Monitoring Stability of Monoclonal Antibodies by Cation-Exchange Chromatography

**INTRODUCTION**

Monoclonal antibody (MAb) microheterogeneity can be attributed to glycosylation, oxidation, mutation, phosphorylation, amino terminal modifications (e.g., to pyroglutamate), incomplete processing of the C-terminus,\(^1\) and asparagine (Asn) deamidation.\(^2\) These variations in protein composition occur in many types of proteins and can impact their activity and stability as biotherapeutics.\(^3\)–\(^8\) Monitoring stability of therapeutic proteins and peptides is regarded as essential for demonstrating safety and efficacy of these drugs and is required by FDA and other regulatory agencies.

Shelf-life studies of proteins and peptides typically monitor a variety of degradation products by ion-exchange chromatography. For example, succinimide formation at aspartate in basic fibroblast growth factor has been monitored using ion-exchange chromatography.\(^9\) It has also been useful in the quantification of oxidized methionine (Met\(^{122}\)) granulocyte-colony stimulating factor (G-CSF), oxidized Met\(^{127/136}\), formyl-Met G-CSF, and partially reduced G-CSF.\(^10\) Also, recombinant human deoxyribonuclease I containing deamidated Asn\(^{74}\) has been separated from its non-deamidated form using ion-exchange chromatography.\(^11\)

Proteins isolated from mammalian cell culture can include C-terminal variants, possibly formed by carboxypeptidases that cleave the protein’s C-terminus. Recombinant monoclonal antibodies have been shown to have C-terminal heterogeneity with either an arginine (Arg) or lysine (Lys) at the C-terminus. When these C-terminal variants of MAbs are treated with carboxypeptidase B, an exopeptidase, Arg and Lys are cleaved from the C-terminus of both antibody subunits, eliminating C-terminal heterogeneity.\(^12\)–\(^17\) Proteins and peptides containing Asn adjacent to glycine (Gly) are particularly susceptible to Asn deamidation.\(^18\) Deamidation is a common decomposition pathway for proteins and peptides, converting Asn to aspartic acid or isoaspartic acid, and it is expected to occur to varying extents during stability studies. Monitoring the extent of deamidation is of interest to quality control and process development chemists concerned with product quality and stability.

In this Application Note the sample was a humanized MAb that was first treated with carboxypeptidase B to remove the C-terminal lysine charge heterogeneity. This antibody was then subject to forced deamidation conditions\(^19\) and changes were monitored using the ProPac\(^{16}\) WCX-10 column.

**EQUIPMENT**

DX-500 BioLC® liquid chromatography system consisting of:
- GP50 gradient pump
- AD20 variable wavelength absorbance detector
- AS50 Autosampler
- LC30 chromatography oven or AS50 thermal compartment
- PeakNet Chromatography Workstation
- Dionex ProPac WCX-10 cation-exchange column, 250 x 4 mm i.d.

**REAGENTS AND SAMPLES**

Deionized water, using a Milli-Q system (Millipore) or equivalent
- Monobasic sodium phosphate, monohydrate, crystal (J.T. Baker, Inc.)
- Dibasic sodium phosphate, anhydrous, powder (J.T. Baker, Inc.)
- Sodium chloride, ACS grade (VWR Scientific)
- Ammonium bicarbonate, HPLC grade (J.T. Baker, Inc.)
- Sodium azide (Mallinckrodt)
Protease inhibitor cocktail, general use, containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma Chemical Co., Cat. #P2714 or equivalent).

Carboxypeptidase B from porcine pancreas, 216 U/mg, 5.0 mg/mL (Boehringer Mannheim, Cat. #0103233001 or equivalent)

Ribonuclease A from bovine pancreas, 10 mg/mL solution (Sigma Chemical Co., Cat. #R5250 or equivalent)

Humanized monoclonal antibody (MAb) (IgG 1 , 10 mg protein/mL), was a generous gift from a biotechnology company. The donor reported that the light chain has 8 Asn residues with none near glycine. The heavy chain has 19 Asn residues with two adjacent to glycine.

