Separation of mAb Fragments on a High-Resolution HIC Column

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Key Words

Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, papain digestion, Fab and Fc fragments, MAbPac HIC-20

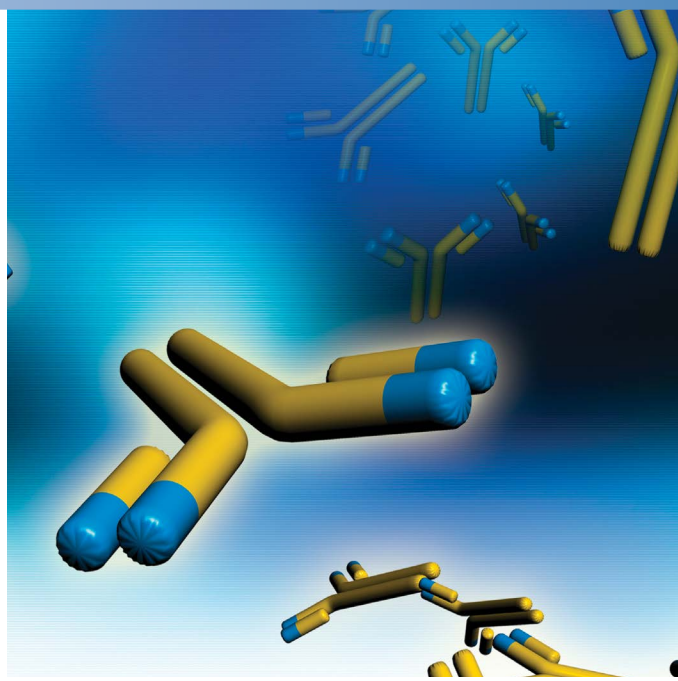
Goal

To demonstrate the separation of Fab and Fc fragments obtained by papain digestion by using a Thermo Scientific™ MAbPac™ HIC-20 column.

Introduction

Monoclonal antibodies (mAbs) are the most prominent and fastest growing class of therapeutic proteins. With their excellent biocompatibility and high specificity, mAbs have been shown to be effective against not only cancer and inflammatory diseases but also rare diseases such as Crohn's disease and paroxysmal nocturnal haemoglobinuria.[1] Recombinant mAbs can be highly heterogeneous due to various biochemical modifications such as sialylation, oxidation, deamidation, and C-terminal lysine truncation. Some of these modifications may reduce stability and efficacy of the drug. Therefore, it is critical to detect, characterize, and quantify impurities, as well as biochemical variants, during production of mAb therapeutics. A variety of HPLC methods are used to assess mAb heterogeneity by analyzing either the intact mAb or fragments after digestion.[2]

Separation of Fab and Fc fragments generated by papain digestion of mAbs is often employed to enhance the detection of some mAb variants. Papain cleaves an antibody into three fragments at the hinge region which produces two Fab fragments and one Fc fragment (Figure 1). Analysis of papain digested mAbs not only allows for the detection of variants that are not visible at the intact mAb level but also provides positional information on whether the modification is on the Fab or Fc fragment. Among various HPLC methods, hydrophobic interaction chromatography (HIC) often provides high-resolution separation of Fab and Fc fragments, thus is generally used for mAb variant analysis.[2-4]



HIC separates proteins in order of increasing hydrophobicity under non-denaturing conditions. Analytes bind to the weakly hydrophobic stationary phase in the presence of high salt concentration and elute off the column as the salt concentration decreases. Unlike reversed-phase liquid chromatography that typically denatures the protein, effectively destroying the native conformation, HIC typically preserves the native structure and bioactivity of the protein, which is useful for downstream functional analysis such as binding and cell-based potency assays. It also allows for the separation on the basis of conformational changes occurring in the native form. In addition, HIC typically provides separation with little or no carryover. Therefore, HIC is not only useful for separation of mAb variants but also valuable as a purification method for mAb products by biopharmaceutical companies.

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This application note describes the use of the new MAbPac HIC-20 column to separate Fab and Fc fragments of mAb therapeutics. The MAbPac HIC-20 column is a wide-pore (1000 Å), 5 µm silica-based HIC column well suited for the separation of high molecular weight mAb variants. The proprietary column chemistry provides high resolution, rugged stability, and desired selectivity for the analysis of mAb heterogeneity. In the examples shown here, high resolution separations of Fab and Fc fragments were achieved and the variants of these fragments were observed as well.

