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OPTIMIZING HPLC FOR PURITY ANALYSIS AND CONFIRMATION OF IDENTITY OF PROTEINS WITH EXACT MASS LC-MS

Thomas E. Wheat; Gillece-Castro, Beth L.; Ziling Lu; and Jeffrey R. Mazzeo
Chemistry Applied Technology; Waters Corporation, Milford, MA USA

ABSTRACT

Chemical degradation or other modifications of proteins may affect the biological properties. A variety of analytical methods have been developed to assess the degree of such modifications in a protein sample, but these techniques are often labor-intensive and time-consuming. It is attractive to consider using measurement of protein molecular weight to determine the degree of modification since any chemical change will alter the molecular weight. Determination of molecular weight using mass spectrometry techniques is a fast and accurate tool for the characterization of recombinant proteins. This technique will be most effective if the several protein species that may be present in the sample are first separated so that they may be individually identified and quantitated. Thereby, the mass spectra are simplified to single species, and the trace components are readily detected. Reversed-phase chromatography provides an aqueous environment for protein separations based primarily on the hydrophobicity. We have studied the behavior of proteins on several reversed-phase packing materials, including non-porous and fully porous particles. The base particles were silica, polymeric, and bridged-ethyl hybrid chemistries. The hydrophobic ligands C4, C8, C18, and Phenyl have each been evaluated. A test set of protein mixtures included acidic, neutral and basic proteins of a range of sizes. The optimized LC analyses were combined with automated high resolution MS for the determination of molecular weights. The samples were also degraded to include the changes likely to be encountered in stability testing, such as, oxidation. Quality of separation was evaluated in terms of the ability of the final method to yield accurate and precise molecular weights of proteins, especially those present in the mixture at low levels. Improving the separation yields improved quantitation.

MATERIALS

Samples:

RNase A, cytochrome c, transferrin, hemoglobin, and enolase were purchased from Sigma Chemical Company, St. Louis
Concentration = 0.1 mg/mL for each protein of mix in aqueous 0.1% formic acid
10µL injections = 1 µg/protein
RNase A oxidation mix = 1:1 untreated and 24 hour H_2O_2 treated 0.05 mg/mL each in normal saline

Sample preparation:

For desalting experiments, all proteins were dissolved in phosphate buffered saline (PBS) at a concentration of 100ng/mL. 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
Mass Spectrometer:	Waters® LCT Premier™
Ionization mode:	ES +
Spectral Processing:	MaxEnt1™

METHODS

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: 100% A - 2min
	Elute: 5-80 % B - 2min
Column:	MassPREP™ On-Line Desalting Cartridge

Chromatography:

Waters 2796 Alliance System
BioSuite™ pPhenyl, 2.0 x 75 mm column
Symmetry® 300 C4, 2.1 x 100 mm column
Flow rate 200 µL/min on BioSuite™ pPhenyl
Flow rate 220 µL/min on Symmetry®300 C4
Buffer A = Aqueous, 0.1% TFA (or 0.1% formic acid if noted)
Buffer B = Acetonitrile, 0.1% TFA (or 0.1% formic acid if noted) with isopropanol (IPA) as noted
Gradient range, 10-60% Buffer B
Gradient time as noted
Temperature 60°C unless noted
2.1 x 10 mm

Mass Spectrometry:

Waters® LCT Premier™
Electrospray Ion Source 100° C
Cone = 30 V
Tunnel 1 = 90 V
Aperture 1 = 15 V

RESULTS

MS ANALYSIS OF PROTEIN MIXTURES

Figure 1: MaxEnt1 Deconvolution of Ribonuclease A Mass Spectra

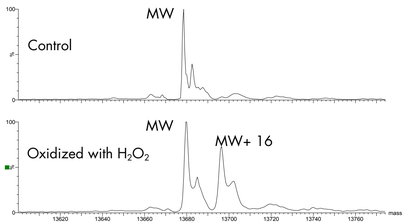

Figure 1: When MaxEnt1™ is applied to the ribonuclease A spectra obtained with infusion, the complexity of the samples is apparent. Both the heterogeneity of the starting material and the effect of oxidation are readily seen. It is interesting to note that the sample of ribonuclease A contains a mixture of native and singly-oxidized material after 24 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 a random oxidation of one of the multiple sites.

OXIDATION OF ENOLASE

Enolase (47kDa) was dissolved in PBS at a concentration of 100 ng/µL. It was incubated for 24 hours at 37°C, and then desalted directly into the LCT Premier. As in the ribonuclease 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 incuba-

Figure 2: Deconvoluted Spectra of Native and Oxidized Enolase

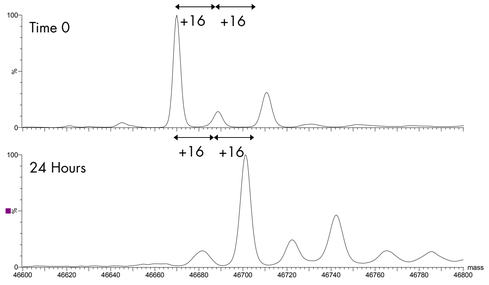

Figure 2: After 24 hours of incubation, the enolase sample no longer contains any native protein. This protein has five potential oxidation sites so a mixture of species might have been expected. However, the only form corresponding to oxidation events is a +32. Again, the technique does not discriminate between two labile methionines and a forced sulfone formed at a single site.

