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In-Source Charge Reduction Enhances Top-Down Characterization of Denatured Proteins

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Introduction

Denatured proteins often yield greater top-down sequence coverage compared to native structures.

However, denatured proteins exhibit signal dilution amongst a wide charge envelope which limits precursor abundance available for top-down MS[1].

Further, congested MS/MS spectra make it difficult to confidently assign fragmentation[2].

Here, we applied in-source chemical charge reduction to allow precise control over charge distribution to achieve native-like charge states from denatured proteins without using salt buffers such as ammonium acetate (Figure 1).

Using ion mobility, we investigate if charge-reduced proteins retain their extended structures, which is favorable for top-down analysis.

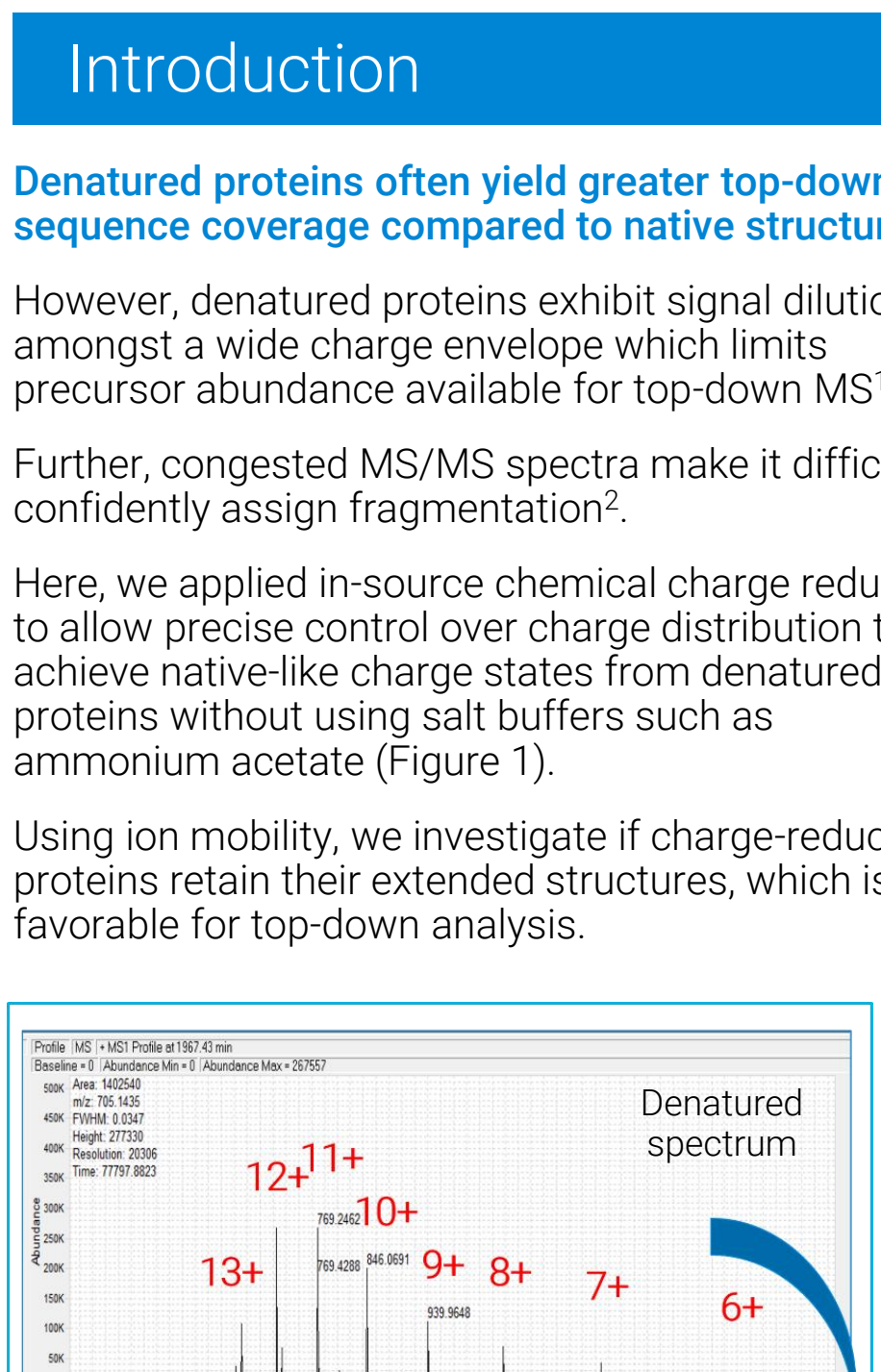

Figure 1- MS1 spectra of ubiquitin showing in-source charge reduction and then top-down fragmentation of the 3^+ precursor.

Experimental

Sample preparation

Ubiquitin, carbonic anhydrase and bovine serum albumin were obtained from Sigma Aldrich. Peirce Intact Protein Standard Mix was obtained from Thermo Fisher Scientific. Lyophilized purified proteins were obtained and reconstituted in 15% ACN with 0.1% formic acid for denatured experiments and 100mM ammonium acetate for native experiments.

In-source charge reduction

