Monitoring Protein Deamidation by Cation-Exchange Chromatography

INTRODUCTION

A common structural modification of recombinant proteins is the deamidation of asparagine (Asn) residues.[1,2] This modification occurs in a variety of protein-based pharmaceuticals, including human growth hormone, tissue plasminogen activator, hirudin, monoclonal antibodies, acidic fibroblast growth factor, and interleukin-1, with varying effects on the activity or stability of the therapeutic protein.[3-10] Determining the deamidation of Asn residues in recombinant proteins is a significant challenge for analytical and protein chemists in the quality control and process departments at biotechnology and pharmaceutical companies.[11]

This application note describes the use of the ProPac® WCX-10 column in a bio-inert HPLC system to monitor the products of protein deamidation. The ProPac WCX-10, a weak cation-exchange column, is well suited for the separation of protein variants produced by posttranslational modifications. 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.[12] The UltiMate® 3000 Titanium System is an HPLC whose flow path 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.

In the example presented here, deamidated variants of ribonuclease A (RNase A) are separated from the native protein in less than 20 min on a ProPac WCX-10 weak cation-exchange column. This application note also compares the results of using a phosphate-based buffer to that of a 4-morpholineethanesulfonic acid (MES) -based buffer.

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)

TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK™ 10-port 2-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

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

Sodium phosphate, dibasic (J.T. Baker; P/N 4062-01)

Sodium phosphate, monobasic monohydrate (J.T. Baker; P/N 3818-01)

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

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

Ammonium bicarbonate (Sigma-Aldrich; P/N A6141)

Ribonuclease A, Type III-A from bovine pancreas, minimum 85% (Sigma-Aldrich; P/N R5125)

CONDITIONS

Column:	ProPac® WCX-10 Analytical 4 x 250 mm (P/N 054993). Different columns were used with the phosphate-based and the MES-based buffers
Flow Rate:	1.00 mL/min
Inj. Volume:	10 µL (partial loop, no wash between re-injections)
Autosampler Temp:	5 ºC
Column Temp:	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:	~ 130 bar (~1900 psi)
Mobile Phase:	MES-based buffers A: 20 mM MES, pH 5.7 B: 20 mM MES, 1.0 M sodium chloride, pH 5.7 Phosphate-based buffers A: 10 mM sodium phosphate, pH 6.0 B: 10 mM sodium phosphate, 1.0 M sodium chloride, pH 6.0
Gradient:	Linear, 4-70% B in 30 min (both buffer systems)

Method:

Time (min)	A(%)	B(%)	Comments
–10.00	96.0	4.0	Equilibration
0.00	96.0	4.0	Sample Injection
30.00	30.0	70.0	End Gradient
40.00	25.0	75.0	Column Regeneration
42.00	96.0	4.0	Re-equilibration

PREPARATION OF SOLUTIONS AND REAGENTS

Both sets of mobile phases used in this application note are prepared using concentrated reagent solutions. The MES buffers are prepared by diluting an MES concentrate solution (with or without added sodium chloride) with DI water and adjusting the resulting solution pH to 5.7 with concentrated sodium hydroxide. Slightly different volumes of sodium hydroxide may be required to produce 2 L volumes of mobile phases A and B. The sodium phosphate mobile phases are prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions (with or without added sodium chloride) with DI water to yield pH 6.0. The following procedure is a recommended starting point for obtaining the desired mobile phases, but some deviation from this formula may be necessary after checking the pH. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic concentrate solutions added. The combined total volume of phosphate concentrate solutions should remain at 100 mL to produce 2 L of 10 mM sodium phosphate mobile phases. Do not adjust the pH of the sodium phosphate mobile phases by adding NaOH or HCl.

Concentrated Solutions

All concentrated solutions are filtered through a 0.2 µm filter and stored at 5 ºC until needed.

400 mM MES

Dissolve 78.07 g MES in water to a final solution volume of 1.0 L.

