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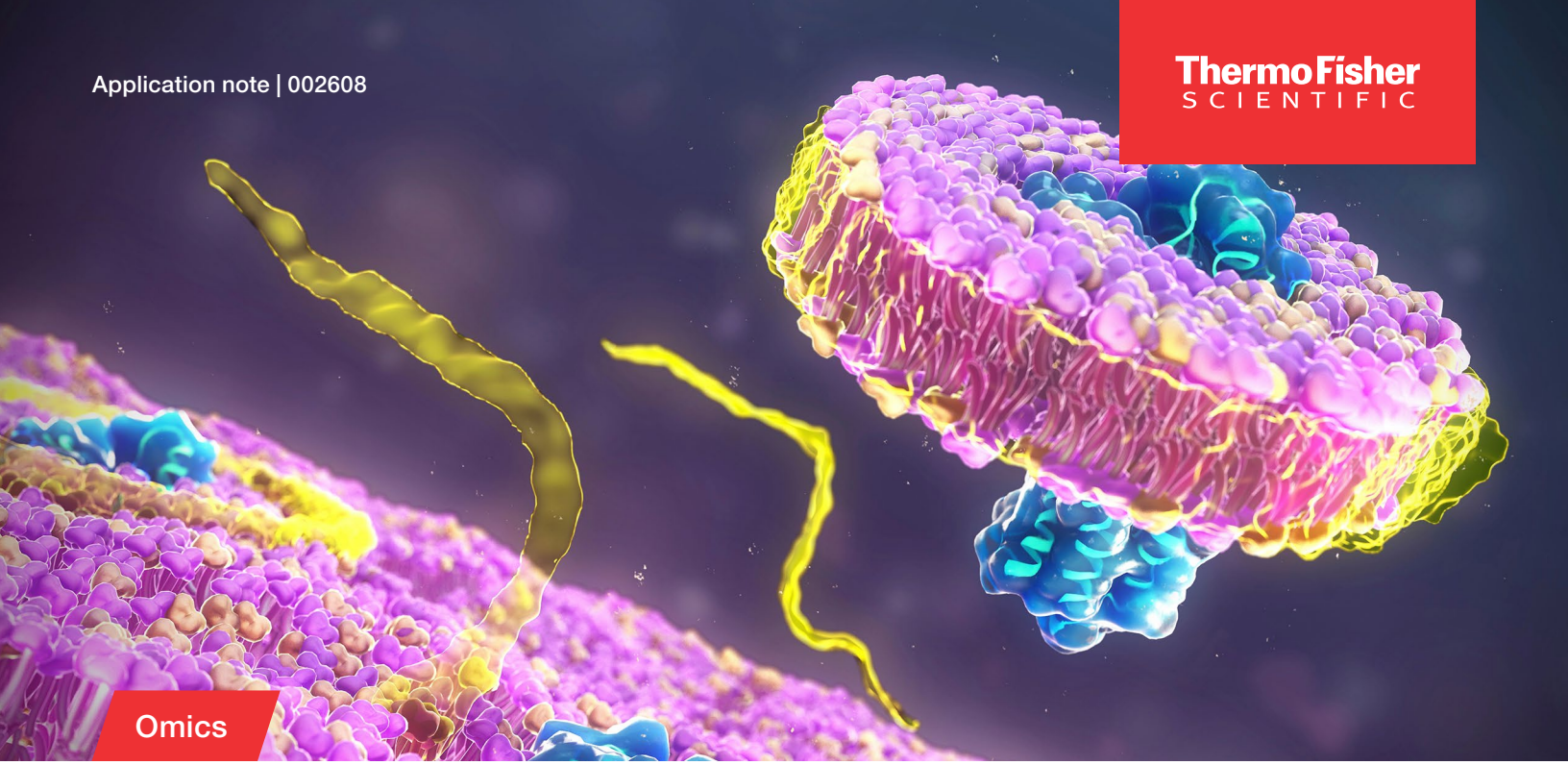

Omics

Crosslinking mass spectrometry analysis of membrane proteins in SMALPs

Authors

Yi He[1], Greg J. Dodge[2], Barbara Imperiali[2], Rosa Viner[1]

