

# Monitoring Protein Deamidation by Cation-Exchange Chromatography

## INTRODUCTION

A common structural modification of recombinant proteins is the deamidation of asparagine (Asn) residues. 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.<sup>1-6</sup> 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.<sup>7</sup>

This Application Note describes the use of the ProPac™ WCX-10, a weak cation-exchange column that 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. In practice, the ProPac WCX-10 weak cation exchange resin affords minimal band spreading and high selectivity.<sup>8</sup> In the example presented here, deamidated variants of ribonuclease A are separated from the native protein in less than 15 min on a ProPac WCX-10 weak cation-exchange column.

## EQUIPMENT

Dionex DX-500 BioLC® Liquid Chromatograph  
GP50 Gradient Pump  
AD20 Variable Wavelength Absorbance Detector  
LC25 Chromatography Enclosure  
AS50 Autosampler (with 50- $\mu$ L sample loop)  
PeakNet Chromatography Workstation

## REAGENTS AND STANDARDS

Sodium phosphate, monobasic and dibasic, analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, USA)  
Sodium chloride, analytical-reagent grade (Fluka, Ronkonkoma, NY, USA)  
High-purity deionized water prepared with a Milli-Q system (Millipore, Bedford, MA, USA)  
Ribonuclease A (Sigma, St. Louis, MO, USA)

## PREPARATION OF SOLUTIONS AND REAGENTS

Two eluents are used for this chromatography: 10 mM sodium phosphate (pH 6.0) and 10 mM sodium phosphate (pH 6.0) 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 6.0. The following procedure is a recommended starting point for obtaining the desired eluents, but some deviation from this formula may be necessary after checking the pH, when using reagents in other labs. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total volume of monobasic and dibasic solution should remain at 100 mL to produce 10 mM sodium phosphate for 2 L of eluent.

## 2 M Sodium Chloride

Dissolve 116.90 g sodium chloride in water, and fill to a final volume of 2.0 L. Filter through a 0.45- $\mu$ m filter.

## 200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) in 1000 mL of water. Filter through a 0.45- $\mu$ m filter. Store frozen until needed.

## 200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ ) in 1000 mL of water. Filter through a 0.45- $\mu$ m filter. Store frozen until needed.

## 10 mM Sodium Phosphate, pH 6.0

Combine 12.3 mL of 200 mM dibasic sodium phosphate, 87.7 mL 200 mM monobasic sodium phosphate, and 1900 mL water.

## 10 mM Sodium Phosphate with 1.0 M Sodium Chloride, pH 6.0

Combine 12.3 mL of 200 mM dibasic sodium phosphate, 87.7 mL of 200 mM monobasic sodium phosphate, 1000 mL of 2 M sodium chloride, and 900 mL water.

## CONDITIONS

Column: ProPac WCX-10, 4 x 250 mm

Flow Rate: 1 mL/min

Detection: Absorbance, 280 nm

Eluent: A: 10 mM sodium phosphate, pH 6.0  
B: 10 mM sodium phosphate, 1 M sodium chloride, pH 6.0

Gradient: Linear, 4–70% B in 30 min

Method:

