High Salt Gradient Analysis of Post-Translational Modifications - Deamidation Monitoring

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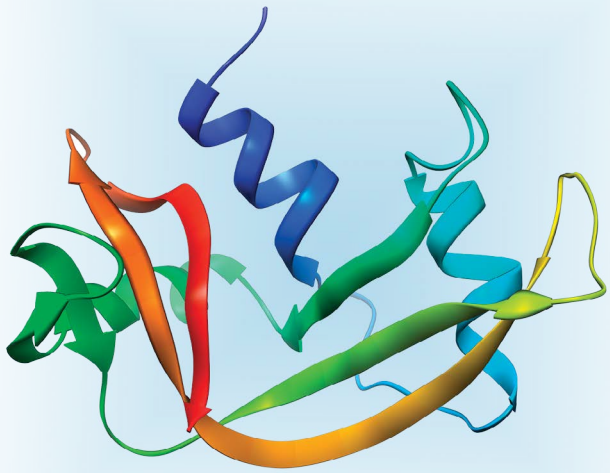
Key Words
Biopharma, biopharmaceuticals, Vanquish Flex UHPLC, variable wavelength detection, charge variant analysis

Goal
Analyzing deamidation variants of ribonuclease A using cation exchange chromatography and providing a baseline separation of post-translational modifications that differ by a few amino acid changes.

Introduction
Therapeutic proteins have a major role in today's medical treatment for a wide array of diseases. The diversity and efficacy of biotherapeutics makes them ideally suited for the treatment of various diseases such as rheumatoid arthritis, multiple sclerosis, and several types of cancer. Due to the complexity of biotherapeutics, they are prone to post-translational modifications that have to be characterized. One of the most common non-enzymatic post-translational modifications in therapeutic proteins is the deamidation of asparagine residues.[1] It has been reported that deamidation can lead to allergic reactions[2] as well as a reduction of the half-life, pharmacological dynamic, stability,[3] or even a loss of the biological function. Thus it is crucial to characterize and control deamidation products in biopharma processes to ensure a high quality product.

The analysis of deamidation products in recombinant proteins is a significant challenge because they differ in only a few amino acids. Ion-exchange chromatography is the method of choice for the separation of charge variants. The Thermo Scientific™ ProPac™ WCX-10 column provides high resolution and minimizes secondary (nonionic) interactions to ensure best selectivity and is able to resolve samples that differ in only one charged residue. For complete biocompatibility of the system we use the Thermo Scientific™ Vanquish™ Flex UHPLC system. The fluidics are optimized for dealing with biomolecules and high buffer concentration to give robustness and high confidence in data evaluation.



In this application we show an easy deamidation monitoring using the Vanquish Flex UHPLC system with UV detection. In less than 20 minutes, the native protein is separated from the deamidated variant on a ProPac WCX-10 column.

Experimental

Vanquish Flex UHPLC system consisting of:
- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (6732.0110)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Semi-micro Flow Cell, Biocompatible, 2.5 µL/7 mm (P/N 6077.0300)

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Column	ProPac WCX-10 analytical 4 × 250 mm (P/N 054993)
Mobile Phase A: Mobile Phase B:	10 mM sodium phosphate in water, pH 6.0 10 mM sodium phosphate, 1 M sodium chloride in water, pH 6.0
Flow Rate	1 mL/min
Injection Volume	10 µL
Sample	Deamidated ribonuclease A (bovine pancreas)
Detection	280 nm; Data Collection Rate: 10 Hz; response time: 0.4 s
Data Processing	Thermo Scientific™ Chromeleon™ CDS Software 7.2 SR3
Gradient	0–30 min: 4–70% B; 30–40 min: 70–75% B; 40–42 min: 75–4% B; 42–45 min: 4% B

Sample Preparation

The deamidation was induced by a 1% ammonium bicarbonate solution. For this, 15 mg of ribonuclease (bovine pancreas) were dissolved in 1 mL starting conditions to get a final solution of 15 mg/mL. Next, 334 µL of the protein solution, 100 µL of 10% ammonium bicarbonate (w/v), and 566 µL of deionized water were combined in a 2 mL tube to create a final ribonuclease A concentration of 5 mg/mL. The tube was placed in a thermo shaker at 37 °C; aliquots were taken after 10 minutes and 24 hours.

Results and Discussion

A salt gradient was used to perform the separation of two deamidation variants. As previously shown, deamidation is a slow process.[4] The ribonuclease A treated with ammonium bicarbonate for 10 minutes was used as baseline to have the same sample preparation steps for both samples. The identification of the asparagine residues reaction was verified using the increased peaks after 24 h incubation times of ammonium bicarbonate as shown in Figure 1. Baseline separation with a resolution R of 1.6 for the two main products was achieved (peak 1 and 2). Therefore, a qualitative and quantitative analysis of a post-translational modification is feasible. Within 24 hours of incubation with ammonium bicarbonate the two main deamidation products increased by 389% (peak 1) and 97% (peak 2) compared to the baseline reference.

Conclusion

In this application we separated different forms of ribonuclease A, where we had introduced deamidation. The Vanquish Flex system with variable wavelength detector and a high-resolution, ion-exchange gradient method was able to resolve various species and to identify the deamidation product by comparing two differently treated ribonuclease samples exposed for different time periods.

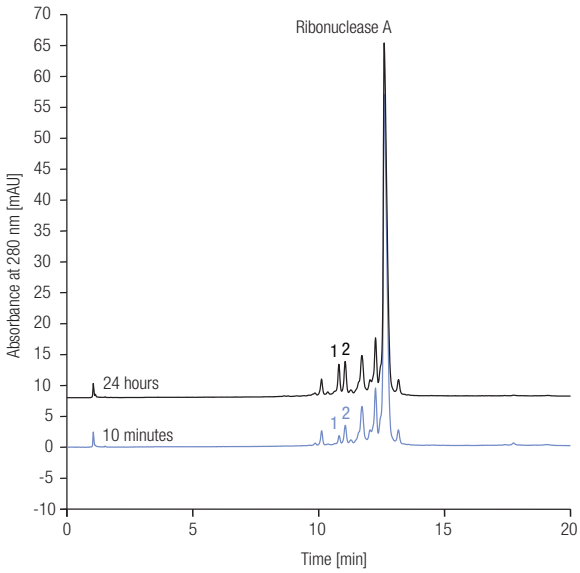

Figure 1. Deamidation products of ribonuclease A.

References

1. Soulby, A.J.; Heal, J.; Barrow, M.P.; Roemer, R.A.; O'Connor, P.B. Does Deamidation Cause Protein Unfolding? A Top-Down Tandem Mass Spectrometry Study, *Protein Science*, DOI 10.1002/pro.2659.
2. Denery-Papini, S.; Bodinier, M.; Larré, C.; Brossard, C.; Pineau, F.; Triballeau, S.; Pietri, M.; Battais, F.; Mothes, T.; Paty, E.; Moneret-Vautrin, D-A. Allergy to deamidated gluten in patients tolerant to wheat: specific epitopes linked to deamidation. *Allergy*, **2012**, *67*, 1023–1032.
3. Cleland, J.L.; Powell, M.F.; Shire, S.J. *Critical Reviews in Therapeutic Drug Carrier Systems*, **1993**, *10*(4), 307-377.
4. Dionex (now part of Thermo Fisher Scientific) Application Note 125: Monitoring Protein Deamidation by Cation-Exchange Chromatography [Online] http://www.dionex.com/en-us/webdocs/4458-AN125-Cation-Exchange-26Jun09-LPN1045-02.pdf