In-source charge reduction was accomplished by taking advantage of the dual nebulizers in the Agilent dual AJS electrospray ionization source. Protein samples of 1-10 µM were introduced using direct infusion or liquid chromatography while dimethylaminopropylamine (base) was infused through the second nebulizer. Protein charge distributions were controlled by modulating base flow rates. (Figure 2)

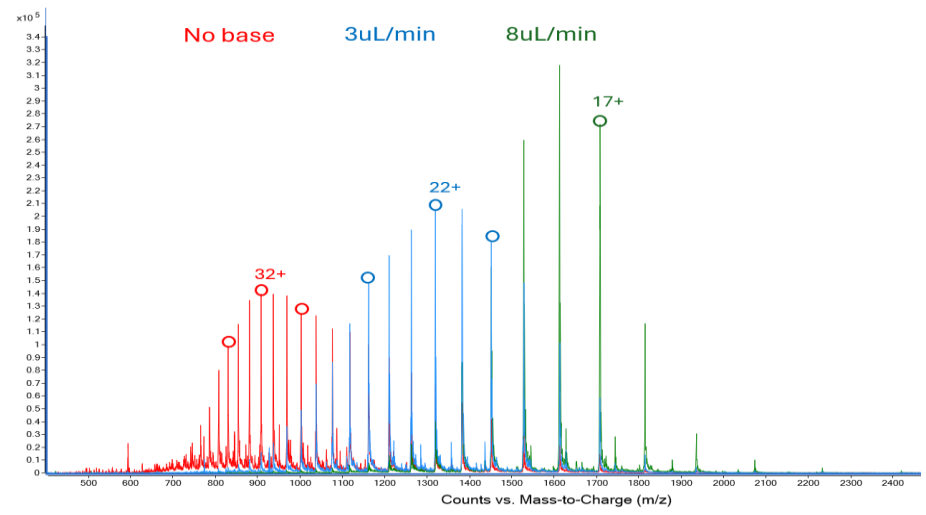

Figure 2- Intact spectrum of carbonic anhydrase through direct infusion with and without base. The charge states marked with circles indicate charge states that were isolated for top-down analysis.

Instrumentation

An Agilent 1290 Infinity II Bio LC System with an PLRP-S reversed phase column was used to separate protein mixtures. Top-down analysis was performed using the Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent ExD cell. An Agilent 6560 Ion Mobility LC/Q-TOF was used for protein drift time and collision cross section (CCS) measurements. All IM measurements were performed with the Drift Tube using nitrogen gas.

Software

Mass Hunter Acquisition software v 11.0 was used for data acquisition. Protein fragmentation patterns were analyzed using ExDViewer v 4.6.12. Mass Hunter Qualitative analysis 12.0 was used for visualizing charge shifts in the MS1 spectra. Agilent IM-Browser 10.0 was used for processing IM-data and Excel was used to create CCS plots.

Results and Discussion

Charge reduction of protein using direct infusion and denaturing chromatography

Reducing the charge envelope of a denatured proteins leads to decongestion of both MS1 and MS2 spectra.

