Disulfide Mapping Using Accurate-Mass ETD and MS^n Techniques

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Overview

Purpose: Disulfide mapping with minimal sample preparation and data interpretation

Methods: Following limited proteolysis, beta lactoglobulin peptides were analyzed using electron transfer dissociation (ETD)-triggered MS^3 experimental paradigm and disulfide bonds were identified by multiple software platforms

Results: We have demonstrated a workflow for discovery of disulfide bonds using ETD triggered MS^n techniques.

Introduction

The biological function of proteins is a result of a number of factors including conformation, alternative splicing and a plethora of known and unknown modifications. Among the important contributors to tertiary and quaternary structure is disulfide linkages. Disulfide linkages influence not only the structural integrity of proteins but also their biological functions. Characterization of these linkages is important in many biological assay protocols. Protein structural characterization continues to present major challenges for the biological mass spectrometry community. Traditional collision induced dissociation (CID) is inefficient at cleaving disulfide bonds and produces difficult-to-interpret heterogeneous MS/MS ion populations. We present a complimentary approach leveraging multiple software platforms along with a novel ETD triggered MS^3 experimental paradigm.

Methods

Sample Preparation

Beta lactoglobulin was used as a model protein to study disulfide linkages in these experiments. The native protein was partially digested using trypsin for limited proteolysis. This method obviates the need for reduction/alkylation and extensive sample manipulation. The UniProt entry for beta lactoglobulin is shown below. The disulfide bonds from the UniProt entry were taken from two literature references.[1, 2]

UniProt ID P02754:

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        10         20         30         40         50         60
MKCLLLALAL TCGAQALIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE

        70         80         90        100        110        120
ELKPTPEGDL EILLQKWENG ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL

       130        140        150        160        170
FCMENSAEPE QSLACQCLVR TPEVDDEALE KFDKALKALP MHIRLSFNPT QLEEQCHI
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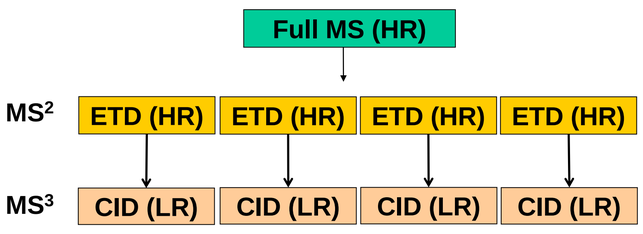
Disulfide bond	82 ↔ 176	Ref.13 Ref.19	
Disulfide bond	122 ↔ 137	Alternate	Ref.13
Disulfide bond	122 ↔ 135	Ref.13 Ref.19	

Data Acquisition

An aliquot of the digest was loaded onto a microcapillary analytical column. Peptides were gradient-eluted and analyzed using a hybrid ion trap- Orbitrap™ mass spectrometer for ETD triggered CID MS^3 experiment. In this method, the mass spectrometer was utilized to acquire high-resolution precursor ion spectra with top 4 high-resolution tandem mass spectra (MS/MS). Also, each MS/MS spectrum was followed by a CID MS^3 spectrum of the most intense ion from the corresponding MS/MS scan.

FIGURE 1. Schematic view of an ETD triggered MS3 experimental paradigm. HR represents high resolution data detected by the Orbitrap mass spectrometer while LR represents low resolution data acquired in the ion trap.



Data Analysis

For disulfide mapping, the data were first analyzed using Thermo Scientific Proteome Discoverer software version 1.3. A workflow was created to search the CID MS^3 spectra using SEQUEST, with the precursor mass for the spectrum chosen to be the MS2 fragment that triggered the MS3 event. Thermo Scientific ProSightPC software version 2.0 was subsequently used to confirm the peptides identified by the Proteome Discoverer™ software by searching the high resolution ETD MS/MS spectra using the “Delta M” error tolerant search mode with a precursor mass tolerance of 3000 Da and a fragment tolerance of 10 ppm.

Results

Unlike collision-induced dissociation (CID), ETD favors fragmentation of disulfide linkages.[3] For disulfide bound peptide, we expected that ETD fragmentation will favor dissociation of the two peptides at the disulfide bond over backbone cleavage, producing abundant fragments corresponding to the unlinked peptides. Figure 2 shows one such case, where the two major fragments in the MS/MS spectrum correspond to two tryptic fragments from beta-lactoglobulin that are known to be disulfide bound.

These peptides could then be isolated and fragmented using CID in the ion trap for subsequent identification by database search. The high mass measurement accuracy of the precursors (Figure 3) and the high sequence coverage of the CID MS^3 data lead to very confidently identified disulfide bound peptides.

FIGURE 2. ETD MS^2 spectrum of disulfide linked peptides. ETD dissociated the two peptides linked by a disulfide bond, producing intense fragments corresponding to the unlinked peptides. Masses corresponding to the dissociated chains were among the most abundant species since ETD favors fragmenting disulfide bonds.

