Disulfide Mapping Using Label-Free Differential Analysis

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Overview

Purpose: To discover disulfide bonds in a purified protein sample.

Methods: Samples digested with trypsin with and without subsequent reduction and alkylation were compared using LC-MS and label-free analysis software.

Results: We demonstrate a workflow for discovery and verification of disulfide bonds using LC-MS and label-free analysis methods. Method robustness appears to be sensitive to sample preparation.

Introduction

Disulfide cross-linkages formed by the oxidation of the thiol groups of cysteines play an important role in the folding and stability of many proteins. Knowledge of the disulfide linkages in a protein provides insight into protein structure. Determination of the disulfide bonds in recombinant or synthetic proteins is also important, since formation of the correct disulfide cross linkages are an indication of proper folding and function. Experimentally, protein structure can be determined with time consuming methods such as Xray crystallography or NMR spectroscopy.

In this presentation, we discuss a method of determining disulfide bonds using LC-MS and label-free differential analysis (Thermo Scientific SIEVE software version 1.3). The method is based upon accurate mass derived from full scan data – no MS/MS fragmentation data were used.

When using accurate mass information for identification assignment, mass degeneracy is often a concern; that is, molecules with the same or similar mass may introduce ambiguity. Because of the mass accuracy of the Thermo Scientific Exactive mass spectrometer and the simple samples (purified proteins), mass degeneracy was never a problem.

The full-scan accurate-mass approach is preferred because the data are easier to interpret and are independent from the identification efficiency of fragmentation interpretation software such a Mascot or SEQUEST.

We present a work-in-progress, whereas, the samples we have chosen are pedagogical, but well studied and understood. Our approach in developing this method is to start with simple proteins that may be validated with previously reported results.

Method

Samples

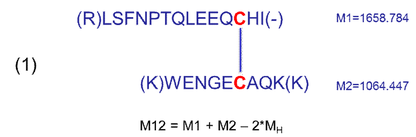
In this presentation analysis methods are emphasized. We focus upon a simple protein sample of beta lactoglobulin of length 178 and a mass of *ca* 20kDa. Beta lactoglobulin contains six cysteines and three disulfide bonds (Figure 1).

FIGURE 1. Beta lactoglobulin disulfide bonds

Disulfide bond	82 ↔ 176	Ref.13 Ref.19	
Disulfide bond	122 ↔ 137	Alternate Ref.13	
Disulfide bond	122 ↔ 135	Ref.13 Ref.19	

Sample Preparation

Purified protein samples were weighed and dissolved in a buffer of 8M Urea 150mM Tris-HCl 2.5% n-propanol, pH 8.5 to denature the proteins.



Two equal fractions were created. The first fraction was reduced with DTT to break disulfide bonds and create free cysteine groups. The sample was then alkylated with iodoacetic acid to modify all cysteines in a way that prevented disulfide bonds from forming. Trypsin was then added to the first fraction for protein digestion into peptides. The second fraction was treated with trypsin only (no digestion alkylation).

The fractions were then divided into four equal aliquots and placed in a 96 well plate for LC-MS acquisition.

Liquid Chromatography

Samples were injected at 150 µL/min onto a Thermo Scientific Hypersil GOLD 5 micron 1mm x150 mm column using a Thermo Scientific Surveyor Autosampler with a pump. Solvents were Fisher Optima LC-MS grade Water with 0.2% formic acid buffer A, acetonitrile 0.2% formic acid buffer B. A gradient from 3% B to 30% B was used to separate the polypeptides. The ion source was a Thermo Scientific Ion Max source with a standard ESI probe fitted with a 32-gauge metal needle.

Mass Spectrometry

The Exactive™ mass spectrometer was tuned on Angiotensin I, T'ed into a 150 µL min flow of 15% B. Tune parameters were 20 on sheath, 3 on aux, with a spray voltage of 3.8 kV and a capillary temp of 300 °C. Scan parameters, 60K resolution with 3 microscans and lock mass were enabled. The divert valve was used during the first 5 minutes to prevent salts and urea in the digests from contaminating the source.

Data Analysis

The acquired data were processed using SIEVE™ software V1.3 in an "A vs B" differential analysis experiment with technical replicates. The SIEVE workflow first performed a chromatographic alignment using full scan spectral shape (no peaks). Frames (a.k.a. features) were constructed based upon prominent peaks throughout the full data set. Identification assignment was based upon the accurate mass database described above using the SIEVE MZLookup tool. Replicates were required to achieve a CV<20%.

