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Characterization of recombinant monoclonal antibody variants detected by hydrophobic interaction chromatography and imaged capillary isoelectric focusing electrophoresis

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ABSTRACT

In-depth characterization of the commonly observed variants is critical to the successful development of recombinant monoclonal antibody therapeutics. Multiple peaks of a recombinant monoclonal antibody were observed when analyzed by hydrophobic interaction chromatography and imaged capillary isoelectric focusing. The potential modification causing the heterogeneity was localized to F(ab′)2 region by analyzing the antibody after IdeS digestion using hydrophobic interaction chromatography. LC-MS analysis identified asparagine deamidation as the root cause of the observed multiple variants. While the isoelectric focusing method is expected to separate deamidated species, the similar profile observed in hydrophobic interaction chromatography indicates that the single site deamidation caused differences in hydrophobicity. Forced degradation demonstrated that the susceptible asparagine residue is highly exposed, which is expected as it is located in the light chain complementarity determining region. Deamidation of this single site decreased the mAb binding affinity to its specific antigen.

1. Introduction

Recombinant monoclonal antibodies (mAbs) are subject to various modifications. Enzymatic modifications occur mainly during the cell culture stage due to the presence of host cell proteins including enzymes under physiological conditions. On the other hand, non-enzymatic reactions occur during all stages of the entire manufacturing process including cell culture, purification and storage. Those modifications turn the otherwise homogeneous molecules expected from a single light chain gene and a single heavy chain gene into highly heterogeneous molecules at the time of batch release. Modifications during long-term storage, accelerated or other forced degradation conditions further increase the molecular heterogeneity. In-depth characterization of the heterogeneity provides understanding of the major modifications or degradation pathways of the molecule and is critical to the successful development of mAb therapeutics by implementing appropriate control strategy, optimizing formulation and storage conditions.

Because charge variants are the most commonly observed source of heterogeneity for mAbs, charge-based methods are routinely used to monitor the mAb charge profile at the time of batch release and during stability. Using the main variant as the reference point, variants with relatively lower isoelectric point (pI) are defined as acidic variants. While variants with relatively higher pI are defined as basic variants. The main variant usually contains mAb with N-terminal pyroglutamate (pyroGlu), neutral oligosaccharides, and complete removal of the C-terminal lysine (Lys). Acidic variants are composed of antibodies with modifications such as sialylation [1–5], asparagine (Asn) deamidation [2,3,6–12], glycation [3,13], and in rare cases, modification of arginine (Arg) residues by methylglyoxal [14], or sulfation of tyrosine (Tyr) residue [15]. Basic variants are composed of mAbs with C-terminal Lys [1,2,6,7,11,16] and uncycled N-terminal glutamine (Gln) [2,17–19], the presence of partial leader sequence [11,20], C-terminal amidation [11,21,22], succinimide formed from the isomerization of aspartate (Asp) residues [7,23–25], smaller oligosaccharides [26], methionine (Met) oxidation [27,28], and incompletely formed disulfide bonds [18].

MAb variants have also been commonly observed by hydrophobic interaction chromatography (HIC). Because HIC separates proteins that differ in hydrophobicity [29], mAb variants with modifications that impact hydrophobicity directly or indirectly by causing structural changes, are expected to be separated. Studies have demonstrated that HIC is capable of separating antibody variants with free thiols [25,30], Asp isomerization and Asn deamidation products [7,25,30–33], and

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oxidation of tryptophan (Trp) and Met residues [30,34]. In the case of Asp isomerization, mAbs containing isoAsp have been shown to result in either an earlier [7,35] or later [25,31,36] elution compared to the native mAbs. Considering the difference between Asp and isoAsp is minimal, it is possible that the introduction of a methylene group by isoAsp to the peptide backbone may cause local conformational changes, which result in differences in hydrophobicity. On the other hand, in the case of deamidation, the conversion of the neutral side chain of Asn to the negatively charged side chains of Asp and isoAsp may account for the earlier elution of mAbs with deamidated products compared to the original Asn [7].

In the current study, multiple variants of a mAb were observed when analyzed by HIC. LC-MS analysis of the collected HIC fractions identified deamidation of an Asn residue in the light chain complementarity determining region (CDR) as the major cause of the observed multiple peaks by HIC. The HIC profile was similar to the profile observed by imaged capillary isoelectric focusing electrophoresis (icIEF), suggesting deamidation of the same Asn residue can account for difference in both hydrophobicity and charge. Deamidation of this Asn residue was mainly driven by solvent pH and caused a substantial reduction in antigen binding affinity.

2. Experimental

2.1. Materials

The recombinant monoclonal IgG1 antibody was expressed in a Chinese hamster ovary (CHO) cell line and purified at Alexion (New Haven, CT). Acetonitrile, dithiothreitol, guanidine hydrochloride, iodoacetic acid, and trifluoroacetic acid (TFA) were purchased from Sigma (St, Louis, MO). IdeS enzyme, FabRICATOR, was purchased from Genovis (Cambridge, MA). Trypsin and chymotrypsin were purchased from Promega (Madison, WI).