In-source charge reduction achieves native-like charge states with excellent ionization efficiency and is compatible with both direct infusion (Figure 2) and denaturing standard flow chromatography (Figure 3-4).

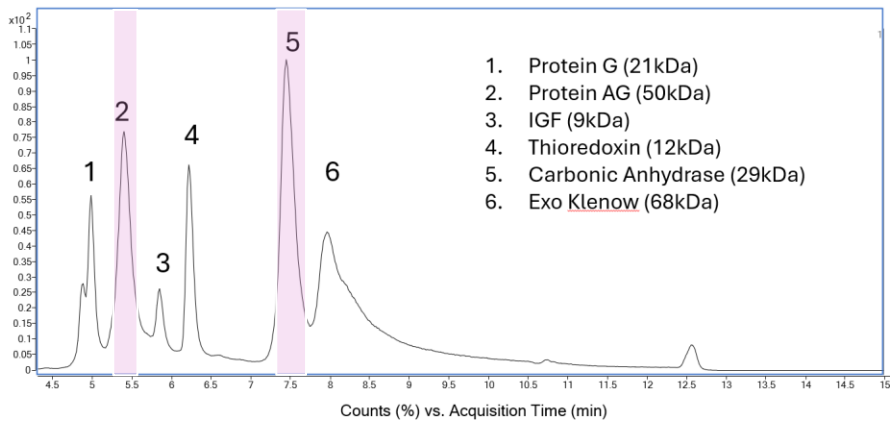

Figure 3- LC chromatograph of Pierce Protein Standard Mix under charge reducing conditions. The spectra from the signals highlighted in pink are shown in Figure 4.

Impact on top-down sequence analysis

Top-down analysis of charge reduced carbonic anhydrase (CA) precursors yielded comparable sequence coverage to denatured charge states (Figure 5).However, lower charge precursors produced cleaner MS2 spectra, improving IonScores (Figure 6). Combining fragmentation results from 29^{+}, 22^{+}, and 16+ precursors increased the total CA sequence coverage to 78% (Figure 7). This suggests that combining results from denatured and charge reduced precursors can enhance top-down coverage by isolating a wider range of abundant charge states.

