Optimization of Crosslinked Peptide Analysis on an Orbitrap Fusion Lumos Mass Spectrometer

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Poster Note 64763

ABSTRACT

Purpose: To improve identification of intra- and inter-protein interactions though analysis of chemically crosslinked peptides.

Methods: Different amine-reactive, homobifuctional crosslinkers including disuccinimidyl suberate (DSS), bis-sulfosuccinimidyl suberate (BS3), disuccinimidyl sulfoxide (DSSO)[1] and disuccinimidyl dibutyric urea (i.e. NHS-BuUrBu-NHS)[2] (Figure 1) were compared for protein crosslinking labeling efficiency and crosslinked peptide identification using MS^2 and MS^3 fragmentation methods. A Thermo Scientific™ Orbitrap™ Fusion Lumos™ Tribrid™ mass spectrometer was used for crosslinked peptides analysis. Data analysis was performed by Thermo Scientific™ Proteome Discoverer™ using a XlinkX[3] software node.

Results: For both DSSO and BuUrBu, we identified over 40 BSA inter-crosslinked peptides using MS^2-MS^3 approach compared to less than 20 using MS^2 CID for DSSO. We also compared these crosslinkers using an *E. coli* whole cell lysate. Our results show an increase number of identified peptides after crosslinking using the MS^2-MS^3 in combination with EThcD method compared to CID/EThcD MS^2 method.

INTRODUCTION

Chemical crosslinking 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. In this study, we evaluated traditional non-cleavable and MS-cleavable crosslinkers for crosslinked peptide analysis using an Orbitrap Fusion Lumos mass spectrometer. For MS-cleavable crosslinkers, we also compared different types of fragmentation (CID, ETD) and levels of tandem mass spectrometry (MS^2 vs. MS^3). Our data provided insight to the relative performance of different crosslinking compounds and acquisition parameters relevant for improving identification of protein-protein interaction sites.

MATERIALS AND METHODS

Sample Preparation

BS3, DSS, DSSO and BuUrBu were used to crosslink 2mg/ml BSA solublized in 50mM HEPES pH 8 for 1hr at various molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 1M Tris pH 8 and analyzed by SDS-PAGE or reduced, alkylated and digested with trypsin for MS analysis. Protein and peptide concentrations were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit and the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay, respectively. *E. coli* lysates were crosslinked using 20-fold molar excess of crosslinker to protein before reduction, alkylation and digestion. Peptides were fractionated using a Thermo Scientific™ HyperSep™ Retain CX column (30mg) with an increasing step gradient of ammonium acetate (e.g. 50mM, 150mM, 250m M, 250mM, 500mM, 1M). Fractionated samples were desalted using C18 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 x 75 µm over a 1 hr. 4-40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The crosslinked BSA and *E. Coli* cell lysates samples were analyzed on the Orbitrap Fusion Lumos and Thermo Scientific™ Q Exactive™ HF mass spectrometers. Additional LC and MS settings are shown in Table 1.

Data Analysis

Spectral data files were analyzed by XlinkX 2.0 or Thermo Scientific™ Proteome Discoverer™ 2.2 software using the XlinkX node 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 was searched against a Swiss-Prot® *E.coli* or BSA databases with a 1% FDR criteria for protein spectral matches. For MS^2-MS^3 methods, a linear–peptide search option (using MS^3 scans for identification and MS^2 scan for detection of crosslinked peptides) was used for XlinkX database searching. The XlinkX standard enumeration search option was used for data acquired using the MS^2 methods (e.g. CID, ETD, EThcD).[3]

Figure 1. Structures of non-cleavable and MS-cleavable crosslinkers used for protein – protein interaction analysis.

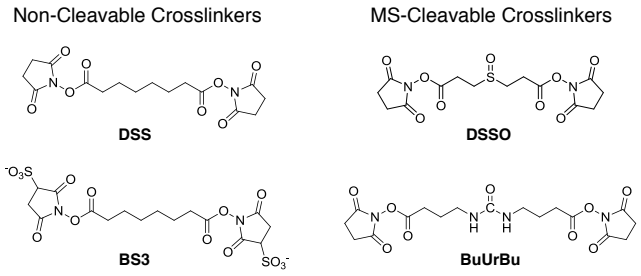

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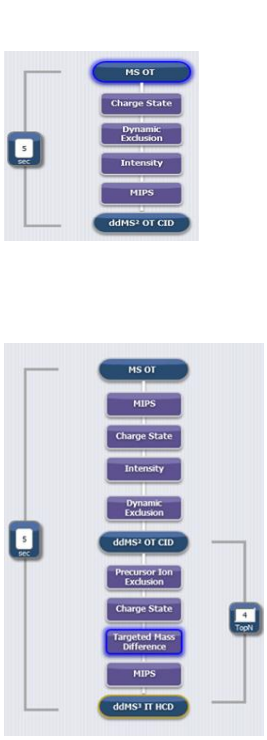
Table 1. LC-MS acquisition and database search parameter settings.

