In-line separation by capillary electrophoresis prior to top-down mass spectrometric analysis enables sensitive characterization of proteoforms

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Overview

- **Purpose** – Improve the top-down analysis of proteoforms from protein complexes and whole proteomes through efficient front-end CE separations
- **Methods** – Capillary zone electrophoresis (CZE)-based separation using CESI-MS, the integration of CE and ESI into a single dynamic process, and intact protein MS/MS
- **Results** – CESI-MS facilitates separation, identification, and quantification of intact proteoforms, including their post-translational modifications

Introduction

Front-end separation has been a challenge for top down proteomics. Extensive multiple-stage separation systems have been developed to catalogue intact proteins at the whole proteome level. However, functional information might be obscured when the components of protein complexes and proteoforms are separated into distinct fractions. To study specific protein complexes that are usually present at low levels in cells, we developed a sensitive and efficient CE based top down platform that provides an in-depth characterization of each subunit of a protein complex. Using this platform, we studied the yeast Dam1 complex which is vital to chromosome segregation during mitosis. We also employed this method to analyze a fractionated lysate from a moderately complex organism, *Pyrococcus furiosus (Pfu)*.

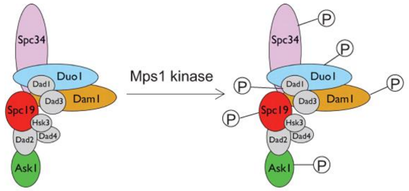


Figure 1: Representation of yeast Dam1 complex. The Dam1 complex is a component of the outer yeast kinetochore that has important functions in chromosome segregation during mitosis. Phosphorylation of the protein Dam1 by Mps1 kinase is required for coupling of the kinetochore to the plus-ends of microtubules. *In vitro* kinase assays and bottom-up proteomics were previously used to identify most phosphorylation sites.

Methods

To perform efficient front-end separation and ionization of intact proteins prior to mass spectrometry, CESI-MS was performed using a SCIEX CESI 8000 coupled to a Thermo Elite MS. The CESI capillary was covalently-coated using polyethylenimine to suppress protein-wall interactions.

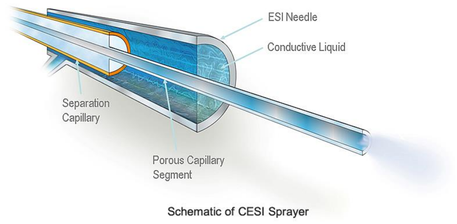


Figure 2: Schematic of CESI sprayer. A fused-silica capillary is etched at the tip until porous and inserted into a metal ESI needle. A conductive liquid is pumped between the porous tip and metal needle. When an ESI voltage is applied, electrical contact is made with the background electrolyte (BGE) in the CESI separation capillary by the conductive liquid and transport of ions through the porous tip. The inherent low flow rate from electroosmotic flow (EOF), ~20 nL/min, has improved ionization efficiency and reduced ion suppression, beneficial for intact protein analysis.

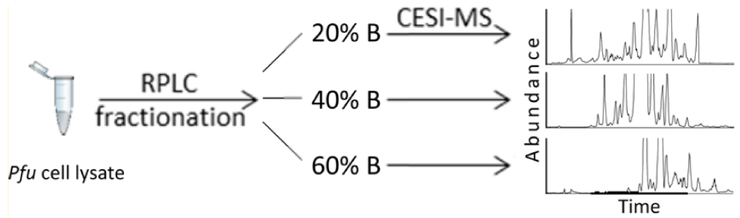
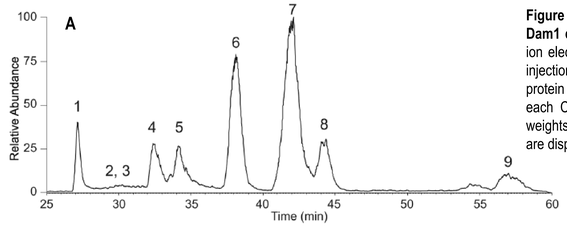


Figure 3: *Pfu* cell lysate containing ~2050 proteins was fractionated and analyzed by CESI-MS.

Top-down analysis of intact Dam1 complex proteins



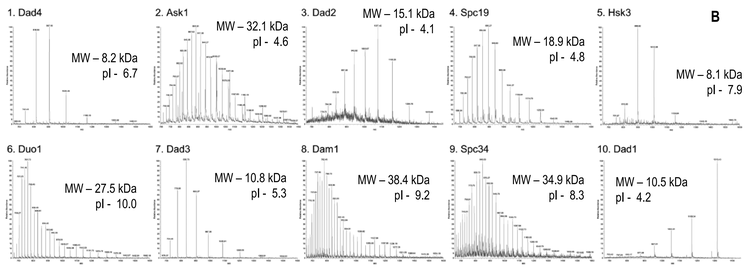


Figure 4: CESI-MS analysis of intact Dam1 complex proteins. (A) Extracted ion electropherogram (XIE) from 2.5 ng injection and separation. (B) Intact protein MS spectra from integration of each CE peak. Theoretical molecular weights (MW) and isoelectric points (pI) are displayed.