2.00 M Sodium Chloride

Dissolve 233.76 g sodium chloride in water to a final solution volume of 2.0 L.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate ($Na_2HPO_4 \cdot H_20$) in water to a final solution volume of 1.0 L.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in water to a final solution volume of 1.0 L.

Mobile Phase Solutions

All mobile phases are filtered through a 0.2 µm 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.

20 mM MES, pH 5.7 (A)

Combine 100 mL of 400 mM MES with 1900 mL DI water. Add 700 µL of 50% sodium hydroxide solution.

20 mM MES with 1.0 M Sodium Chloride, pH 5.7 (B)

Combine 100 mL of 400 mM MES, 1000 mL of 2.0 M sodium chloride, and 900 mL DI water. Add 700 µL of 50% sodium hydroxide solution.

10 mM Sodium Phosphate, pH 6.0 (A)

Combine 9.5 mL of 200 mM dibasic sodium phosphate, 90.5 mL of 200 mM monobasic sodium phosphate, and 1900 mL DI water.

10 mM Sodium Phosphate with 1.0 M Sodium Chloride, pH 6.0 (B)

Combine 40.0 mL of 200 mM dibasic sodium phosphate, 60.0 mL of 200 mM monobasic sodium phosphate, 1000 mL of 2.0 M sodium chloride, and 900 mL DI water.

Stock Standards

RNase A - 15 mg/mL solution in degassed, filtered DI water. Aliquot 400 µL volumes and store at –40 ºC until ready to use.

Ammonium bicarbonate – prepare a 10% w/v solution in filtered DI water. The measured pH of this solution is 8.0. Store at 5 ºC until ready to use.

Sample Preparation

Combine 334 µL of 15 mg/mL RNase A, 100 µL of 10% ammonium bicarbonate and 566 uL of DI water in a 1.5 mL microcentrifuge tube to make a 5 mg/mL RNase A solution in 1% ammonium bicarbonate. Incubate this solution at 37 ºC. Periodically withdraw 50 µL aliquots and freeze these aliquots. When ready to analyze the samples, thaw the aliquots, dilute five-fold with the appropriate Mobile Phase A (20 mM MES or 10 mM sodium phosphate), and place in the 0.3 mL autosampler vials. The final protein concentration is 1.0 mg/mL. This forced deamidation of RNase A follows the method described by Di Donato et al.[13] Controls consisting of 5 mg/mL RNase A and 1% ammonium bicarbonate were incubated with the samples and processed as described above.

RESULTS AND DISCUSSION

Separation

Figure 1 compares the chromatographic profiles for native RNase A run on the ProPac WCX-10 weak cation-exchange column using either MES- or phosphate-based buffers. The resolution between the two deamidation products (peaks 1 and 2 in Figure 1) is 1.37 using phosphate-based mobile phases and is 2.94 with the MES-based mobile phases.Visual inspection of the two chromatograms (see Figure 1A) shows more chromatographic detail with the MES mobile phases, increasing the ability of the system to separate more RNase A variants in a single chromatographic analysis. In contrast, Di Donato et al. reported the separation of the Asn[67] deamidation products of RNase A by cation-exchange chromatography followed by hydrophobic interaction chromatography to resolve the two deamidation variants.