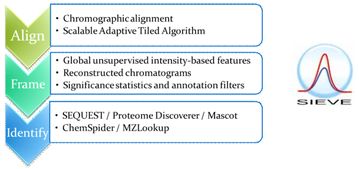


FIGURE 2. A disulfide folded peptide displayed in sequence (2) is shown in this view of SIEVE software where the red trace is from the digested fraction.

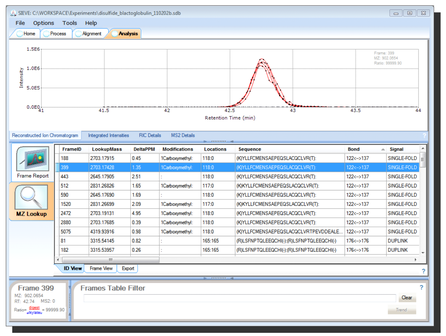
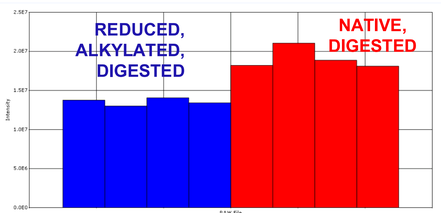
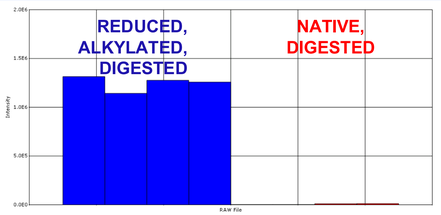


FIGURE 3. SIEVE results demonstrating characteristic intensity signal shapes of four technical replicate injections for A) Non-cysteine containing peptides, B) Carboxymethyl-modified cysteine containing peptides, and C) Unmodified peptides.

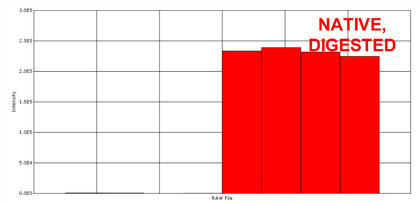
A. Non-cysteine containing peptides – Detection levels of non-cysteine containing peptides were expected to be similar. The peptide sequence shown here is (R)TPEVDDEALEK(F) and is located at position 139.



B. Reduced cysteine containing peptide – Peptides containing a reduced and alkylated cysteine will generally not appear in the sample digested under native (=non-reducing) conditions. The peptide shown here is (K)KYLLFCMENSAEPEQSLACQCLVR(T), located at position 117.

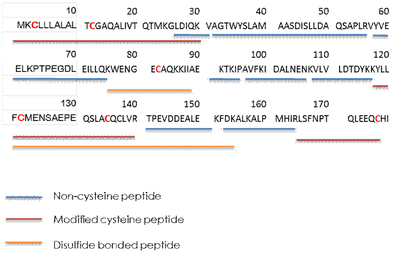


C. Native cystine containing peptides – a dimer of the peptide (R)LSNPTQLEEQCHI(-) located at position 165 was identified and can be attributed to a side reaction during sample preparation.

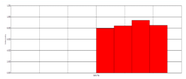


Results

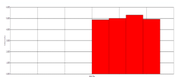
FIGURE 4. The protein coverage for alkylated cysteine containing peptides (red), non-cysteine containing peptides (blue), and disulfide bonded peptides (yellow) for beta lactoglobulin are shown below.



As is evident from the data, the method provided complete sequence coverage. The bond between cysteines 82? 122 (not previously reported in the literature) was confirmed . The bonds between 122? 137, 122? 135 and 135? 137 were generally confirmed but cannot be distinguished since this peptide was not cleaved between cysteines. The bond between 176? 82 was also confirmed.



(K)YLLFCMENSAEPEQSLACQCLVR(T)
Folded peptide



Bond between 82 ? 122

Conclusion

We have demonstrated a workflow for the discovery and verification of disulfide bonds using label-free LC-MS methods. The method worked well for peptides that were well separated by an enzymatic cleavage point; however, it was not possible to distinguish intra-molecular bonds in peptides bonds in peptides with three or more cysteines.

One caveat of the method was the observation of prominent signals for peptide dimers. This may be an indication of insufficient sample preparation chemistry. Although the samples were treated with additional DTT post reduction/alkylation, (in order to quench the alkylation reaction and return the sampled to a reduced state), free sulfhydryl containing cysteine peptides apparently remained.

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