

Identification of Disulfide-Linked Peptides by LC-MALDI Using Extracted Fragment Chromatograms

Protein Characterization using the AB SCIEX TOF/TOF™ 5800 System

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The intact three dimensional structure of proteins is essential for their biological function. Important to the stability of the tertiary structure are intra-molecular disulfide bonds. The structure of proteins is dynamic and important biological processes like protein-protein interactions or enzyme-substrate binding can lead to a change in the tertiary structure, which may result in the cleavage and reconnection of disulfide bridges. The identification and characterization of disulfide bridges is therefore a very important tool for the investigation of such processes. Here we present a way to identify and characterize disulfide bridges using an LC-MALDI workflow and extracting fragment ion chromatograms.

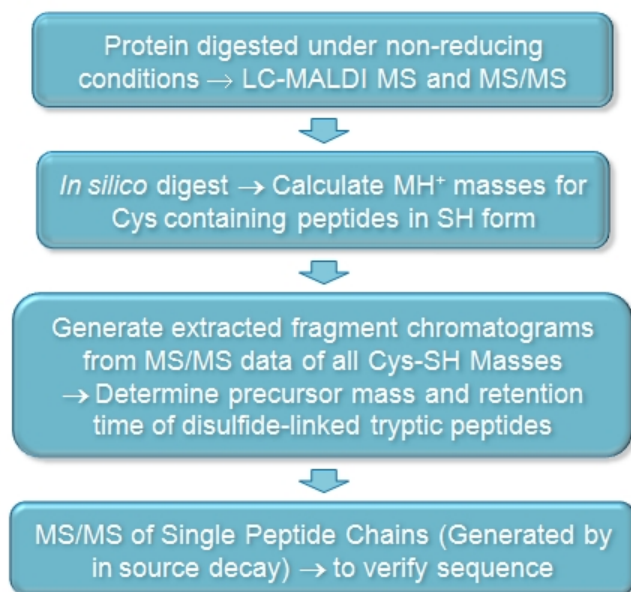


Figure 1. Workflow Summary. Disulfide-bridged proteins are digested under non-reducing conditions and run using the LC MALDI workflow on the TOF/TOF™ 5800 System. From the in silico digested protein, all the possible MH⁺ masses of Cysteine-containing peptides are calculated. By generating extracted fragment chromatograms from the MS/MS data for each peptide mass, the precursor mass and retention time of disulfide linked tryptic peptides present are determined. Individual Cys peptides are generated during in-source decay (ISD) from the disulfide-linked peptides and MS/MS on these ISD generated species can be done to verify the peptides involved in the disulfide bridge.

Key Features of TOF/TOF™ 5800 System for Disulfide Bridge Characterization

- Identification and characterization of disulfide-bridged peptides can be performed using the LC-MALDI workflow and the AB SCIEX TOF/TOF™ 5800 system.
- With the TOF/TOF™ optics and collision cell, the 5800 System is capable of performing both low-energy and high-energy MALDI CID MS/MS
- Generating many more useful fragments for the characterization of proteins and peptides
- Disulfide-linked peptides display a typical pattern of ion signals in MS/MS mode
- PeakExplorer™ Software enables screening for the disulfide bridged peptides using extracted fragment chromatograms (Figure 1).

Material and Methods

Sample Preparation: Standard proteins (Alpha-2-HS Glycoprotein, BSA, Beta-Lactoglobulin) were digested with trypsin (Promega, U.S.A.) under non-reducing conditions.

Chromatography: The protein digests were separated using an Acclaim PepMap 75 μm ID x 15 cm length column (Dionex) at a flow rate of 300 nL/min. The buffers being used were: Buffer A - 5% acetonitrile in water with 0.1% TFA and Buffer B - 80% acetonitrile in water with 0.085% TFA. Peptides were desalted for 5 min with 0.1% TFA / 2% acetonitrile on the precolumn, followed by a gradient from 5 to 50% solvent B in 30 min. Fractionation of the peptides was done with a Probot micro fraction collector (Dionex). *a*-Cyano-4-hydroxycinnamic acid (CHCA, 2 mg/mL in 70% acetonitrile) was continuously added to the column effluent via a μ -tee mixing piece at a flow rate of 900 nL/min. Fractions were collected for 20 seconds and spotted on a blank MALDI plates (AB SCIEX) using a 52 x 32 geometry.