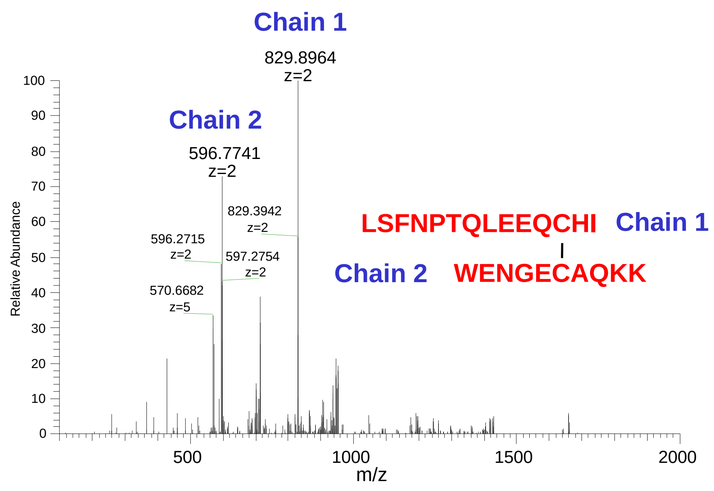
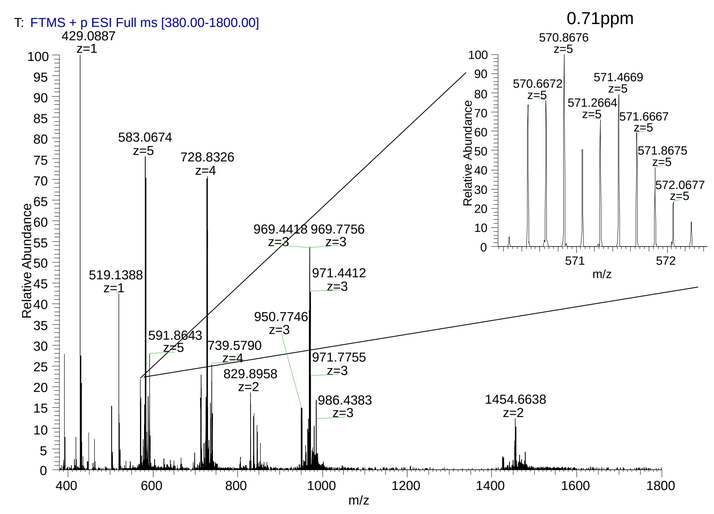


FIGURE 3. Detection of disulfide linked peptides LSFNPTQLEEQCHI and WENGECAQKK in full MS scan.



Disulfide Mapping Using Proteome Discoverer Software

When searching only the ETD MS/MS data by SEQUEST, no cysteine-containing peptides were identified as shown in the sequence map of beta-lactoglobulin shown in Figure 4.

FIGURE 4. Protein coverage by ETD MS^2 spectra. Green highlights represent the portions of the sequence where peptides were identified with high confidence.

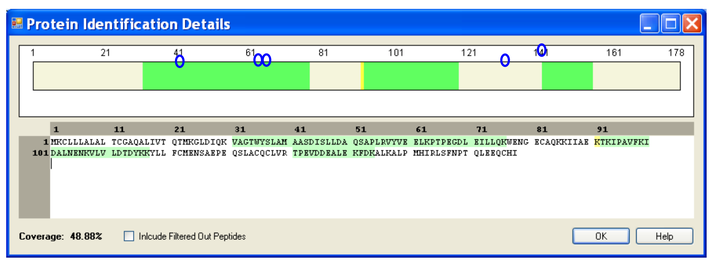


Figure 5 shows the sequence coverage when the CID MS3 are included, where an additional cysteine-containing peptide at the C-terminus was identified. Figure 6 shows the Proteome Discoverer software results highlighting the C-terminal peptide detected by MS^3.

FIGURE 5. Protein coverage by combined ETD MS/MS and CID MS3 spectra.

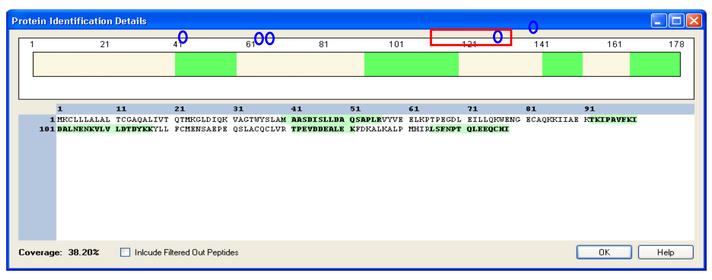
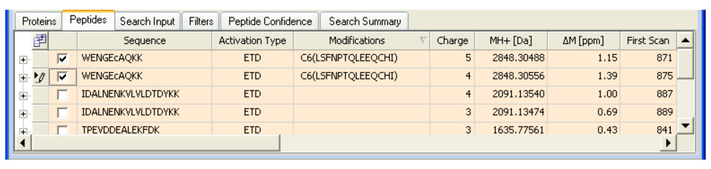


FIGURE 6. From CID MS^3, identification of the cysteine-containing peptide LSFNPTQLEEQCHI using SEQUEST in Proteome Discoverer software. The upper panel shows a list of peptides identified by CID MS^3 spectra including LSFNPTQLEEQCHI; bottom panel demonstrates a CID MS^3 spectrum for LSFNPTQLEEQCHI with fragment ions annotated.