[1]Thermo Fisher Scientific, San Jose, CA, USA

[2]Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA

Keywords

Crosslinking mass spectrometry, XL-MS, membrane protein, SMALP, DSS, tBu-PhoX (TBDSPP), IMAC-enrichable, Proteome Discoverer software, XlinkX node, Orbitrap Eclipse mass spectrometer

Goal

Develop an end-to-end XL-MS workflow for membrane proteins in membrane mimetics

Introduction

Membrane proteins play an essential role in several biological processes like ion transport, signal transduction, and electron transfer. Understanding their three-dimensional structure is of great interest to scientists in both academia and industry. Many techniques have been used to elucidate the structure of membrane proteins, including X-ray crystallography, cryogenic electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) spectroscopy, but they all have their disadvantages and limitations. Crosslinking mass spectrometry (XL-MS) is a rapid, high-resolution structural technique that has grown dramatically, becoming a key method for characterizing protein higher-order structure and mapping protein-protein interaction networks. However, the XL-MS analysis of membrane proteins, especially in membrane mimetics, remains a significant challenge. This is largely due to obstacles associated with their extraction process and instability outside of their native lipid environment. Membrane proteins are usually extracted or solubilized in detergents, nanodiscs, or styrene-maleic acid lipid particles (SMALPs), which are not compatible with downstream MS analysis. Several issues arise, including incomplete solubility and restricted enzyme accessibility, limiting the amount of information obtained from XL-MS analysis. Currently, there is no standardized XL-MS workflow for membrane proteins.

Another significant limitation of XL-MS is the poor identification rates of crosslinked peptides due to their relatively low abundance, which impedes the wider adoption of this technique for more complex protein samples. To overcome this issue, crosslinkers containing an additional affinity group have been designed to enrich low-abundant crosslinked peptides. PhoX (DSPP, disuccinimidyl phenyl phosphonic acid)[1] and tBu-PhoX (TBDSPP, *tert*-butyl disuccinimidyl phenyl phosphonic acid)[2] are trifunctional amine-reactive crosslinkers with a phosphonic acid group that can be enriched using immobilized metal affinity chromatography (IMAC) or TiO_2 beads.

In this study, we developed an optimized XL-MS workflow for membrane proteins in SMALPs using two membrane-permeable crosslinkers, Thermo Scientific™ phospho-enrichable crosslinker tBu-PhoX[2] and DSS (disuccinimidyl suberate).

Experimental

Methods and materials

Parameter	Value
Samples	Membrane protein *S. enterica* WbaP in SMALPs
Crosslinkers	tBu-PhoX (P/N A52287), DSS (P/N 21555)
Proteases and reagents	Trypsin (P/N 90057), DDM (P/N 89902)
Enrichment and desalting	Thermo Scientific™ Pierce™ TiO_2 Phosphopeptide Enrichment Tips (P/N 88303) Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns (P/N 89852)
Protein and peptide concentration assay	Thermo Scientific™ Pierce™ BCA Protein Assay Kit (P/N 23225) Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (P/N 23290)
Column	Thermo Scientific™ EASY-Spray™ HPLC column, 75 µm × 25 cm (P/N ES902)
Column temp (°C)	40
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in 80% acetonitrile
Mass spectrometer	Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer, ICSW 4.0 SP1
Liquid chromatography	Thermo Scientific™ Vanquish™ Neo UHPLC system
Data analysis	Thermo Scientific™ Proteome Discoverer™ 3.0 software and XlinkX node 3.0

Separation conditions

Retention (min)	Flow (nL/min)	%B
5	300	3
55	300	25
65	300	40
67	300	98
78	300	98

Mass spectrometry nanospray conditions

Parameter	Value
Spray voltage (V)	2,000
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	275

MS conditions

Parameter	Value
MS^1 scan	**Orbitrap**
Resolution	60,000
AGC	100%
Max. injection time (ms)	118
Charge state	3–8
MS mass range (*m/z*)	380–1,400
Intensity threshold	$5e^4$
RF lens	30
MS^2 scan	**Orbitrap HCD**
Resolution	30,000
AGC	200%
Max. injection time (ms)	70
Charge states	3–8
First mass (*m/z*)	120
Isolation width	1.6
NCE (%)	SCE 21, 26, 31
Top speed (s)	5

Sample preparation

Membrane protein *S. enterica* WbaP in SMALPs was provided in 20 mM HEPES buffer pH 7.0 by G. Dodge and B. Imperiali[3] at 0.6 mg/mL. 5 mM DSS or 2 mM tBu-PhoX in DMSO was added to the solution and incubated for 1 hour at room temperature. Reactions were quenched with 20 mM of Tris-HCl, pH 8.0, for 15 min. Part of the crosslinked protein was separated on a denaturing gradient gel (Invitrogen™ NuPAGE™ 4–12% BisTris gel) and then stained with InstantBlue™ Coomassie Protein Stain (Abcam). Bands of crosslinked proteins were excised and prepared for enzymatic digestion.