2.2. Hydrophobic interaction chromatography

A Waters Alliance high performance liquid chromatography (HPLC) system and a hydrophobic interaction chromatography (HIC) column (ProPac HIC-10, 4.6 × 150 mm, Thermo Scientific, Sunnyvale, CA) were used for separation of the antibody variants. Mobile phase A is composed of 1 M sodium sulfate in phosphate buffered saline (PBS) at pH 7.4. Mobile phase B is PBS at pH 7.4. The column was run at a flow rate of 0.5 mL/min at ambient temperature. Proteins eluted off the column were monitored using UV absorption at 280 nm.

For analysis of intact antibody, samples were loaded at 50% mobile phase A and 50% mobile phase B. After 5 min, proteins were eluted off the column by increasing mobile phase B from 50% to 90% over 20 min. The column was then washed using 100% mobile phase B for 5 min and then equilibrated using the loading condition. Fractions corresponding to each peak were collected manually under the UV280 nm guidance. The collected fractions were concentrated using Amicon ultra-4 centrifugal devices (Millipore, Burlington, MA) with a molecular weight cut-off of 30 kDa and then analyzed using the same HIC condition to determine the fraction purity.

For analysis of IdeS digested antibody, samples were loaded at 80% mobile phase A and 20% mobile phase B. After 5 min, proteins were eluted off the column by increasing mobile phase B from 20% to 90% over 35 min. The column was then washed using 100% mobile phase B for 5 min and then equilibrated using the loading condition.

2.3. IdeS digestion

Samples were diluted using PBS, pH 7.4, to 2 mg/mL. Each vial of IdeS (5000 units) was reconstituted in 200 μL water. Digestion was

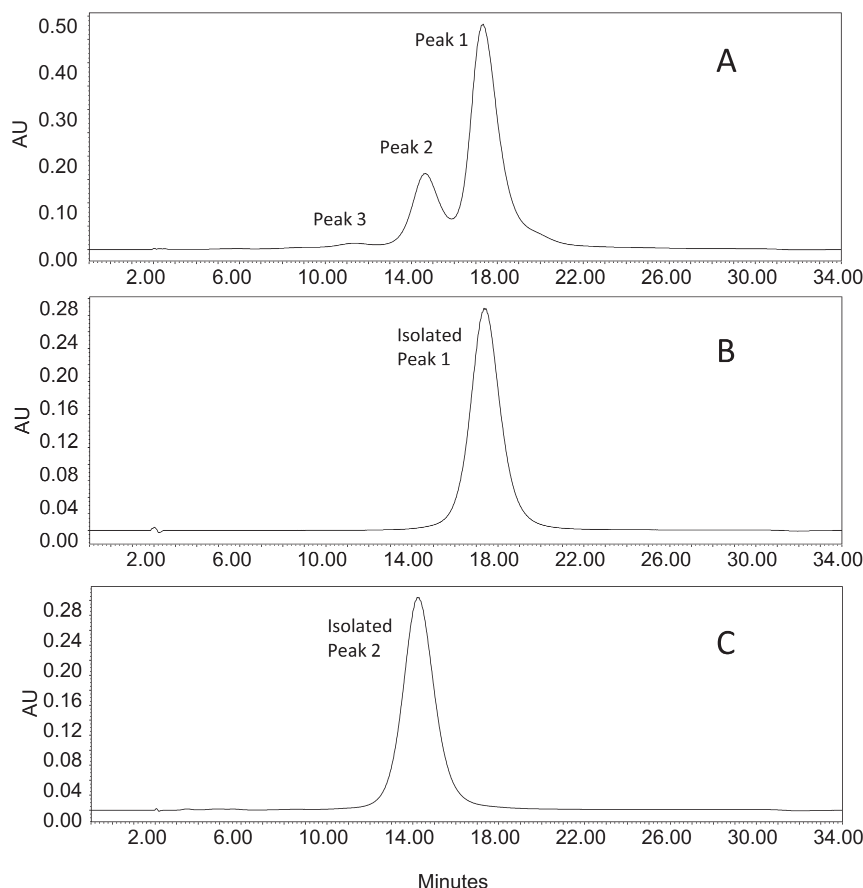

Fig. 1. HIC chromatograms of a mAb (A) and isolated fractions corresponding to the major peak (B), and the earlier eluting peak (C). The small peak prior to peak 2, labeled as peak 3, was only collected for BiaCore analysis. The chromatograms were obtained by injection of 200 μg of the bulk material (A) or 100 μg of each of collected fractions corresponding to the major peak (B) or the earlier eluting peak (C).

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