PTM site localization and isomer differentiation of phosphorylated peptides

Tunable electron activated dissociation (EAD) MS/MS using the SCIEX ZenoTOF 7600 system

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Protein phosphorylation is an important post-translational modification (PTM) as it is involved in a large variety of dynamic cellular processes. However, PTM site localization and quantification of phosphopeptides by collision induced dissociation (CID) MS/MS can be challenging, and phospho-peptides can exhibit a partial neutral loss of the phospho group (-98 Th). Phospho-isomer differentiation and subsequent precise PTM site localization can be achieved by measuring isomer-specific ions containing the actual modification (direct evidence), or by measuring differentiating fragment ions that do not contain the modification (indirect evidence). Depending on the peptide sequence, detecting near complete fragment ion series, and more particularly the challenging fragment ions that would define the peptide C- and N- termini, can be necessary for PTM-site localization, such as for pS-56 and pS-59 of the NDUFA10 subunit of mitochondrial Complex I[1] (Figure 1). The benefits of electron activated dissociation (EAD) versus CID were first explored for malonylation PTM.[2]

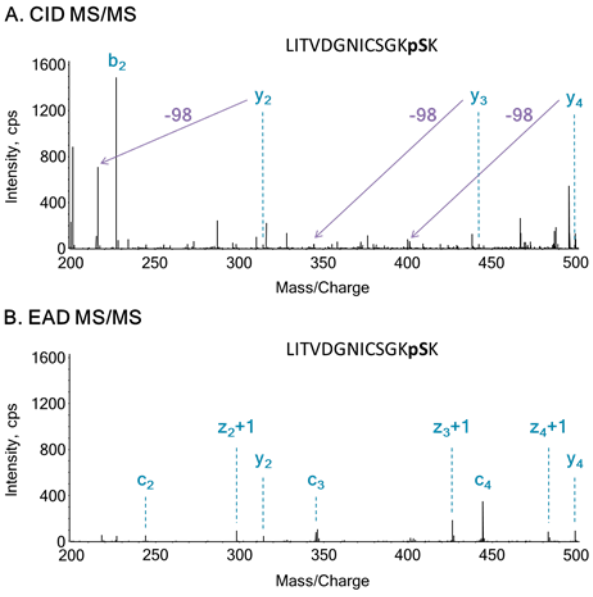

Figure 1. MS/MS spectra of LITVDGNICSGKpSK peptide analyzed with CID and EAD fragmentation. The phosphorylated peptide at m/z 505.58 (z=3) was analyzed in (A) CID mode and (B) EAD mode (kinetic energy, KE = 2). CID fragmentation resulted in low abundant and noisy PTM-specific differentiating ions (y and prominent y-98, no detected b ions). However, comprehensive EAD MS/MS generated distinct z and c fragment ions that provided evidence for definitive PTM site localization.

In this study, the use and benefits of EAD fragmentation for phosphopeptide analysis, site-localization and differentiation, and MS/MS-based phosphopeptide quantification were evaluated.

Key features of EAD for phosphoproteomics

- Efficient electron activated dissociation (EAD)[3] generates strong and distinct PTM site localization ions, enabling phospho-isomer differentiation
- Tunable kinetic energy (KE) for EAD MS/MS allows for selection of KE that provides the highest fragment ion abundance, while not inducing neutral loss from the phosphoryl group (-98 Th)
  - Generation of strong PTM-containing site localization ions, even small z+1 ions (z_2+1, z_3+1 and z_4+1) and high c ions (c_{10}, c_{11} and c_{12})[4]
  - The optimal KE values are different between different types of modifications, phosphorylation and malonylation[2]
- Using the Zeno trap gives up to ~10x increase in intensity for key site-localizing fragment ions (small z+1 ions and high c ions)

- Preliminary quantification using EAD high-resolution MRM (MRM^{HR})[5] indicates good linearity over concentrations interrogated
- Detailed characterization and MS/MS-based quantification of the modified peptides analyzed by EAD MS/MS using Skyline.

Methods

Sample preparation: Phosphorylated synthetic peptides were obtained from Princeton Biomolecules Corporation and diluted in simple matrix for analysis.