For the rest of the samples, *N*-dodecyl β-D-maltoside (DDM, 10% stock) was added to the sample to a final concentration of 1% and the mixture was incubated on ice for 30 min. To remove SMALP, $MgCl_2$ (50 mM stock) was added to a final concentration of 4 mM, and the resulting solution was incubated at 4 °C for 1 hour, and then centrifuged at 21,000 ×g at 4 °C for 1 hour. The supernatants were transferred to fresh microfuge tubes, diluted 1:1 with 0.1% SDS, 25 mM DTT and incubated at 50 °C for 1 hour.

Chloroacetamide was added to 25 mM and incubated at room temperature for 30 min in the dark before acetone precipitation overnight at -20 ºC. The samples were washed twice with 90% acetone and the pellet was vortexed with 25 mM ammonium bicarbonate until re-solubilized. Enzymatic digestion was carried out with either trypsin in 0.1% RapiGest™ SF (Waters) (1:20 ratio) or pepsin (1:50 ratio). The trypsin digestion was stopped after 16 hours with 1% formic acid (FA). Crosslinked peptides were desalted using Pierce peptide desalting spin column and dried. The tBu-PhoX crosslinked peptides (60 µg) were enriched using the TiO_2 Phosphopeptide Enrichment Tips according to the manufacturer's instructions and incubated before enrichment with 2% TFA for 1 hour at 37 °C to remove the *tert*-butyl protection group.

Data analysis

The raw data files were analyzed using Proteome Discoverer 3.0 software with XlinkX node 3.0 using non_clevable or non_cleavable_fast search algorithms for crosslinked peptides and the SEQUEST™ HT search engine for linear peptides and loop-links/mono-links with the following settings:

- MS^1 ion mass tolerance: 10 ppm
- MS^2 ion mass tolerance: 20 ppm
- Maximum number of missed cleavages: 2
- Minimum peptide length: 6
- Maximum modifications: 4
- Peptide mass: 500–8,000 Da

Carbamidomethylation (+57.021 Da) of cysteines was used as a static modification. PhoX or DSS crosslinked mass modifications for lysine or the protein N-terminus and methionine oxidation (+15.995 Da) were used as variable modifications. Data were searched for crosslinks against a protein database generated from protein identifications using WbaP sequence and the *E. coli* proteome fasta retrieved from UniProt™. The false discovery rate (FDR) was set to 1% at crosslinked spectra matches (CSM) and crosslink levels. The XlinkX score was set using dynamic scoring based on the highest decoy scoring CSM. Post-processing and visualization were carried out using the XMAS plug-in for ChimeraX[4].

Results and discussion

Optimization of crosslinking protocol for membrane proteins

A major challenge in membrane protein XL-MS analysis is sample preparation. Due to issues such as limited solubility, the presence of lipids or detergents, and, as a result restricted enzyme accessibility, the currently preferred method to perform XL-MS analysis is in-gel digestion. However, this approach usually provides a limited number of crosslinked peptides.[5] Moreover, no protocol has been developed for crosslinking membrane proteins in SMALPs. In this study, we used two crosslinkers DSS and tBu-PhoX (Figure 1) to achieve maximum coverage in both extra- and intra-membrane regions. Both crosslinkers can penetrate the membrane or lipid layer with space arms of 11.4 Å or 4.8 Å, respectively. The DSS crosslinker is a bifunctional reagent reactive to amine groups. The tBu-PhoX crosslinker is a trifunctional reagent with two amine-reactive groups and a phosphonic acid as the affinity group for enrichment using phospho-enrichment methods such as IMAC or TiO_2.

