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Combined Top-down and Bottom-up Proteomics using Capillary Electrophoresis-Mass Spectrometry

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ABSTRACT

Purpose: Develop a combined top-down and bottom-up workflow to resolve the stoichiometry and locations of PTMs

Method: The integrated platform of microfluidic capillary electrophoresis-mass spectrometry (CE-MS) allows combined workflow versatility

Results: CE separation conditions for both peptides and proteins has been optimized, and MS/MS analysis with bottom-up and top-down are verified. Beta-casein is tested on this platform with combined approach. 11 proteoforms are able to be resolved through the intact analysis. The position of natural variants is identified by the bottom-up peptide mapping. High abundance of 5 phosphorylation sites are confirmed via the top-down analysis. A comprehensive CE-MS solution is introduced here to resolve the proteins of interest for the better understanding of complex biological systems.

INTRODUCTION

Bottom-up proteomics has been widely used for resolving the sequence of a single protein or components of protein complexes. Bottom-up approaches for single protein characterization enables the localization of PTMs via digested peptides; however, the stoichiometry of PTMs are not able to be resolved easily. Top-down approaches have proved to be a better solution for understanding combinatorial PTM profiles. A combined top-down and bottom-up proteomics workflow is a promising solution for comprehensive protein characterization. Current analytical strategies mainly rely on liquid chromatography–mass spectrometry (LC-MS) for top-down and bottom-up analysis. However, protein and peptide separation requires different LC platforms; therefore, the combined workflow using LC-MS is usually time consuming.

In this study, we introduce a new platform of CE-MS for proteomics research. On this platform, intact proteins and peptides can be monitored on the same microfluidic CE chip, and top-down and bottom-up analysis can be combined on one platform. Furthermore, CE even provides superior separation efficiency on intact proteoforms. Overall, the 18-min workflow including 3 min for intact, 3 min for top-down, and 8 min for bottom-up is designed to provide comprehensive information for resolving the heterogeneous proteins functioning in the biological systems.

MATERIALS AND METHODS

Sample

15 PRTC peptide mix, digested cytochrome c, fish allergens, and hemoglobins are validated for the CE separation. Cytochrome c, carbonic anhydrase, KRAS, and beta-casein are also tested for the combined workflow.

Test Method

One CE chip, two sample vials, and three MS methods enable the mass spectrometric analysis in 18 min.

Data Analysis

The data analysis of intact protein, peptide mapping, and top-down is performed with BioPharma Finder 3.0 software.

- **CE-MS Workflow:** **Using one CE chip in a sequential run, instead of two LC columns in separate setups**

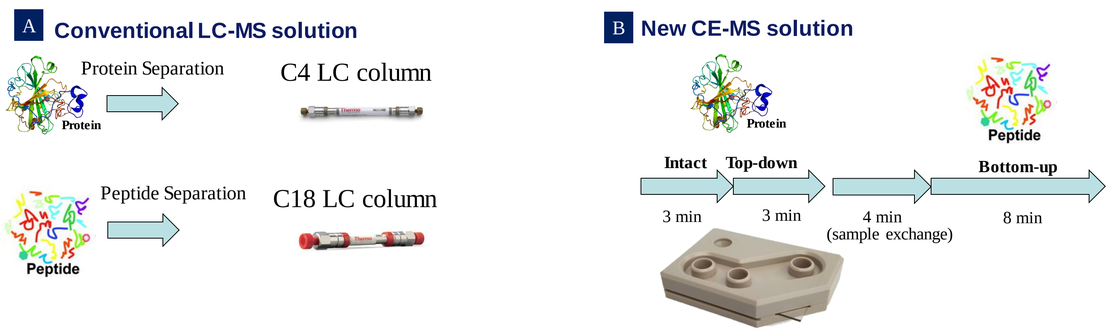


Figure 1. Schematics of combined top-down and bottom-up workflow. A) Conventional LC-MS workflow requires two LC platforms. One C4 column for protein separation, and one C18 column for peptide separation. B) New CE-MS workflow only requires one CE chip. Intact, top-down, and bottom-up analysis can be integrated into 18-min analysis.

- **Instrumentation:** **Complete hardware and software solutions from Thermo Scientific™**

ZipChip CE System | Q-Exactive HF MS | BioPharma Finder

Figure 2. The instruments used in this study including ZipChip™ CE system, Q-Exactive™ HF mass spectrometer, and BioPharma Finder™ software 3.0 are listed below.

