In-Depth Characterization of Intact Protein Standards Using HRAM Top Down Mass Spectrometry with Multiple MSMS Strategies

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ABSTRACT

Purpose: Demonstrate unique characteristics and effectiveness of various dissociation mechanisms for intact protein identification and characterization.

Methods: Collection and analysis of high resolution CID, HCD, ETD, and UVPD data on various proteins at various energies or reaction times.

Results: Each fragmentation mechanism generates unique data that, together, maximizes sequence coverage for improved protein identification and proteoform characterization. Considerations for optimizing each dissociation mechanism with respect to proteins representing a MW range from 9kDa to 50kDa are presented.

INTRODUCTION

Complete and accurate characterization of intact proteins by mass spectrometry is both possible and increasingly popular today thanks to the latest technological developments made in LC and MS hardware, instrument control software, and data processing software. Here we demonstrate the dissociative behavior of four proteins from the recently released Pierce™ Intact Protein Standard Mix representing a MW range of 9kDa to 50kDa, with four different modes of ion dissociation (CID, HCD, ETD, and UVPD) available on the Orbitrap™ Fusion™ Lumos™. For each dissociation mode, we test three different normalized collision energies or reaction/ irradiation times. We aim to illustrate attributes of each of these modes on intact proteins, and ultimately inform method development for top down proteomics applications. While we focused here was on single mode techniques to highlight the specific uniqueness of each mode of dissociation, mixed mode dissociation techniques (ex. EThcD) are also available and can be highly beneficial for both identification and structural characterization.

Ion trap CID employs m/z selective slow heating to produce b- and y- type product ions via many low energy-imparting collisions with He atoms, resulting in minimal secondary dissociation of product ions. This is advantageous, unless post translational modification (PTM) loss is the primary fragmentation pathway. HCD also produces b- and y- type ions through “fast heating” induced relatively fewer, but higher energy-imparting collisions with N_2 gas molecules in a non-m/z selective manner. This makes subsequent over-fragmentation of product ions a risk, but also overcomes the limitation presented by primary loss of labile PTMs. By contrast, ETD generates c- and z- type ions through the abstraction of electrons from a donor reagent anion. To accommodate the resulting radical site, the cation almost instantaneously undergoes rearrangement leading to bond cleavage without internal energy transfer. As such, PTMs are preserved by this mode of dissociation. Intact charge reduced dissociation products from lower charge state precursors can at times dominate spectra, however mild activation of these species through techniques such as EThcD can overcome this limitation. Finally, UVPD, the most recently introduced mode of dissociation on the Orbitrap™ Fusion™ Lumos™ is initiated by irradiation of the precursor ions with photons from a 213nm UV laser, proceeds though multiple dissociation pathways. This results in formation of a-, b-, c-, x-, y-, and z- type fragment ions, many only observed with this mode of dissociation. .

MATERIALS AND METHODS

Pierce intact protein standard mix (A33526) was purchased from Fisher Scientific and each vial was reconstituted in 100ul HPLC grade water prior to use. Proteins were separated over a 20minute gradient (**Figure 1a**) at 200ul/min using a Dionex Ultimate 3000 UHPLC system fitted with a 2.1 mm MabPac™ RP LC column. Solvent A was 0.1% formic acid in LCMS grade water (Fisher Scientific LS118-1) and solvent B was 0.1% formic acid in LCMS grade acetonitrile (Fisher Scientific LS120-1). Full scan MS data was collected at 15k resolution in the Orbitrap, with alternating targeted MS2 scans at either 60k (CID, HCD) or 120k resolution (ETD, UVPD). A single charge state of each protein near the center of the charge envelope was selected at random for isolation and fragmentation. As such, precursor charge state selection within a protein is not considered here, though it can be a major variable affecting extent of dissociation. In all cases, precursors were isolated by the quadrupole using a 3Da window. For ETD, anion target value was reduced to 5e4 to reduce reaction kinetics in an attempt to avoid over-fragmentation of large highly charged precursors. Data was collected in a targeted fashion, and MS2 were manually averaged, then deconvoluted using Xtract in QualBrowser. Xtracted raw files were submitted to ProSightPC 4.1 for fragment ion assignment. The Pierce Intact Protein Standard Mix database (.pscw) was downloaded directly from the Proteinaceous database warehouse (http://proteinaceous.net/database-warehouse/).

