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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 (SCX) spin columns for cross-linked peptide analysis using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer.

Methods: Different amine-reactive, homobifuctional crosslinkers were used to crosslink BSA (monomer) and yeast enolase (homodimer) proteins. Crosslinked samples were reduced, alkylated and digested with trypsin 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[1] 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 as SEC based fractionation.

INTRODUCTION

Chemical cross-linking in combination with mass spectrometry is a powerful method to determine protein-protein interactions. This method has been applied to recombinant and native protein complexes and, more recently, to whole cell lysates or intact unicellular organisms in efforts to identify protein-protein interactions on a global scale. However, this method suffers from low identification rates without enrichment/fractionation, as the typical yield of cross-linked peptides is less than 1 % of total identified peptides. In this study, we evaluated multiple, widely used enrichment/fractionation techniques and benchmarked newly developed SCX spin columns for cross-linked peptide analysis using an Orbitrap Fusion Lumos mass spectrometer.

MATERIALS AND METHODS

Sample Preparation

Disuccinimidyl suberate (DSS), disuccinimidyl sulfoxide (DSSO)[2] and disuccinimidyl dibutyric urea (DSBU)[3] (Table 1) were used to crosslink 2mg/ml BSA and yeast enolase (Sigma Aldrich) solubilized in 50mM HEPES pH 8 for 1hr at 100 molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 1M Tris pH 8. BSA was reduced, alkylated, acetone precipitated and digested with trypsin before MS analysis. Enolase was first desalted using an Amicon® centrifugal filter unit (30 kDa, EMD Millipore) and then digested with trypsin. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ Quantitative Colorimetric Peptide Assay, respectively. Peptides were fractionated using a polymer-based SCX spin columns with an increasing step gradient of sodium chloride (e.g. 20mM, 100mM, 500mM). Cross-linked peptides were also pre-fractionated on SCX stage tips (Thermo Fisher Scientific) according to manufacturing instructions and SEC Superdex™ Peptide PC column (GE Health) as described.[4] SCX fractionated samples were desalted using a 70% ACN elution step on Pierce™ High pH Reversed-Phase Peptide spin columns before LC-MS/MS analysis.

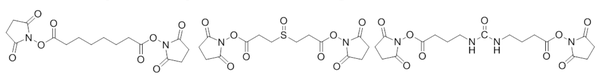
Liquid Chromatography and Mass Spectrometry

Samples were separated by RP-HPLC using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system connected to a Thermo Scientific™ EASY-Spray™ column, 50 cm × 75 µm over a 45 min 2-28% gradient (A: water, 0.1% formic acid; B: acetonitrile, 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 using the XlinkX node(Figure 1) for crosslinked peptides and SEQUEST®HT search engine for unmodified and dead-end-modified peptides. Carbamidomethylation (+57.021 Da) used as a static modification for cysteine. Different crosslinked mass modifications for lysine were used as variable modifications for lysine in addition to methionine oxidation (+15.996 Da). Data were searched against a database contained the Uniprot/SwissProt entries of the model proteins with/out common contaminants with a 1% FDR criteria for protein spectral matches. For MS2-MS3 methods, a linear–peptide search option (using MS3 scans for identification and MS2 scan for detection of crosslinked peptides) was used for XlinkX database searching. The XlinkX standard enumeration search option was used for data acquired using non-cleavable crosslinker DSS and the MS2 methods (e.g. CID, ETD, EThcD).[1] Results visualization and distance restraints were performed using *xiNET*[5] and PyMOL 1.8 software (Schrodinger LLC).

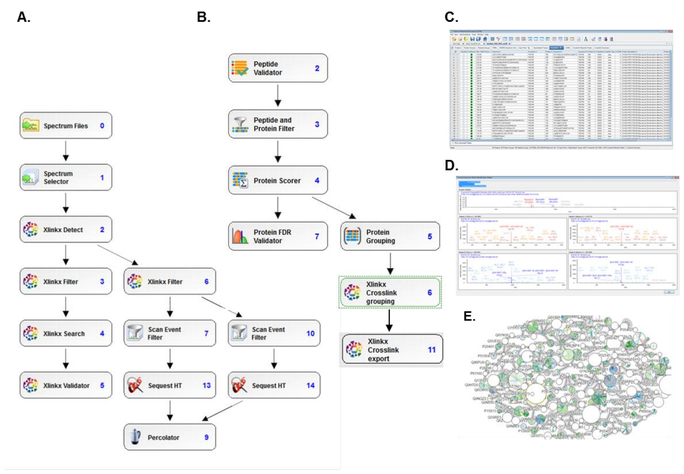
Table1. Orbitrap Fusion Lumos MS crosslinker specific LC-MS acquisition parameter settings.



