DIONEX

Application Note 127

Monitoring Monoclonal Antibody Heterogeneity by Cation-Exchange Chromatography

INTRODUCTION

During the development and production of therapeutic proteins, characterization of structural variants is a critical challenge. C-terminal processing of lysine residues on the heavy chain of monoclonal antibodies (MAbs) is a common structural variation that demands careful analysis.[1-7] As a result of this processing, C-terminal lysine or arginine residues are often absent in proteins isolated from mammalian cell cultures, even though their presence may be expected on the basis of the gene sequence. This discrepancy, which is common in plasma-derived proteins, results from the activity of one or more basic carboxypeptidases. Incomplete protein processing results in charge heterogeneity which is readily identified by cation-exchange chromatography on the Dionex ProPac® WCX-10, a weak cation-exchange column. The packing in this column is a unique pellicular resin with a hydrophilic coating and carboxylate functional groups on grafted linker arms. The physicochemical properties of this support eliminate secondary (nonionic) interactions between the protein analytes and the stationary phase, affording minimal band broadening and high selectivity.[8] The UltiMate® 3000 Titanium System is an HPLC whose flow path is ensures that neither solvents nor sample are in contact with stainless steel materials, removing concerns about iron and other transition metals contaminating the column and samples. This application note describes a method for analyzing a humanized IgG_1 MAb for C-terminal lysine variants. These variants are baseline resolved from the native antibody using the ProPac WCX-10 column and a 4-morpholineethanesulfonic acid (MES)-based mobile phase. Other acidic and basic variants are also better resolved than had been observed with traditional phosphate-based mobile phases buffered at pH 7, enabling the detection of additional MAb variants, each with heavy chain C-terminal lysine heterogeneity.

EQUIPMENT

Dionex UltiMate 3000 Titanium System consisting of:

SRD-3600 Solvent Rack with 6 Degasser Channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2-L glass bottles for each pump

LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability

WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020) with 50 µL biocompatible sample loop

TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK ten-port two-position valves (P/N 5723.0025) for added productivity

VWD-3400 Variable Wavelength Detector (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)

Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromeleon® Chromatography Data System

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent Nylon filter)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

0.3 mL polypropylene (Vial Kit, P/N 055428) injection vials with caps

Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

4-Morpholineethanesulfonic acid (MES) hydrate, minimum 99.5% titration (Sigma-Aldrich; P/N M8250)

Sodium chloride, crystal (J.T. Baker; P/N 4058-05)

Sodium hydroxide solution, 50% W/W (Thermo Fisher Scientific; P/N SS254)

Carboxypeptidase B, chromatographically purified, ≥ 170 U/mg, 5.0 mg/mL (Worthington Biochemical Corporation; P/N LS005305) or equivalent

Humanized monoclonal IgG_1 antibody (a generous gift from a biotechnology company)

CONDITIONS

Method

Column: ProPac WCX-10 Analytical 4 × 250 mm (P/N 054993)

Flow Rate: 1.00 mL/min

Inj. Volume: 10 µL (partial loop)

Sampler Temp: 5 ºC

Column Temperature: 30 °C

Detection: Absorbance, 280 nm (absorbance at 214 and 254 nm also collected)

Data Collection Rate: 1.0 Hz

Noise: 12-24 µAU

Typical System Operating Backpressure: ~ 125 bar (~1830 psi)

Mobile Phase: A: 20 mM MES, 60 mM sodium chloride, pH 5.6
B: 20 mM MES, 240 mM sodium chloride, pH 5.6

Gradient: Linear, 20% B for 2 min, 20–50% B from 2–52 min

Gradient Method

Time (min)	A(%)	B(%)	Comments
–20.00	80.0	20.0	Equilibration
0.00	80.0	20.0	Sample Injection
2.00	80.0	20.0	Sample Binding
52.00	50.0	50.0	End Gradient
52.10	0.0	100.0	Column Regeneration
57.00	80.0	20.0	Re-equilibration

PREPARATION OF SOLUTIONS AND REAGENTS

All mobile phases are filtered through a 0.2 µm nylon filter under vacuum to remove particulates and to degas prior to their use. The mobile phases are blanketed under 34–55 kPa (5-8 psi) of helium headspace to reduce the growth of opportunistic microorganisms, and maintain a low concentration of dissolved air.

20 mM MES, 60 mM Sodium Chloride, pH 5.6 (Mobile Phase A)

Combine 7.81 g of MES, 7.01 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

20 mM MES, 240 mM Sodium Chloride, pH 5.6 (Mobile Phase B)

Combine 7.81 g of MES, 28.04 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

Sample Preparation

MAb samples were diluted to 10 mg/mL using filtered, degassed water. One 100 µL aliquot of the MAb solution was combined in a microcentrifuge tube with 0.5 µL of thawed carboxypeptidase B solution using a micropipette while another 100 µL aliquot was combined with 0.5 µL of DI water. Both sample and control were incubated at 37 ºC for 2 h. After the 2-h incubation period, sample and control were diluted 5-fold with Mobile Phase A and analyzed.

