Confident PTM site localization of immunoaffinity-enriched peptides from cell lysates using electron activated dissociation (EAD)

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In this technical note, we describe the confident site localization of tyrosine phosphorylation and lysine ubiquitination in antibody-enriched proteomes using electron activated dissociation (EAD) MS/MS. Collision-induced dissociation (CID) mass spectrometry is universally used to identify and characterize post-translationally modified peptides in proteomic samples. However, for longer chain peptides and peptides that contain labile modifications, CID may not produce sufficient fragments for localization of the modification and full sequence coverage of the peptide backbone. In contrast, fragmentation using EAD on the ZenoTOF 7600 system typically results in fragments that allow for confident assignment of the modification site, even in longer peptides.

Key features of PTM characterization using PTMScan® enrichment and EAD fragmentation on the ZenoTOF 7600 system

- Fragmentation by EAD on the ZenoTOF 7600 system results in information-rich spectra for the confident identification of phosphotyrosine modification and ubiquitination in proteomic samples
- EAD allows for site-specific localization of PTMs, even in long chain peptides or peptides with multiple candidate modification sites
- PTMScan kits provide highly specific enrichment of modified peptides using immunoaffinity precipitation, for subsequent LC-MS/MS analysis

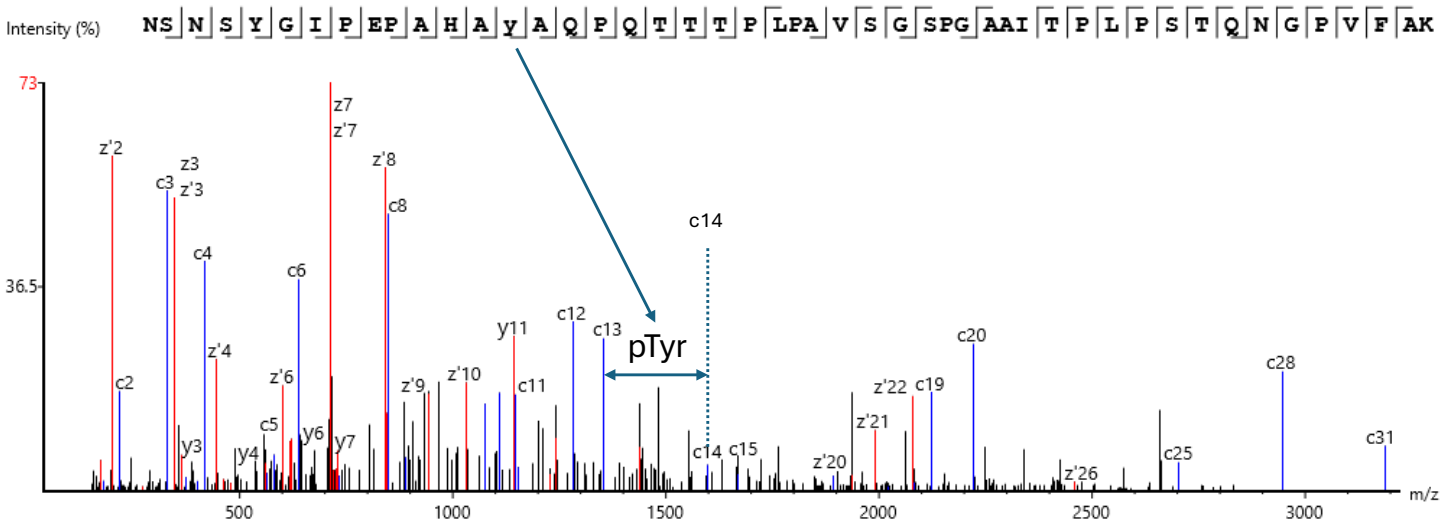

Figure 1: Confident localization of pY207 in CRK-like protein from a phosphotyrosine enriched sample. The EAD MS/MS spectrum shows a high degree of peptide backbone sequence coverage for this long peptide consisting of 48 residues. Location of phosphorylation was confidently assigned to Y207 based on the mass difference of the c13 and c14 ions despite the presence of an additional candidate residue at Y198.

