

Separation and Analysis of Intact Prostate Specific Antigen (PSA) and its Proteoforms by CESI-MS Under Native and Denaturing Conditions.

- *The ability to separate, ionize and detect intact PSA using the CESI 8000 Plus High Performance Separation-ESI Module coupled to high mass resolution mass spectrometry under native and denaturing conditions*
- *Improved ionization efficiency of PSA results in the identification of almost 2-fold greater PSA proteoforms detected*

Marcia R. Santos,¹ Chitra K. Ratnayake,¹ David M. Horn,² Barry L. Karger,³ Alexander R. Ivanov,³ and Rosa Viner²

1 SCIEX, Brea, CA

2 Thermo Fisher Scientific, San Jose, CA

3 Northeastern University, Barnett Institute of Chemical and Biological Analysis, Boston, MA

Overview

Despite recent advances in intact protein mass spectrometry (MS), challenges around detection and analysis of these molecules remain. Currently, most studies are performed by infusion of highly purified proteins or by immunoaffinity analysis. This is mainly due to the lack of high-efficiency front-end separation tools that would allow for direct analysis of these molecules. Capillary electrophoresis (CE) is an established and powerful technique for intact protein analysis, particularly in the characterization of biologics under both native and denaturing conditions. The integration of CE and ESI into a single dynamic process within the same device (deemed CESI) provides the means to perform highly efficient protein separation and ionization in the ultra-low nanoflow regime (~25 nL/min) simply using an open capillary tube. Thus CESI-MS is ideally suited for intact protein analysis under native or denaturing conditions by MS.

In this study, we used a neutral surface capillary to enable the separation of proteoforms of PSA under both native and

denaturing conditions, aiming to identify and quantify the number of proteoforms that can be detected in comparison with direct infusion by nanoLC. The top 20 PSA proteoforms were identical in experiments performed under both native and denaturing conditions, where HexNac₄Hex₅dHex₁NeuAc₂ was identified as the most predominant glycan form, agreeing nicely with an ABRF Interlaboratory study from 2012.¹ The combination of infusion² and CESI-MS experiments using both native and denaturing

conditions resulted in the identification and quantification of 56 glycoforms. The total ion electropherograms acquired by CESI-MS performed under denaturing and native conditions resulted in the detection of only 4–5 predominant peaks. However, the deconvolution analysis of the CESI-MS data under native conditions revealed a possible 236 proteoforms in contrast to only 127 identified by direct infusion. Due to the increased signal-to-noise ratio under native conditions, we were able to identify additional proteoforms compared to the analysis under denaturing conditions. These hybrid proteoforms migrated noticeably later than the sialylated/complex glycan species.

Introduction

Prostate specific antigen (PSA) is a ~30 kDa glycoprotein, containing 1 N-glycosylation site at Asn69 that is secreted by the epithelial cells of the prostate gland. Despite the difficulties in validating antibodies and their possible cross-reactivity, the traditional and most robust method to detect and quantify protein biomarkers has been immunoaffinity analysis. In this work, we demonstrate a simple and fast method for the profiling of PSA proteoforms under native or denaturing conditions using CESI-MS. CESI-MS operates at ultra-low nL/min flow rates offering several advantages, including increased ionization efficiency and a reduction in ion suppression (Figure 1A and Figure 1B).

In this study, we have detected more than 200 PSA proteoforms, using CESI coupled with high-sensitivity, high-resolution mass spectrometry compared to 127 proteoforms identified by nanoLC infusion.



Figure 1B. The OptiMS sprayer connected to a Thermo Scientific Nanospray II ion source.

Results

CESI-MS under denaturing conditions

Separation of 4 predominant resolved peaks was achieved with denatured PSA in 10% acetic acid (pH 2.2) using CESI-MS (Figure 2A). A corresponding ion density map illustrates the complexity of each peak (Figure 2B). The amount of PSA injected in this experiment was 4.2 ng, which represents only 1.6% of the total capillary volume. However, the corresponding ion density maps (Figure 3B) and raw and deconvoluted mass spectra (Figure 4A and Figure 4B) show numerous proteoforms and much higher complexity of the sample.

Analysis of the deconvoluted spectra (Figure 3B) corresponding to each CESI-MS peak (Figure 3A) revealed the presence of over 202 proteoforms that were predominantly complex, hybrid structures. These data also indicate that the separation and migration order of PSA molecular species appears to be based on the hydrodynamic volume and charge of the proteoforms. In this experiment, we confidently identified 36 glycoforms by high-resolution, accurate-mass HRAM MS1 spectra.