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%]
- Tris (hydroxymethyl) aminomethane (Fisher Scientific P/N T395-500)
- Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8\ 2H_2O$, ≥ 99%)
- L-cysteine (Fisher Scientific P/N AC173600250)
- Papain, lyophilized (≥10 units/mg protein)

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Preparation of 2× papain digestion buffer

Dissolve 0.485 g of Tris, 0.060 g of EDTA, and 0.024 g of L-cysteine in 15 mL of DI water. Adjust the pH to 7.6 using HCl and bring the volume to 20 mL with DI water.

Papain digestion

Prepare a 2 mg/mL papain solution in DI water. Then mix the following in a microtube and incubate it for 4 h in a 37 °C water bath:

mAb solution (5 mg/mL)	80 µL
Papain (2 mg/mL)	4 µL
Water	16 µL
2× Papain digestion buffer	100 µL

Add 200 µL of mobile phase A before HPLC analysis. The final concentration of mAb is 1 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 equipped with a micro flow cel (P/N 5074.0010)
Columns	MAbPac HIC-20, 4.6 × 100 mm (P/N 088553)
	MAbPac Protein A, 4 × 35 mm (P/N 082539)
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% sodium hydroxide (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 1 (Standard 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

Gradient 2 (Shallow gradient)

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Flow rate	1 mL/min
Run time	20 min
Temperature	30 °C
UV detector wavelength	280 nm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

Two different therapeutic mAbs were digested with papain resulting in Fab and Fc fragments. The separations of these fragments were carried out using the MAbPac HIC-20 column, and the data was compared with that of intact mAbs. Initially, these samples were tested with 2 M ammonium sulfate as the starting mobile phase. For both mAb1 and mAb2, Fab and Fc fragments were well resolved in 20 minutes (Figures 2b and 3b). Separation was further optimized by simply reducing the starting ammonium sulfate concentration to 1.2 M (by adjusting the gradient) while maintaining the overall gradient time (Figures 4b and 5b). Addition of organic solvent did not further improve the resolution (data not shown).

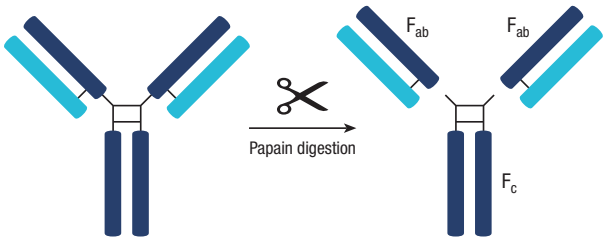

Figure 1. Schematic representation of papain digestion of monoclonal antibody

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:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

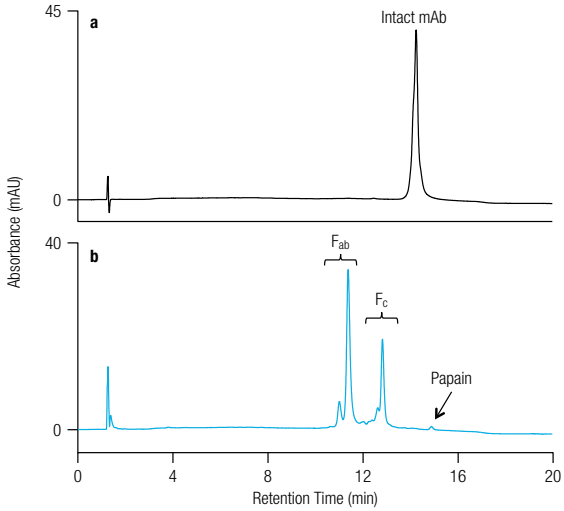

Figure 2. Separation of mAb1 and papain digested mAb1 using standard gradient

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:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