Chromatography: A NanoLC 425 system plumbed for microflow chromatography (5 µL/min) was used and operated in direct injection mode. The analytical column used was a 0.3 mm x 150 mm (2.6 µm particle size) Phenomenex Omega Polar column. Column temperature was controlled at 30°C. Short gradients of 8 minutes were used.

Mass spectrometry: All data was acquired using a SCIEX ZenoTOF 7600 system and an OptiFlow Turbo V ion source[6] equipped with the microflow probe and 25 µm electrodes. The system is equipped with the electron activated dissociation (EAD) cell which enabled hot electron capture dissociation (hot ECD) to be performed in a targeted manner on the phosphorylated peptides. MRM^{HR} data were collected using a TOF MS scan of 250 msec and MS/MS accumulation times of 150 msec both in CID and EAD fragmentation modes. Electron current was also ramped to optimize and a final value of 5000 nA was used throughout study. Kinetic energies were ramped and optimized per analyte.

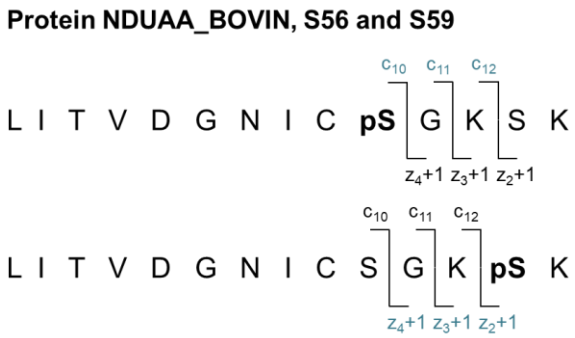

Figure 2. Sequence of the investigated phosphorylated isomeric peptides. Peptides at position 47-60 in bovine NDUFA10 subunit of Complex I (P34942) and carrying phospho-groups on serine S-56 (top) and S-59 (bottom) were investigated. PTM site-specific ions are highlighted in blue.

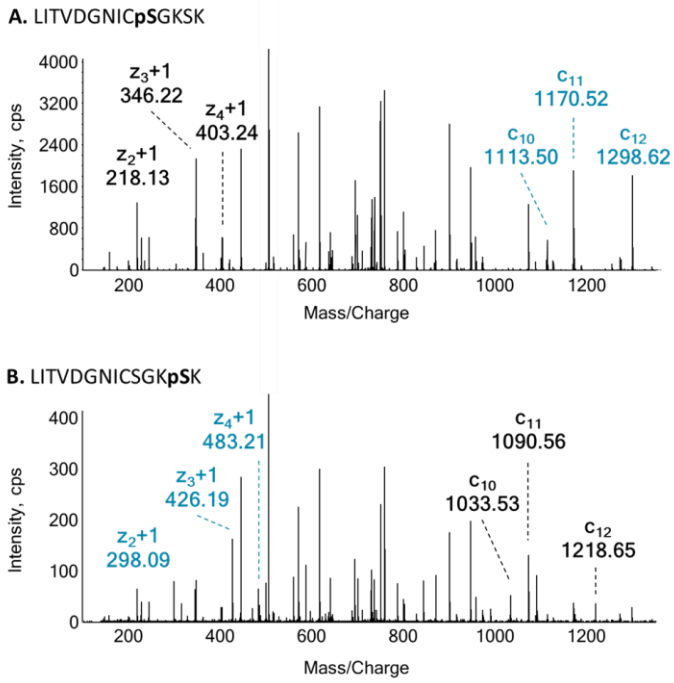

Figure 3. EAD fragmentation enables isomer differentiation. MS/MS spectra of (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K at m/z 505.58 (z=3) analyzed in EAD mode (KE = 2). PTM-containing site-specific ions are indicated in blue, additional differentiating ions in black.