Introduction

Post-translational modifications (PTMs) modulate various cellular processes and are implicated in the progression of many diseases. Modifications can alter the local surface charge and tertiary structure of proteins. These changes can influence enzymatic activity or binding to various ligands. For example, tyrosine phosphorylation creates a protein binding domain to the Src homology-2 domain that recruits specific proteins. This, in turn, mediates various cell signaling events that are responsible for regulating proliferation, differentiation, adhesion, and immunity[1]. Dysregulation of phosphotyrosine signaling is observed in various diseases like cancer, making modulation of this PTM a therapeutic target[2,3]. Other modifications, like ubiquitination, influence protein half-life through the ubiquitin-proteasome degradation pathway, which also regulates cell death[4]. Manipulation of the ubiquitin-proteasome pathway through proximity-inducing therapeutics like PROTACs and molecular glues has recently gained interest for the elimination of specific target proteins implicated in various diseases through cellular degradation[5,6]. Consequently, knowledge of the modification of specific residues helps researchers better understand their influence on cellular functions, and it also helps guide drug discovery in targeting specific residues or protein surfaces. However, characterization of PTMs can be challenging because of the low abundance of PTM peptides compared to their non-modified counterparts. Proteins can also be modified at several sites, including residues within close primary sequence proximity.

EAD has previously been shown to enable both site specific characterization of phosphorylated and glycosylated peptides containing multiple candidate modification sites and the ability to discern positional isomers[7,8]. Here, we set out to demonstrate the effectiveness and sensitivity of EAD fragmentation for confident PTM localization from low proteomic loads for phosphotyrosine and ubiquitin remnant K-ε-GG-containing peptides after antibody enrichment. Antibody enrichment has been proven to be an effective way to simplify proteomic mixtures, enabling the detection of low-level PTMs by LC-MS/MS[9-12].

Methods

Cell lines: HCT-116 cells were from the American Type Culture Collection (Manassas, VA). The cells were cultured in the appropriate media supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C with 5% CO_2. Cells were harvested and processed further at about 80-90% confluency.
Cell lysate preparation: HCT-116 cells were washed 2x with cold PBS. The cell pellets were snap-frozen and stored at -80°C until subsequent use. Cell lysis was carried out in urea lysis buffer containing 8M Urea, 200mM HEPES, pH 8.5, 1X Protease and Phosphatase inhibitors (CST #5872). The cell lysate was sonicated three times for 20 seconds each at 15W output power with a 1 min cooling between bursts. Sonicated lysates were centrifuged at 20,000 g for 15 min at RT. Supernatants were collected, and total protein concentration was determined using a Pierce BCA assay kit. The total protein amount equivalent to 3 mg was reduced with 4.5 mM DTT for 30 min at 55°C. Reduced samples were alkylated with 10 mM iodoacetamide for 30 min at room temperature in the dark. The samples were diluted 1:4 with 20 mM HEPES, pH 8.5 with 1mM $CaCl_2$, and digested overnight at room temperature with TPCK-treated trypsin at 37.5:1 of substrate to enzyme ratio followed by Lys-C (Wako-Chem) digestion for 4 hrs at 37°C. Digestion was stopped by adding 0.5 ml of 20% TFA for ~1% TFA final. Samples were incubated for 15 minutes at 4°C to allow any lipid to precipitate out of solution. Upon incubation, samples were centrifuged at 4500 rpm for 10 min at 10°C to pellet any insoluble. Clarified peptides were desalted over SEP PAK C18 columns from Waters. Peptides were eluted with 50% acetonitrile in 0.1% TFA, dried under vacuum, and stored at 80°C until subsequent use.
Immunoaffinity Purification: Modified peptides (phosphotyrosine and ubiquitinated peptides) were immunoprecipitated using PTMScan HS Phospho-Tyrosine (P-Tyr-1000) Kit (Cat# 38572, Cell Signaling Technology) and PTMScan HS Ubiquitin/SUMO Remnant Motif (K-e-GG) Kit (Cat# 59322, Cell Signaling Technology), respectively[10-12]. The immunoprecipitation (IP) was performed according to the manufacturer's protocol. Briefly, 3 mg of digested peptides were dissolved in 1 mL of 1x IP buffer (50 mm MOPS, 10 mm Na_2HPO_4, 50 mm NaCl, pH 7.2, Cell Signaling Technology). Peptides were incubated with PTMScan motif kits for 2 h at 4 °C on a rotator. The peptide-beads mixture was centrifuged at 2,000 g for 5 seconds and washed 2 times in 1x IP wash buffer,

followed by 3 washes in water. The modified peptides were eluted 2x with 50 µl of 0.15% TFA and desalted on STAGE tips with C18 cores (Empore, Sigma). Enriched peptides were dried using a speed vac and stored at -20°C until the next use.