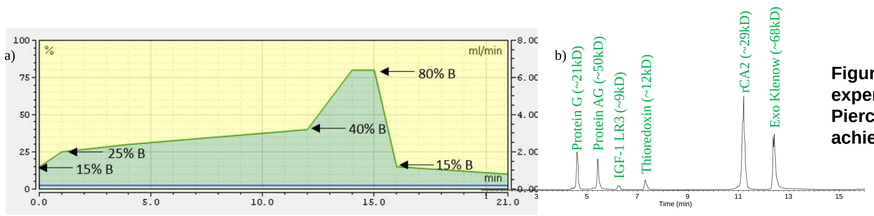

Figure 1. a) Gradient profile used in experiments. b) typical chromatogram for Pierce intact protein standard mix achieved using settings described above.

RESULTS

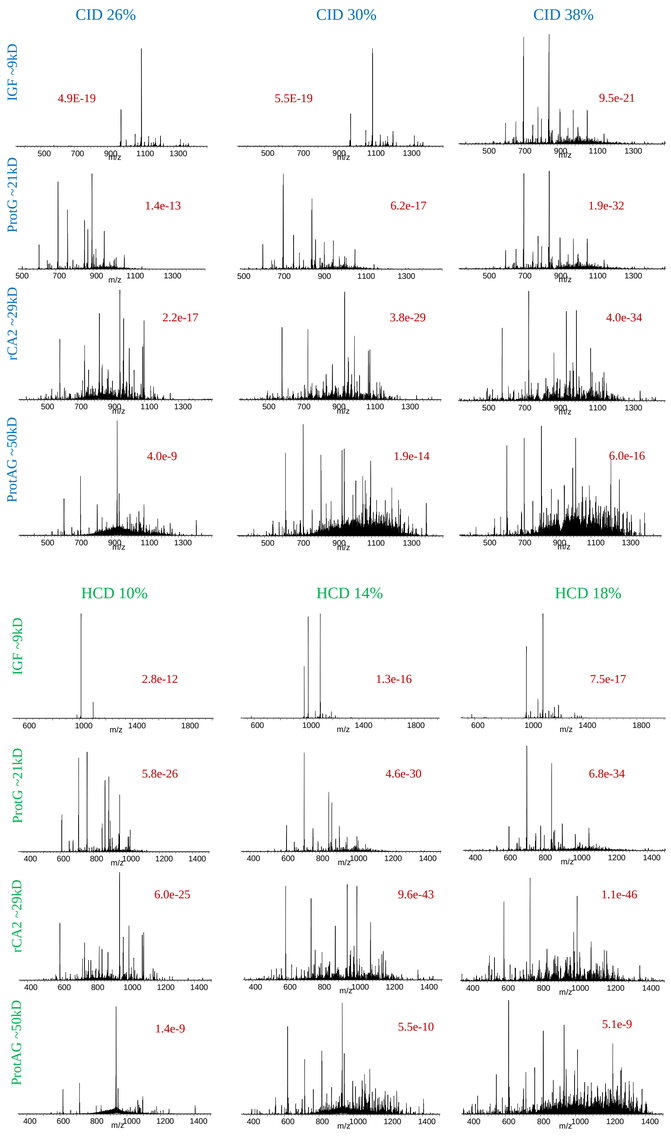

Figure 2: CID analysis of 4 different proteins ranging in MW from 9kD to 50kD, at 3 different collision energies. Inset numbers in red are ProSightPC P-scores.

For top-down analysis if non-modified intact proteins, CID provides the benefit of limited secondary fragmentation, over-fragmentation, and formation of internal fragments that is consistent across the mass range.

Figure 3: HCD analysis of 4 different proteins ranging in MW from 9kD to 50kD, at 3 different collision energies. Inset numbers in red are ProSightPC P-scores.

Provided that energies are carefully chosen to avoid over-fragmentation, this mode of fragmentation is efficient across the mass range, and provides well resolved fragments regardless of presence of PTMs.