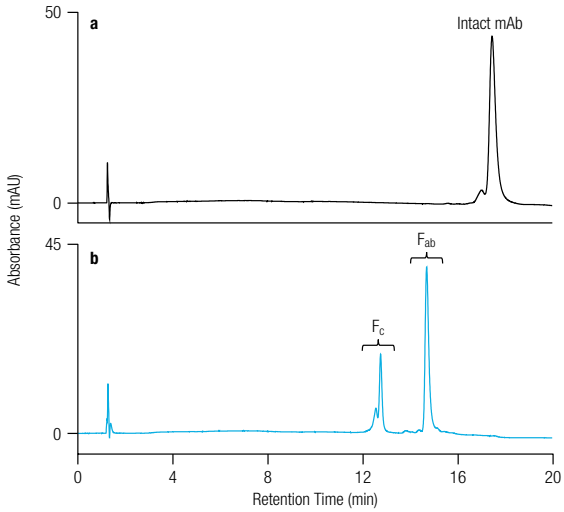

Figure 3. Separation of mAb2 and papain digested mAb2 using standard gradient

Since two Fab fragments and one Fc fragment are generated when a mAb is digested with papain, the peak assignment was done based on peak area. Further confirmation of the peak assignment was carried out using the MAbPac Protein A column (data not shown).

For mAb1, the Fab fragment is less hydrophobic than the Fc fragment and thus eluted first; for mAb2, the opposite trend is observed. Compared to corresponding intact mAbs (Figures 2a and 3), mAb fragments were more hydrophilic in both cases. The intact mAb1 chromatogram showed an asymmetric peak most likely due to the presence of unresolved variants (Figure 4a). After papain digestion, minor variant peaks in front of both Fab and Fc fragments were observed. The separation of these variant peaks was improved with a shallower gradient (Figure 4b). For mAb2, the intact mAb chromatogram shows a small peak in front of the main mAb peak (Figure 5a) and one variant peak is observed in front of the Fc fragment (Figure 5b).

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	60	40
	0.0	60	40
	1.0	60	40
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

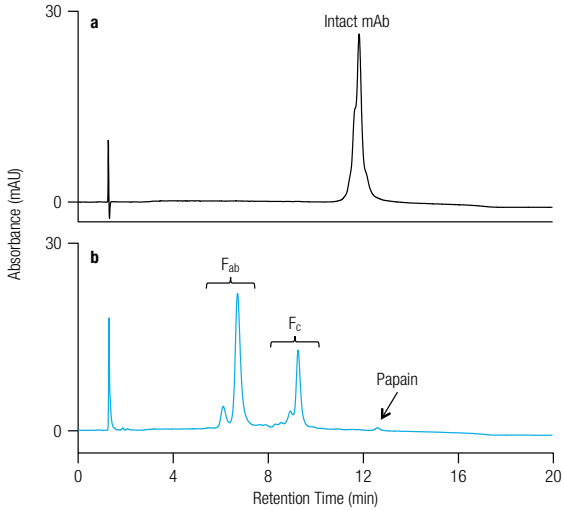

Figure 4. Separation of mAb1 and papain digested mAb1 using shallow gradient

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	60	40
	0.0	60	40
	1.0	60	40
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

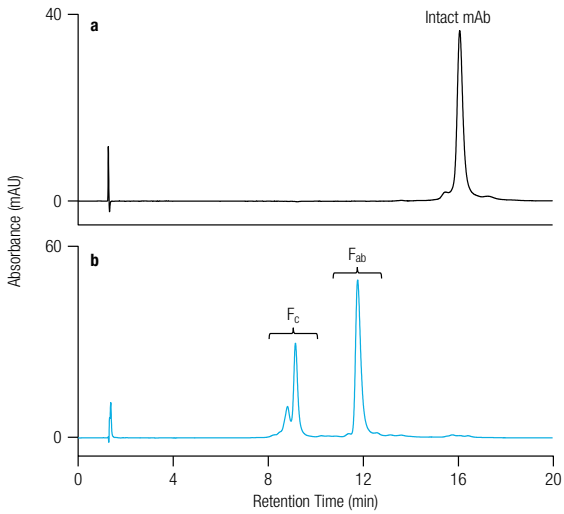

Figure 5. Separation of mAb2 and papain digested mAb2 using shallow gradient

Conclusion

- The MAbPac HIC-20 column provides the desired selectivity for separation of Fab and Fc fragments from the native mAb.
- The MAbPac HIC-20 column is a valuable tool for the analysis of mAb variants.

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