A dynamic chemical modification for the mass of the peptide LSFNPTQLEEQCHI that included loss of two hydrogen [M-2H] was created in Proteome Discoverer software and the ETD MS/MS data was searched again using this modification with SEQUEST. As seen in Figure 7, the complimentary chain for the disulfide linked peptides was confidently identified as WENGECAQKK.

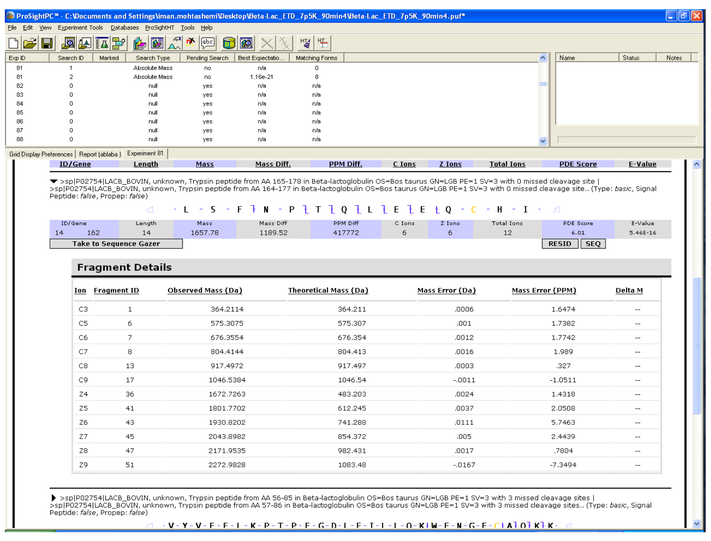
FIGURE 7. From ETD MS^2, WENGECAQKK was identified as the other chain of the disulfide linked peptides using LSFNPTQLEEQCHI with loss of two hydrogen [M-2H] as a dynamic chemical modification.



Disulfide Mapping Using ProSightPC Software

ProSightPC software does not have a “variable” modification search mode for unknown post translational modification identification. Instead, it can searches for any single modification of any mass using a “delta-M” mode. In delta-M mode, another set of fragments are matched to the sequence: b + “delta-M” and y + “delta-M”. The delta-M is calculated as the mass difference between the measured precursor and the matched sequence. In this case, to each chain of the disulfide linked peptides, mass of the other chain with loss of two hydrogen would be the calculated delta mass. In the example shown in Figure 8, the peptide LSFNPTQLEEQCHI was very confidently identified by ProSightPC software with an E-value of 5.46e-16 from the ETD MS/MS data with a delta mass calculated to be 1189.52, which is 2 Da smaller than the theoretical mass of WENGECAQKK. This further confirms the disulfide bond detected by the CID MS^3 method and Proteome Discoverer software.

FIGURE 8. Identification of peptide LSFNPTQLEEQCHI by ProSightPC software using the delta-M mode. E-value was 5.46e-16 and the delta mass was 1189.52 Da. This confirmed the disulfide bond detected by CID MS3 and Proteome Discoverer software.



Conclusion

Here we present a comprehensive approach leveraging multiple software platforms along with a novel ETD triggered MS^3 experimental paradigm.

- ETD fragmentation dissociated the two peptides linked by a disulfide bond, producing intense fragments corresponding to the unlinked peptides which could then be isolated and fragmented by CID for identification.
- Without knowledge a priori of the disulfide linkages, we could detect inter-peptide disulfide linkages using this method. In this case, disulfide linkage between Cys82 and Cys176 was identified unambiguously.
- Analysis of the data using Proteome Discoverer software allowed the unlinked peptide in the CID MS3 spectra to be identified and then to be used as a modification to identify the linked peptide in the ETD MS^2 spectrum.
- Analysis of the data using delta-M mode in ProSightPC software also allowed identification of the interpeptide disulfide linkage.

References

1. McKenzie HA, Ralston GB, Shaw DC. Location of sulfhydryl and disulfide groups in bovine -lactoglobulins and effects of urea. *Biochemistry*. **1972** Nov 21;11(24):4539-47.
2. Kuwata K, Hoshino M, Forge V, Era S, Batt CA, Goto Y. Solution structure and dynamics of bovine beta-lactoglobulin A.. *Protein Sci.* **1999** Nov;8(11):2541-5.
3. Zubarev RA, Kruger, NA, Fridriksson EK, Lewis MA, Horn DM, Carpenter BK, McLafferty FW. Electron capture dissociation of gaseous multiply-charged proteins is favored at disulfide bonds and other sites of high hydrogen atom affinity. *J Am. Chem. Soc.* **1999**; 121 (12):

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