DSS Spacer Arm 11.4Å | DSSO Spacer Arm 10.3Å | DSBU Spacer Arm 12.5Å

Settings	DSSO MS2 –MS3	DSS MS2	DSBU MS2
LC gradient	2-28% in 45min	2-28% in 45min	2-28% in 45min
MS 1	OT	OT	OT
Resolution	120K	120K	120K
Target value	4e5	4e5	4e5
Max injection time	100	100	100
Top Speed	5 sec	5 sec	5 sec
MS²	OT CID	OT EThcD	OT HCD
Charge states	3-8	3-8	3-8
Isolation width	1.6	1.6	1.6
NCE	25	SA15	SCE 30±5%
Resolution	30K	60K	30K
Target value	5e4	2e5	5e4
Max injection time	100ms	250ms	70ms
Targeted Mass MS³	IT HCD	n/a	n/a
Isolation width	2		
NCE	30		
Resolution	Rapid		
Target value	2e4		
Max injection time	150 ms		

Figure 1. The processing (A) and consensus (B) XlinkX workflows in Proteome Discoverer 2.2 software includes a separate crosslinkers results tab (C), spectra annotation (D) and *xiNET* data visualization (E) using csv and database files generated by Xlink Crosslink export node



RESULTS

Figure 2. BSA crosslinked peptides enriched by Size Exclusion Chromatography (SEC). Separation profiles shows collected fractions, number of total and unique non-redundant crosslinked peptides identified for DSS (A), DSSO(B) and DSBU(C) crosslinkers vs original sample using instrument methods as in Table 1. For each SEC experiment 100 µg of BSA tryptic digest was loaded on the column and 1 µg of each fraction was analyzed by LC/MS. Most cross-linked peptides were identified in first fractions (e.g. 0 and 1) as expected.[4] 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 SCX spin column performance.