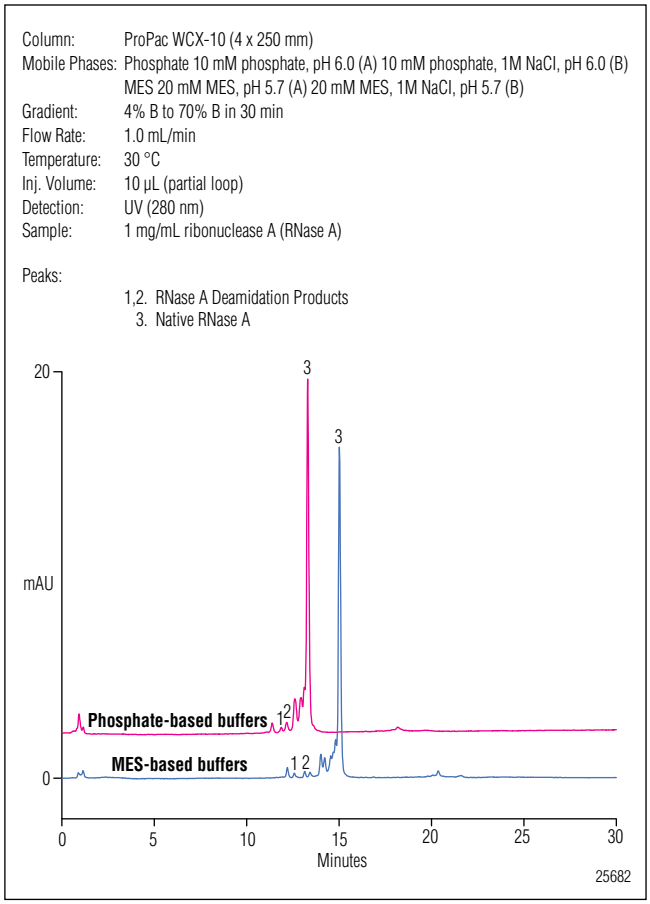

Figure 1: Full chromatogram. Comparison of the chromatography using a ProPac WCX-10 column with phosphate-based vs. MES-based mobile phases for ribonuclease A and its deamidation products.

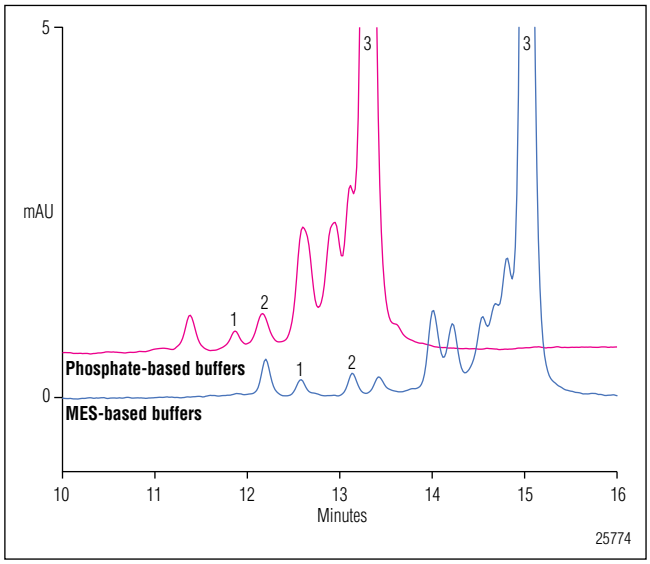

Figure 1A: 10–16 min region showing improved deamidation product resolution for MES-based vs. phosphate-based mobile phases.

Figure 2 displays the chromatographic profiles of samples taken during the 37 ºC incubation of RNase A in 1% ammonium bicarbonate. The deamidation peaks (12.5 and 13.1 min) increase in abundance while the native RNase A peak abundance (15.0 min) decreases. RNase A stability in aqueous solution at 37 ºC is demonstrated by observing little RNase A peak area change in the control (37 ºC incubation of RNase A in the absence of ammonium bicarbonate) over the 425 h incubation period (data not shown). This sample of untreated RNase A (Figure 1) shows additional peaks, including ones at the same retention times as the deamidation variants. It is likely that the untreated RNase A standard contains small amounts of deamidated RNase A.

Deamidation Monitoring

Insight into the deamidation kinetics can be gleaned by plotting the peak area of the deamidated forms of RNase A as a function of incubation time. Figure 3 shows the results of this plot (normalized to the RNase A peak area at time = 0 h) for both the full 425 h time course study (Figure 3A) and the initial 169 h (Figure 3B). Initially, the deamidation reaction followed first order kinetics.

