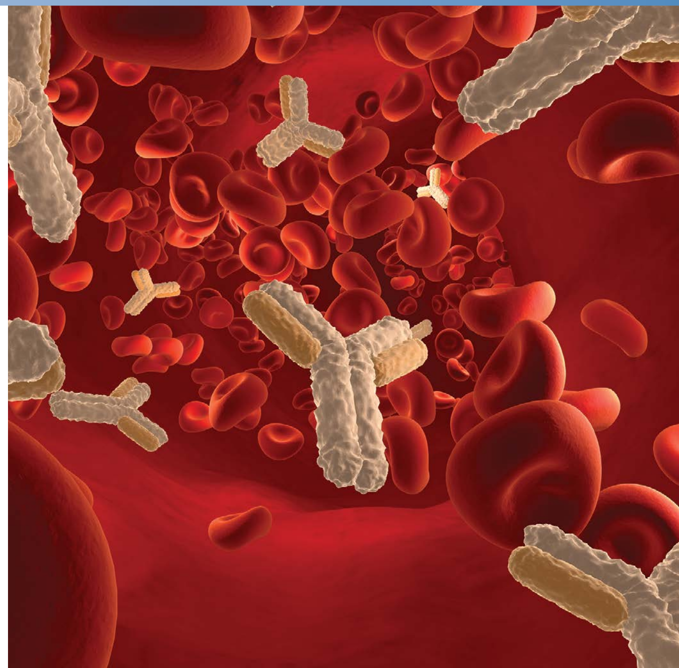
Application Note 21069

Separation of Monoclonal Antibody (mAb) Oxidation Variants on a High-Resolution HIC Column

Julia Baek and Xiaodong Liu, Thermo Fisher Scientific, Sunnyvale, CA, USA



Key Words

Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, oxidation, MAbPac HIC-20

Goal

To demonstrate the separation of oxidized mAb variants from their native form using Thermo Scientific™ MAbPac™ HIC-20 columns.

Introduction

Monoclonal antibody (mAb) drugs are a rapidly growing class of biotherapeutics that target various diseases including autoimmune disorders, cardiovascular diseases, infectious diseases, and cancers.[1] Recombinant mAbs are subject to a variety of biochemical modifications during processing, delivery, and storage. Some of these modifications have been shown to affect the safety and efficacy of mAb therapeutics, increasing the importance of analytical methods to detect mAb variants. Among these modifications, oxidation of exposed amino acid residues such as tryptophan (Trp) and methionine (Met) is a major concern in therapeutic mAb stability studies. A number of researchers have reported oxidation of mAbs and the adverse effect on product shelf life and bioactivity.[2] Oxidation of amino acid residues on a mAb can alter the hydrophobic nature of the mAb by either the increase in polarity of the oxidized form or the resulting conformational change. Therefore, hydrophobicity-based HPLC methods such as reversed-phase liquid chromatography (RPLC) and hydrophobic interaction liquid chromatography (HIC) are often used to characterize oxidized mAb products.[2]

Several recent studies have indicated that the Thermo Scientific™ ProPac™ HIC-10 column provided desired selectivity for oxidized mAbs, as an excellent alternative to RPLC.[3] HIC is a method that separates proteins, including monoclonal antibodies based on molecular hydrophobicity. The HIC mobile phase usually consists of a salting-out agent, which at high concentration retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted by decreasing the salt concentration. In contrast to RPLC, HIC mobile phases typically contain little or no organic solvent at physiological pH levels, which allows the protein to preserve its native structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC.[4]

The MAbPac HIC-20 column is a high-resolution, silica-based HIC column designed for the separation of mAbs and mAb variants. Its unique proprietary column chemistry provides high resolution, rugged stability, and desired selectivity for the analysis of mAbs and related variants. Here we describe the separation of oxidized variants of two mAbs on MAbPac HIC-20 columns.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Isopropanol (Fisher Scientific P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98.0%)
- Ammonium sulfate ($(NH_4)_2SO_4$, ≥99.0%)
- Hydrogen peroxide (H_2O_2) (Fisher Scientific P/N H325-500)
- 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Fisher Scientific P/N AC40156-0250)
- Sodium chloride (NaCl ≥99.5%)
- Sodium acetate trihydrate (Fisher Scientific P/N 50-877-058)

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Two therapeutic mAbs were provided by a customer.

Preparation of 2× oxidation buffer

Dissolve 2.014 g of 360 mM sodium chloride and 0.136 g of 10 mM sodium acetate in 90 mL of DI water. Adjust the pH to 5.0 with 10 N HCl. Bring the final volume to 100 mL with DI water and filter the solution with a 0.22 µm filter.

Preparation of H_2O_2 oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2× oxidation buffer. Then add H_2O_2 to a final concentration of 0.01% (v/v) and incubate the sample for 24 h at room temperature. Dilute the oxidized mAb solution in half with mobile phase A (2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0) before analysis. The final oxidized mAb concentration is 1.25 mg/mL.