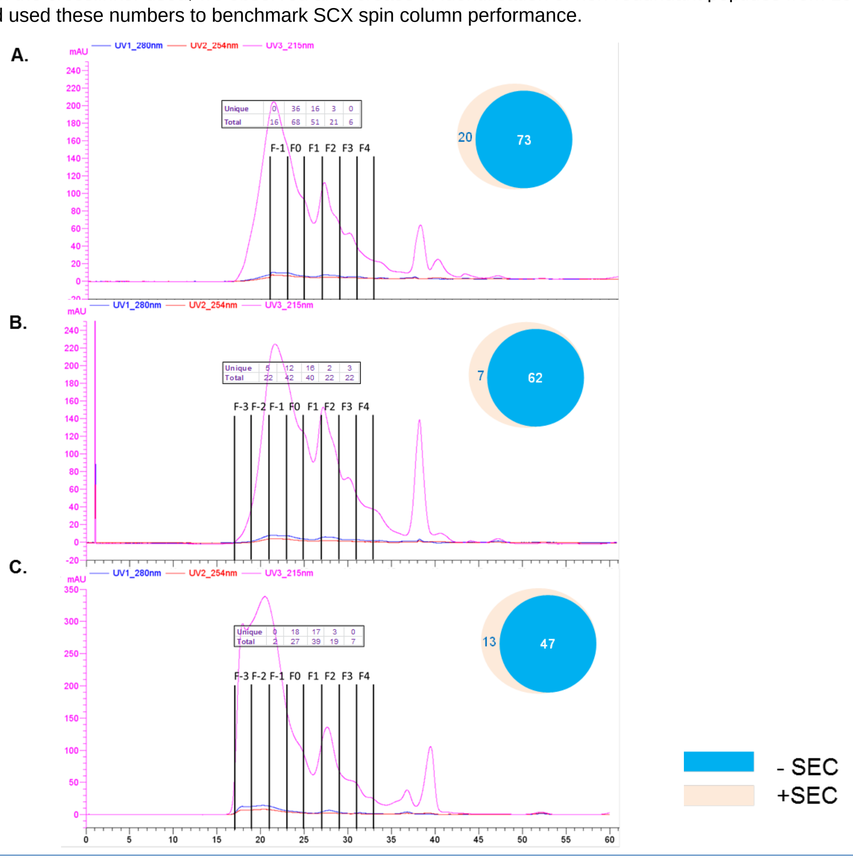


Figure 3. BSA crosslinked peptides enrichment by SCX spin columns. A) SCX enrichment workflow. B) Non-redundant crosslinked peptides identified from original BSA digest or from mixture of control BSA digest (no crosslink) with BSA crosslinked sample in 100: 1 ratio to mimic typical 1% presence of crosslinked peptides. For each experiment, 1 µg of BSA digest was analyzed by LC/MS using methods as shown in Table 1. Using newly developed SCX spin columns, we identified a similar number of crosslinked peptides in only 2 fractions (C) as by SEC (Figure 2).

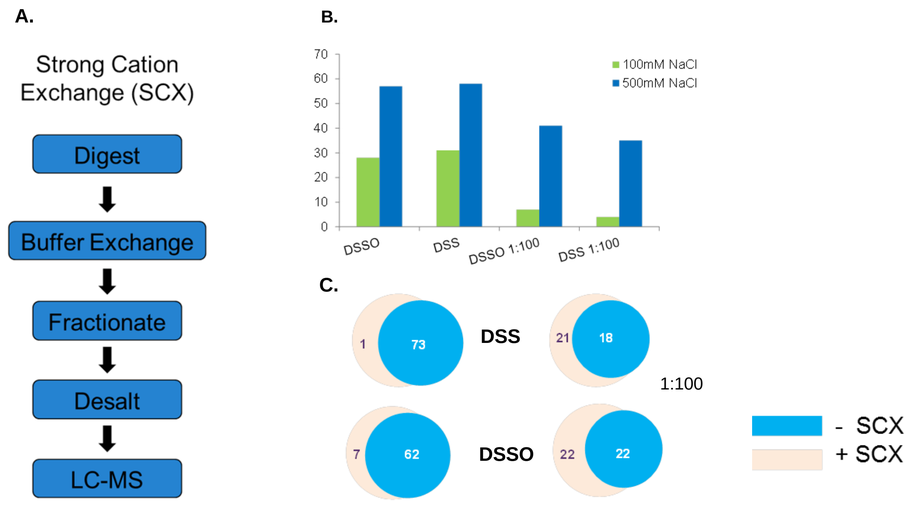


Figure 4. Comparison of BSA DSSO cross-linking enrichment by SEC and SCX. A) Charge distribution peptides identified in 100 mM and 500 mM NaCl fractions showing higher charge of crosslinked peptides in higher salt fraction. B) Distribution of precursor molecular weight (MW) in SEC fractions 0 and 1(Figure 2, B) showing higher MW of crosslinked peptides vs. standard proteolytic peptides. C) Venn diagram of SEC/SCX/Original sample. As expected, there is significant overlap of identified crosslinked peptides after enrichment. D) Lys-Lys contacts identified by SEC and SCX for DSSO crosslinking experiments mapped onto BSA homology structure with distances (Cα-Cα) less than 35Å. Visualization performed using PyMol 1.8 software.