	Orbitrap Fusion Lumos MS Parameters	Q Exactive HF MS Parameters
LC gradient	6-40% in 45min	4-40% in 65min
Full MS	OT	OT
Resolution	120K	120K
Target value	2e5	3e6
Max injection time	100	50
Top N	5 sec	15
MS^2	OT CID	OT HCD
Isolation mode	Quadruple	Quadruple
Isolation width	1.6	1.4
NCE	25	30, SID 15-25
Resolution	30K	15K
Target value	5e4	1e5
Max injection time	100ms	100ms
MS^3 SPS	IT HCD	
Isolation width	2	
NCE	30	
Resolution	Rapid	
Target value	2e4	
Max injection time	120 ms	
Search parameters	XlinkX, SequestHT	XlinkX, SequestHT
Precursor tolerance	10ppm	10ppm
Fragment tolerance	0.02Da	0.02Da
Static	Carbamido-methyl (C)	Carbamido-methyl (C)
Dynamic	Oxidation (M) DSS, DSSO or BuUrBu(K)	Oxidation (M) DSS, DSSO or BuUrBu (K)

Figure 2. MS acquisition used for MS^2 (A or MS^2-MS^3 (B) fragmentation methods.



RESULTS

Figure 3. Comparison of BSA crosslinking efficiency by SDS-PAGE. Different crosslinkers were incubated with BSA at molar excess of crosslinker to protein (e.g. 20, 100 or 500-fold). Crosslinking efficiency is shown by decreased mobility by SDS-PAGE and varied by crosslinker type, solubility and concentration.

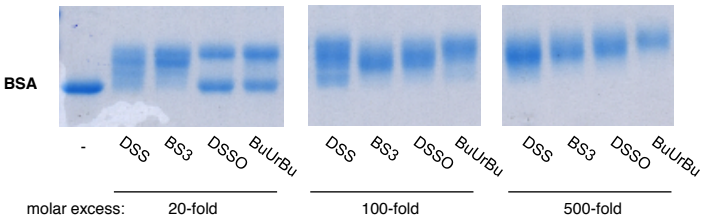


Figure 4. BSA crosslinked peptide spectra identified by MS^2-MS^3 method and XLinkX using DSSO (A) or BuUrBu (B) crosslinkers. XlinkX uses unique fragment ion patterns of MS-cleavable crosslinkers (purple annotation) to detect and filter crosslinked peptides for a crosslinked database search.

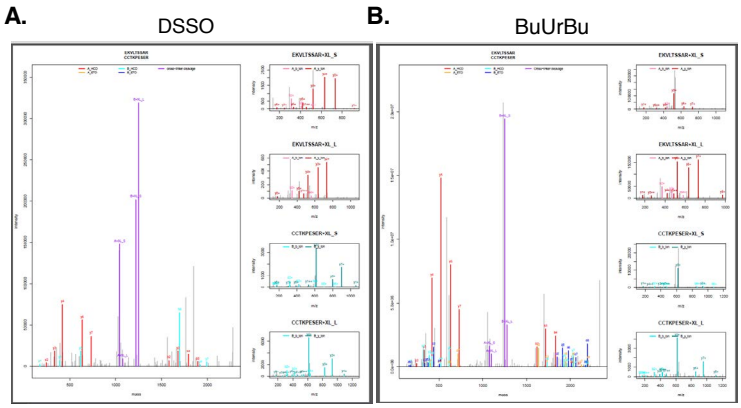


Figure 5. Graph showing number of BSA crosslinked peptides identified using different non-cleavable (BS3,DSS) and cleavable crosslinkers (DSSO, BuUrBu) for various MS^n methods. Both BS3 and DSS had similar numbers of crosslinked peptides identified for CID and EThcD methods. BuUrBu had more crosslinked peptides identified by CID and SID-HCD on a Q Exactive HF MS. Although DSSO, had the fewest crosslinked peptides identified by CID and HCD, it had the most for the MS^2-MS^3 method if the linear-peptide search mode[3] is used . All crosslinkers showed similar numbers of identified crosslinked peptides by EThcD.

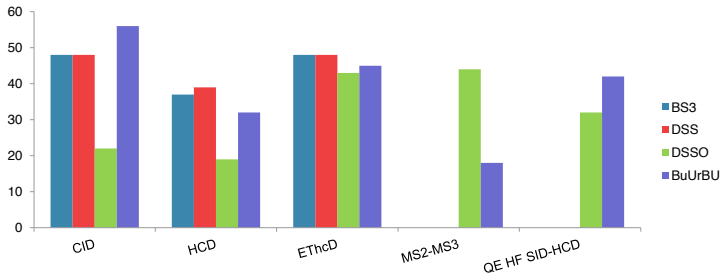


Figure 6. The processing (A) and consensus (B) XlinkX workflows in Proteome Discoverer 2.2 software including a separate crosslinkers results tab (C) and spectra annotation (D).

