Characterize Fab and Fc Fragments by Cation-Exchange Chromatography

Application Note

Biologics and Biosimilars

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Abstract

This application note describes the use of the Agilent Bio MAb weak cation-exchange column in measuring the mAb fragments obtained by papain digestion and the subsequent mass spectrometric characterization of the generated Fab and Fc variants following peak collection.

Introduction

Monoclonal antibodies (mAbs) are a rapidly growing class of therapeutics used in the treatment of cancer and autoimmune diseases. Cation-exchange chromatography (CEX) is a powerful technology for the characterization of these molecules.

Another application note described the CEX separation of the charge variants of the intact monoclonal antibody trastuzumab [1]. Trastuzumab, marketed as Herceptin since 1998, is in widespread use in the treatment of HER2 positive breast cancer. The work presented here describes the use of the Agilent Bio MAb weak cation-exchange (WCX) column to separate charge variants of papain digested trastuzumab and the subsequent characterization of the Fab and Fc variants by mass spectrometry (MS).

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Experimental

Materials

Acetonitrile and water were obtained from Biosolve B. V. (Valkenswaard, The Netherlands). NaCl, 2-(N-morpholino)ethanesulfonic acid (MES), papain, and *tris*(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland).

Sample preparation

Herceptin was incubated at 37 °C for 2 hours after adding papain. Following incubation, the papain digest buffer was exchanged to CEX mobile phase A and subsequently diluted to 2 mg/mL. The pH-stressed mAb was obtained by incubating Herceptin at 37 °C for 3 days in 1 M *tris* pH 9.0. Collected CEX fractions were optionally reduced at room temperature using TCEP to a final concentration of 15 mM.

Instrumentation

Cation-exchange measurements were performed on:

- Agilent 1100 Series Degasser (G1322A)
- Agilent 1100 Series Binary Pump (G1312A)
- Agilent 1100 Series Autosampler (G1367A)
- Agilent 1200 Series Thermostat for autosampler (G1330B)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Diode Array Detector (G1315A)
- Agilent 1200 Series Analytical Fraction Collector (G1364C)

LC/MS measurements were performed on an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF with Agilent Jet Stream LC/MS (G6540A) and an Agilent 1290 Infinity LC System equipped with:

- Agilent 1290 Infinity Binary pump (G4220B)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat for autosampler (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A)

Software

- Agilent OpenLAB CDS ChemStation revision C.01.05 (35)
- Agilent MassHunter for instrument control (B05.01)
- Agilent MassHunter for data analysis (B06.00)
- Agilent BioConfirm software for MassHunter (B06.00)

Instrumental conditions

Cation-exchange conditions

Column:	Agilent Bio MAb NP5, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Mobile phase:	A) 20 mM MES pH 5.6 B) 20 mM MES pH 5.6 + 300 mM NaCl
Flow rate:	170 µL/min
Injection volume:	16 µL (32 µg on-column)
Column temperature:	30 °C
Gradient:	Time (min) % B 0 2 39.5 80 40 100 50 100 50.5 2 80 2
Detection:	UV at 214 nm/8 nm
Fraction collection:	Time-based

Online desalting LC/MS conditions

Cartridge:	On-line desalting cartridge, 2.1 × 10 mm
Mobile phase:	A) 0.1% formic acid in water (v/v) B) 0.1% formic acid in acetonitrile (v/v)
Flow rate:	400 µL/min
Injection volume:	20 µL
Needle wash solvent:	60% Acetonitrile, 35% water, 5% isopropanol
Autosampler temperature:	7 °C
Gradient:	Time (min) % B 0 5 0.5 5 2 80.0 3 80.0 3.10 5 5 5
Q-TOF source:	Agilent Jet Stream positive ionization mode
Drying gas temperature:	300 °C
Drying gas flow rate:	8 L/min
Nebulizer pressure:	35 psig
Sheath gas temperature:	350 °C
Sheath gas flow rate:	11 L/min
Nozzle voltage:	1,000 V
Capillary voltage:	3,500 V
Fragmentor voltage:	200 V
Q-TOF detection:	Mass range 3,200 amu
Data acquisition range:	500 to 3,200 *m/z*
High resolution mode	(4 GHz)
Data acquisition rate:	1 spectrum per s
Profile acquisition	
Diverter valve:	Time (min) 0 Flow to waste 1 Flow to MS 3.5 Flow to waste

Results and Discussion

The structures of an antibody and its Fab and Fc fragments generated following papain digestion are displayed in Figure 1. Figure 2 shows the UV 214 nm CEX profiles of the papain digest of nonstressed and pH-stressed Herceptin obtained on the Agilent Bio MAb weak cation-exchange column. Due to the lower isoelectric point of the Fab and Fc fragments compared to the intact antibody, the pH of the mobile phase needs to be lowered compared to the mobile phase buffer at pH 7.65 used for intact mAb CEX analysis. An MES buffer at pH 5.6 was used to obtain optimal separation of the Fab and Fc.

The CEX peaks indicated in Figure 2 were collected and further characterized by MS following online desalting.