CONCLUSION

The high efficiency of the ProPac WCX-10 column, coupled with an inert, titanium HPLC system, allows for the rapid analysis of protein deamidation variant forms. Using MES-based mobile phases improve peak resolution when compared to typical phosphate-based mobile phases.

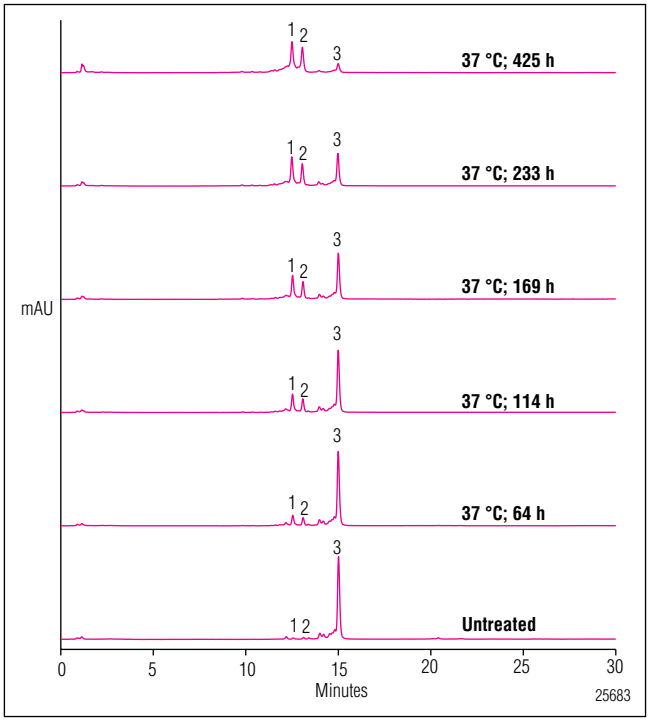

Figure 2: Use of MES-based mobile phases to separate ribonuclease A and its two deamidation products during the time course of forced deamidation. Ribonuclease A (5 mg/mL) was incubated in 1% ammonium bicarbonate at 37 ºC, aliquots diluted 5-fold with mobile phase A (see Figure 1 for conditions).

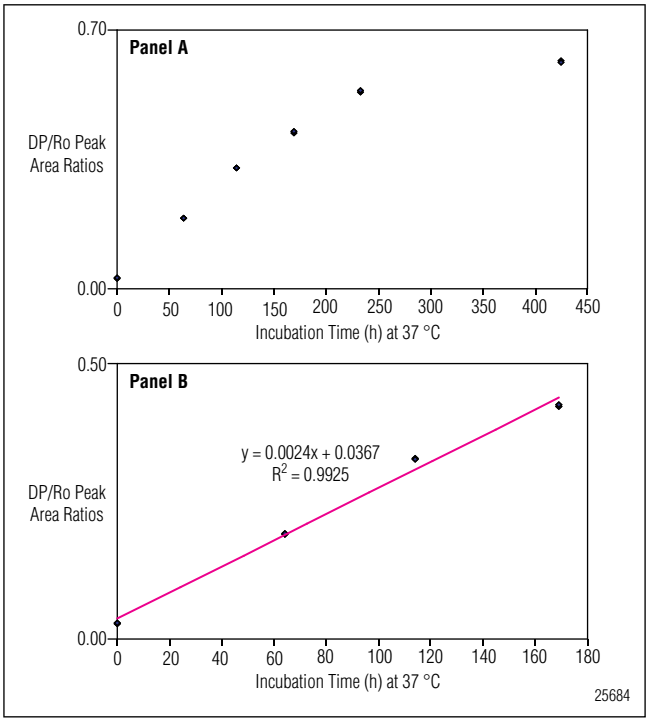

Figure 3: Formation of deamidation products (DP) of ribonuclease A as a function of time (Ro is the initial RNase A peak area at T=0 h). A – Plot displayed of the full time course (425 h). B – Plot displayed of the first 169 h. Duplicate injections for each time point.

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