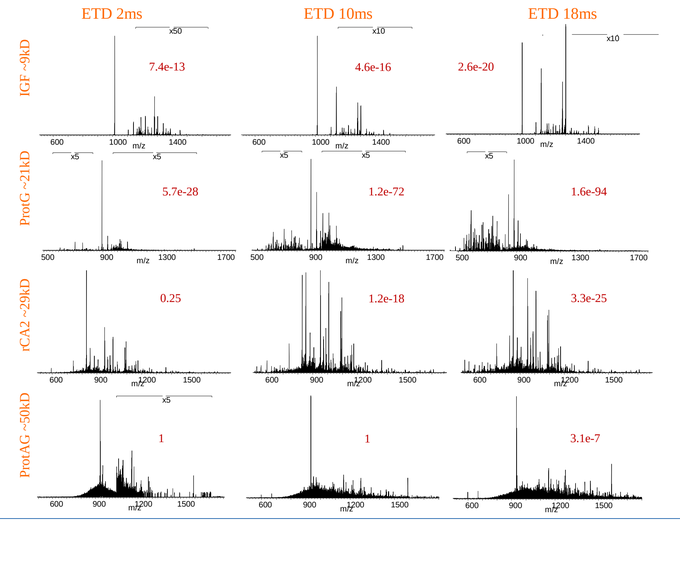

Figure 4: ETD analysis of 4 different proteins ranging in MW from 9kD to 50kD, at 3 different reaction times. Inset numbers in red are ProSightPC P-scores.

ETD spectra of the smaller proteins are extremely rich, however because reactions proceed at rates proportional to the square of the precursor charge state, overfragmenation of larger proteins is common and evidenced by the high, unresolvable baseline seen in all ProtAG spectra.

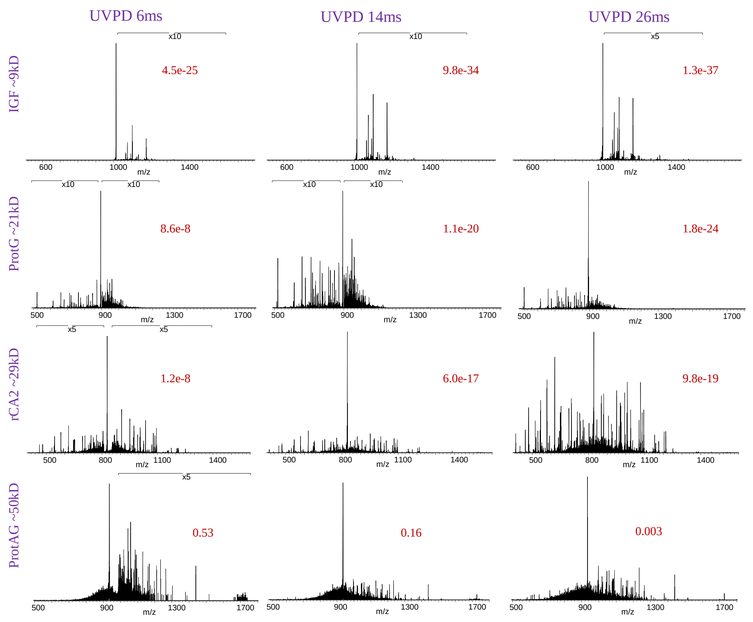

Figure 5: UVPD analysis of 4 different proteins ranging in MW from 9kD to 50kD, at 3 different irradiation times. Inset numbers in red are ProSightPC P-scores.

Dissociation here happens at a speed proportional to the MW of the precursor, and as such we see rich spectra produced for the smaller proteins, but a high unresolved baseline for ProteinAG, indicating overfragmentation.