Chromatography: Preparation of samples for chromatographic separation using the Evosep One system was performed according to the Evosep instructions. Briefly, C18 EvoTips were loaded with 20 µL acetonitrile, spun in a centrifuge at 800 g for 1 min. Next, EvoTips were dipped in IPA for 10 seconds, loaded with 20 µL deionized water, and spun in a centrifuge at 800 g for 1 min. Peptides enriched from a total of 3 mg proteins were loaded onto the EvoTip, and two wash steps were performed using 20 µL and 100 µL deionized water. Based on the total ion chromatogram, approximately 20 ng of enriched peptides was estimated to be loaded on column. The EvoSep One was operated using the 30 samples per day (SPD) LC gradient. The 30 SPD EvoSep method utilizes a 0.5 uL/min flow rate, an overall gradient length of 44 min, and a total 48 min cycle time. Mobile phase A consisted of 0.1% formic acid in deionized water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. All solvents were LC-MS grade and purchased from Sigma-Aldrich. The EV-1137 column (C18, 15 cm x 150 µm ID, 1.5 µm particle diameter) was used, heated to 40 °C using the column heater attached to the OptiFlow Turbo V ion source.

Mass Spectrometry: All analyses were performed using the SCIEX ZenoTOF 7600 system with an OptiFlow Turbo V ion source. Data for the structural characterization of all peptides were acquired using DDA (data dependent acquisition). An electron potential of 7 eV, a beam current of 5500 nA, and a 20 ms reaction time were used for the EAD fragmentation. Additional parameters are shown in Table 1.

Data processing: PEAKS Studio 12.5 software (Bioinformatics Solutions Inc.) was used for data analysis. Database searches were performed using a FASTA file for the human proteome downloaded from UniProt (www.uniprot.org). Search parameters are summarized in Table 2.

Table 1. Source, gas, and ZenoTOF 7600 system conditions.

Parameter	Setting
Curtain gas (CUR)	35 psi
Ion source gas 1 (GS1)	12 psi
Ion source gas 2 (GS2)	35 psi
CAD gas (CAD)	7
Source temperature (TEM)	150 °C
Ion spray voltage (IS)	4000 V
Declustering potential (DP)	60 V
Max. number of candidates	15
Exclusion	6 seconds after one occurrence
TOF MS Accumulation time	0.1 s
TOF MS Collision energy (CE)	10 V
TOF MS mass range	100-1500 amu
TOF MS/MS Accumulation time	0.035 seconds
TOF MS/MS CE	12 V
TOF MS/MS mass range	100-3000 amu
Time bins to sum (TOF MS; TOF MS/MS)	4;4
Zeno trap for MS/MS	On
Zeno trap threshold	20000

Table 2. PEAKS Studio software search parameters used.

Parameter	Setting
Precursor mass	10 ppm
Fragment ion	0.02 Da
Digest mode	Semi-specific
Missed cleavage	2
Max. allowed variable PTM per peptide	2
Peptide length	6-50
Deep learning boost	off
Peptide FDR	1%
Protein FDR	1%

Results: Analysis of phosphotyrosine-modified peptides

Out of 269 phosphorylated peptides identified in the tyrosine phosphorylation enriched sample, 258 peptides (96%) from 146 protein groups were identified with phosphorylation of one or more tyrosines. 170 of these peptides are considered "confident identifcations" (AScore ≥ 15 and Ion Intensity ≥ 2%) by the PEAKS Studio software.

As serine and threonine phosphorylation are considered to be much more prevalent (86% and 12% respectively)[3] than tyrosine phosphorylation, it is clear that the PTMScan technology very efficiently enriched for the lower abundant tyrosine-phosphorylated peptides.

Figure 1 shows fragmentation of a 48 residue peptide witihin CRK-like protein using EAD. For long chain peptides with multiple proline residues, CID often provides insufficient peptide backbone fragmentation. However, here with EAD, the MS/MS spectrum shows a high degree of peptide backbone sequence coverage and a confident phosphorylation site assignment could be made at Y207. In addition to the two tyrosine residues, this peptide contains several serine and threonine residues within the interior of its sequence, all reported as phosphorylation sites according to phosphosite.org. In this case, no peptides with phosphorylation on serine and/or threonine were found, confirming the specificity of the PTMScan technology to enrich peptides phosphorylated on tyrosine residues.