Mass Spectrometry: MS and MS/MS analysis of offline separated peptides were performed using the AB SCIEX TOF/TOF™ 5800 system. After screening of all LC-MALDI sample positions in MS mode, the fragmentation of automatically selected precursors was performed at a collision energy of 1kV with collision gas air at a pressure of about 1×10^{-6} Torr.

Distinct Fragmentation Pattern for Detecting Disulfide Linked Peptides

MALDI high energy CID fragmentation of disulfide-linked peptides leads to a typical MS/MS pattern of ion signals¹, which are the result of Cysteine and Cysteine in different states of oxidation. Within the MS/MS spectrum of a selected precursor of two disulfide-linked peptides, at least one peptide chain can be detected that has the intact peptide mass with Cys in the SH form. The mass of peptide CDSSPDSAEDVR (m/z 1280) is observed in this case (Figure 2). The mass differences of the characteristic ion signal patterns observed correspond to the intact peptide mass of one of the Cys peptide components, and that mass with either -32 Da or -2 Da, or +32 Da for the singly charged peptide fragments (Figure 2, bottom).

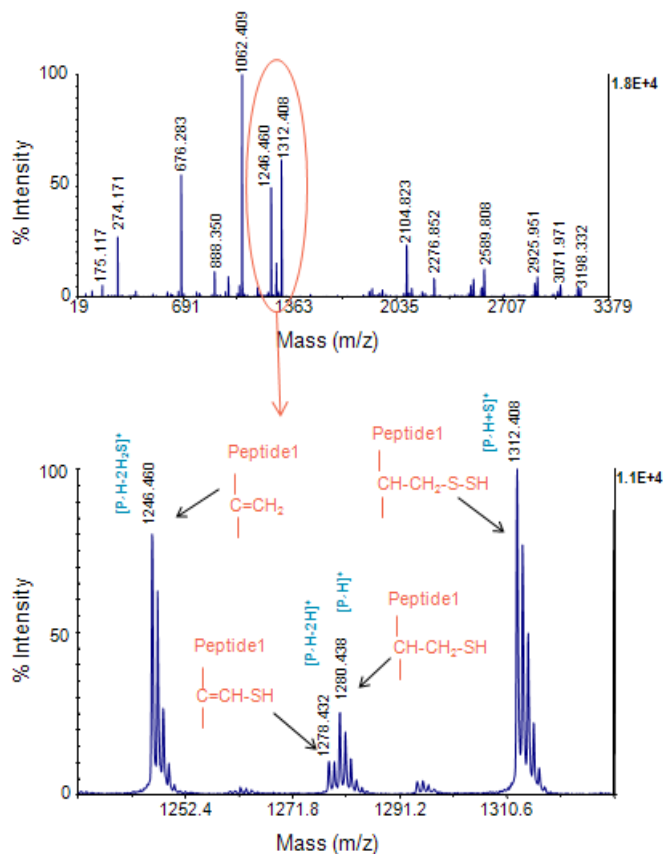


Figure 2. MS/MS Spectrum of the Disulfide-Linked Tryptic Peptides (Cys114-Cys132) from Alpha-2-HS-Glycoprotein. The top spectrum shows the full MS/MS spectrum of the peptide at m/z 3198.4 Da, which is Peptide P1 with amino acid sequence CDSSPDSAEDVR crosslinked to Peptide P2 with amino acid sequence QQTQHAVEGDCDIHVLK. The bottom pane is zoomed in to a more narrow mass range to show the typical fragmentation pattern observed in MS/MS spectra, which is one of the individual peptide chains intact and its different cysteine forms.

Screening MS/MS Data to Detect Disulfide Bridged Peptides

As an example of the workflow for the characterization of disulfide bridges, we show here a tryptic digest (non-reducing conditions) of Alpha-2-HS-Glycoprotein. An LC-MALDI analysis (MS and MS/MS mode) was performed resulting in an analysis of all peptides with highest possible sequence coverage. On the basis of the typical fragmentation pattern of disulfide linked peptides as outlined in Figure 2, the PeakExplorer™ (a software tool for the processing of LC-MALDI MS and MS/MS data) was used to generate extracted fragment chromatograms, using the MH⁺ ion signal of the Cys-containing peptide, as theoretically calculated with the Cys in SH-form. These extracted fragment chromatograms present an easy way to screen for the precursor mass of all cysteine containing peptides with intra – and intermolecular disulfide bridges (Figure 3).