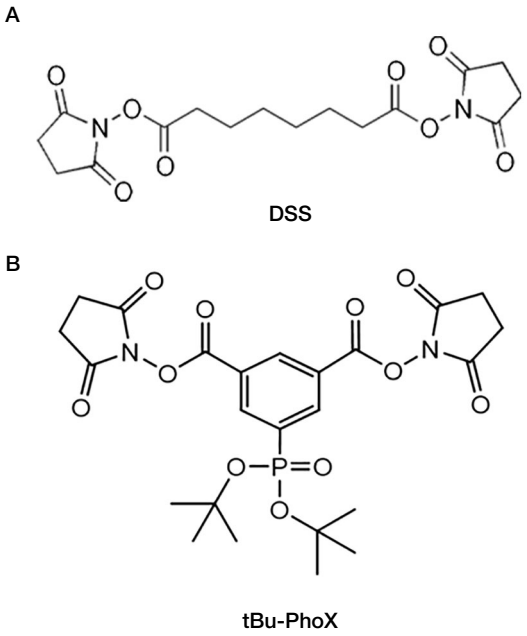

Figure 1. Structures of crosslinkers used in the study. (A) DSS, (B) tBu-PhoX

WbaP, a homodimer membrane protein, is a bacterial phosphoglycosyl transferase. It catalyzes the transfer of phospho-galactose from UDP-galactose onto undecaprenol phosphate (UndP), forming UndPP-galactose. WbaP used in this experiment was expressed in *E. coli* cells, purified, and solubilized in SMALPs. The crosslinking reaction was carried out in 20 mM HEPES buffer containing intact WbaP-SMALPs to ensure that membrane proteins stayed in their native conformation. After crosslinking, we either submitted samples to SDS-PAGE or performed a pre-clearance step in solution to remove SMALPs for the downstream MS analysis (Figure 2).

Optimization of digestion conditions

We compared two different workflows (in-gel digestion vs. in-solution digestion). For the in-gel digestion workflow, samples were separated on an SDS-PAGE gel, and the protein dimer band was excised for digestion. For the in-solution digestion workflow, samples were buffer-exchanged into 1% DDM and then reduced and alkylated before digestion. The results are summarized in Table 1. In both workflows, we were able to successfully remove SMALPs and achieve sequence coverages of more than 80%. Compared to in-gel digestion, the in-solution digestion provided better sequence coverage and a 1.5–2-fold increase in CSM (149 vs. 62), and unique XL identifications (49 vs. 32). Importantly, using the optimized digestion conditions described in the Methods section, we were able to achieve a sequence coverage of 93.86% (Table 1). Detailed information is shown in Figure 3. The only region that was not identified is part of the transmembrane domain.

We further evaluated XL-MS results from two membrane permeable crosslinkers (DSS vs. tBu-PhoX) and two digestion enzymes (trypsin vs. pepsin) from the in-solution digestion results. We identified 42 DSS crosslinks and 32 PhoX crosslinks in *S. enterica* WbaP using the XlinkX node in Proteome Discoverer software. The overlap of unique crosslinking sites between DSS and tBu-PhoX modification was ~80% (Figure 4A). More DSS crosslinks were identified, mainly due to the more flexible and longer linker of DSS (11.4 Å in DSS vs. 4.8 Å in tBu-PhoX). In contrast, crosslinks from trypsin and pepsin digestion were complementary with only one crosslink overlapped between samples (Figure 4B). Pepsin and trypsin have different digestion specificities, which help to improve sequence coverage and crosslinking identification rates.

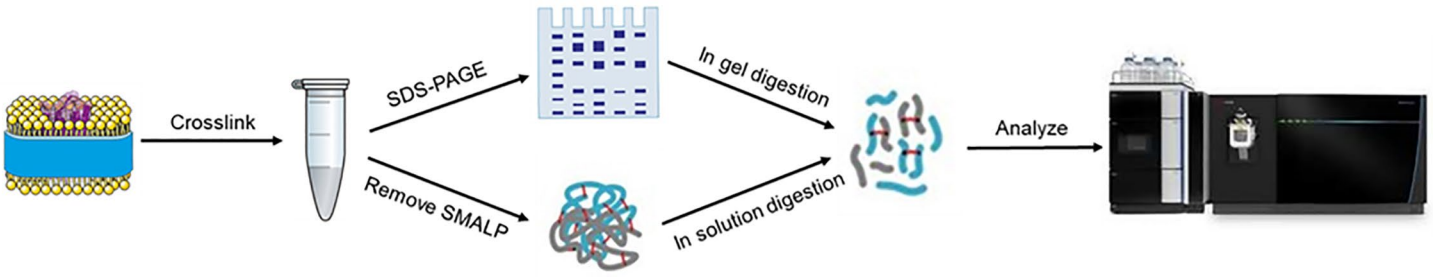

Figure 2. Crosslinking analysis of membrane protein in SMALP

Table 1. Summary of search results from in-gel and in-solution digestion using trypsin and pepsin enzymes