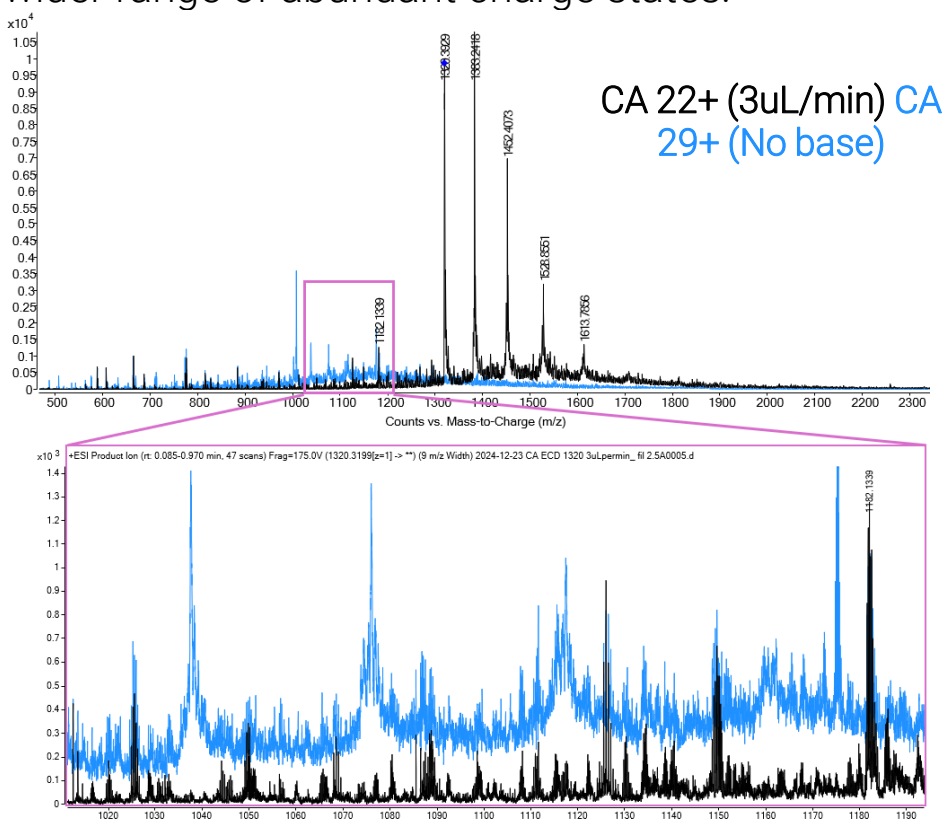

Figure 6- Overlaid ECD fragmentation spectra of 22^{+} (black) and 29^{+} (blue) carbonic anhydrase.

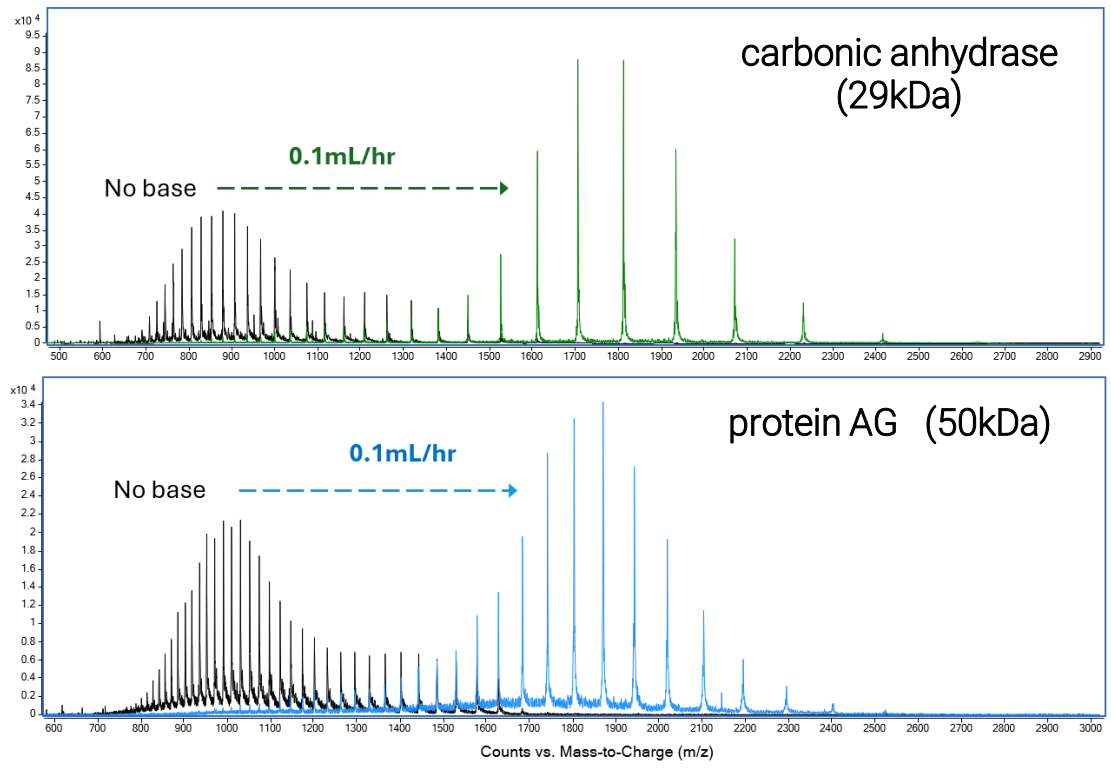

Figure 4- In spray charge reduction of proteins from LC eluant. Top spectrum shows reduction of carbonic anhydrase (29kDa) while the bottom spectrum shows reduction of protein AG (50kDa).

