Enrichment Strategies for Improvement of Mass Spec Analysis of Chemical Cross-linked Peptides.

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ABSTRACT

Purpose: To evaluate multiple, widely used enrichment/fractionation techniques and benchmark newly developed strong cation exchange (SEC) spin columns for cross-linked peptide analysis using a Thermofisher Scientific™ Orbitrap Fusion Lumos™ TMT™ mass spectrometer.

Methods: Different anti-enzyme, homobifunctional crosslinkers were used to crosslink BSA (monomer) and yeast enolase (homo-dimer) proteins. Crosslinked samples were reduced, alkylated, trypsin digested with tryptic best before MS analysis. Cross-linked peptides were pre-fractionated on SCX stage tips, SCX spin columns or a peptide size exclusion chromatography (SEC) column. An Orbitrap Fusion Lumos mass spectrometer was used for crosslinked peptide analysis. Data analysis was performed with Thermo Scientific™ Proteome Discoverer® 2.2 software using a XlinkX™ software node.

Results: Newly designed spin columns containing a polymer-based strong cation exchange resin were used for selective enrichment of cross-linked peptides. Our simplified 2 fractions salt step gradient enabled identification a similar number of cross-linked peptides using an Orbitrap Fusion Lumos mass spectrometer.

MATERIALS AND METHODS

Sample Preparation

Disuccinimidyl-suberate (DSS), disuccinimidyl suberate (DSSD) and disuccinimidyl dihydroxide us (DSSU) (Table 1) were used to crosslink 2 nmol BSA and yeast enolase (Sigma Aldrich) stabilized in 30 mM Hepes pH 8 for 1 h at 100 molar excess of crosslinker to protein. After crosslinking reactions were quenched with 100 mM Tris pH 9. After BSA was reduced, alkylated, isotopically precipitated and digested with trypsin before MS analysis. Enolase was first desalted using an Amicon Ultra centrifugal filter (30 kDa, EM Diblo) and then digested with trypsin. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ QuantiFluor™ Quantitative Colorimetric Peptide Assay, respectively. Peptides were fractionated using a polymer-based SEC spin columns with an increasing step gradient of sodium chloride (e.g. 20 mM, 100 mM, 500 mM). Cross-linked peptides were also pre-fractionated on SCX stage tips (Thermo Fisher Scientific) according to manufacturing instructions and SEC Super™ Peptide PC column (GE Health) as described. SCX fractionated samples were desalted using a 70% ACN elution step on Pierce™ High pH Reversed-Phase Peptide spin column before LC MS/MS analysis.

Liquid Chromatography and Mass Spectrometry

Samples were separated by RP-HPLC using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system connected to a Thermo Scientific™ Q-Exactive™ mass spectrometer. MS/MS: 70 ps was at 4.0-4.5% (0.01 ppm) and 0.1% formic acid. B. subtilinke, 0.1% formic acid at 300 nL/min flow rate. The crosslinked BSA and enolase samples were analyzed on the Orbitrap Fusion Lumos mass spectrometer. Specific LC and MS settings are shown in Table 1.

Data Analysis

Spectral data files were analyzed using Proteome Discoverer 2.2 software with the XlinkX node (Figure 1) for crosslinked peptides and SEQUEST™ search engine for unmodified and dead-end modified peptides. Carbamidomethylation (+57.021 Da) was used as a static modification for cysteine. Different crosslinked mass modifications for lysine were used as variable modifications for lysine in the XlinkX standard-enumeration search option was used for data acquired using non-cleavable crosslinkers DSS and the MS2 methods (e.g. C0, E0, E0C0, E0C0C0, E0C0C0C0). Results visualization and distance analysis were performed using Xmem™ and PyMSASC™, 1.8 software (Schindelin LLC).

RESULTS

Figure 2. BSA crosslinked peptides enriched by Size Exclusion Chromatography (SEC). Separation profiles shows collected fractions, number of total and uniquely non-redundant crosslinked peptides identified for DSS (A), DSSD(B) and DSSU (C) crossliners as original sample methods as in Table 1. For each SEC experiments 100 µg of BSA tryptic digest was loaded in the sample column and 1/4 of each fraction was analyzed by LCMS. Most cross-linked peptides were identified in first fractions (e.g. 1 and 2) as expected. Depending upon the crosslinker used, we observed an increase in identification of non-redundant peptides from 10% to 30% after SEC fractionation and used these numbers to benchmark SEC tip column performance.

Figure 3. BSA crosslinked peptides enrichment by SCX spin column. (A) BSA enrichment workflow. (B) Non-redundant crosslinked peptides identified from original BSA digested and from mixture of protein BSA digested (no crosslinking) with BSA crosslinked sample in 100:1 ratio. No nitric isoptical 1:1 presence of crosslinked peptides. For each experiment, 1/4 of BSA digested was analyzed by LCMS using methods as shown in Table 1. Using newly developed SCX spin columns, we identified a similar number of cross-linked peptides in only 2 fractions (C) as by SEC (Figure 2).

Figure 4. Comparison of BSA DSSO cross-linking enrichment by SEC and SCX. A) Charge distribution peptides identified in 100 and 500 mM NaCl fractions showing higher charge of crosslinked peptides in higher salt fraction. B) Distribution of precursor molecular weight (MW) in SEC fractions 1 and 2 (Figure 2). B) showing higher MW of crosslinked peptides vs. standard proteolytic peptides. C) Lane diagram of SCX/SEC/Digested sample. As expected, there is significant overlap of identified crosslinked peptides after enrichment. (D) Lys contacts identified by SEC and SCX for DSSO crosslinking experiments mapped onto BSA homology structure with distance (C-Ca) less than 3.5Å. Visualization performed using PyMSASC™ 1.8 software.

Figure 5. DSS & DSSO crosslinking mapping of yeast enolase. Venn diagram (A) and (Table S) of combined SCX & SEC DSS/DSSO identified crosslinked peptides. Many crosslinked peptides as in case of BSA were identified using DSS O-crosslinking. Yeast enolase is a homodimer containing 2αM per subunit (C, D). Using distance contour analysis we were able to distinguish intra- from inter beta. Crosslinking was generated using ANTDIC™ and visualization was performed using PyMSASC™ 1.8 software. Lys Lys contacts identified by SEQUA SCX DSSO crosslinking experiments mapped onto enolase 1 homology structure with distance (C-Ca) less than 3.5Å.

CONCLUSIONS

- MS analysis of DSS and DSSO cross-linked peptides resulted in numbers of BSA and enolase cross-linked peptides comparable to those obtained from DSSO identification of non-crosslinked DSS and crosslinked DSBU cross-linked peptides. The differences in identification is more likely due to differences in MS2 ionization efficiency.
- Cross-linked cross-links are more advantageous than noncovalent cross-links for diluted and complex samples due to improved FDR calculations based on MS1 integration and/or the presence of reporter ions for MS2 crosslinkers.
- A simple 2 step salt gradient SCX fractionation enriched cross-linked peptides similar to SEC fractionation for BSA and enolase.
- Proteome Discoverer 2.2 software with XlinkX was used to confidently identify crosslinked peptides for PyMSASC structure overlay.

REFERENCES


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TRADEMARKS/LICENSING

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