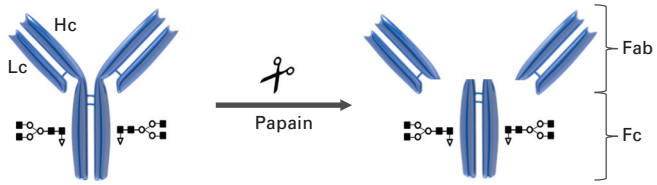

Figure 1. Structure of a monoclonal antibody and its Fab and Fc fragments. A mAb is composed of two light (Lc) and two heavy chains (Hc) connected through disulfide bonds. Papain cleaves the mAb at the hinge region with the generation of the Fab and Fc fragment. The Fc region is N-glycosylated and contains complex bi-antennary glycans.

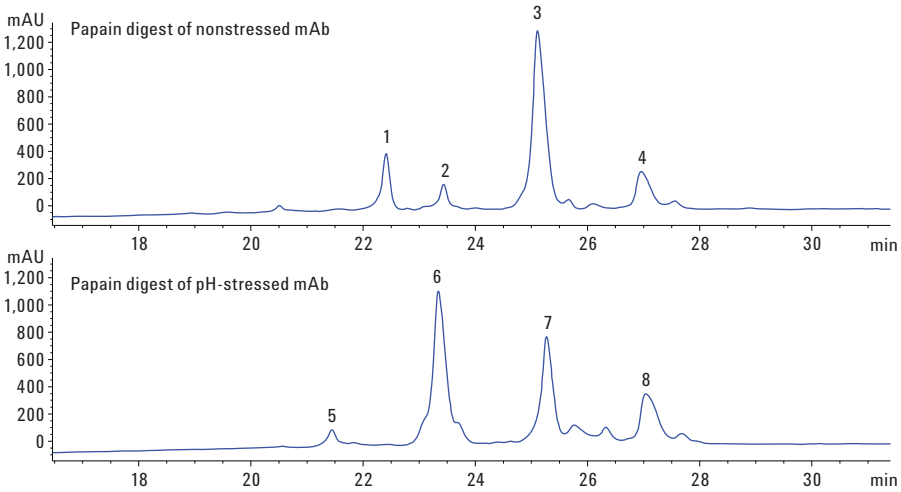

Figure 2. Cation-exchange separation of charge variants of papain-digested pH-stressed and nonstressed Herceptin. The annotated peaks were collected and further characterized using mass spectrometry following online desalting.

The deconvoluted spectra of the four collected CEX peaks of the nonstressed sample are shown in Figure 3. The spectra indicate that the Fab and Fc fragments correspond to, respectively, CEX peaks 3 and 4. The typical Fc glycosylation pattern is apparent in CEX peak 4. The 1 Dalton mass difference between CEX peaks 2 and 3 is indicative of a deamidation of the Fab fragment. Indeed, a deamidation involving the conversion of asparagine to aspartic acid renders the Fab fragment more acidic, explaining its earlier elution. CEX peak 1 is identified as the Fab fragment with loss of the basic tripeptide KTH at the papain cleavage site. This represents an artifact of the sample preparation.

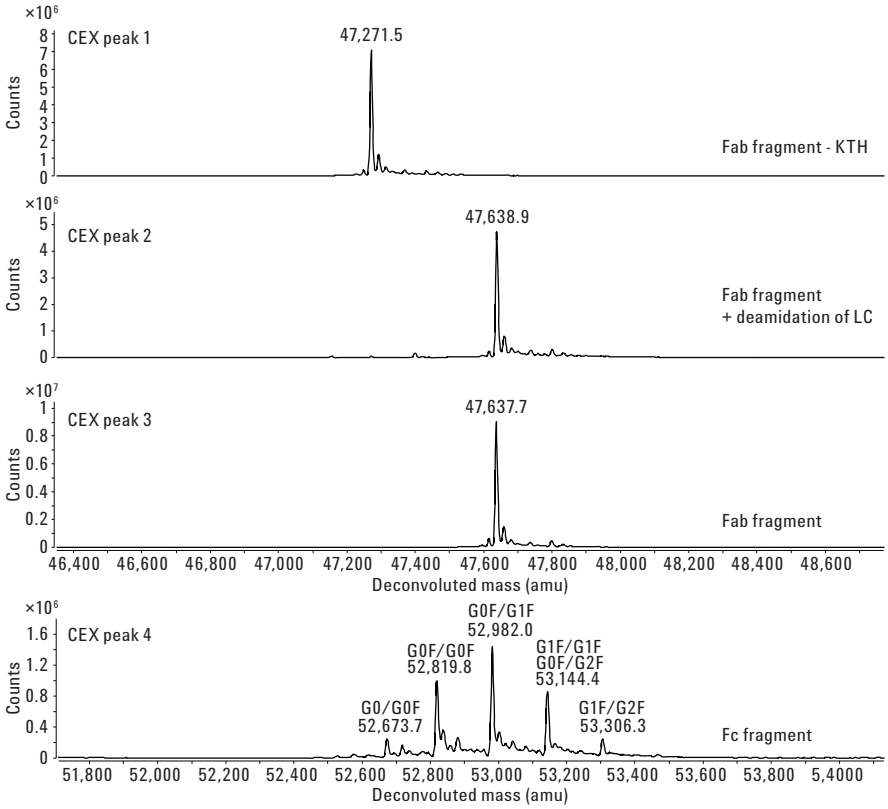

Figure 3. Deconvoluted spectra of the four collected cation-exchange peaks of nonstressed papain digested Herceptin. The abbreviations G0, G0F, G1F, and G2F refer to the N-glycans attached to the Fc fragment.