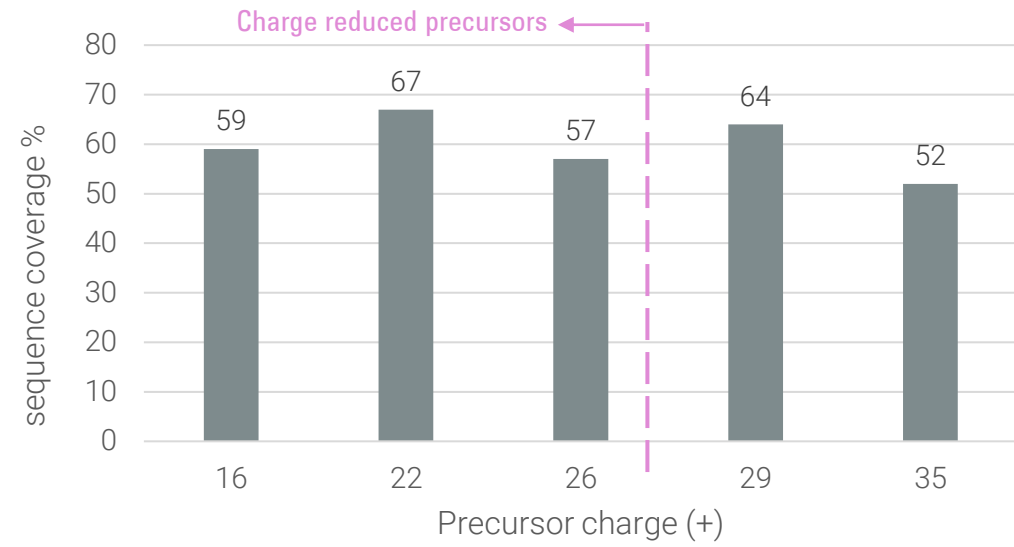

Figure 5- Carbonic anhydrase sequence coverage percent as a function of charge state.

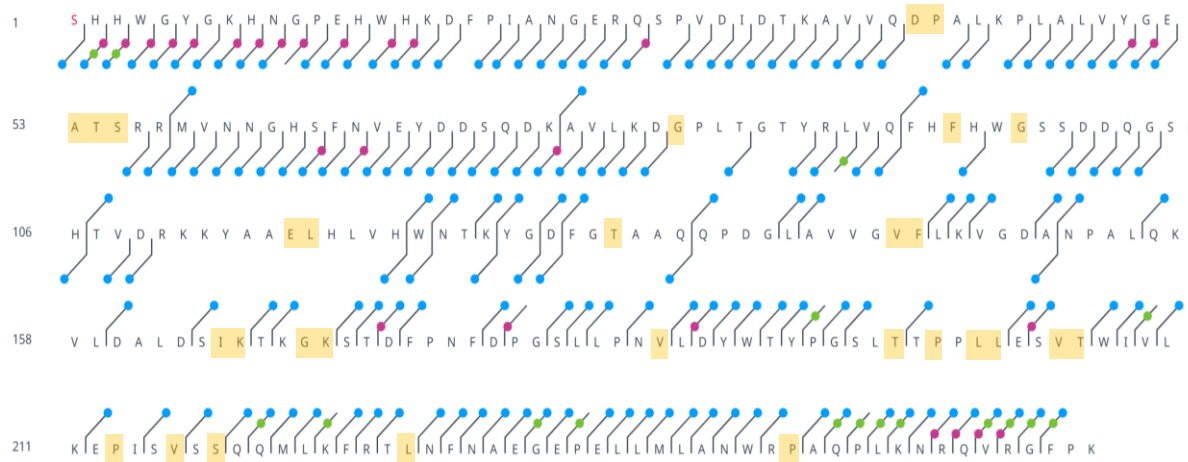

Figure 7- The sequence map of the 22^{+} carbonic anhydrase precursor. Yellow boxes illustrate cleavage sites that were unique to the 16^{+} and/or the 29^{+} precursor. Combining coverage from three precursors yielded 78% sequence coverage.

Results and Discussion

Structural analysis of denatured precursors before and after in-source charge reduction.

Figures 8 shows the CCS plots for CA 20^+ and BSA 25^+ charge states with and without base.