Figure 3: Concentration Dependence of Enolase Oxidation

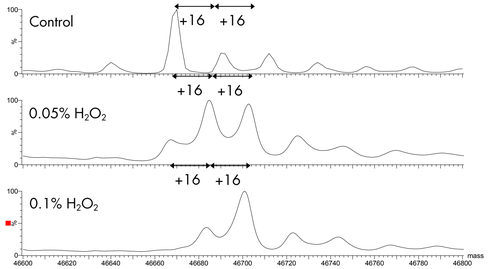

Figure 3: Samples of enolase incubated at two different concentrations of hydrogen peroxide for four hours. Under these conditions, the oxidation intermediates can be discriminated. The results are consistent with two labile methionines of the five total.

Figure 4: Analysis of Oxidized Proteins

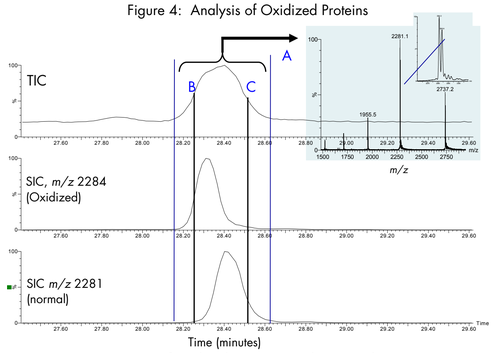

Figure 4: A 1:1 mixture of oxidized RNase A and untreated RNase A was separated on a Symmetry300 C4 column. The protein forms eluted from 28.15—28.63 minutes. When the summed mass spectra over this peak are examined (Top), a pair of closely spaced *m/z* species are observed. Single mass chromatograms were extracted for the two most intense signals, 2281 and 2284. These SIC's correspond to the 6-charge state of native and oxidized RNase A, and the chromatograms show partial separations of these species.

Figure 5: Analysis of Oxidized Proteins

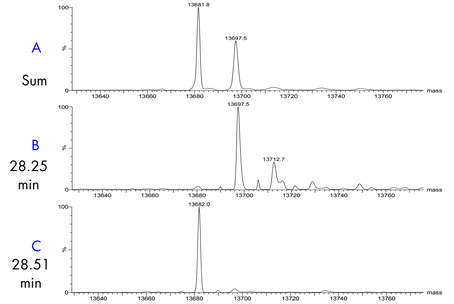

Figure 5: RNase A peak of mixed oxidized and untreated protein eluted from the Symmetry C4 column. The molecular weights were deconvoluted from spectra over the entire peak, A, an early eluting spectrum, B, and a later eluting spectrum, C (See figure 4). Spectra B and C are only 9 seconds apart, but they show no co-elution. The spectra were deconvoluted with MaxEnt1™ and yield the correct molecular weights separated by 16 Daltons. These results demonstrate that the singly oxidized protein elutes prior to the untreated protein, and that chromatographic separations can improve spectral quality for intact proteins.

SEPARATION OF PROTEIN MIXTURES

Figure 6: Effect of Temperature

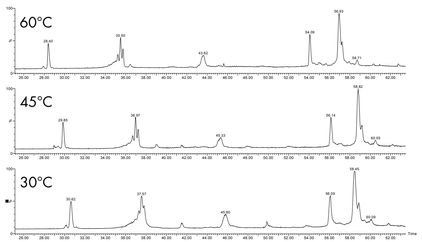

Figure 6: Reversed phase chromatography of proteins is usually performed at elevated temperature to ensure good peak shape and recovery. As shown here, the proteins elute earlier in the gradient. With this test sample, the peaks are sharper, and better resolution is possible with increased temperature. The effect is, however, relatively small. It is necessary to consider additional parameters for greater improvements in resolution.

Figure 7: Effect of Organic Solvent

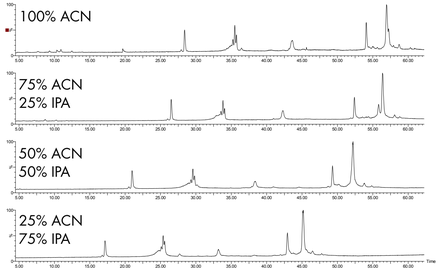

Figure 7: Effect of Organic Solvent: Mixing *iso*-propanol with acetonitrile also reduces retention of proteins. In contrast to temperature effects, a change in selectivity can be observed with the mixed organic solvents. This is most apparent for the peaks near 55min. The effect does not, however, suggest a general mechanism for greatly improving reversed phase protein separations.

CONCLUSIONS

- The molecular weights of the individual proteins in a mixture can be determined by combining exact mass measurement with the MaxEnt1 deconvolution algorithm.
- Automated on-line desalting produces high resolution spectra for use in these determinations.
- This process can be used to detect modifications of proteins as small as a single oxidation.
- Chromatographic separations can simplify the protein spectra so that more information can be extracted.
- Improved separation mechanisms are desirable.

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