1,3,9]. This indicates that the presence of the C-terminal Lys affects the elution profile of antibodies from ion exchange columns only by direct alteration of charge. The effect of sialic acid on elution is also based on net charge differences. Sialic acid introduces a negative charge and antibodies with oligosaccharides containing sialic acid will elute earlier on cation exchange columns. Removal of sialic acid with sialidase treatment results in elution of antibodies consistent with antibodies containing asialylated oligosaccharides [3]. Secondly, the same modification located at different positions of the antibody can have an effect on the retention time. For example, antibodies with the same number of pyroglutamate residues elute at different retention times depending on the position of the pyroglutamate [2]. Positional effects on elution profile have also been reported for deamidation [9]. The positional effects are likely due to the fact that antibodies are not perfectly symmetrical as demonstrated by Sapphire et al. [14,15], which would lead to differences in local surface charges that interact with the column matrix. Lastly, modifications can affect elution by affecting conformation. This is shown by comparing the retention times of antibodies with either aspartate or isoasparate residues located at the same position. From the chemical point of view, isoaspartate is slightly more acidic than aspartate and the antibody containing isoaspartate would be expected to elute earlier from a cation exchange column. However, antibodies with aspartate elute earlier than antibodies with isoaspartate [11]. Isoaspartate introduces an additional methane group to the peptide backbone compared to aspartate, therefore it may cause more significant structural changes than aspartate to the antibody. Although the effects of modifications on column retention time can be classified, in reality, chromatographic behavior of monoclonal antibodies is likely due to simultaneous contribution of multiple factors. Therefore, the behavior of monoclonal antibodies on column chromatography is not always predictable based on the chemical nature of the modifications alone.

In this study, a recombinant humanized monoclonal antibody produced in human embryonic kidney (HEK) 293 cell line during the research stage was analyzed by weak cation exchange chromatography. The major peaks were analyzed by enzymatic digestion and mass spectrometry to determine their composition. It was found that the multiple peaks were due to different glycosylation states of the antibody.

2. Experimental

The recombinant humanized monoclonal IgG1 antibody was expressed in HEK 293 cell line by transient expression and purified using protein A chromatography at Abbott Bioresearch Center (Worcester, MA).

A Shimadzu HPLC and a weak cation exchange WCX-10 column (250 mm × 4 mm) from Dionex (Sunnyvale, CA) were used for analyzing charge variants of this antibody. Mobile phase A was 10 mM sodium phosphate, pH 6.0. Mobile phase B was 10 mM sodium phosphate, 500 mM sodium chloride, pH 6.0. The samples were injected at 90% mobile phase A and 10% mobile phase B. After running at the initial condition for 5 min, the percentage of mobile phase B was increased to 50% within 20 min. The column was then washed by increasing mobile phase B to 100% within 5 min and running at 100% mobile phase B for 5 min. The percentage of mobile phase B was decreased to 5% in 5 min. The column was equilibrated at 5% mobile phase B for 10 min before the next injection. The flow-rate was set at 1 mL/min. Protein elution was monitored at UV 280 nm and 214 nm. Results were reported in mV and the instrument was set at 2.5 AU/V.

Fractions of the recombinant monoclonal antibody were collected using a semi-preparative WCX-10 column (250 mm × 9 mm) from Dionex. The mobile phases and gradient were the same as described previously for the analytical WCX-10 column except a 5 mL/min flow rate was used. The fractions were collected and concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA) with a molecular weight cut-off of 10 kDa. The purity of the fractions was analyzed using the analytical WCX-10 column procedure.

For deglycosylation, the recombinant monoclonal antibody and the collected fractions were digested with PNGaseF (2.5 mU/μL, Prozyme, San Leandro, CA) at a ratio of approximately 1 μL enzyme: 200 μg antibody. *N*-Octylglucoside (Roche, Indianapolis, IN) was included in the sample preparation to a final concentration of 1% (w/v) to facilitate the removal of N-linked oligosaccharides. Digestion was allowed to proceed at 37 °C for 18 h. The samples were reanalyzed using analytical WCX-10 chromatography, and fractions were collected using the semipreparative WCX-10 column.

For molecular weight determination, the samples were first diluted to 0.5 mg/mL and then reduced with 10 mM dithiothreitol (DTT) (Sigma, St. Louis, MO) at 37 °C for 30 min. Molecular weights of the reduced samples were determined by LC–MS. An Agilent HPLC (Santa Clara, CA) and a protein C4 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 A pore size) were used to desalt, separate and introduce samples into a Q star mass spectrometer (Applied Biosystems, Framingham). Five micrograms of each sample was loaded at 95% mobile phase C (0.02% TFA, 0.08% FA in Milli-Q water) and 5% mobile phase D (0.02% TFA, 0.08% FA in acetonitrile). After running at 5% mobile phase D for 5 min, proteins were eluted off the column by increasing mobile phase D to 65% within 35 min. The column was washed by increasing mobile phase D to 95% in 5 min and then decreasing to 5% in another 5 min. The column was equilibrated at 5% mobile phase D for 10 min before the next injection. The flow rate was set at 50 μL/min and the column oven was set at 60 °C. The mass spectrometer scan range was set at a range of *m/z* 800–2500. The IonSpray voltage was set at 4500 V and the source temperature was set at 350 °C.

For peptide map, fractions of the deglycosylated antibody were denatured with 6 M guanidine hydrochloride in 100 mM Tris, pH 8.0, reduced with 10 mM DTT at 37 °C for 30 min and then alkylated with 25 mM iodoacetic acid (Sigma) at 37 °C for another 30 min. The samples were diluted six fold using Milli-Q water. Trypsin (Worthington, Lakewood, NJ) was added to the samples at an enzyme:antibody ratio of 1:50 (w/w) and the samples were incubated at 37 °C for 18 h. Proteolysis was stopped by adding 1N HCl. The Agilent HPLC and a C18 column (Vydac, 250 mm × 1 mm i.d., 5 μm particle size, 300 A pore