DETECTION AND MEASUREMENT OF PROTEIN MODIFICATION AND DAMAGE IN COMPLEX SAMPLES USING EXACT MASS MEASUREMENT

Waters

Ziling Lu, Beth L. Gillece-Castro, Thomas E. Wheat, Laetitia Cravello, and Jeffrey R. Mazzeo
Waters Corporation; 34 Maple Street; Milford, MA01757

INTRODUCTION

Therapeutic proteins can undergo structural modifications during production, storage, and stability testing. Such changes can affect safety and efficacy. Therefore, reliable determination and continuous monitoring of protein properties are essential. Since structural changes are reflected in molecular weight, measuring this property is one convenient way to monitor modifications. Methods including electrophoresis, chromatography, and ultracentrifugation have been used; however, measurements with those techniques have relative errors from 10-100%. This large error range is due to these methods being affected by properties other than molecular weight such as conformation, Stokes' radius, and hydrophobicity. Mass spectrometry is a technique whereby more exact molecular weight information can be obtained. However, the proteins are often dissolved in buffers containing non-volatile salts. These salts cause ion suppression and adduct formation in ESI-MS complicating molecular weight determination, as shown in Figure 1. Many techniques have been developed for removing salts from proteins. These methods include SEC, centrifugal ultrafiltration and buffer exchange. These methods are limited to off-line use and are often labor intensive. An alternative technique has been developed that can be used either off-line or on-line coupled directly to a mass spectrometer. The MassPREP™ On-Line Desalting Cartridge is a 2.1 x 10 mm device, packed with polymer sorbent. Its performance has been demonstrated using acidic (bovine serum albumin), basic (cytochrome c), and large globular (monoclonal IgG1) protein samples. The results of this study show no sample carryover and excellent lifetime for
repeated sample analysis under the conditions tested. This technique yields accurate direct measurement of molecular weight of biopharmaceutical proteins.

The MassPREP ™On-Line Desalting Cartridge has been applied to some real analytical problems in protein biopharmaceutical analysis. The oxidation of model proteins dissolved in saline is monitored in a forced degradation experiment as an example of the technique could be applied in a process development or a stability testing experiment.

Figure 1:Effect of Salt in ESI-MS of Protein

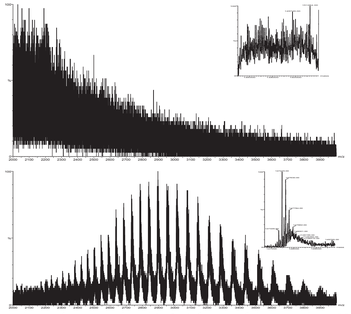

Figure 1: Desalting of a monoclonal IgG. Infusion of the sample in PBS gives no interpretable mass spectrum. After desalting, a useful spectrum is obtained.

METHODS

Sample preparation:
Ribonuclease A and enolase were obtained from Sigma Chemical Co. St. Louis, Mo. All proteins were dissolved in phosphate buffered saline (PBS) at a concentration of 100ng/µL. A therapeutic antibody was kindly provided by Pierre Fabre Medicaments - CIPFI. Samples were oxidized by exposure to hydrogen peroxide as described in the figure legends.

Instrumentation:

HPLC System:	Waters Alliance® Bioseparations Module
Needle Wash Solution:	35:5:60 H_2O:IPA:ACN
Number of Needle Washes:	Single
Needle Wash Time:	15 sec needle exterior / 3 sec inject port
Diverter Valve:	Figure 2
Mass Spectrometer:	Waters Micromass® LCT Premier
Ionization mode:	ES +
Spectral Processing:	MaxEnt1™

On-Line Desalting Method:

Eluent A:	H_20 with 0.1% Formic Acid
Eluent B:	Acetonitrile with 0.1% Formic Acid
Flow:	0.4 mL/min
Gradient elution:	Load: 5% B - 2min Elute: 5-80 % B - 2min
Column:	MassPREP™ On-Line Desalting Cartridge 2.1 x 10 mm

Figure 2: Diverter valve for the on-line protein desalting method.

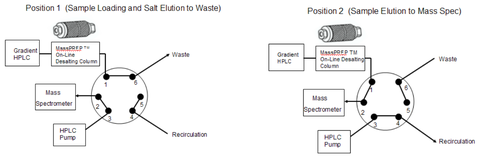

Figure 3: Elution of Proteins from MassPREP™ On-Line Desalting Cartridge

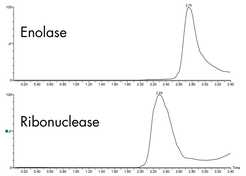

Figure 3: The elution of proteins from the desalting cartridge shows some variation in elution time for different proteins. Useful separations are not observed on this small cartridge, particularly when used in a fast desalting mode.

RESULTS

OXIDATION OF RIBONUCLEASE A

Ribonuclease A (13kDa) was dissolved in phosphate buffered saline at a concentration of 100ng/µL. It was incubated for 16 hours at 37°C, and then desalted directly into the LCT Premier.

Figure 4: Mass Spectra of Native and Oxidized Ribonuclease

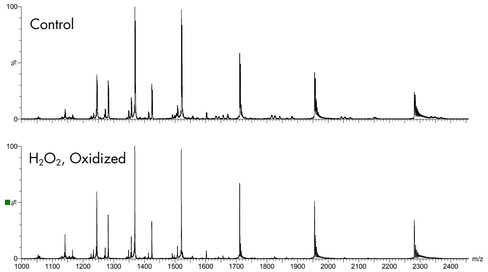

Figure 4: The solutions of ribonuclease in PBS give typical protein spectra after desalting. There are, however, no immediately obvious distinguishing features between raw spectra obtained from the native and the oxidized material. Complete analysis requires deconvolution.