- **CE Separation of Peptides:** **Clean separation of peptides in 8 min**

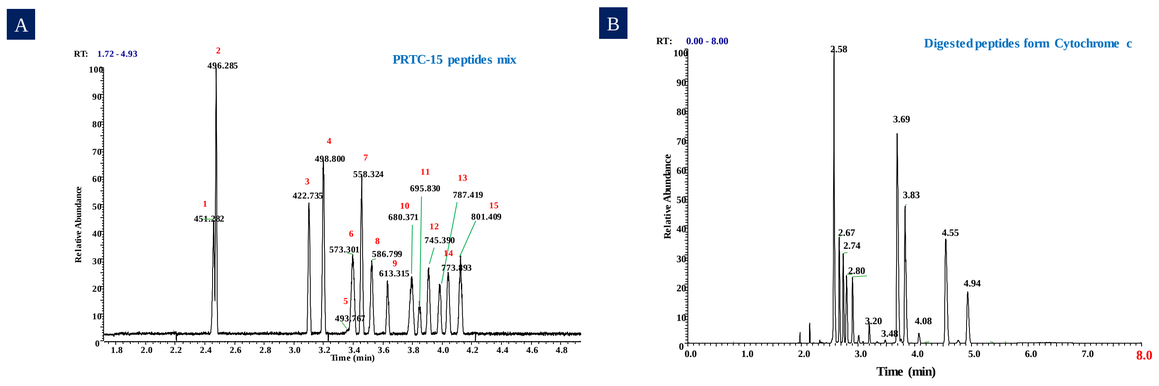


Figure 3. The peptide separation is demonstrated with the mixture from A) PRTC 15 peptide mixture and B) digested peptides from Cytochrome c.

- **CE Separation of Proteoforms:** **Superior protein isoform separation efficiency at the intact protein level**

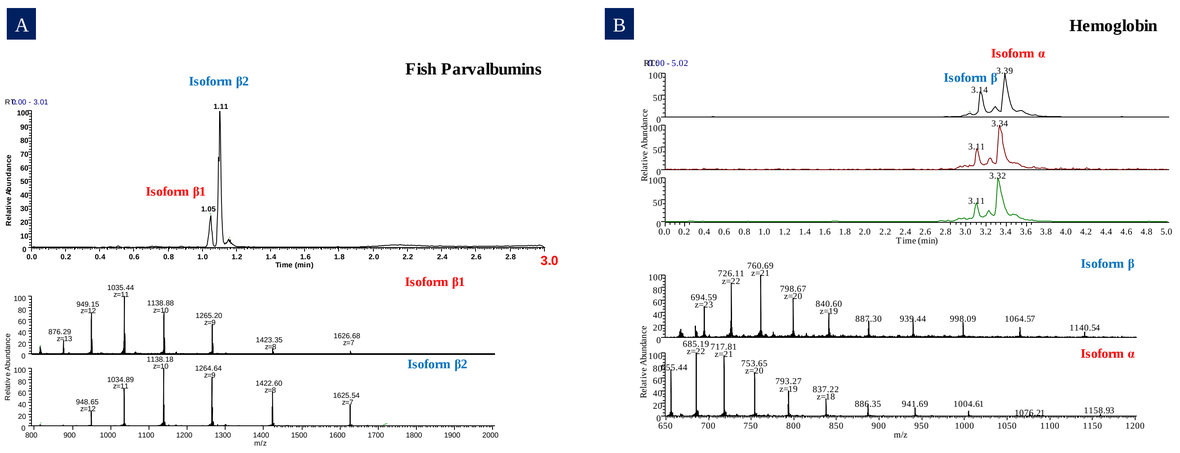
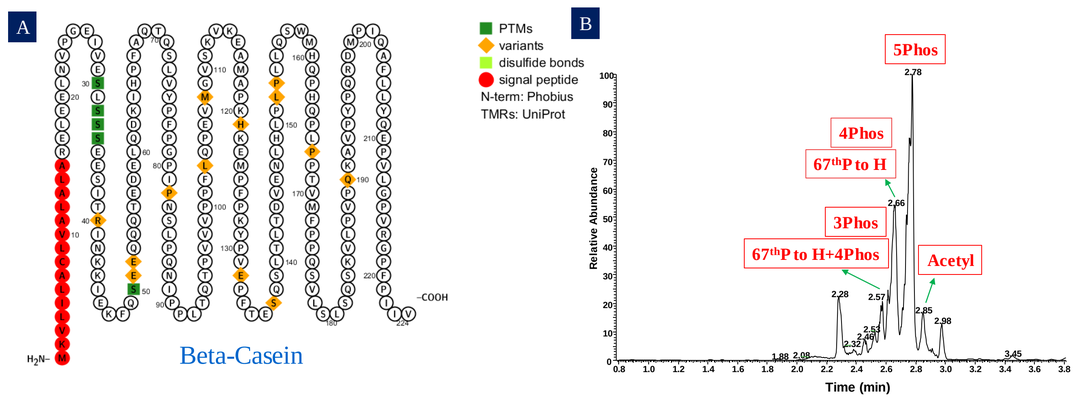