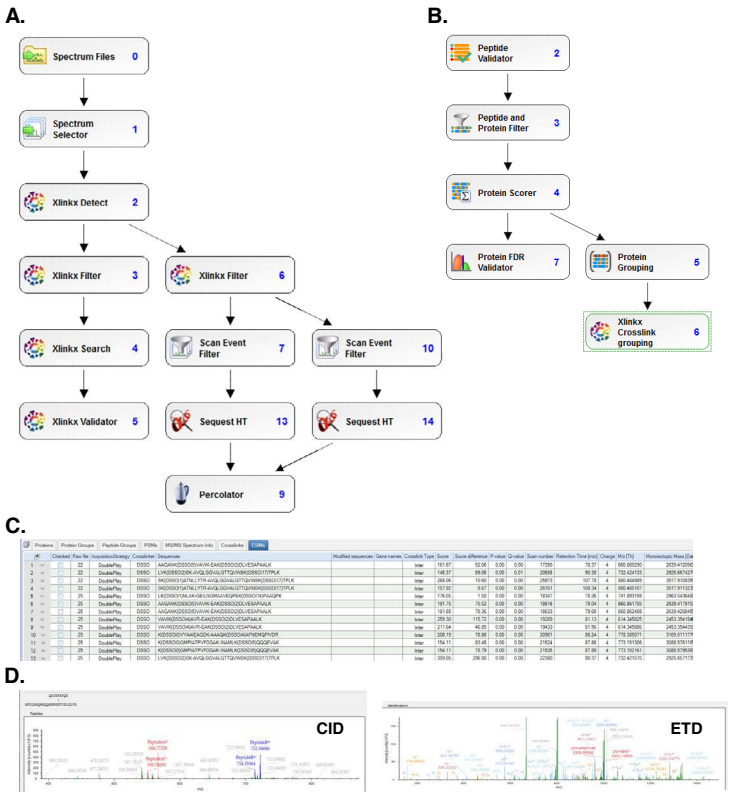


Figure 7. Comparison of different EThcD energies for crosslinked peptide fragmentation. Increasing EThcD fragmentation energy results in different fragment ion intensity in MS/MS spectra (A) and unique identified crosslinked peptides (B).

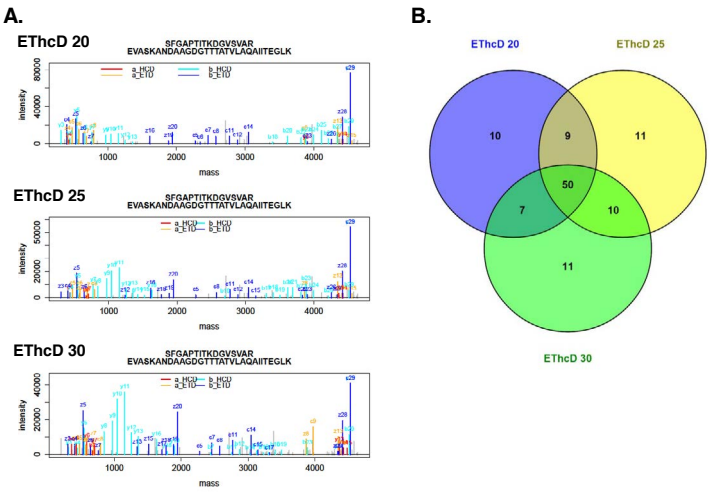
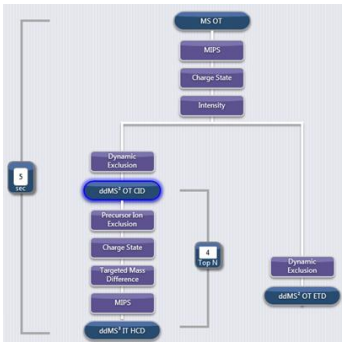
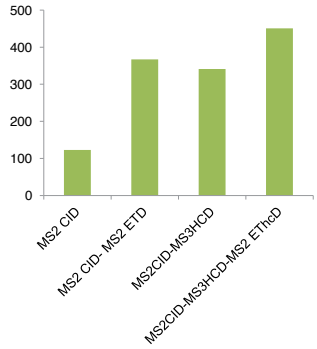


Figure 8. ***E.coli*** **cell lysate crosslinked peptides identified using different instrument methods and XlinkX software. MS²-MS³ method combined with MS² EThcD (A) provided most identifications (B) compared to MS² CID, MS² CID-MS3 ETD, or MS² CID-MS³ HCD methods.**

A.



B.



CONCLUSIONS

- MS-cleavable crosslinkers, DSSO and BuUrBu, crosslink BSA with slightly lower efficiency than non-cleavable crosslinkers, DSS and BS3, possibly due to small differences in crosslinker length or solubility.
- DSS, BS3 and BuUrBu worked well for CID, HCD and EThcD MS² fragmentation methods. However, DSSO resulted in the most identified BSA crosslinked peptides using the a combination of MS², MS³ spectral sequence information and XlinX.
- Different EThcD energies not only changed MS/MS fragment ion intensities but also resulted in different identified crosslinked peptides.
- For more complex *E. coli* crosslinked samples, using the MS²-MS³ in combination with EThcD method resulted the most identified crosslinked peptides compared to other methods.

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ACKNOWLEDGEMENTS

The authors would like to thank Kai Fritzemeier (Thermo Fisher Scientific, Germany) and Richard Scheltema (University of Utrecht) for their work on integrating XLinkX software as a node in Proteome Discoverer 2.2.