Method	Sequence Coverage	XLs-DSS	CSMs-DSS	XLs-tBuPhoX	CSMs-tBuPhoX
In gel	81.96%	26	52	13	16
In solution	93.86%	42	116	32	93

WSHPQFEKGGGSGGGSGGSAWSHPQFEKSSGVDLGTENLYFQSNAMDNIDNKYNPQLCKIFLAISDLIFFNLALWFSLGCVYFIFDQVQRFIPQDQLDTR
VITHFILSVVCVGWFWIRLRHYTYRKPFWYELKEIFRTIVIFAIFDLALIAFTKWQFSRYVWVFCWTFALILVPFFRALTKHLLNKLGIWKKKTIILGSGQNARG
AYSALQSEEMMGFDVIAFFDTDASDAEINMLPVIKDTEIIWDLNRTGDVHYILAYEYTELEKTHFWLRELSKHHCRSVTVVPSFRGLPLYNTDMSFIFSHEV
MLLRIQNNLAKRSSRFLKRTFDIVCSIMILIIASPLMIYLWYKVTRDGGPAIYGHQRVGRHGKLFPCYKFRSMVMNSQEVLKELLANDPIARAEWEKDFKLK
NDPRITAVGRFIRKTSLDELPQLFNVLKGDMSLVGPRPIVSDELERYCDDVDYYLMAKPGMTGLWQVSGRNDVDYDTRVYFDSWYVKNWTLWNDIAILF
KTAKVVLRRDGAY

Figure 3. The sequence coverage of *S. enterica* WbaP (93.86%)

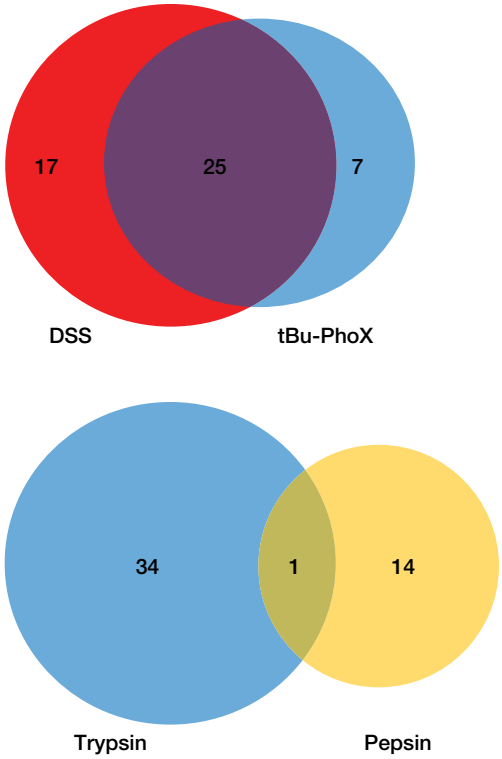

Figure 4. Venn diagrams showing the overlap of crosslinking sites identified using (A) two crosslinkers, (B) two different enzymes for digestion

Mapping crosslinked sites

The overall locations of identified crosslinked sites in *S. enterica* WbaP using DSS and tBu-PhoX are shown in Figures 5A and 5B, respectively. Both reagents labeled primarily lysine residues in the extracellular domain of the membrane proteins. The majority of the DSS crosslinks are localized to the solvent-accessible areas of the phosphoglycosyl transferase (PGT)[3] domain, while several of the tBu-PhoX crosslinks are localized to the membrane-adjacent regions at the putative dimer interface (Figure 5). As shown in Figure 5C, the distances between the majority of the crosslinks were under 30 Å and tBu-PhoX crosslinks demonstrated shorter average distances. These results are in agreement with the different spacer lengths of the two crosslinkers (Figure 1). We also noticed a small number of crosslinks with extremely long distances of 60–80 Å, suggesting the presence of partially unfolded protein.