Data processing: MRM^{HR} data were processed using SCIEX OS software 2.1 using both Explorer and Analytics modules, and Skyline (version 3.6).[7]

EAD fragmentation enables direct PTM site localization

Two isomeric phosphorylated peptides from bovine mitochondrial NDUFA10 subunit of Complex I (P34942), LITVDGNIC**pS**GKSK and LITVDGNICSGK**pS**K, were synthetized with a phospho-serine at position S-56 and S-59 respectively (Fig. 2) (hereafter referred to as LITV-pS-56 and LITV-pS-59 respectively).
In order to effectively characterize PTM peptides, it is important to detect the 'intact' fragment ion(s) containing the modification to confidently determine its type and localization site. Here, the modification sites of the two isomers are two amino acids apart, close to the peptide c-terminus, respectively at positions pS-56 and pS-59 on the 14 aa-long peptides making these modifications difficult to characterize. Therefore, near complete coverage of the ion series in both directions is required to localize the PTM site.

However, in CID small y-ions and large b-ions are often challenging to detect, and in addition CID MS/MS induces some neutral loss of H_3PO_4 (-98 m/z) from the labile phosphorylation group (Figure 1 and 4B-D). Fortunately, MRM^{HR} analysis using EAD MS/MS enabled detection of PTM-containing, site-specific ions, such as c_{10} to c_{12} for LITV-pS-56 and z_2+1 to z_4+1 for LITV-pS-59, in blue (Figure 3) with very good intensity, providing direct evidence for the phosphorylation site on each peptide (Figure 4A-C). Additional differentiating, non-PTM containing ions were also observed, such as z_2+1 to z_4+1 for LITV-pS-56 and c_{10} to c_{12} for LITV-pS-59, in black (Figure 3). Altogether, these results allow confident discrimination of both isomers.

MRM^{HR} assays using EAD MS/MS uniquely provided quantification of phosphopeptides using PTM-site specific ions (Figure 4A and C) which have very strong signal. Whereas, when using CID MS/MS, the required differentiating ions are very low abundance (Figure 4B and D) which negatively impacts quantification sensitivity in addition to not providing definitive discrimination between the two peptide isomers.

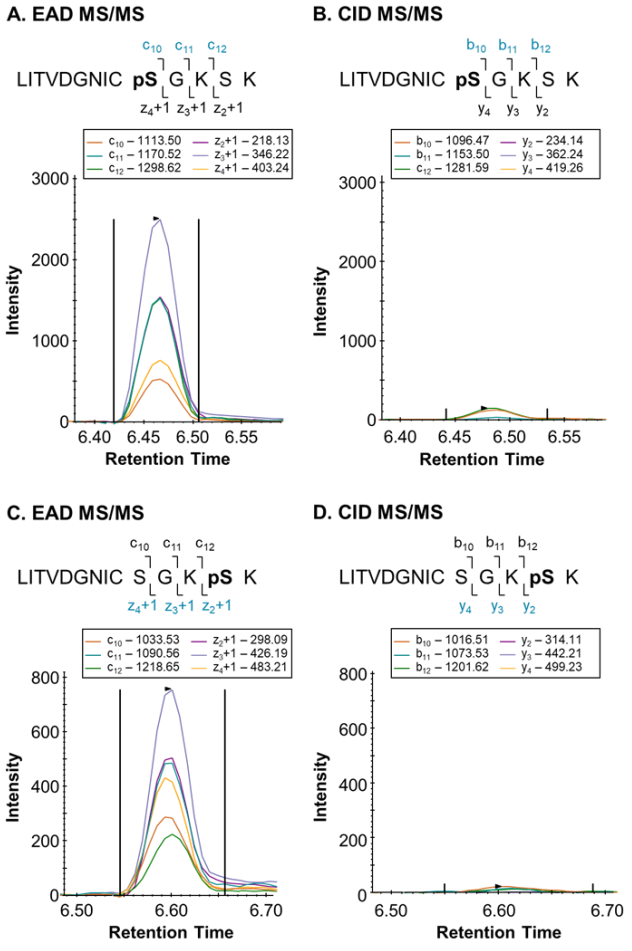

Figure 4. EAD fragmentation enables preserving the labile phospho-group. Differentiating ions of phosphorylated isomers (A, B) LITVDGNIC**pS**GKSK and (C, D) LITVDGNICSGK**pS**K analyzed in (A, C) EAD (KE = 2) and (B, D) CID modes were extracted in Skyline. In EAD mode, the labile phospho-group is preserved.