Figure 5: MaxEnt1 Deconvolution of Ribonuclease A Mass Spectra

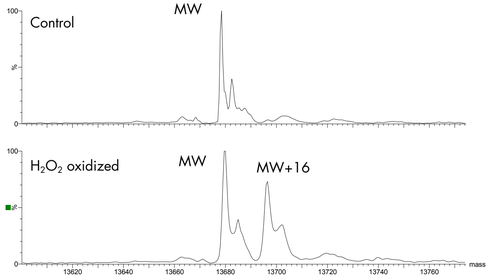

Figure 5: When MaxEnt1™ is applied to the ribonuclease spectra shown above, the complexity of the samples is apparent. Both the heterogeneity of the starting material and the effect of oxidation are readily seen. It is interesting to note that the sample of ribonuclease A contains a mixture of native and oxidized material after 16 hours. No other oxidation states are apparent. Since this sample has four possible oxidation sites, it appears that they are not all equally reactive. It is not possible to decide from this forced degradation experiment whether there is one rapidly oxidized site or random oxidation of one or more sites.

OXIDATION OF ENOLASE

Enolase (47kDa) was dissolved in PBS at a concentration of 100ng/µL. It was incubated for 24 hours at 37°C, and then desalted directly into the LCT Premier. As in the ribonuclease A experiment, the product reached a steady state. To determine whether the analytical technique could discriminate intermediate stages in the oxidation process, samples were incubated with different concentrations of hydrogen peroxide and evaluated after four hours of incubation.

Figure 6: Deconvoluted Spectra of Native and Oxidized Enolase

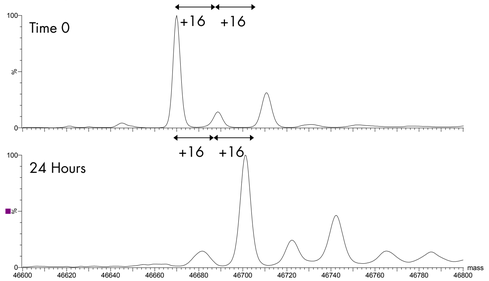

Figure 6: After 24 hours of incubation, the enolase sample no longer contains any native protein. This protein has five potential oxidation sites so a mixture of species might have been expected. However, the only form corresponding to oxidation events is a +32. Again, the technique does not discriminate between two labile methionines and a forced sulfone formed at a single site.

Figure 7: Concentration Dependence of Enolase Oxidation

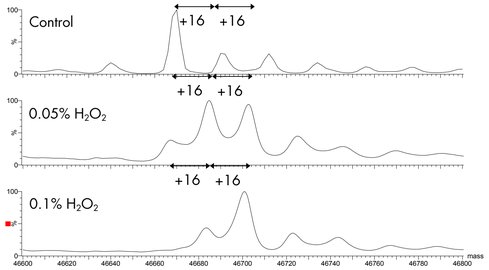

Figure 7: Samples of enolase incubated at two different concentrations of hydrogen peroxide for four hours. Under these conditions, the oxidation intermediates can be discriminated. The results are consistent with two labile methionines of the five total.

DESALTING OF A THERAPEUTIC ANTIBODY

A therapeutic antibody was kindly provided by Pierre Fabre Medicaments - CIPF. For these experiments, the MassPREP™ On-Line Desalting Cartridge was mounted directly on the diverter valve of the mass spectrometer, and the procedure was executed manually using a syringe. First, in load position, the cartridge was washed with about 1mL of 5% acetonitrile, 95% water and 0.1% FA. Then, a sample aliquot (100µL at 1µM) was injected. The absorbed sample was desalted with about 1mL of 5% acetonitrile, 95% water and 0.1% FA. The valve was switched to the inject position, and the sample was eluted at 50µL/min with a solution of 50% acetonitrile, 50% water and 0,1% FA.

Figure 8: Spectra and Deconvolution of the Mass Spectra of an Antibody

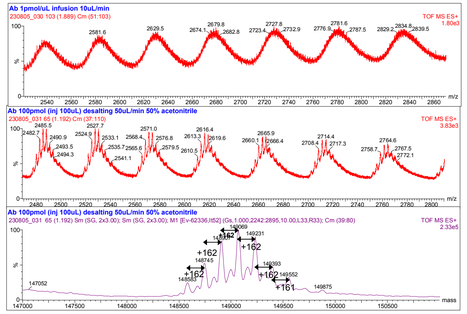

Figure 8: When the antibody sample is directly infused (Top), the charge state envelope can be discerned. The signal-to-noise of this spectrum is too low for highly discriminatory deconvolution. The spectrum obtained as the antibody elutes from the Desalting Cartridge (Middle) immediately shows the fine structure in the charge state envelope. This indicates that there are multiple molecular species in the sample. When the spectrum of the desalted sample is deconvoluted, the species are apparent. The multiple glycosylation states of the antibody can be distinguished in this way.

CONCLUSIONS

- High resolution mass spectra can be obtained from protein samples eluted from the MassPREP™ On-Line Desalting Cartridge in less than five minutes.
- The spectra can be deconvoluted with MaxEnt1™ to identify the components of the mixture.
- This approach is used to monitor oxidation of ribonuclease A (13kDa) and enolase (47kDa) in forced degradation experiments.
- The technique is shown to be suitable for producing sufficiently high signal-to-noise spectra for identifying the glycosylation states of a therapeutic antibody

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