It can be challenging to assign the exact site of modification for phosphopeptides containing candidate residues that are in close proximity or consecutive in the sequence. Figure 2 shows an example of a tyrosine phosphorylated peptide, in this case from the ERBB receptor feedback inhibitor 1 protein. This peptide includes 4 tyrosine residues, two of which are adjacent. The EAD fragmentation spectrum allowed for the assignment of the phosphorylation to position 394 based on the observed mass difference between the c5 and c6 fragment ions.

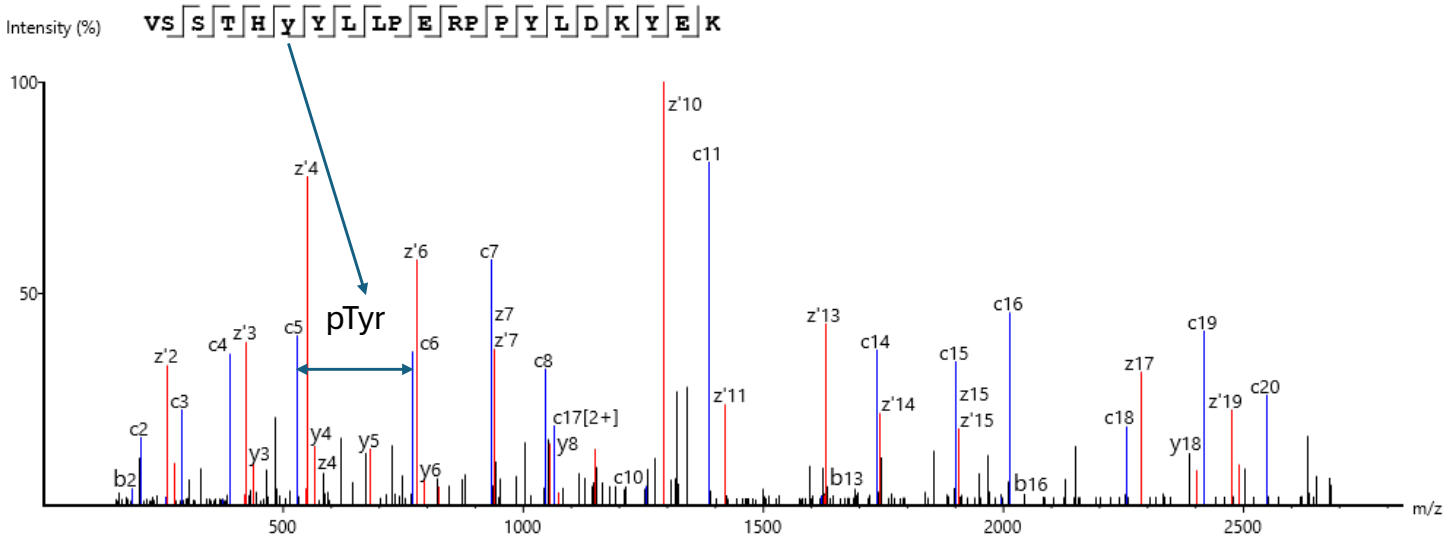

Figure 2: Confident localization of pY394 in ERBB receptor feedback inhibitor 1 protein. The EAD MS/MS spectrum shows a high degree of peptide backbone sequence coverage and a confident assignment of the site of phosphorylation based on the mass difference between the c5 and c6 ions compared to that of c6 and c7.

Results: Analysis of ubiquitinated peptides

Ubiquitination can be identified by a mass shift of 114.04 Da from the diGly remnant (K-ε-GG) of ubiquitin after digestion. Since ubiquitin is conjugated to lysine resides, tryptic peptides containing this modification are inherently more challenging to characterize with CID, as the basic lysine side chain often results in cleavages close to the lysine residue, resulting in insufficient fragment ions for sequencing and ubiquitination localization. In addition, modified lysine residues result in longer peptides, which can be challenging to obtain comprehensive backbone fragmentation. Alternatively, EAD can help to identify peptides with ubiquitin remnants and localize the site of ubiquitination in peptides with multiple lysine residues.