Targeted EAD MRM^{HR} for detailed characterization

MRM^{HR} is a MS/MS-based targeted acquisition strategy for accurate quantification that offers the possibility to process and refine data post-acquisition using dedicated tools as Skyline. First, near complete c- and z+1-ion series were extracted for the two isomeric peptides in Skyline as shown in Figure 5A and B. When extracting all possible fragment ions (PTM-site specific as well as ions that are in common between the two phospho-site isomers) two chromatographic peaks are detected that correspond to the two isomers that were obtained. However, when only PTM site specific fragment ions are extracted for the corresponding peptide isomers in each case, for pS-56 (Figure 5C) and for pS-59 in (Figure 5D), it is possible to unambiguously differentiate these isoforms with the pS-56 isomer eluting at 6.45 min and the pS-59 isomer eluting at 6.6 min.

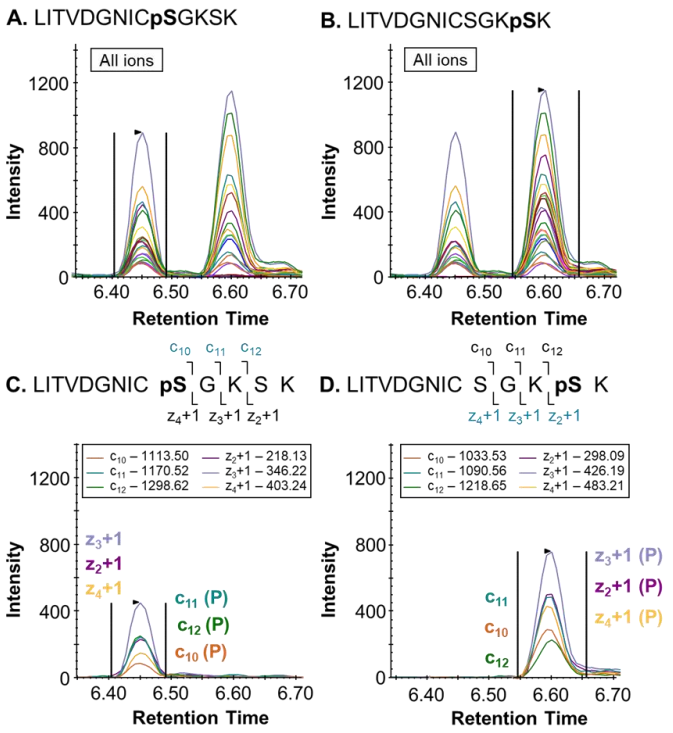

Figure 5. The extraction of site-specific fragment ions in Skyline from EAD data enables PTM localization sites. (A, B) Near complete c- and z+1-ion series were obtained with the EAD dissociation for the phosphorylated isomers (A) LITVDGNICpSGKSK and (B) LITVDGNICSGKpSK. Post-acquisition data refinement in Skyline enables differentiating the two isomeric peptides (C) LITVDGNICpSGKSK and (D) LITVDGNICSGKpSK using only discriminating ions.

Targeted EAD MRM^{HR} data processing in Skyline offers a detailed characterization of the modified peptides of interest. Currently, Skyline computes c- and z+1-ions that can be visualized and used for precise MS/MS quantification.

Tunable kinetic energy for EAD MS/MS preserves labile phospho-groups

The kinetic energy during EAD MS/MS can be tuned on the ZenoTOF 7600 system so that the fragmentation parameters can be customized to favor both the preservation of the labile phospho-group and to generate optimal sensitivity. In this experiment, kinetic energies (KE) were ramped from 0 to 11. For the two phosphopeptide isomers, the optimal KE value was 2, which generated high intensity differentiating and site-specific fragment ions that contained the intact PTM, while generating very limited background noise (Figure 6). Increased KE values, above 7, resulted in some neutral loss for the differentiating ions for LITV-pS-56. To note, the KE-dependent abundance patterns vary slightly for the illustrated, site specific ions between the isomeric peptides. The neutral loss of -98 Th is only observed on the c-ions when using the very high KE values. Thus, the tunable KE allows for the generation of methods that will both preserve the site-specific fragment ions (KE 2) and induce neutral loss (KE 11) for complete characterization of a modified peptide.

