Application Note 1084

Separation of Papain Digests of Oxidized Monoclonal Antibodies

Dai Zhenyu,[1] Xu Qun,[1] Lina Liang,[1] and Jeffrey Rohrer[2]
[1]Thermo Fisher Scientific, Shanghai, People's Republic of China;
[2]Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
MAb Heterogeneity/Oxidation, Hydrophobic Interaction Chromatography (HIC), ProPac HIC-10 Column

Goal
To develop a HIC method for separation/enrichment of oxidized monoclonal antibody (MAb) species for further MAb modification characterization

Introduction
Many MAbs have been developed as human therapeutics and several others are under development. Monoclonal antibodies are subject to various post-translational modifications and are susceptible to degradation during storage and delivery. Degradation proceeds through various mechanisms including oxidation, deamidation, isomerization, and cleavage of peptide bonds. Both post-translational modification and degradation lead to a variety of charge and hydrophobic variants. Due to the potential impact of these modifications to MAb safety and efficacy, a variety of liquid chromatography (LC) methods have been developed to fully characterize and monitor MAb heterogeneity.

Cation-exchange chromatography (CEX) and size-exclusion chromatography (SEC) are routinely used for characterizing and release testing therapeutic antibodies, but these techniques may not be enough to fully characterize the low-level population of MAb variants that are a result of modification or degradation. Hydrophobic interaction chromatography (HIC), a method that is partially or fully orthogonal to CEX and SEC, provides the possibility of separating and purifying low-abundance MAb fractions in stability studies. With a relatively high column capacity and nondenaturing mobile phase conditions (i.e., little or no organic solvent), HIC-separated fractions will typically retain protein activity and are thus amenable to subsequent analysis.[1]

Among these modifications, oxidation—especially oxidation of methionine (Met) and tryptophan (Trp)—is a main concern and focus in therapeutic MAb stability studies. Oxidation of Met and Trp changes hydrophobicity of the MAb surface and HIC has been shown to separate oxidized MAbs from the native protein.[2]

Equipment, Software, and Consumables
- Thermo Scientific™ Dionex™ UltiMate™ 3000 x2 Dual Biocompatible Analytical LC system, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
  - TCC-3000SD Thermostatted Column Compartment
  - DAD-3000 Diode Array Detector with 13 µL Flow Cell
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.20
- Standard RC Dialysis Tubing, 10 kD MW (Spectrum Laboratories P/N 132592)

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Reagents and Standards

- Deionized (DI) water, 18.2 M -cm resistivity
- 2-Propanol (Optima™), ≥99.9% (Fisher Scientific P/N A464-4)
- Sodium phosphate monobasic anhydrous (NaH_2PO_4, Fisher Scientific P/N S397-500)
- Ammonium sulfate ($[NH_4]_2SO_4$, Fisher Scientific P/N A702-500)
- Sodium acetate trihydrate, HPLC (Fisher Scientific P/N S220-1)
- Sodium chloride (NaCl), Crystalline/Biological, Certified (Fisher Scientific P/N S671-500)
- 2,2'-Azobis(2-methylpropionamidine) dihydrochloride/2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), 98% (Fisher Scientific P/N AC40156-0250)
- Hydrogen peroxide (H_2O_2), 30% (Fisher Scientific P/N H325-500)
- Hydrochloric acid ([HCl], 25% v/v [1+3], Fisher Scientific P/N SA812-1)
- Sodium hydroxide (NaOH) solution, 50% w/w/Certified (Fisher Scientific P/N SS254-500)
- Tris (hydroxymethyl) aminomethane (Fisher Scientific P/N T395-500)
- L-Cysteine, 99+% (Fisher Scientific P/N AC173600250)
- Papain, Lyophilized (Fisher Scientific P/N LS003119)
- Ethylenediaminetetraacetic acid (EDTA), 99.5+% (Fisher Scientific P/N BP118-500)

Conditions (Applicable to Figures 1–4)