A PTMScan kit was used to enrich peptides containing the ubiquitin remnant motif (K- ε -GG)[11,12]. In the enriched sample, 3,416 ubiquitinated peptides were identified from 4,403 peptides, highlighting the effectiveness of the antibody-based enrichment. Of all the ubiquitinated peptides, 1,930 are considered "confident identifcations" (AScore ≥ 15 and Ion Intensity ≥ 2%) by the PEAKS Studio software.

Figure 3 shows the fragmentation of a modified peptide from the large ribosomal subunit protein eL6. EAD resulted in a high degree of fragmentation throughout the peptide backbone, enabling a confident identification and site localization of ubiquitination for this peptide that contained multiple lysine residues.

Conclusions

- Fragmentation by EAD coupled with the Zeno trap on the ZenoTOF 7600 system resulted in the identification and localization of tyrosine phosphorylation for > 250 peptides in an immunoaffinity enriched digested cell lysate sample demonstrating its usefulness for low load samples
- PTMScan® kits provide highly specific enrichment of low abundant modified peptides as demonstrated by the high quality of the EAD spectra where 96% of the peptides contained phosphotyrosine
- For long chain peptides and for peptides containing multiple candidate modification sites, EAD fragmentation offers increased confidence to identify and localize PTM(s) as shown for phosphotyrosine and ubiquitin remnant modifications
- Knowledge of specific sites of modification will improve understanding of cellular processes and improve development of safe and effective therapeutics

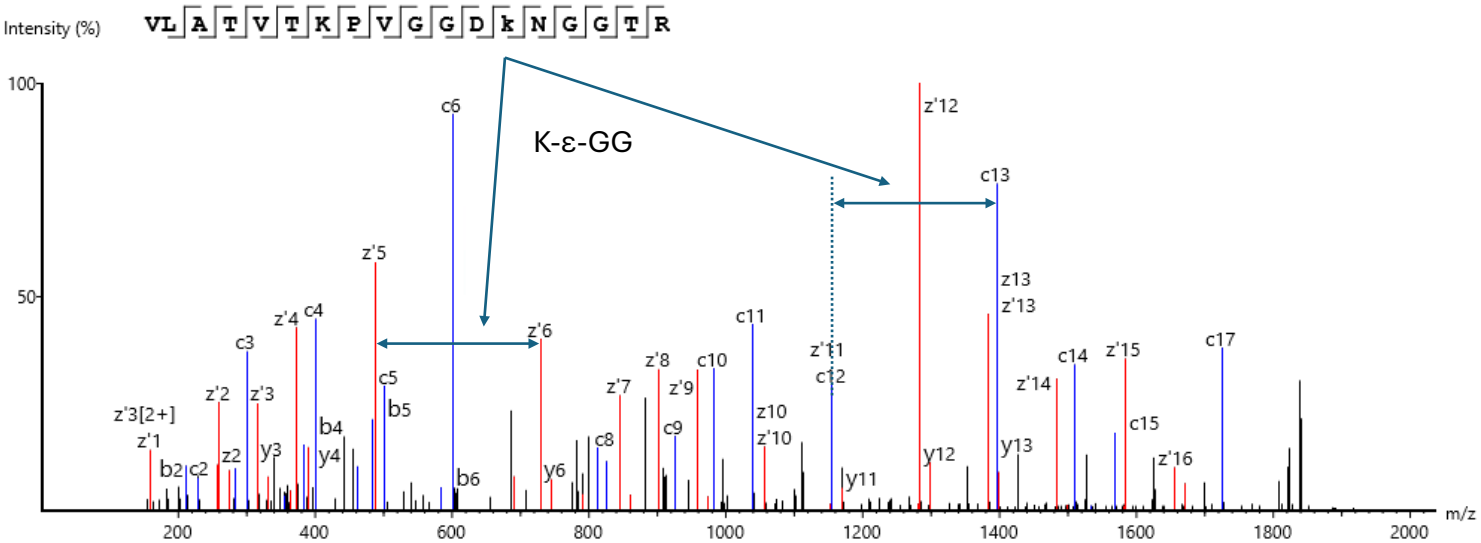

Figure 3: Confident localization of ubiquitination at position 100 in large ribosomal subunit protein eL6 . The EAD MS/MS spectrum shows a high degree of peptide backbone sequence coverage and a confident assignment of the site of ubiquitination, both from the z' and c fragment ions.

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