Preparation AAPH oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2× oxidation buffer. Then add AAPH to a final concentration of 1 mM and incubate the sample for 24 h at 40 °C. Dilute the oxidized mAb solution in half with mobile phase A (2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0) before analysis. The final oxidized mAb concentration is 1.25 mg/mL.

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS LC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a micro flow cell
Column(s)	MAbPac HIC-20, 4.6 × 100 mm (P/N 088553)
	MAbPac HIC-20, 4.6 × 250 mm (P/N 088554)
Mobile phase A	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) and 264.2 g of ammonium sulfate in 800 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Mobile phase B	100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) in 900 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Gradient	Specified in Figures 1–4
Flow rate	1.0 or 0.5 mL/min
Temperature	30 °C
UV detector wavelength:	280 nm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

A recent study on protein oxidation showed that hydrogen peroxide (H_2O_2) and t-butyl hydroperoxide (t-BHP) primarily oxidize Met residues, while 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and H_2O_2 + Fe(II) oxidize both Met and Trp residues. AAPH was reported to be more efficient in oxidizing Trp on a model protein.[5] In this study, H_2O_2 and AAPH were used as oxidants to induce Met and Trp oxidation on mAb samples. Using the standard HIC mobile phase condition, the MAbPac HIC-20 was able to differentiate oxidized mAb variants from the untreated mAb (Figure 1). Oxidation of the mAb with H_2O_2 resulted in two peaks with lower retention times, presumably by introducing conformational changes. Over time, the intensity of peak 1 continued to increase as the intensity of peak 2 decreased (data not shown). It is most likely that peak 1 has more residues oxidized than peak 2. As the oxidation continues, concentration of the variant with more sites oxidized increases.

For the AAPH oxidized mAb sample, a broader peak with two non-resolved shoulders where H_2O_2 oxidized variants (peaks 1 and 2) elute was observed. We speculate that AAPH oxidized both Met and Trp residues. The oxidation of a more buried Trp can lead to a more dramatic structural change that may cause the mAb to partially unfold. Partially unfolded mAb is more likely to have more conformational variations and result in a broader peak. This phenomenon has been reported previously with another mAb sample on ProPac HIC-10 column.[3] To optimize the resolution of the chromatograms, conditions using a lower starting salt concentration and addition of organic solvent into both mobile phases were investigated. Both approaches reduced the retention time. However, reducing the starting ammonium sulfate concentration to 1 M improved the resolution of the two oxidized mAb variants (Figure 2), while addition of isopropanol in both mobile phases reduced the resolution (data not shown). Mobile phases containing sodium acetate pH 5.2 buffer were also examined, but no improvement on the separation of the mAb and its oxidized variants was exhibited (data not shown).

Column: **MAbPac HIC-20**, 5 µm
Format: 4.6 × 100 mm
Mobile Phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile Phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Flow Rate: 1.0 mL/min
Inj. Volume: 10 µL
Temp.: 30 °C
Detection: UV (280 nm)
Sample: mAb (1.25 mg/mL)
H_2O_2 oxidized mAb (1.25 mg/mL)
AAPH oxidized mAb (1.25 mg/mL)

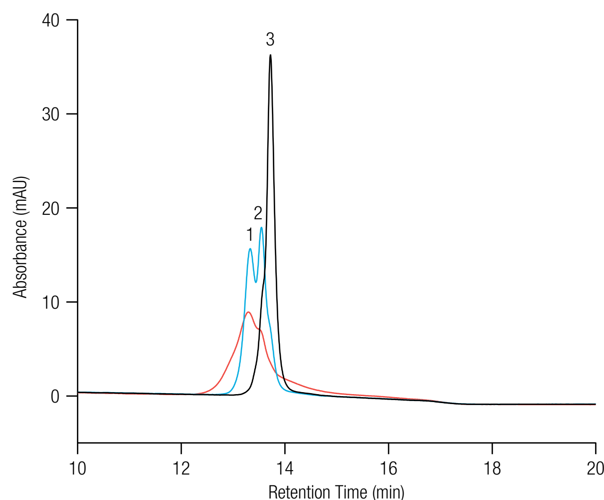

Figure 1. Separation of oxidized mAb1 using standard condition

Column: **MAbPac HIC-20**, 5 µm
Format: 4.6 × 100 mm
Mobile Phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile Phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-5.0	50	50
0.0	50	50
1.0	50	50
15.0	0	100
20.0	0	100

Flow Rate: 1.0 mL/min
Inj. Volume: 10 µL
Temp.: 30 °C
Detection: UV (280 nm)
Sample: mAb (1.25 mg/mL)
H_2O_2 oxidized mAb (1.25 mg/mL)
AAPH oxidized mAb (1.25 mg/mL)