**CONDITIONS**

Column: Dionex ProPac WCX-10 cation-exchange column, 250 x 4 mm

Flow Rate: 1 mL/min

Inj. Volume: 10 µL

Detection: UV, 254 nm

Eluents: A: 10 mM Sodium phosphate, pH 7.0
B: 10 mM Sodium phosphate with 1.0 M Sodium chloride (pH 7.0)

Gradient Program:

<table>
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<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
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<tr>
<td>45.0</td>
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**PREPARATION OF SOLUTIONS AND REAGENTS**

Two eluents are used for chromatography: 10 mM sodium phosphate (pH 7.0) and 10 mM sodium phosphate (pH 7.0) with 1.0 M sodium chloride (NaCl). The sodium phosphate buffer system was prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions with water to attain the desired pH 7.0. The relative amount of monobasic and dibasic sodium phosphate solution added differed between eluent with or without sodium chloride because NaCl is not normally pH neutral. The following procedure is a recommended starting point for obtaining the desired eluents, but some deviation from this formula may be necessary, after checking pH, for the reagents used in other labs. If the pH is not 7.0, adjust the proportions of monobasic and dibasic solutions added. The combined total amount should remain 100 mL to produce 10 mM sodium phosphate.

**2 M Sodium Chloride**

Dissolve 116.90 g of sodium chloride in water, and fill to a final volume of 1.0 L. Filter through a 0.45-µm filter.

**200 mM Sodium Phosphate, Dibasic**

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1.0 L of water. Filter through a 0.45-µm filter. Store frozen until needed.

**200 mM Sodium Phosphate, Monobasic**

Dissolve 27.60 g monohydrate monobasic sodium phosphate in 1.0 L of water. Filter through a 0.45-µm filter. Store frozen until needed.

**10 mM Sodium Phosphate, pH 7.0**

Combine 61 mL 200 mM dibasic sodium phosphate, 39 mL 200 mM monobasic sodium phosphate, 1900 mL water.

**10 mM Sodium Phosphate with 1 M Sodium Chloride, pH 7.0**

Combine 83.3 mL 200 mM dibasic sodium phosphate, 16.7 mL 200 mM monobasic sodium phosphate, 1000 mL 2 M sodium chloride, 900 mL water.

**SAMPLE PREPARATION**

**10% Ammonium bicarbonate buffer, pH 8.2**

Dissolve 5.0 g ammonium bicarbonate with 40 mL water. Adjust pH with concentrated HCl to pH 8.2. Adjust volume to 50 mL with additional water.

**1% Sodium azide**

Dissolve 20 mg sodium azide in 2.0 mL water. *Caution: sodium azide is very toxic. Use necessary precautions to protect against exposure.*

**10X Concentrate Protease Inhibitor Cocktail**

Reconstitute the protease inhibitor cocktail vial contents with 10 mL water. Cocktail concentrate consists of 20 mM AEBSF, 14 µM E-64, 1.3 mM Bestatin, 10 µM Leupeptin, 3 µM Aprotinin, and 10 mM EDTA.
Ammonium Carbonate-Azide-Protease Inhibitor Cocktail (ACAPIC)

Combine: 1.05 mL 10% ammonium bicarbonate
0.53 mL 1% sodium azide
0.11 mL protease inhibitor cocktail (10X concentrate)
8.31 mL water

MAb Sample Preparation

Combine 35 µL of 10 mg/mL MAb solution with 1.0 µL carboxypeptidase B, and incubate for 2 h at 37 °C. Remove 2.0 µL and dilute protein to 0.5 mg/mL in 36.9 µL 10 mM phosphate buffer; analyze a 10 µL injection.

Combine the remaining 34 µL of the carboxypeptidase B-treated antibody solution with 627 µL of ACAPIC. Final deamidation conditions consist of 0.5 mg/mL MAb, 1% ammonium bicarbonate, 0.05% sodium azide, 210 µM AEBSF, 150 µM E-64, 14 µM Bestatin, 0.1 µM Leupeptin, 0.03 µM Aprotinin, and 100 µM EDTA. Incubate at 37 °C and remove 100 µL aliquots at 0 and 50 h, analyzing 10 µL of each aliquot. Ribonuclease A and water were treated in the same manner as the MAb solution to serve as a deamidation positive control and blank, respectively.