Column:	Thermo Scientific™ ProPac™ HIC-10, 4.6 × 250 mm (P/N 074197)		
Mobile Phase:	**Formula 1** A. 0.1 M NaH_2PO_4, 2 M $(NH_4)_2SO_4$, 7% isopropanol, pH 7.0 B. 0.1 M NaH_2PO_4, 7% isopropanol, pH 7.0 **Formula 2** A. 20 mM Sodium acetate, 2 M $(NH_4)_2SO_4$, 7% isopropanol, pH 5.0 B. 20 mM Sodium acetate, 7% isopropanol, pH 5.0		
Flow Rate:	0.6 mL/min		
Inj. Volume:	50 µL		
Temperature:	30 °C		
UV Detection:	Absorbance at 280 nm		
Gradient:	Time (min)	A%	B%
	0	100	0
	40	0	100
	50	0	100

Preparation of Solutions

Preparation of Mobile Phases

Formula 1

For Mobile Phase A, dissolve 11.156 g NaH_2PO_4 and 245.780 g $(NH_4)_2SO_4$ in 750 mL DI water, adjust the pH to 7.0 with 50% NaOH solution, bring the volume to 930 mL with DI water, then bring the volume to 1000 mL with isopropanol.

For Mobile Phase B, dissolve 11.156 g NaH_2PO_4 in 900 mL DI water, adjust the pH to 7.0 with 50% NaOH solution, bring the volume to 930 mL with DI water, then bring the volume to 1000 mL with isopropanol.

Formula 2

For Mobile Phase A, dissolve 2.531 g sodium acetate trihydrate and 245.780 g $(NH_4)_2SO_4$ in 750 mL DI water, adjust the pH to 5.0 with 10 N HCl solution, bring the volume to 930 mL with DI water, then bring the volume to 1000 mL with isopropanol.

For Mobile Phase B, dissolve 2.531 g sodium acetate trihydrate in 900 mL DI water, adjust the pH to 5.0 with 10 N HCl solution, bring the volume to 930 mL with DI water, then bring the volume to 1000 mL with isopropanol.

Samples

The MAb samples were generous gifts from two customers.

Preparation of 2× MAb Dilution Buffer

Dissolve 2.014 g sodium chloride and 0.136 g sodium acetate in 90 mL DI water, adjust the pH to 5.0 with 10 N HCl, and bring the volume to 100 mL with DI water. The buffer will contain 360 mM sodium chloride and 10 mM sodium acetate.

Preparation of Oxidized MAb Using Hydrogen Peroxide

To generate H_2O_2-oxidized antibody, first dilute the MAb to 8 mg/mL with DI water, then make a 1:1 dilution with 2× MAb dilution buffer. The solution will have 180 mM sodium chloride, 5 mM sodium acetate, a pH of 5.0, and a MAb concentration of 4 mg/mL. Subsequently, add H_2O_2 to a final concentration of 0.01% (v/v) and incubate the sample for 24 h at room temperature.

Preparation of Oxidized MAb Using AAPH

To generate an AAPH-oxidized antibody, first prepare a buffer-diluted MAb solution as described above. Add AAPH to a final concentration of 1 mM and incubate the sample for 24 h at 40 °C. Store the oxidized MAb at –20 °C before papain digestion or analysis.

MAb Papain Digestion Procedure

Preparation of dialysis buffer

Dissolve 24.228 g tris, 1.169 g EDTA, and 1.211 g cysteine in 1900 mL DI water; adjust the pH to 7.6 with HCl, and bring the volume to 2000 mL with DI water. The buffer will contain 100 mM tris/HCl, 2 mM EDTA, and 5 mM cysteine.

Dialysis of MAb samples

Using the 10 kD dialysis tubing, dialyze the oxidized and original MAb samples against 2 L of 100 mM tris/HCl, 2 mM EDTA, and 5 mM cysteine at a pH of 7.6 for 24 h at room temperature. Change the dialysis buffer once after the first 12 h.

Papain digest

To start the digest, add 1 mg/mL papain solution (in the dialysis buffer) to the dialyzed MAb samples. The papain/MAb ratio will be 1:100. Gently mix and incubate for 4 h at 37 °C. Store the digested samples at –20 °C before analysis.

Results and Discussion

Separation of Intact MAb and Oxidized MAb

In vitro incubation of a MAb with oxidation agents such as H_2O_2 and t-butyl hydrogen peroxide is generally accepted as a simulation of oxidative stress conditions. These agents predominantly generate Met oxidation, with Trp oxidation frequently absent. Recently AAPH has been shown to generate significant Trp oxidation on parathyroid hormone.[3] Because Met and Trp are the main amino acid residues susceptible to oxidation in storage and delivery, H_2O_2 and AAPH were chosen to produce oxidative stress.