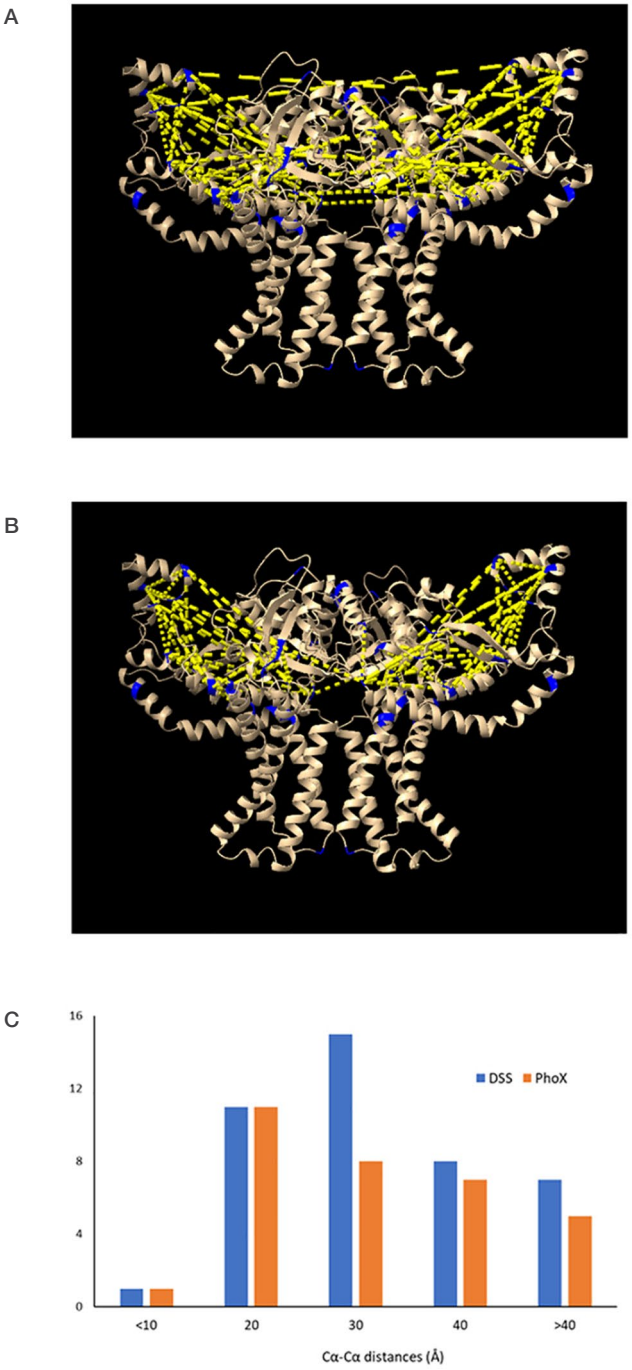

Figure 5. Crosslinking sites identified in *S. enterica* WbaP dimer are visualized in XMAS[4] for DSS (A) and tBu-PhoX (B). Lysine residues in the structure are highlighted in blue. (C) Histogram of Cα-Cα distances for DSS (dark blue) and tBu-PhoX (orange).

Importantly, four inter crosslinks were observed with high confidence (Figure 6, highlighted in red) in our data. The distances were compared in both intra- and inter-situations, and only three of them were valid when the linkage was between the subunits (Table 2). Specific crosslinks between Lys148-Lys273 and Lys232-Lys273 were identified in both DSS and tBu-PhoX modifications.

The MS^2 spectrum of Lys148-Lys273 crosslinked peptide is displayed in Figure 7, and all the major fragment peaks were assigned in the spectrum, providing unambiguous identification of the linkage sites. Taken together, this result provides strong evidence for the dimer structure and detailed information on the interface between subunits of the WbaP protein.