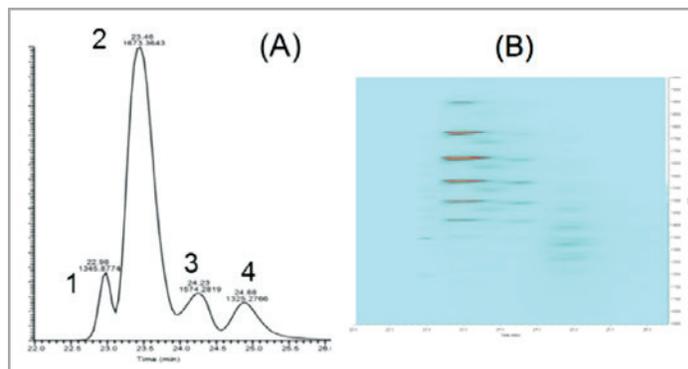


Figure 2. (A) Base ion electropherogram of PSA under denaturing conditions showing the 4 resolved peaks. (B) Corresponding ion density map demonstrates the CE migration and m/z patterns of the detected proteoforms.

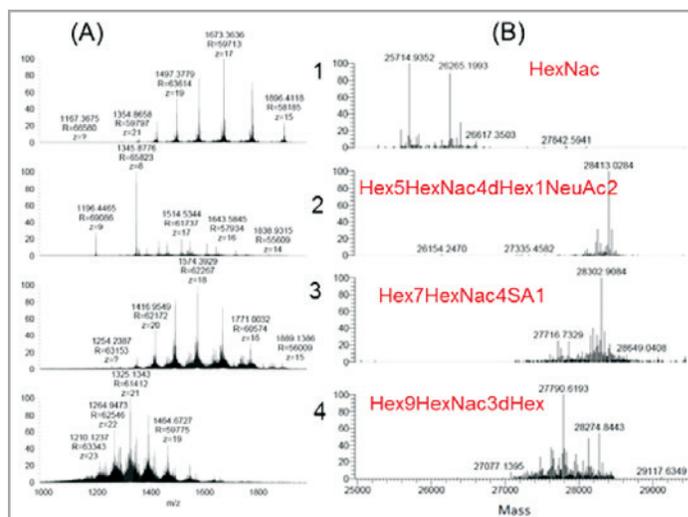


Figure 3. PSA profiling under denaturing conditions (panel A) and corresponding deconvoluted spectra (panel B).

CESI-MS under native conditions

Separation of PSA under native conditions yielded results similar to that of reduced PSA, suggesting that this separation is based on its hydrodynamic volume and charge distribution. CESI separation was performed using 40 mM ammonium acetate (pH 7.5) as the background electrolyte, resulting in 5 main peaks (Figure 4A). The corresponding ion density map again illustrated the complexity of PSA (Figure 4B). In this experiment, the amount of PSA injected was 2.9 ng, representing only 1.14% of the total capillary volume.

Analysis of the deconvoluted spectra (Figure 5B) for each peak revealed the presence of over 236 proteoforms, with the main sialylated biantennary isoform eluting in peak 2. The overall separation profiles under native and denaturing conditions are very similar, strongly suggesting that this separation is based on the hydrodynamic volume and charge distribution of the protein isotope envelope. Under native conditions, we confidently identified 38 glycoforms by HRAM MS1 spectra.

From the 56 identified main glycoforms, 19 species were common to the 3 approaches. By CESI-MS under denaturing and native conditions, we identified 8 and 12 unique glycoforms, respectively. In the infusion experiment, we detected only

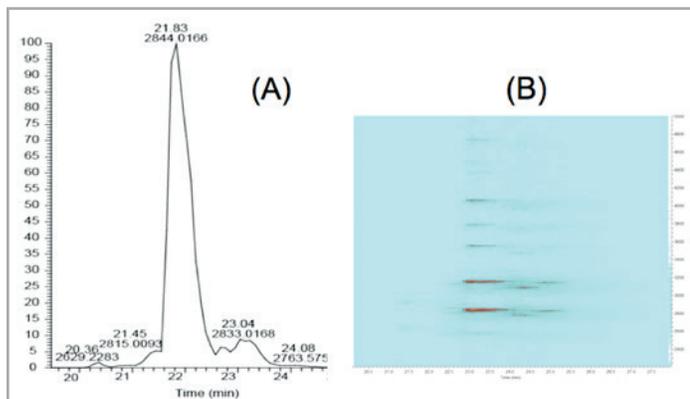


Figure 4. (A) Base ion electropherogram of PSA under native conditions, showing 5 distinct peaks. (B) Density ion map of a separation of PSA under native conditions highlighting the CESI migration patterns of proteoforms.