Analysis of Dam1 complex *in vitro* phosphorylation

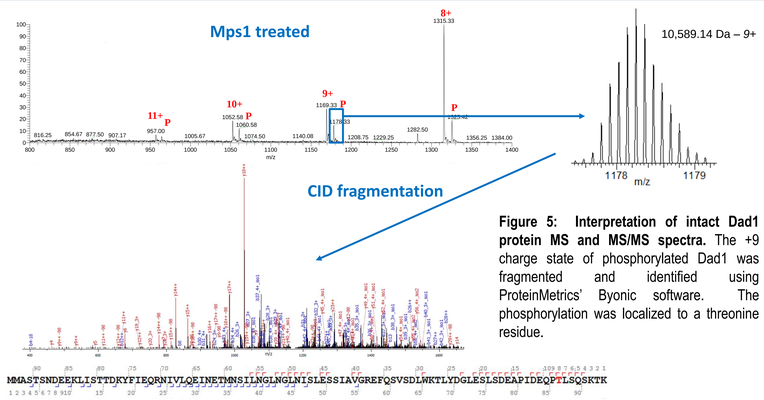


Figure 5: Interpretation of intact Dad1 protein MS and MS/MS spectra. The +9 charge state of phosphorylated Dad1 was fragmented and identified using ProteinMetrics' Byonic software. The phosphorylation was localized to a threonine residue.

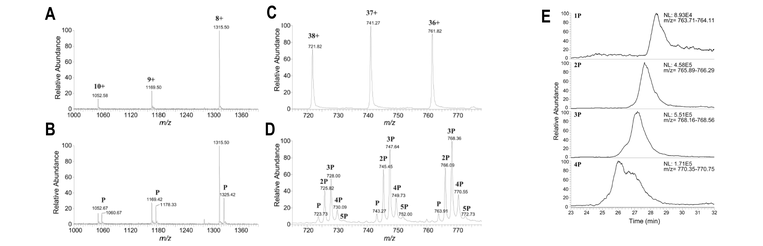


Figure 6: Detection, separation, and quantitation of phosphorylation stoichiometry by CESI-MS. Comparison of the phosphorylation states of untreated Dam1 complex subunits (A) Dad1p and (C) Duo1p, and Mps1p kinase-treated Dam1 complex subunits (B) Dad1p and (D) Duo1p on selected charge states. The phosphorylated proteoforms were labeled with #P, where # is the number of phosphorylation states. (E) XIEs of the four major phosphorylation forms (1P, 2P, 3P, 4P at charge state 36+) for Duo1p following *in vitro* Mps1p kinase treatment.

Identification of proteoforms from a whole cell lysate

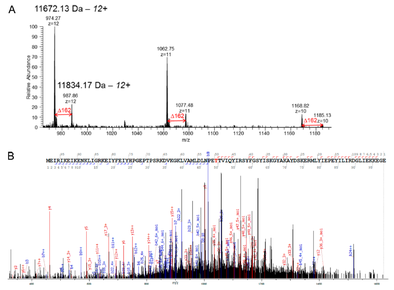


Figure 7: Characterization of hexose/maltose modified *Pfu* protein Q8U442 (30S ribosomal protein S24e). (A) Orbitrap mass spectrum of unmodified and modified forms of Q8U442. (B) HCD fragmentation spectrum for precursor 987.86^{12+} and graphical fragmentation map generated with ProteinMetrics' Byonic.

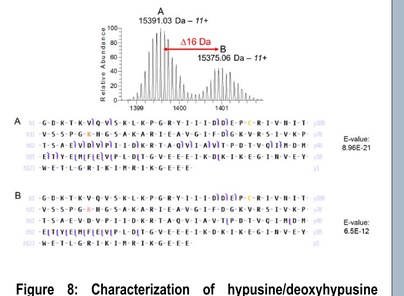


Figure 8: Characterization of hypusine/deoxyhypusine containing protein translation initiation factor 5A (IF5A) in *Pfu*. Graphical fragmentation maps generated with ProSightPC are shown for (A) deoxyhypusinated IF5A and (B) hypusinated IF5A. This modification was not previously known in this commonly studied *Pfu* model organism.

Figure 8: Identification of disulfide bonds on 50S ribosomal protein Q8TZI4 in *Pfu*. Graphical fragmentation maps generated with ProSightPC are shown for consideration of the (A) reduced protein form with a 4 Da mass matching error and the (B) non-reduced protein form with two disulfide bonds, resulting in a 0.1 Da mass matching error.

Conclusions

- CESI-MS is ideally suited for the sensitive top-down analysis of intact proteins
- PEI coating of the CESI capillary facilitates efficient protein separations over a wide range of isoelectric points and sizes
- CZE separation of intact proteoforms allows for generation of high quality MS and MS/MS spectra for sequence and PTM identification
- Localization and quantification of phosphorylation by CESI-MS can serve as a MS-based *in vitro* kinase assay at the intact protein level
- Common and previously unknown proteoform post-translational modifications can be found using top-down proteomics with CESI-MS

References

1. Han X, Wang Y, Aslanian A, Fonslow BR, Graczyk B, Davis TN, Yates JR 3rd, In-Line Separation by Capillary Electrophoresis Prior to Analysis by Top-Down Mass Spectrometry Enables Sensitive Characterization of Protein Complexes, *J. Proteome Res.*, **2014**, 13, 6078−6086.
2. HanX, Wang Y, Aslanian A, Bern M, Lavallée-Adam M, Yates JR 3rd, Sheathless Capillary Electrophoresis-Tandem Mass Spectrometry for Top-Down Characterization of *Pyrococcus furiosus* Proteins on a Proteome Scale, *Anal. Chem.*, **2014**, 86 (22), 11006–11012.

Acknowledgements

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