Figure 4. The separation of proteoforms is demonstrated with A) fish allergen parvalbumins and B) hemoglobins

RESULTS

- **Intact Protein Analysis:** **Stoichiometry of proteoforms, including PTMs, is determined by the intact analysis**

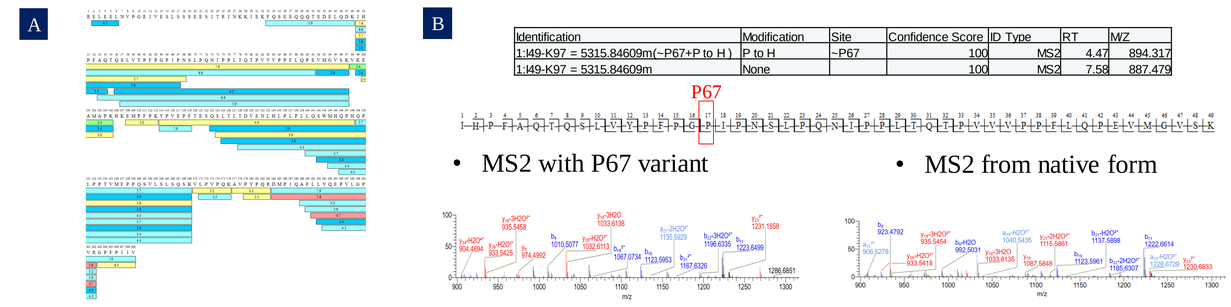


C

Protein Name	Modification	Average Mass (Da)	Theoretical Mass (Da)	Mass Error (ppm)	Relative Abundance	RT Range (min)
beta_casein	5Phos	23982.30	23983.02	29.9	100.00	2.659 - 2.872
beta_casein	1xP to H, 5Phos	24022.36	24023.04	28.0	48.25	2.533 - 2.707
beta_casein	1xH2O loss, 5Phos	23964.58	23965.00	17.7	35.21	2.631 - 2.899
beta_casein	Acetylation (N-term), 5Phos	24025.50	24025.05	18.6	17.24	2.738 - 2.919
beta_casein	1xP to H,1xH2O loss, 5Phos	24005.00	24005.02	1.0	13.86	2.631 - 2.773
beta_casein	4Phos	23902.62	23903.04	17.3	10.43	2.631 - 2.707
beta_casein	1xH2O loss,1xminus phosphrylation, 5Phos	23884.62	23885.02	16.6	7.24	2.631 - 2.697
beta_casein	1xP to H, 4Phos	23942.94	23943.06	5.0	5.15	2.533 - 2.600
beta_casein	1xAcetylation (N-term),1xP to H, 5Phos	24064.43	24065.07	26.8	3.99	2.719 - 2.833
beta_casein	1xAcetylation (N-term),1xH2O loss, 5Phos	24007.55	24007.04	21.5	3.60	2.816 - 2.909
beta_casein	3Phos	23823.73	23823.06	28.2	0.66	2.553 - 2.590

Figure 5. Intact protein analysis is shown with the example of beta-casein. A) Schematic of beta-casein structure with the signal peptide, PTMs, and variants. B) CE separation of intact beta-casein C) Identified proteoforms including the information of intact mass, stoichiometry of modifications, and their associated migration times.