RESULTS AND DISCUSSION

The ProPac WCX-10 weak cation-exchange column was assessed for its ability to separate humanized IgG_1 variants. As shown in Figure 1 this antibody not only produces three major peaks but also separates a number of other variants, both more acidic as well as more basic variants compared to the three major peaks. The MES-based buffers resolved additional details not seen when using phosphate-based buffers, an observation sometimes reported when separating other protein variants.[9]

To verify that the three major peaks were due to variations in C-terminal lysine content, the IgG_1 sample was treated with carboxypeptidase B, an exopeptidase that specifically cleaves C-terminal lysine residues. A comparison of the two chromatographic traces in Figure 2 shows a disappearance of the second and third major peaks, which are proposed to contain one and two terminal heavy chain lysine residues, respectively. The disappearance of peaks 2 and 3 was accompanied by a corresponding increase in the area under the first major peak, the variant with no terminal lysine. Additionally, the pattern for the minor peaks eluting before the first major peak is maintained, suggesting that these peaks do not contain terminal heavy chain lysine residues. The complex chromatographic pattern of peaks eluting after the three major peaks is also simplified with carboxypeptidase B incubation, showing MAb variants, each with heavy chain C-terminal lysine heterogeneity, are present in the original sample.

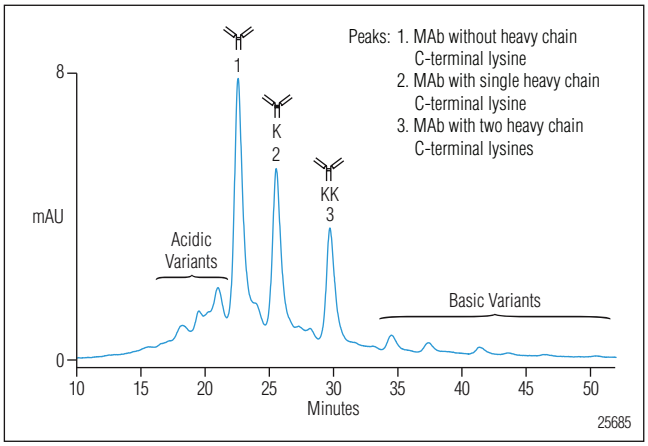

Figure 1. Separation of acidic and basic terminal lysine variants of an IgG_1 monoclonal antibody using the ProPac WCX-10 column and MES-based buffers on the UltiMate 3000 Titanium System.

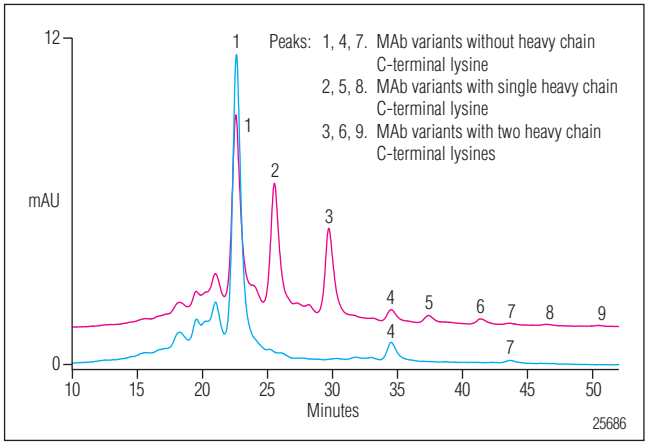

Figure 2: Analysis of IgG_1 monoclonal antibody before (upper trace) and after (lower trace) treatment with carboxypeptidase B for 2 h at 37 ºC.

CONCLUSION

The ProPac WCX-10 weak cation-exchange column, used with MES-based buffers on an inert titanium system, produced detailed chromatograms of variants of an IgG_1 MAb, including baseline separation of C-terminal lysine variants. These results demonstrate that this chromatographic system is well suited for analyses such as quality control monitoring of protein-based therapeutics that require high efficiency, high resolution separation of closely eluting proteins.

REFERENCES

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LIST OF SUPPLIERS

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 269-926-6171, http://www.gastmfg.com.

Mallinckrodt Baker, 222 Red School Lane, Phillipsburg NJ 08865, U.S.A. Tel: 800-582-2537 http://www.mallbaker.com

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 800-625-4327, http://www.nalgenunc.com

Praxair, 39 Old Ridgebury Road, Danbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany +49-2293-305-0, http://www.sarstedt.com

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A. Tel: 800-325-3010, http://www.sigma.sial.com

Thermo Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60133, U.S.A. Tel: 800-766-7000, http://www.fishersci.com .

Worthington Biochemical Corp., 730 Vassar Ave., Lakewood, NJ 08701, U.S.A. Tel: 800-445-9603 http://www.worthington-biochem.com