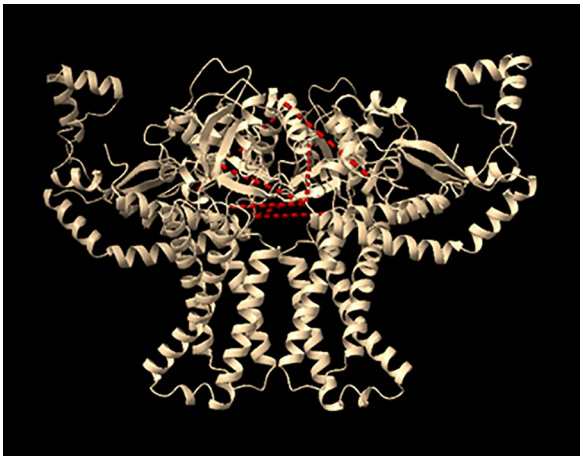

Figure 6. Inter-crosslinks (highlighted in red) identified in WbaP dimer structure

Table 2. Inter-crosslinks confirming homodimer structure of WbaP

Residue 1	Residue 2	Distance –intra (Å)	Distance –inter (Å)
Lys81	Lys273	**28.4**	**20.7**
Lys148	Lys273	43.2	**20.8**
Lys232	Lys273	46.3	**13.0**
Lys222	Lys422	42.2	**26.6**

*A number in bold indicates that the distance is within the maximum distance of the crosslinker[6].

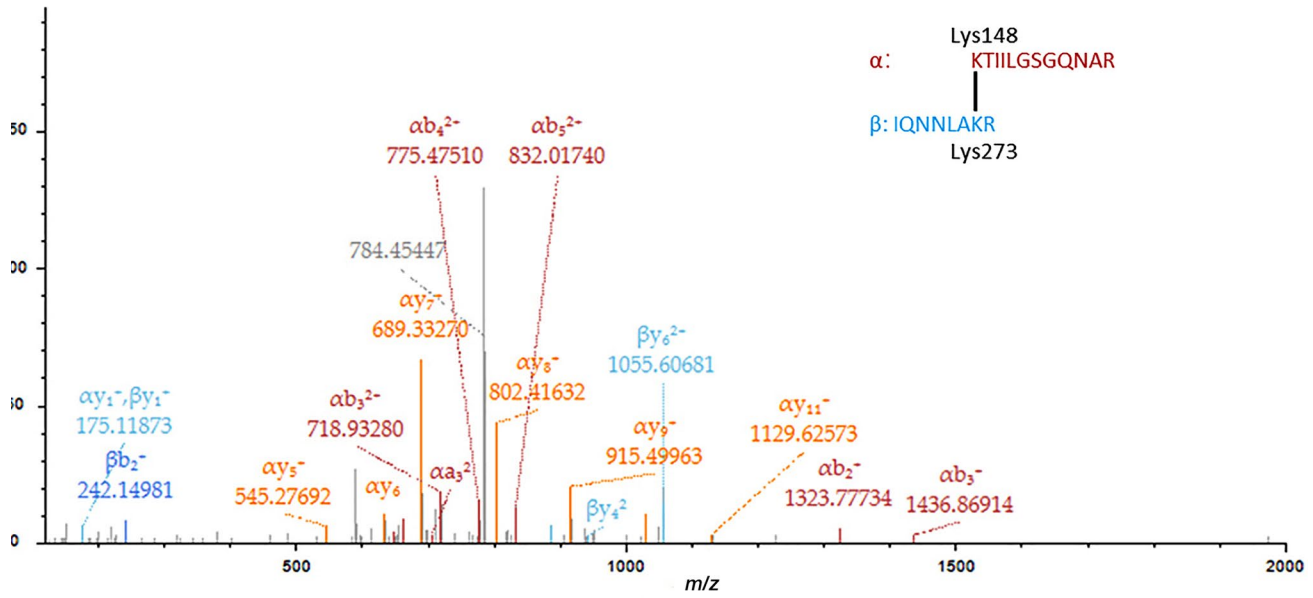

Figure 7. XlinkX identification of crosslinked peptide between Lys148 and Lys273

Conclusions

- An end-to-end XL-MS workflow for membrane proteins in SMALPs was developed using two membrane-permeable crosslinkers DSS and tBu-PhoX.
- The in-solution digestion workflow enables better sequence coverage and more unique crosslinked site identifications compared to the traditional in-gel digestion approach.
- Employing the optimized crosslinking workflow, four inter-chain crosslinks were identified with high confidence, confirming the predicted dimer interface of WbaP.

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