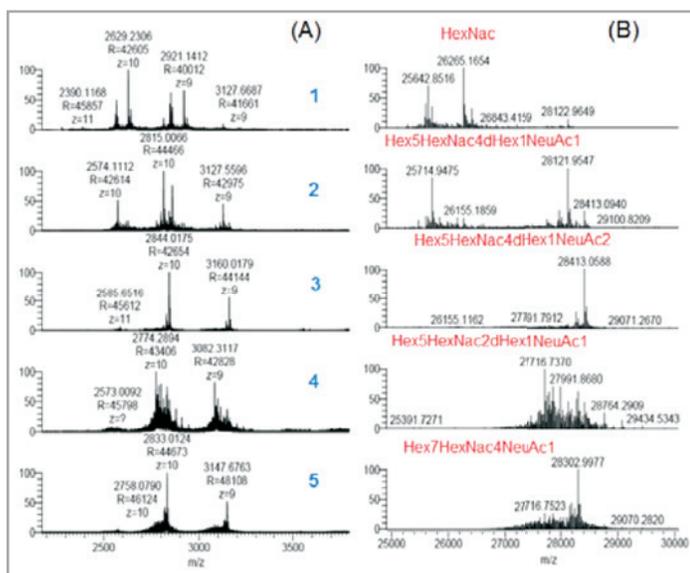


Figure 5. Mass spectra of PSA peaks under native conditions (panel A) and corresponding deconvoluted spectra (panel B).

6 unique species, although we expected to see many more glycoforms due to the much greater amount of sample injected. Since the infusion experiment was performed under native conditions in which the protein is still folded, it may not be exposed to enough protons to allow for adequate ionization. In the CESI-MS separation, however, even though less material was introduced into the capillary, the separation allowed for better ionization in the native state leading to a greater number of unique glycoforms detected. Additionally, no sample preparation was required other than sample dilution.

Infusion experiments

Using a direct infusion strategy, we characterized the PSA sample without (Figure 6A) and with (Figure 6B) all ion fragmentation (AIF) using the Exactive Plus EMR system. Fragment ions, mostly from N and C-termini, generated by AIF enabled PSA identification (Figure 6C). We achieved sequence coverage of only 10% because analysis was performed under native conditions, and PSA tertiary structure was likely conserved as it contains 5 disulfide bond interactions. This experiment allowed for the detection and quantification of only 127 proteoforms by MS1.

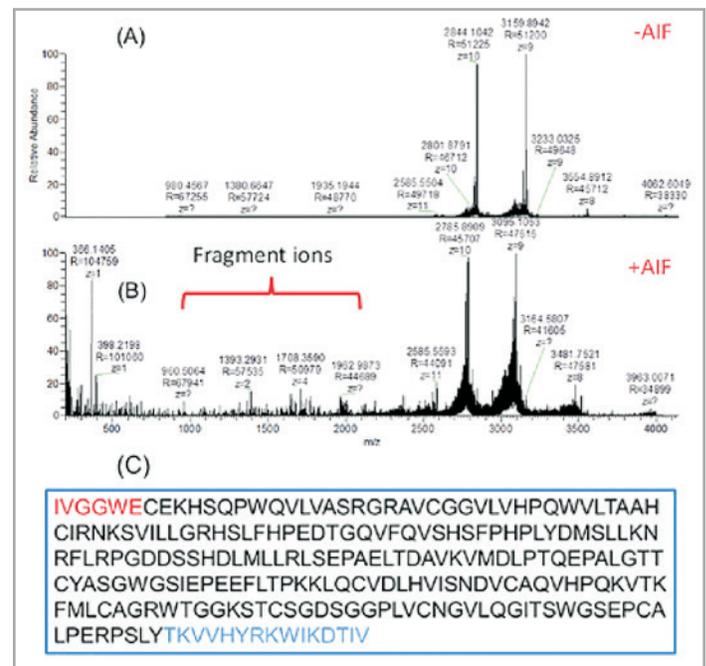


Figure 6. PSA nanoLC infusion analysis under native conditions. (A) MS1 spectrum. (B) AIF spectrum of PSA. (C) PSA sequence, identified b-ions (red) and y-ions (blue) are indicated.

Conclusions

- CESI is a powerful separation and ionization technique for MS analysis of intact glycoproteins under both native and denaturing conditions.
- The neutral coated capillary used in this work allowed for the separation of PSA proteoforms based on their structural heterogeneity.
- CESI-MS coupled with HRAM MS under both native and denaturing conditions offers significant improvement for the identification of glycoforms compared to infusion by nanoLC.
- In this study, we confidently identified and quantified 56 PSA glycoforms via intact/top-down MS analysis. We also identified 236 proteoforms vs. 127 identified by nanoLC-MS.
- The sample amount loaded in the CESI-MS experiments was nearly half of that used in a typical nanoLC infusion Experiment, and yet twice as many proteoforms were detected. This demonstrates the value of CESI as a front-end separation/ionization tool for these molecules.
- No special sample preparation was required other than dilution.

References

1. Leymarie, N. et al. *Mol Cell Proteomics*. 2013 Oct, 12(10), 2935–2951.
2. Viner, R. et al. *From Qualitative to Quantitative: The Evolution of Glycoproteomics*. HUPO 2013, Yokohama, poster 51.

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