Figure 1 shows that the retention time of the H_2O_2-oxidized MAb main peak is quite close to that of the untreated MAb. In the enlarged inset of Figure 1, two peaks that elute earlier than the untreated MAb can be observed after H_2O_2 oxidation. This observation is consistent with a previous report that showed that a MAb oxidized at different Met sites can be separated into more than one peak by HIC.[2] Conversely, the AAPH-oxidized MAb did not show well-resolved peaks, although the MAb oxidized by AAPH showed more retention time/hydrophobicity change than the H_2O_2-oxidized MAb. It is known that Trp residues are not as exposed as Met residues. Thus, modification of buried Trp residues may induce more MAb tertiary structure change that may also be responsible for retention time change.

Separation of MAb and Oxidized-MAb Papain Digests

Digestion of a MAb with papain before analysis is a generally accepted procedure to obtain more information about the sample. As shown in Figure 2, Chromatogram A, Peak 2 can be identified as the antigen-binding fragment (Fab region) and Peak 5 as the nonantigen-binding fragment (Fc region) by comparison to previous literature.[4] Additionally, the digest of the untreated MAb also seems to have some oxidized species (Peaks 3 and 4). In Figure 2, Chromatogram B, AAPH-oxidized Fc (Peaks 3 and 4) increased and unmodified Fc decreased. More than 50% of the original Fab (Peak 2) was oxidized to another peak (Peak 1). In the H_2O_2-oxidized MAb, the main oxidation targets were in the Fc fragment. As shown in Figure 2, Chromatogram C, one of the oxidized Fc fragments (Peak 3) was the major peak among three Fc peaks, whereas no changes in the Fab fragment (Peak 2) were observed after H_2O_2 oxidation.

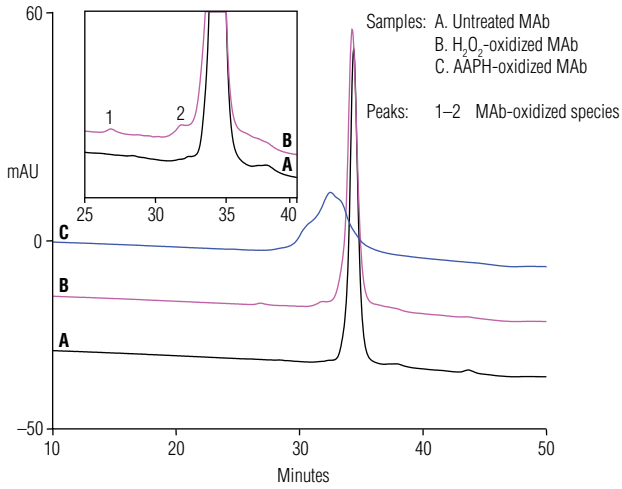

Figure 1. MAb and oxidized MAbs.

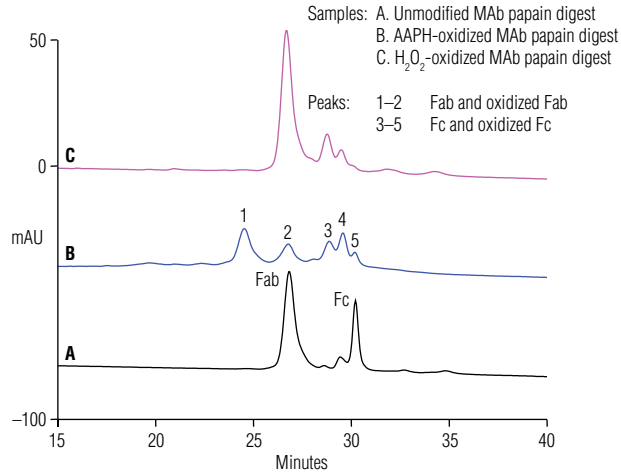

Figure 2. MAb papain digests.