After identification of the Cys-bridge containing peptides, the molecular mass of the second missing peptide chain can easily be calculated via the precursor mass. In order to verify the sequence of the second peptide chain or even to identify an unknown linked peptide, MS/MS data is necessary. Acquisition of the required MS/MS data is made possible by exploiting a characteristic property of disulfide-linked peptides, namely that they are unstable in MALDI. In every MS spectrum, acquired on a LC fraction, not only the MH⁺ peak of the disulfide-linked peptides could be detected but also the MH⁺ peaks of the single peptide chains in their SH-form that are produced by in-source decay. Comparison of the extracted ion chromatograms (Figure

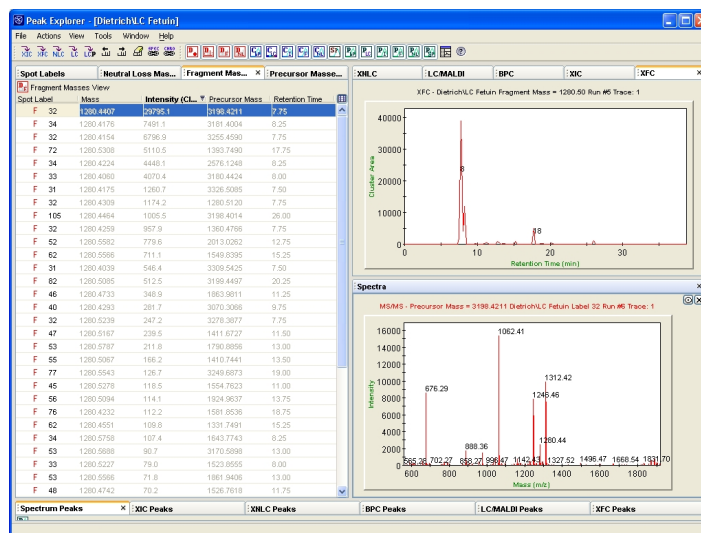


Figure 3. PeakExplorer™ Software for Screening MS and MS/MS Data. Here an extracted fragment chromatogram for m/z 1280.4 Da is shown, which corresponds to 132-143 with Cys132 in SH form from Alpha-2-HS-Glycoprotein. Precursor mass of the linked peptide is 3198.4 Da on LC spot position 32.

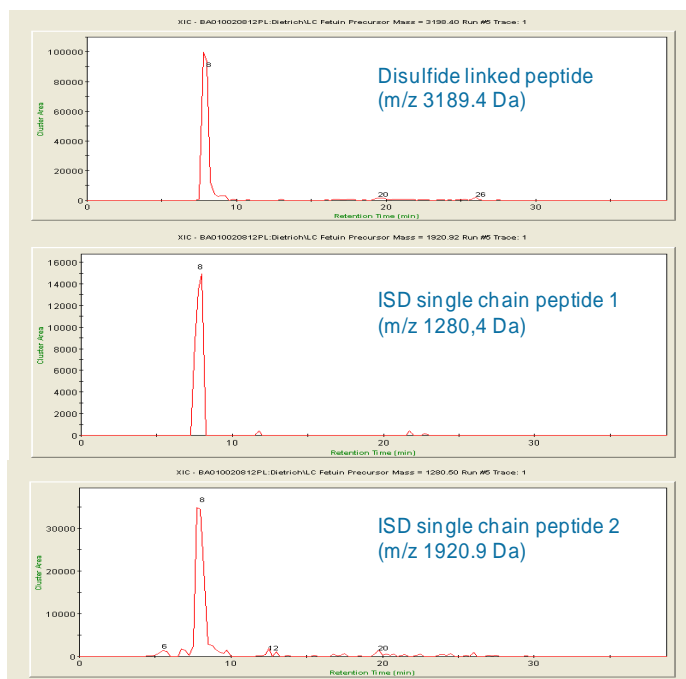


Figure 4. LC-MALDI Analysis and Detection of Disulfide Bridged Peptides by In Source Decay. The extracted ion chromatograms of the disulfide linked peptides (m/z 3189.4 Da) in comparison to the ones of the two single chain peptides (m/z 1280.4 Da and 1920.9 Da) showed a similar retention time. This is because as the disulfide bridged peptide ionizes, it also fragments to produce the single peptide chains, and these will appear as 'co-eluting' peaks.