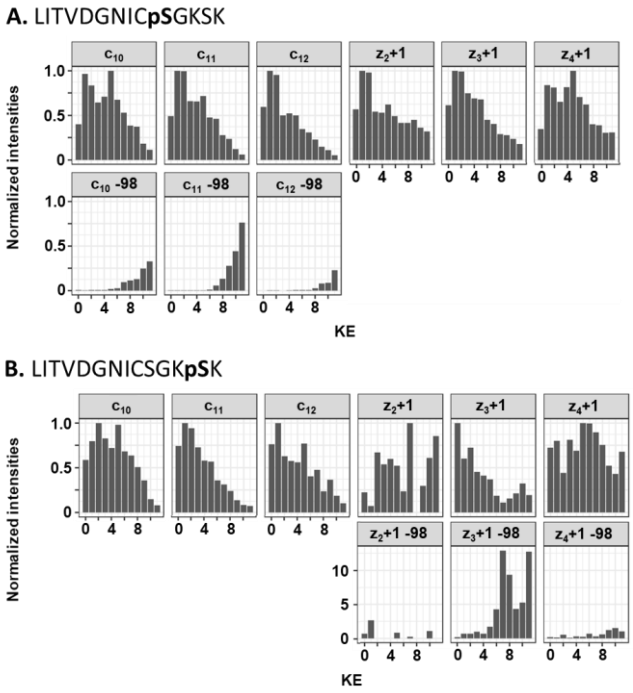

Figure 6. Kinetic energy ramping for EAD MS/MS. Phosphorylated isomeric peptides (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K were analyzed in EAD mode with KE values ranging from 0 to 11. Chromatographic peaks were extracted for 12 fragment ions. For the intact ions (first row), peak area values were normalized to the highest area. For the neutral loss ions (second row), peak area values were normalized to their respective intact ion.

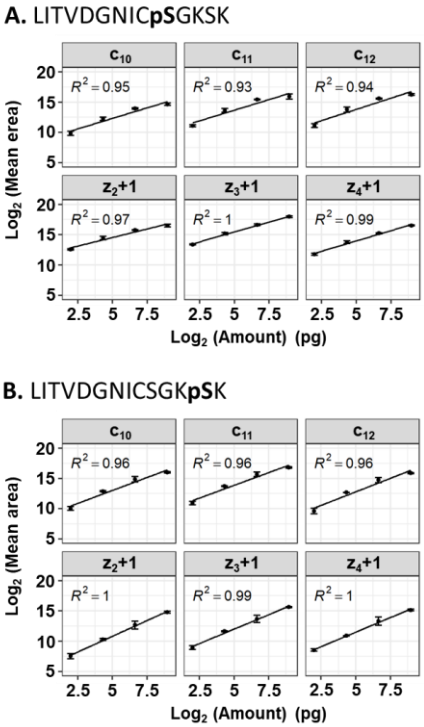

Figure 7. Linear response of fragment ions during EAD MRM^{HR}. Peptides (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K were analyzed in EAD mode (KE = 2) in triplicate and at various loading amounts (4, 20, 100 and 500 pg). Six fragment ions are displayed. The R^2 coefficient for determination of the linear regression is displayed for each ion.

Preliminary linear response for quantification

To get an initial appreciation of the quantitative performances of MRM^{HR} assays using EAD MS/MS, 4-point concentration curves were designed (4, 20, 100 and 500 pg on-column), and each point was injected in triplicate (Figure 7). Six differentiating ions were investigated for each peptide, and each showed good linear response ($R^2 \geq 0.93$). Although the explored dynamic range is limited (2.1 orders of magnitude), this first assessment suggests utility of EAD MS/MS for quantification.