The charge reduced CA 20^+ CCS is 5973 +/- 200 $Å^2$ while structure before reacting with base was 5171 5973 +/- 100 $Å^2$ angstrom. A similar trend is seen for BSA 25^+, where the charge reduced structures are more elongated.

After reacting with base, the initially elongated protein structures of higher charge states are retained for a rather long time.

The rate of structural changes depends on the protein size: the larger the protein the higher probability for the elongated structure to survive inside the mass spectrometer.

Native and charge reduced precursor structure comparison

Figure 9 compares charge reduced structures to native-like structures for 11^+ carbonic anhydrase. These results demonstrate that charge reduced precursors have more elongated structures compared to native structures.

Using collision induced dissociation (CIU), we investigated the unfolded structures of native CA. The maximally unfolded structure of 11^+ CA was recorded at a collision energy of 350V.

Charge reduced precursors have CCS values that are similar to the maximally unfolded CCS value of 11^+ CA obtained with collision induced unfolding.

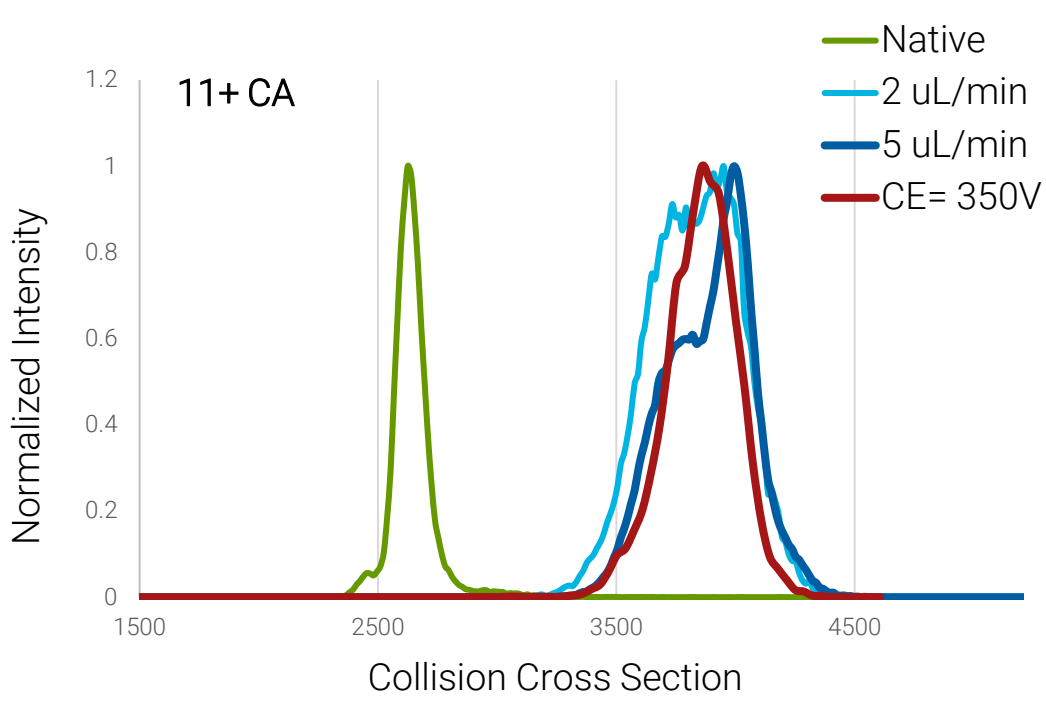

Figure 9- CCS plots for 11^+ carbonic anhydrase under native-like (green) and charge reducing conditions (light/dark blue). The maximally unfolded structure obtained through CIU is plotted in red.