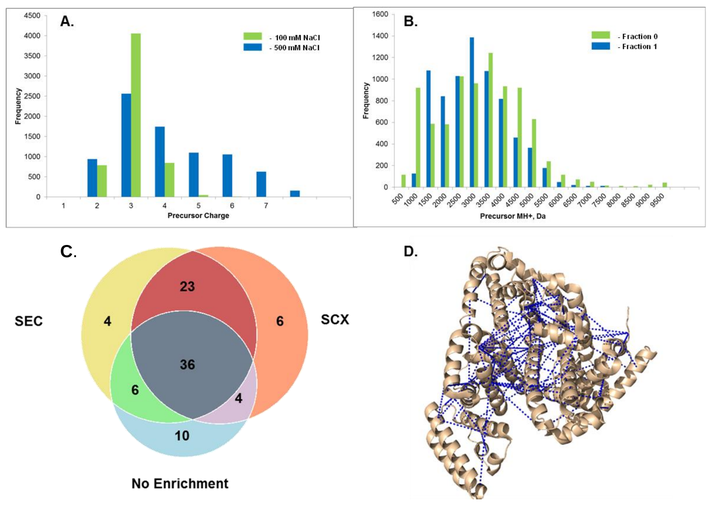
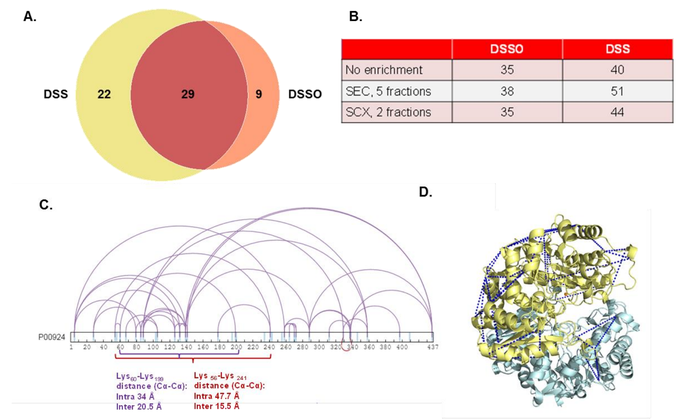


Figure 5. DSS & DSSO crosslink mapping of yeast enolase. Venn diagram (A) and Table (B) of combined SCX & SEC DSS/DSSO identified crosslinked peptides. More crosslinked peptides as in case of BSA were identified using DSS crosslinker. Yeast enolase is a homodimer containing +2Mg^{2+} per subunit (C, D). Using distance constrain analysis we were able to distinguish intra from inter links. Crosslinking map was generated using *xiNET*(C) and visualization was performed using PyMol 1.8(D) software. Lys-Lys contacts identified by SEC& SCX DSSO crosslinking experiments mapped onto enolase 1 homology structure with distances (Cα-Cα) less than 35Å.



	DSSO	DSS
No enrichment	35	40
SEC, 5 fractions	38	51
SCX, 2 fractions	35	44

CONCLUSIONS

- MS^n analysis of DSSO cross-linked peptides resulted in numbers of BSA and enolase cross-linked peptides comparable to those obtained from MS^2 identification of non-cleavable DSS and cleavable DSBU cross-linked peptides. The differences in identification is more likely due to differences in MS acquisition, data analysis, crosslinker length and/or solubility.
- Cleavable cross-linkers are more advantageous than noncleavable cross-linkers for diluted and complex samples due to improved FDR calculations based on MS^n integration and/or the presence of reporter ions for MS cleavable crosslinkers.
- A simple 2 step salt gradient SCX fractionation enriched crosslinked peptide similar to SEC fractionation for BSA and enolase.
- Proteome Discoverer 2.2 software with XlinkX was used to confidently identify crosslinked peptides for PyMol structure overlay.

REFERENCES

1. Liu F, Rijkers D, Post H, Heck AJ. Proteome-wide profiling of protein assemblies by cross-linking mass spectrometry. 2015. *Nat Methods*, **12**(12):1179-84.
2. Kao A, Chiu CL, Vellucci D, Yang Y, Patel VR, Guan S, Randall A, Baldi P, Rychnovsky SD, Huang L. Development of a novel cross-linking strategy for fast and accurate identification of cross-linked peptides of protein complexes. 2011. *Mol Cell Proteomics,* **10**(1): M110.002212.
3. Müller MQ, Dreiocker F, Ihling CH, Schäfer M, Sinz A. Cleavable cross-linker for protein structure analysis: reliable identification of cross-linking products by tandem MS. 2010. *Anal Chem.* **82**(16):6958-68.
4. Leitner A, Reischl R, Leitner A, Reischl R, Walzthoeni T, Herzog F, Bohn S, Förster F, Aebersold R. Expanding the chemical cross-linking toolbox by the use of multiple proteases and enrichment by size exclusion chromatography. 2012. *Mol Cell Proteomics,* **11**(3):1-12.
5. Combe CW, Fischer L, Rappsilber J. xiNET: cross-link network maps with residue resolution. 2015 *Mol Cell Proteomics*, **14**(4):1137-47.

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TRADEMARKS/LICENSING

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