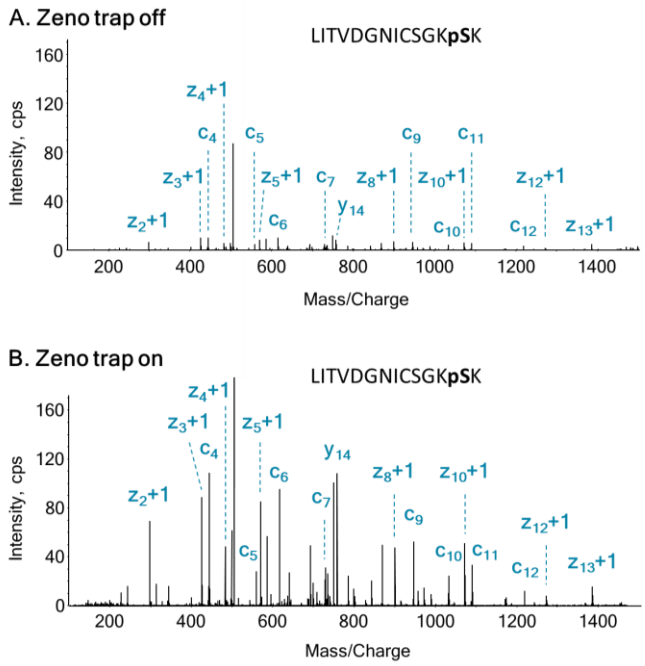

Figure 8. Increased sensitivity using the Zeno trap. MS/MS spectra of LITVDGNICSGK**pS**K peptide (m/z 505.58, z=3) analyzed in EAD mode (KE = 2) (A) without and (B) with the Zeno trap activated. Significant improvements in signal intensity and thus spectral quality was observed while using Zeno MS/MS in combination with EAD, enabling more confident PTM site localization.

Zeno trap improves sensitivity

Finally, to explore the impact of the Zeno trap on quality of EAD MS/MS spectra and sensitivity performances, EAD MS/MS analyses were performed with and without the Zeno trap activated. Analysis with Zeno trap on significantly increases sensitivity and thus generates higher intensity c and z+1 ions (Figure 8), strengthening the confidence for PTM site localization and isomer differentiation. Differentiating fragment ion peaks were extracted, and the ratios of the areas obtained with Zeno trap on and off were determined (Table 1). Sensitivity gains ranging between 7.4 and 14.9 were achieved for the various fragment ions. All the ions benefited from the activation of the Zeno trap, and more particularly the smaller ones, as the average gain was 12.7 for the three investigated small z+1 ions and 9.0 for the high c ions.

Conclusions

In this work, the performance of electron activated dissociation (EAD) on the SCIEX ZenoTOF 7600 system was investigated for the characterization and the quantification of phosphorylated isomeric peptides.

- Specifically tuning the kinetic energy (KE) for the phosphorylated peptide isomers is an added value for determination of PTM site localization, for differentiating isomers, and for improving quantification accuracy
- For the two phosphorylated isomers investigated, an optimal kinetic energy value of 2eV allowed preservation of the labile group and generated fragment ions with site localization evidence
- Activation of the Zeno trap provided large improvements in sensitivity, leading to highly confident PTM characterization
- Using MRM^{HR} and collecting full scan EAD MS/MS fragmentation offers the possibility to refine the extracted chromatographic peaks post-acquisition, using dedicated processing tools such as Skyline
- Preliminary quantitative assessment of EAD MRM^{HR} workflows shows promising performances for the robust and accurate quantification of labile PTMs

Table 1. Gain of MS/MS sensitivity using the Zeno trap. LITVDGNIC**pS**GKSK and LITVDGNICSGK**pS**K peptides were analyzed in EAD mode (KE = 2) with and without the Zeno trap activated, loading various amounts (4 and 20 pg on column). Chromatographic peak areas were extracted and sensitivity changes between Zeno trap on and Zeno trap off were determined.

Ions	LITVDGNIC**pS**GKSK			LITVDGNICSGK**pS**K		
	m/z	Zeno trap on/off 4 pg	Zeno trap on/off 20 pg	m/z	Zeno trap on/off 4 pg	Zeno trap on/off 20 pg
z_2+1	218.13	*14.5*	*12.7*	*298.09*	*10.4*	*12.1*
z_3+1	346.22	*14.9*	*10.8*	*426.19*	*12.7*	*9.2*
z_4+1	403.24	*13.6*	*11.8*	*483.21*	*19.9*	*10.1*
c_{10}	*1,113.50*	*7.4*	*10.6*	*1,033.53*	*8.8*	*9.4*
c_{11}	*1,170.52*	*9.2*	*10.1*	*1,090.56*	*9.9*	*8.5*
c_{12}	*1,298.62*	*8.4*	*9.6*	*1,218.65*	*6.8*	*8.8*

References

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