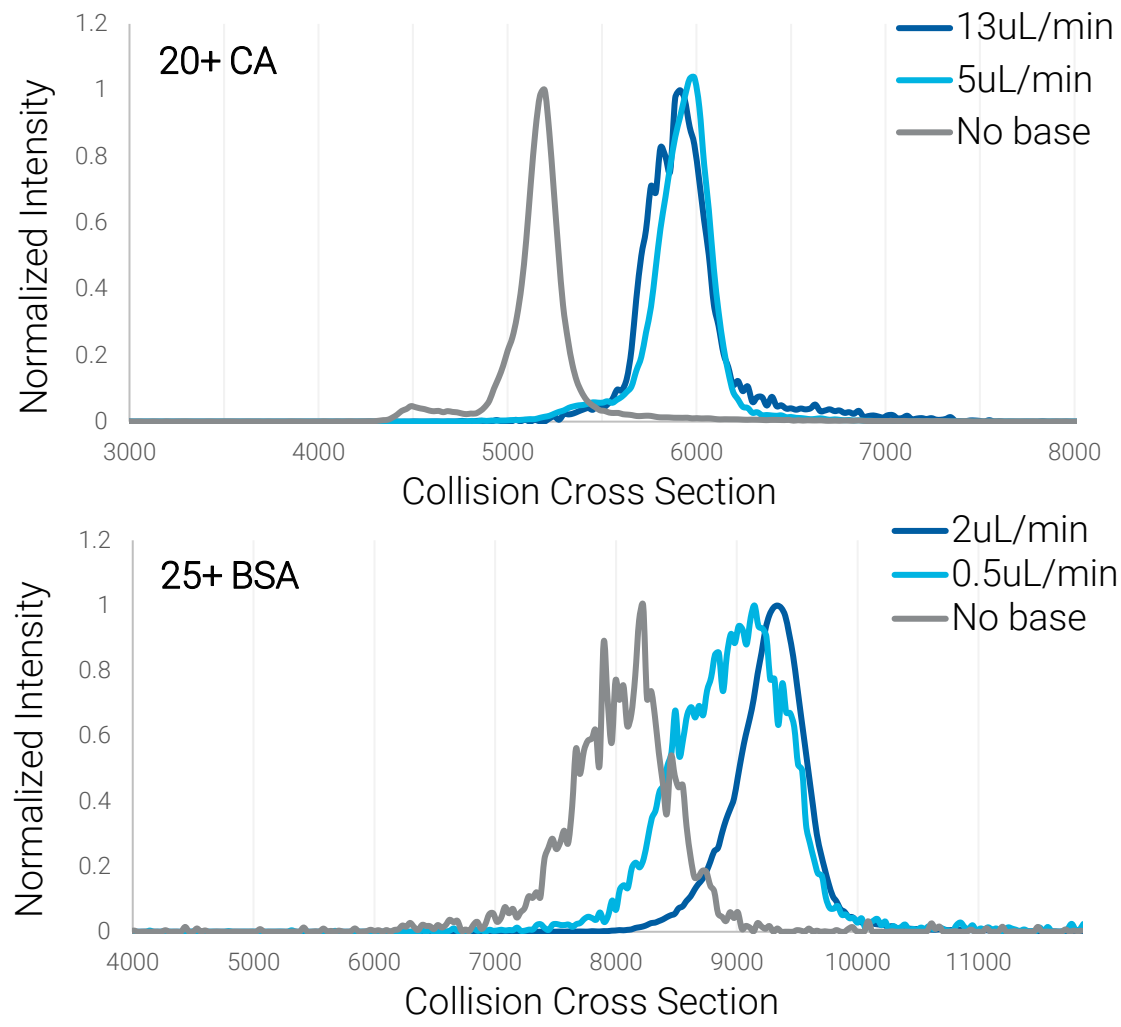

Figure 8- CCS plots for 20^+ carbonic anhydrase (top) and 25^+ bovine serum albumin (bottom) under denatured and charge reduced conditions.

Conclusions

In-source charge reduction enables precise control over protein charge distributions using direct infusion or chromatography.

Charge reduced precursors yield rich ECD fragmentation spectra with improved S/N compared to denatured precursors.

Combining ECD results from denatured and charge reduced precursors improved TD sequence coverage.

CCS analysis using ion mobility reveals that charge reduced precursors retain their extended structure, which are similar to the maximally unfolded structures obtained via CIU.

References

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Conflict of Interest Disclosure

Authors are employees of Agilent Technologies, which sells the instrumentation used in this analysis. As such, the authors disclose a potential conflict of interest, in respect to their commercial involvement.

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