- **Bottom-up Analysis:** **Localization of a natural sequence variant is accomplished by the MS2 peptide mapping**



Identification	Modification	Site	Confidence Score	ID Type	RT	MZ
1:I49-K97 = 5315.84609m(~P67+P to H)	P to H	~P67	100	MS2	4.47	894.317
1:I49-K97 = 5315.84609m	None		100	MS2	7.58	887.479

Figure 6. Bottom-up peptide mapping A) identified peptides listed under the beta-casein sequence B) Localization of a one amino acid polymorphism from a natural sequence variant through MS/MS analysis

- **Top-down Analysis:** **Confirmation of 5 phosphorylation sites**

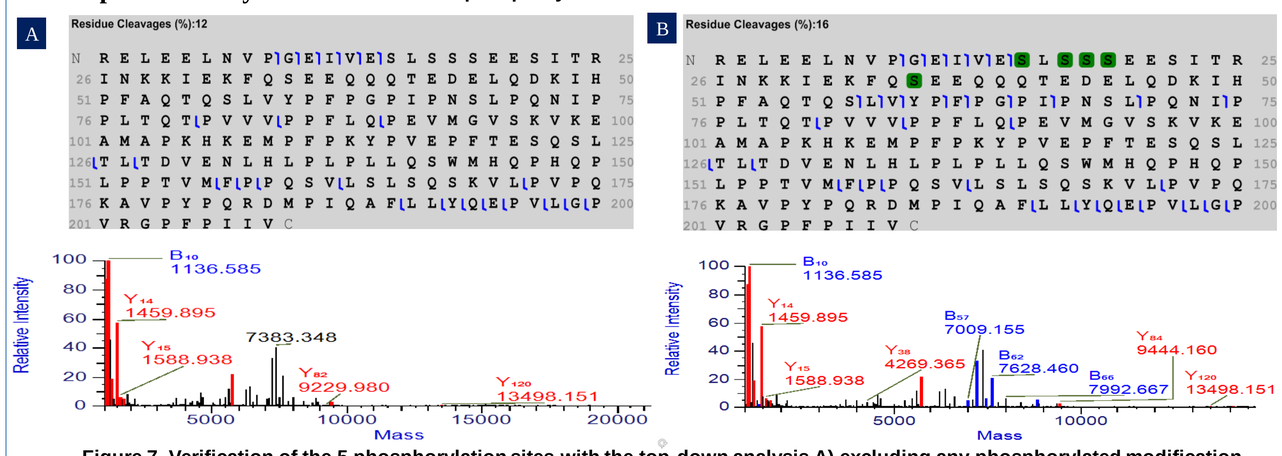


Figure 7. Verification of the 5 phosphorylation sites with the top-down analysis A) excluding any phosphorylated modification B) including 5 phosphorylation sites which are labeled in the green rectangles.

- **Summary**

A

	Intact Protein	Bottom-up	Top-down	
	Proteoforms	Sequence coverage	Residue Cleavage	Modifications
Cytochrome c	1	97%	50%	2
Carbonic anhydrase	1	97%	19%	1
KRAS	10	89%	15%	4
Beta-casein	11	86%	16%	7

B

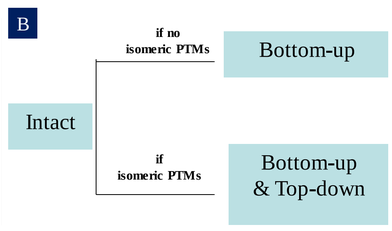


Table 1. A) The table summarizes the combined results from 4 protein samples. B) The following workflow describes the analysis strategy proposed in this study.

CONCLUSIONS

- The CE-MS platform enables intact, top-down, and bottom-up analysis of protein samples in 18 min.
- Proteoforms separation by CE has been demonstrated for the examples of fish parvalbumin, hemoglobin, and beta-casein.
- For beta-casein, stoichiometry of proteoforms are determined by intact analysis. Natural variants can be localized with bottom-up analysis. Phosphorylation sites can be confirmed with top-down analysis.
- This integrated CE-MS platform allows the capture of comprehensive protein information including sequence coverage, PTMs determination, as well as stoichiometry in a single analytical set up without the need of separate LC configurations.

REFERENCES

1. Chien-Hsun Chen[1], Stephane Houel[1], Terry Zhang[1], Brian J. Agnew[2], Shanhua Lin[3], Kai Zhou[1], Jonathan Josephs[1], Aran Paulus[1], and Andreas FR. Huhmer[1] ,” Quick Screening of Intact Antibody and Antibody-Drug Conjugates with Microfluidic Chip Electrophoresis and Mass Spectrometer ”, Thermo Fisher Scientific Application Note 72358 (2017)

2. Erin A. Redman, J. Scott Mellors, Jason A. Starkey, and Michael Ramsey, “Characterization of Intact Antibody Drug Conjugate Variants Using Microfluidic Capillary Electrophoresis – Mass Spectrometry” Anal. Chem. 88, 2220-2226 (2016)

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TRADEMARKS/LICENSING

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