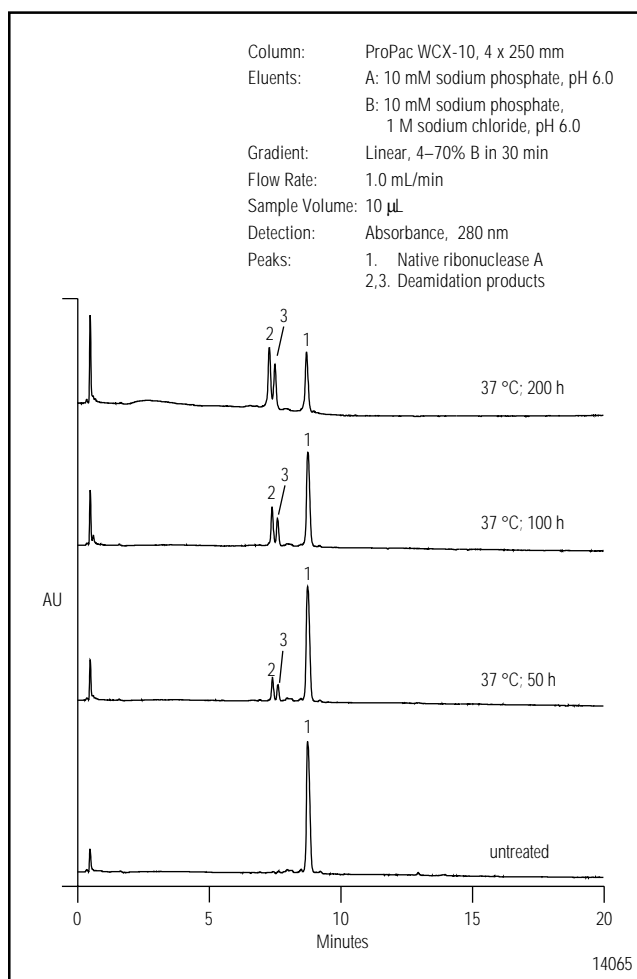
Time	A (%)	B (%)	Comments
Initial	96.00	4.00	Equilibration
0.00	96.00	4.00	Sample Injection
30.00	30.00	70.00	End Gradient
40.00	25.00	75.00	Regeneration
42.00	96.00	4.00	Re-equilibration

## SAMPLE PREPARATION

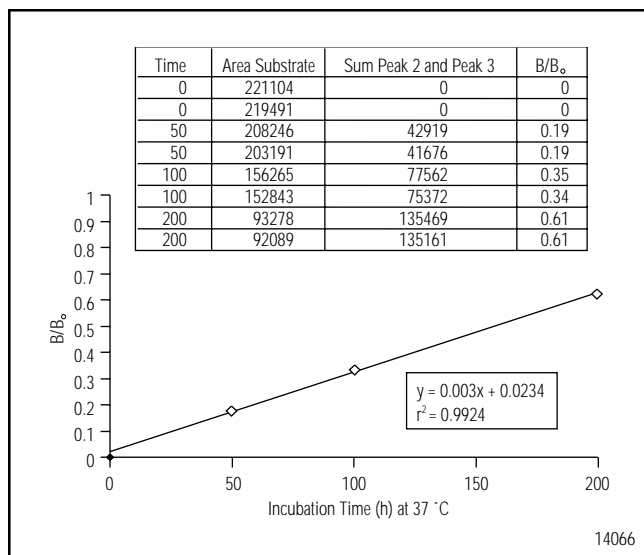
Protein samples were dissolved or diluted in Mobile Phase A at a final protein concentration of 0.5 mg/mL. Forced deamidation of ribonuclease A was achieved using the method described by Donato et al.<sup>9</sup>

## RESULTS AND DISCUSSION

Donato et al. reported the separation of the Asn<sup>67</sup> deamidation products of ribonuclease A by cation-exchange chromatography on Mono S resin followed by hydrophobic interaction chromatography to resolve the two deamidation variants (Asn and isoAsn deamidated at residue 67).<sup>9</sup> In contrast to this two-step procedure, the ProPac WCX-10 column separated deamidation variants (Asp<sup>67</sup> and isoAsp<sup>67</sup>) from native ribonuclease A in a single chromatographic run (see Figure 1). The high efficiency of



**Figure 1** Separation of ribonuclease A and its two deamidation products during the time course of forced deamidation. Ribonuclease A (3 mg/mL) was incubated in 1% ammonium carbonate buffer, pH 8.2, at 37 °C.



**Figure 2** Formation of deamidation products (B) of ribonuclease A (B<sub>0</sub>) as a function of time.

the ProPac WCX weak cation-exchange resin yielded baseline resolution of the variant forms, allowing quantification of each form of the protein as a function of time. A plot of peak areas of the Asn<sup>67</sup> deamidated forms of ribonuclease A as a function of time indicated that the deamidation reaction followed first order kinetics, with a half-time of 159 hours (see Figure 2).

## CONCLUSION

The high efficiency of the ProPac WCX-10 column allows the rapid analysis of protein deamidation with baseline resolution of variant forms. This capability makes the ProPac WCX-10 column well-suited for the rapid monitoring of deamidation in therapeutic proteins.

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