4) for all three m/z values will appear as 'co-eluting' peaks, because the two single chains are generated during ionization of the disulfide bridged form.

MS/MS of the single chain peptides, generated by in-source decay was used to verify the peptide sequence (Figure 5) by a database search using Mascot search engine (MatrixScience) and MS/MS peak annotation in the Data Explorer® Software.

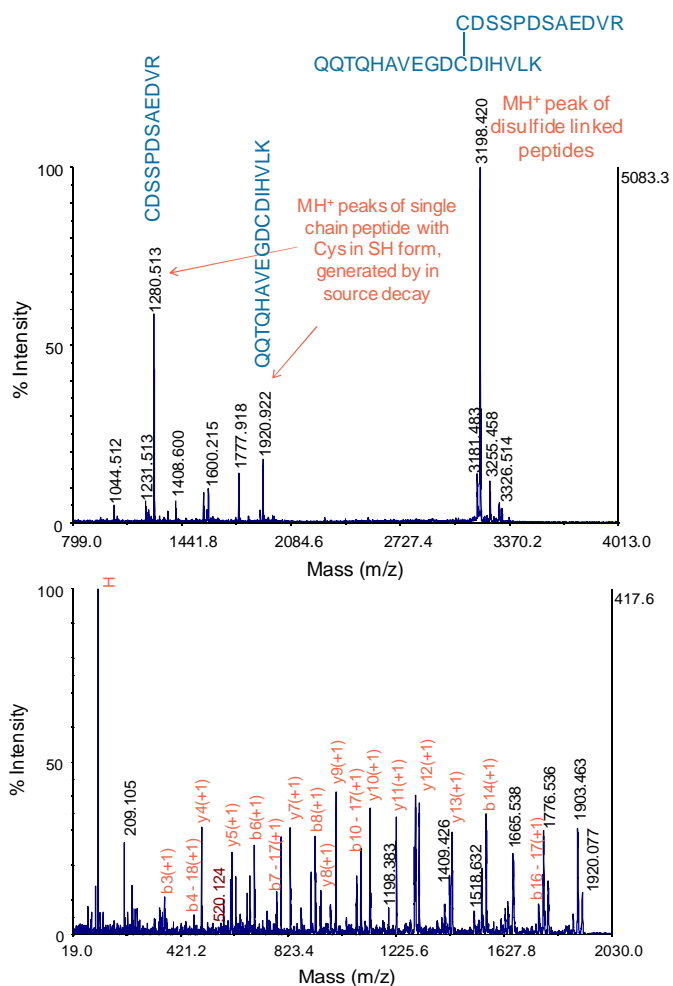


Figure 5: Characterizing LC MALDI Spot 32 from Alpha-2-HS Glycoprotein Tryptic Digest. (Top) The MS spectrum on LC MALDI spot 32 shows not only the MH⁺ peak of the disulfide linked peptides (m/z 3198.4 Da) but also the MH⁺ ion signal of the two single peptide chains P1 and P2 with cysteine in SH form, generated by in source decay (1280.5 Da and 1920.9 Da, respectively). (Bottom) Example of the MS/MS data of the peptide P2 with precursor mass of 1920.9 Da is shown including the annotation of the fragment ion signals from DataExplorer® Software.

Conclusions

The characterization of inter- and intramolecular disulfide bridges is an important tool to characterize changes in the tertiary structure of proteins or protein complexes. Here we present a workflow for characterization based on a LC-MALDI MS/MS analysis of a tryptic digests obtained under non-reducing conditions. Even though it would be possible to search for the theoretical masses of all possible disulfide linked peptides, the screening method shown here using extracted fragment chromatograms can detect the linked peptides in a much faster and more specific way. In addition, it offers the possibility to find completely unknown intra- but also intermolecular disulfide bridges.

Because disulfide linked peptides are unstable during MALDI ionization, partial cleavage of the disulfide linked peptides into their single chain peptides by in source decay can be detected. Due to this effect, a clear identification by direct MS/MS is possible.

References

1. Quinton L, Demeure K., Dobson R., Gilles N., Valérie Gabelica and De Pauw E. (2007) Journal of Proteome Research, 6, 3216-3223.

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