The deconvoluted spectra of the four collected CEX peaks of the pH-stressed sample are shown in Figure 4. The most abundant CEX peak 6 is identified as the deamidated Fab fragment. The spectra reveal an extra mass difference of 1 Dalton between CEX peaks 5 and 6. Additional MS analysis following TCEP reduction of peaks 5 and 6 (Figure 5) demonstrated a deamidation of the heavy chain portion of the Fab fragment on top of the deamidation of the light chain for peak 5.

Conclusions

The Agilent Bio MAb weak cation-exchange column successfully separated charge variants of a papain-digested monoclonal antibody. Following peak collection, charge variants could be further characterized by mass spectrometry in combination with online desalting.

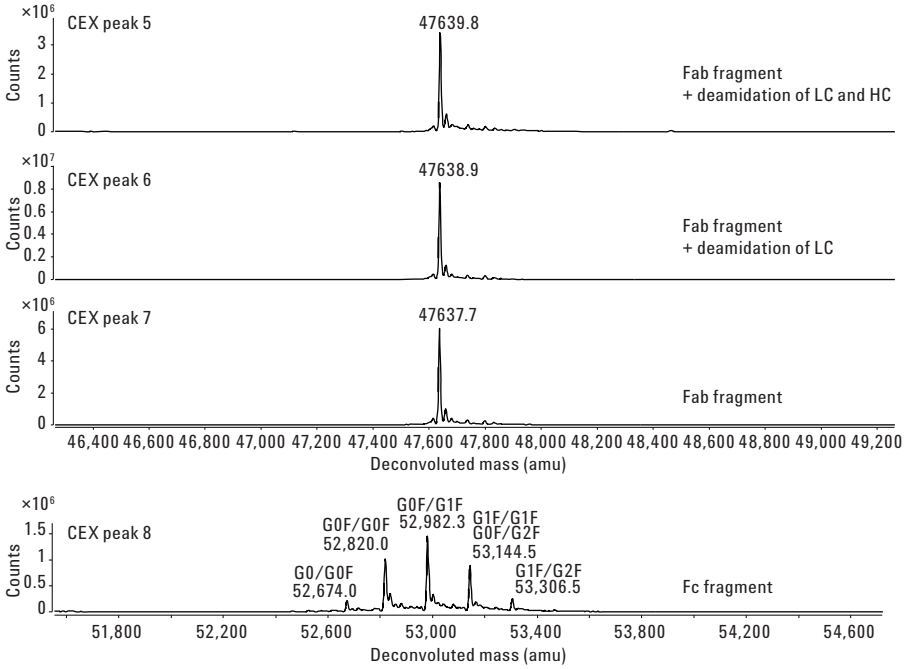

Figure 4. Deconvoluted spectra of the four collected cation-exchange peaks of pH-stressed papain digested Herceptin.

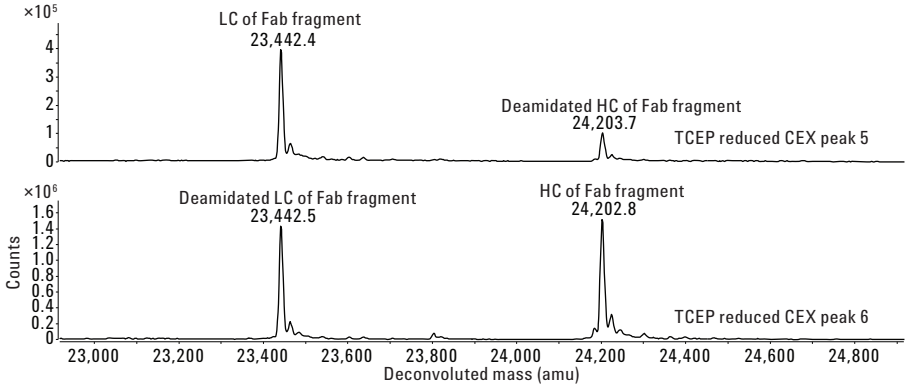

Figure 5. Deconvoluted spectra of TCEP reduced cation-exchange peaks 5 and 6.

Reference

1. I. Vandenheede, E. Dumont, P. Sandra, K. Sandra, M. Joseph. *Characterize mAb Charge Variants by Cation-Exchange Chromatography*. Agilent Technologies, Inc. Application Note, publication number 5991-5273EN, **2014**.

For More Information

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