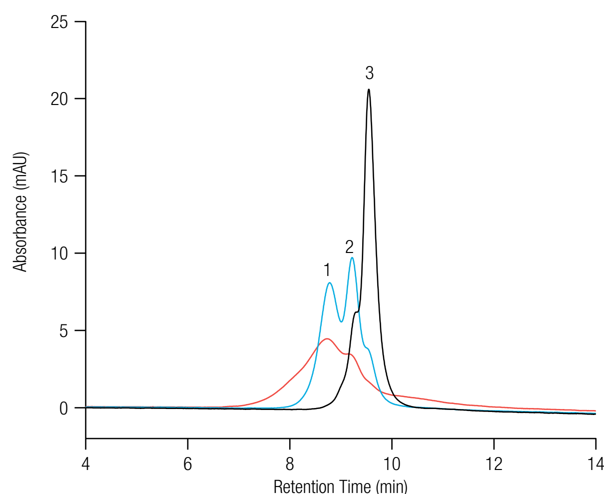

Figure 2. Separation of oxidized mAb1 using lower starting salt concentration

To further improve the resolution, a 4.6 × 250 mm column was employed. Using a flow rate of 0.5 mL/min and a starting ammonium sulfate concentration of 1 M, highest resolution was achieved with a relatively short analysis time (Figure 3). In this chromatogram, two oxidized species are also clearly observed from the untreated mAb chromatogram.

Using a similar condition another oxidized mAb sample was analyzed (Figure 4). Similar to the first mAb, H_2O_2 oxidized mAb2 has two distinct variant peaks and the AAPH oxidized sample has a broad peak with two non-resolved shoulders where the H_2O_2 oxidized peaks (peak 1 and 2) elute. This mAb sample appears to have two small oxidation variants as well as two hydrophobic variants (peak 4 and 5) on the right side of the main peak (peak 3).

Column: **MAbPac HIC-20**, 5 µm
Format: 4.6 × 250 mm
Mobile Phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile Phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Flow Rate: 0.5 mL/min
Inj. Volume: 20 µL
Temp.: 30 °C
Detection: UV (280 nm)
Sample: mAb (1.25 mg/mL)
H_2O_2 oxidized mAb (1.25 mg/mL)
AAPH oxidized mAb (1.25 mg/mL)

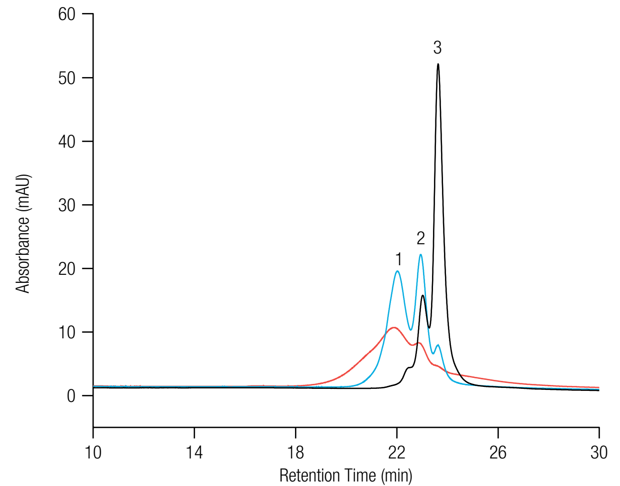

Figure 3. Separation of oxidized mAb1 on a 4.6 × 250 mm column

Column: **MAbPac HIC-20**, 5 µm
Format: 4.6 ×250 mm
Mobile Phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile Phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-6.0	40	60
0.0	40	60
2.0	40	60
30.0	0	100
35.0	0	100

Flow Rate: 0.5 mL/min
Inj. Volume: 20 µL
Temp.: 30 °C
Detection: UV (280 nm)
Sample: mAb (1.25 mg/mL)
H_2O_2 oxidized mAb (1.25 mg/mL)
AAPH oxidized mAb (1.25 mg/mL)

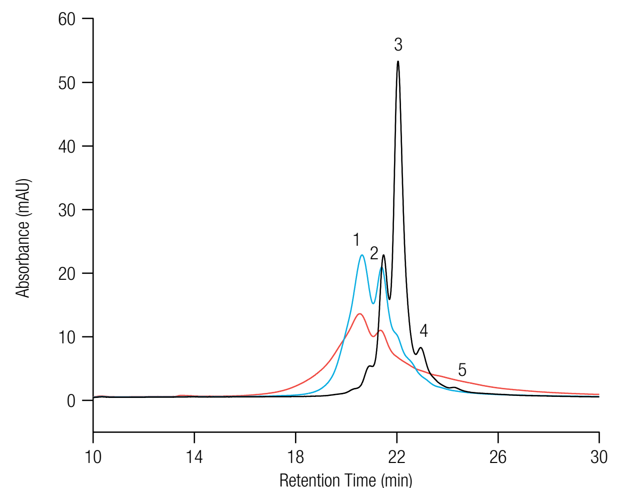

Figure 4. Separation of oxidized mAb2 on a 4.6 × 250 mm column

Conclusion

- The MAbPac HIC-20 column is a high-resolution HIC column that allows the separation of oxidized mAb variants from its native form.
- Using the MAbPac HIC-20 column, separation of oxidized mAb variants can be achieved without further sample processing.
- Best separation was achieved with 0.5 mL/min and 1.0–1.2 M starting salt concentration on a 4.6 × 250 mm MAbPac HIC-20 column.

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