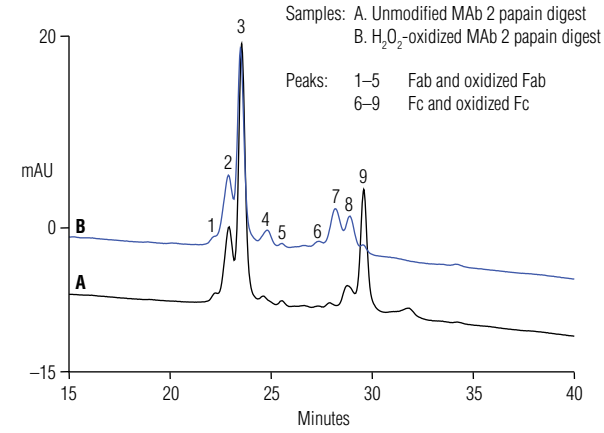

Figure 3. MAb 2 papain digests.

Another MAb (noted as MAb 2 in Figure 3) was oxidized by H_2O_2 and papain digested using the same procedure. The resulting fragments were separated on the ProPac HIC-10 column (Figure 3). This column provides higher resolution for this MAb papain digest. Five Fab fragments (Peaks 1–5) and four Fc fragments were resolved. Both oxidized Fab (Peak 4) and oxidized Fc fragments (Peaks 6–8) increased.

Impact of Mobile Phase pH on Retention of the MAb Fragments

It has been well documented that chaotroptic agents (such as urea and sodium dodecyl sulfate) and organic solvent can change the elution behavior of a MAb. This study included tests of 2 M urea in a number of mobile phases, but no better resolution was observed (data not shown). When comparing acetonitrile and isopropanol as mobile phase additives, 7% isopropanol was similar or slightly better than 7% acetonitrile for improving resolution.

Mobile phases with the pH at 5.0 or 7.0 are frequently used in HIC applications. Data previously shown in this study used pH 7.0 mobile phases. A separation using mobile phases with the pH at 5.0 is shown in Figure 4. Interestingly, the elution order of Fab and Fc fragments is apparently reversed, mainly due to increased retention time of the Fc fragment at pH 5.0. Histidine has an isoelectric point—the pH at which a particular molecule or surface carries no net electrical charge—of ~6.0, so the charge state/hydrophobicity of histidine/MAb may change significantly between a pH of 5.0 and 7.0. This may explain the hydrophobicity change of the Fc fragment. In a comparison of the AAPH-oxidized MAb papain digest (Figure 2, Chromatogram B compared to Figure 4, Chromatogram B), a pH of 7.0 slightly improves resolution, especially between Peaks 1 and 2. In a comparison of the H_2O_2-oxidized MAb papain digest (Figure 2, Chromatogram C compared to Figure 4, Chromatogram C), a pH of 5.0 cannot resolve oxidized Fc from nonmodified Fc.

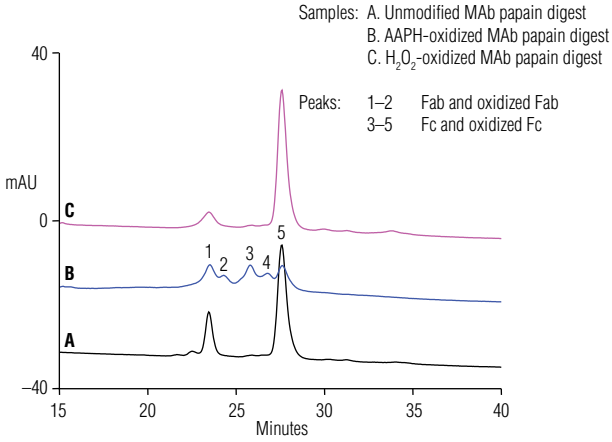

Figure 4. MAb papain digests using pH 5.0 mobile phase.

Conclusion

This study shows that the ProPac HIC-10 column can resolve Fab and Fc fragments generated by papain digestion, as well as oxidized Fab/Fc from native Fab/Fc. Changing the mobile phase pH can affect retention order of the fragments. Due to the orthogonal separation character of HIC to SEC or CEX and its preservation of biomolecule activity, the ProPac HIC-10 column is suitable for enriching a low-level population of modified MAbs or modified papain fragments in MAb therapeutic stability studies.

References

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Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic) 400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 9 3291 0200
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