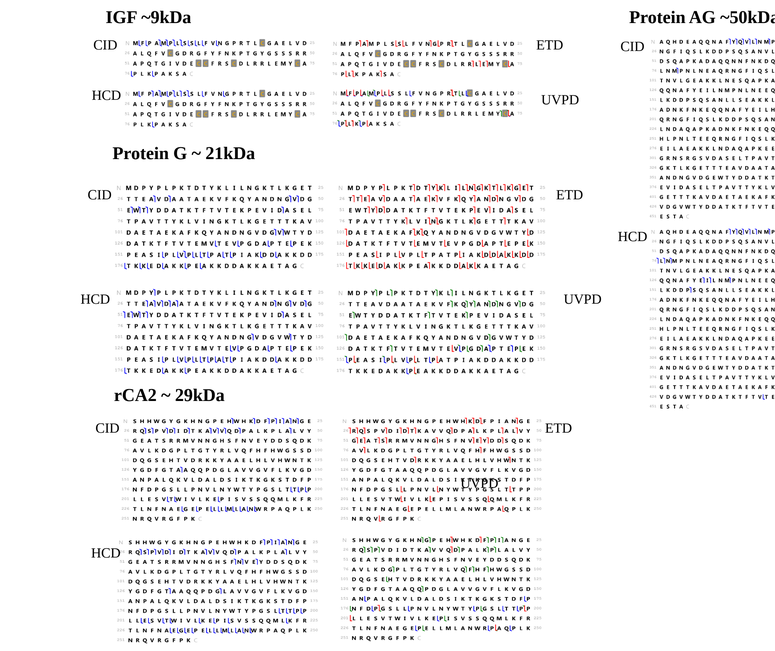

Figure 6: Sequence coverage maps for each of 4 proteins analyzed by each of the 4 modes of dissociation. Each map represents the results from the spectra to the left with the best P-score.

Discussion

The versatility afforded by the Orbitrap™ Fusion™ Lumos™ with respect to the multiple types of available dissociation modes is a clear advantage for top down analyses. Additionally, the Pierce Intact Protein Standard Mix provides an ideal sample for method development (both data acquisition and data analysis) and quality control. In using this sample for method optimization we have highlighted both strengths and weaknesses of our current technology. We are able to obtain extensive sequence coverage for the proteins in the sample up to 30kD on a chromatographic time scale. We do, however, still struggle with MS2 analysis of larger proteins. Multiple challenges contribute to this problem. First, by both ETD and UVPD, larger proteins dissociate much faster than smaller proteins, whether due to their higher charge state, or higher cross section, respectively. In this work, we decreased the anion target value in an attempt to reduce ETD reaction rate (the kinetics of the ETD reaction as we perform it here are first order with respect to anion concentration) and minimize over fragmentation of Protein AG (50kD), though this helped only marginally. Other ion manipulation techniques such as ion parking have been shown to address this problem. Second, larger proteins can of course fragment at more positions, thereby diluting signal among more potential product ions. CID and HCD benefit here from preferential fragmentation at weaker bonds, concentrating signal to fewer possible product ions. Because ETD and UVPD are democratic in their bond cleavage, this is a significant challenge that currently can only be overcome with significant signal averaging. **Figure 7** demonstrates the efficiency of ETD and UVPD on enolase, a 46kD protein, when ~500 transients are averaged.

An added challenge presented by over fragmentation is the production of internal ions. These low abundance, unresolved overlapping product ions create a high baseline that varies across the m/z range. Deconvolution algorithms generally use the averagine model to assign monoisotopic mass, but the large number of overlapping peaks confound such algorithms due to experimental isotopic distributions that deviate too far from theoretical. We continue to work toward addressing these challenges

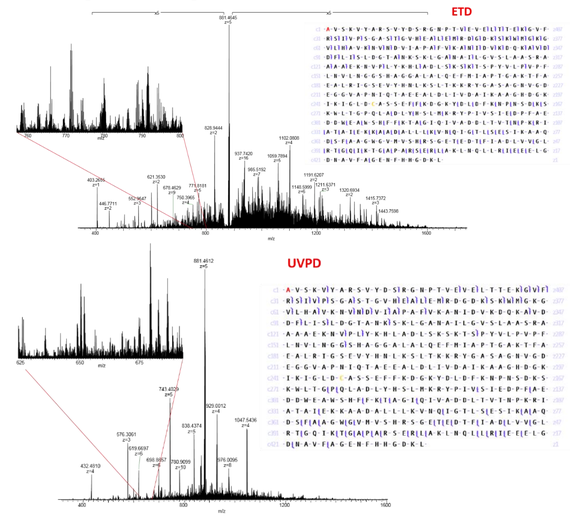

Figure 7: ETD and UVPD of enolase (~46kD); ~500 averaged transients.

CONCLUSIONS

- The multiple modes of dissociation available on the Orbitrap™ Fusion™ Lumos™ present a clear advantage for intact protein identification and characterization, enabling extensive sequence coverage and PTM mapping capabilities.
- The Pierce Intact Protein Standard Mix is an ideal sample for top down method development, optimization, and quality control.
- Many challenges remain in top down analysis, particularly with respect to large proteins. We are actively working to address these.

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ACKNOWLEDGEMENTS

The authors would like to acknowledge John E.P Syka, David Horn, and Tara Schroeder for helpful discussion.

TRADEMARKS/LICENSING