RESULTS AND DISCUSSION

Figure 1A shows the separation of the original MAb with the three classes of variants: 1) no C-terminal Lys (peak 1); 2) a single C-terminal Lys on either one of the two heavy chains (peak 2); and 3) two C-terminal Lys on both heavy chains (peak 3).1 After carboxypeptidase B treatment, which cleaves C-terminal Lys, only a single peak was produced that had a retention time corresponding to one of the three peaks in the original antibody solution (Figure 1B). Ribonuclease A, tested in parallel as a positive control for deamidation, did not show any change upon addition of carboxypeptidase B (data not shown).

The MAb was treated with carboxypeptidase B to make the results of exposure of the antibody to deamidating conditions simpler to interpret. Rather than monitoring the degradation of three peaks, it is simpler to monitor the degradation of a single peak. The carboxypeptidase B-treated antibody (without terminal Lys) was then incubated for 0 and 50 h at 37 °C in 1% ammonium bicarbonate, containing sodium azide to eliminate microbial contamination. The antibody solution also contained a broad spectrum protease inhibitor cocktail to inhibit proteolytic cleavage from trace contaminating proteases that may be present in the carboxypeptidase B preparation.

Figure 2A shows the single peak (peak 8) of the MAb at the start of the incubation (0 h). Sodium azide peak (1.3 min) and protease inhibitor peaks (2.0 and 3.2 min) were also present. These same peaks were present in the buffer control (data not shown). After 50 h, two additional major peaks (peaks 6 and 7) and two minor peaks (peaks 9 and 10) appeared (Figure 2B). The appearance of multiple peaks is expected as a consequence of deamidation,5 but may be due to other thermal degradation pathways. This IgG1 molecule contains two Asn residues adjacent to Gly in each of the two heavy chains. Because Asn adjacent to Gly can be converted to either Asp or isoaspartic acid, 81
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different isoforms are possible. This does not include the less stable cyclic imide intermediate forms, which could increase the number of possible combinations beyond 81.

When more than one Asn-Gly is present in a protein or peptide, peptide mapping is often performed to simplify the interpretation. When more than one Asn-Gly is present in a protein or peptide, peptide mapping is often performed to simplify the interpretation. 20 The protein is cleaved with a site-specific proteases (e.g., trypsin), and then the peptides are separated by reversed-phase HPLC. Fractions are collected and further characterized by amino acid analysis, protein sequencing, and mass spectrometry. These data are correlated to the expected sequence to establish peptide identities. These analyses can confirm the deamidation or oxidation of an amino acid. To further simplify interpretation, proteins can be cleaved into larger fragments by cyanogen bromide and each fragment analyzed by peptide mapping.

In this study, a 50-h exposure to forced deamidation conditions causes the appearance of multiple components contained in at least five peaks. The ribonuclease A positive control for deamidation showed the predicted formation of new peaks, confirming that this experiment replicated forced deamidation conditions. 2 The additional peaks are broadly defined as decomposition products because there was no additional analysis to confirm deamidation of the monoclonal antibody. These experimental conditions, specifically 1% ammonium bicarbonate, are extreme for stability studies, but should suggest the more probable degradation products. Shelf-life studies of protein pharmaceuticals should yield only a small amount of degradation by the most chemically favored pathways.

CONCLUSION

The separations described in this study highlight the resolving ability of the ProPac WCX-10 column for the analysis of protein microheterogeneity. These separations use simple non-toxic eluents and are an alternative to reversed-phase or affinity chromatography for characterizing proteins.

REFERENCES

1. Dionex Application Note 127
2. Dionex Application Note 125

Figure 2. Stability of a MAb under deamidating conditions (1% ammonium bicarbonate). Carboxypeptidase B-treated MAb before deamidation treatment (panel A) and after 50 h of deamidation treatment (panel B).