193 nm ultraviolet photodissociation for the characterization of singly charged proteoforms generated by MALDI

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ABSTRACT:

MALDI imaging allows for the near-cellular profiling of proteoforms directly from microbial colonies, or plant, and mammalian tissues. Despite detecting hundreds of proteoforms, identification of unknowns with only intact mass information remains a distinct challenge, even at with high resolution and mass accuracy. To this end many supplementary methods have been used to create experimental databases for accurate mass matching, including bulk or spatially resolved bottom-up and/or top-down proteomics. Herein we describe the application of 193 nm ultraviolet photodissociation (UVPD) for fragmentation of quadrupole isolated singly charged ubiquitin (m/z 8565) by MALDI-UVPD on an UHMR HF Orbitrap. This platform permitted the high-resolution accurate mass measurement of not just terminal fragments, but also large internal fragments. The outlined workflow demonstrated the feasibility of high resolution top-down analysis of MALDI generated protein ions and the potential towards more comprehensive characterization of proteoforms in MALDI imaging applications.

INTRODUCTION:

Matrix-assisted laser desorption/ionization (MALDI) is a powerful technique for generating low charge state biomolecules from droplet preparations, cultures, or biological tissues. Despite the popularity of MALDI for protein analyses, application and outcomes of intact protein mass spectrometry imaging (MSI) are limited due to the challenge of detecting and annotating protein ions. While time-of-flight (TOF) and

Fourier transform ion cyclotron resonance (FTICR) are commonly used mass analyzers that have both been applied to analyze intact proteins.[1] High mass resolution measurements by FTICR, and recently ultra-high mass range (UHMR) Orbitrap,[2, 3] offer advantages over TOF for more precise identification of proteoforms, distinct forms of proteins potentially harboring multiple post-translational modifications (PTMs).[4, 5]

However, assigning proteoforms solely based on accurate mass can be challenging regardless of the analyzer employed, and often requires supplemental analyses.[6] For example, top-down proteomics (TDP) can deliver accurate mass databases for peak annotation in MALDI; however, proteome coverage between methods may not significantly overlap due to different preparation requirements. Thus, direct fragmentation of ions generated by MALDI is appealing, especially for confident annotations of unknowns in MSI applications. However, traditional dissociation techniques exhibit a charge-state dependence and/or inefficient fragmentation with increased mass.

While MALDI in-source decay (ISD) delivered exciting results,[7] the technique lacks a mass isolation step and thus proteoform selectivity, which can be problematic for complex mixtures. Commonly used activation methods (*e.g.,* collision or electron based) are generally difficult to apply to singly charged protein ions generated by MALDI.[8, 9] Alternatively, photon-based methods such as ultraviolet photodissociation (UVPD) and infrared multiphoton photodissociation (IRMPD) have been demonstrated as viable TDP techniques.[10, 11] UVPD has also been suggested to be less sensitive to precursor charge state and thus has potential for MALDI applications,[12] but UVPD has been almost exclusively applied for electrospray generated ions.[13] First demonstrated three decades ago, 193 nm MALDI-UVPD of protein ions showed great promise.[14] Herein, we report application of MALDI-UVPD to singly charged ubiquitin. We have noted significantly improved peak assignments with the high mass resolution and accuracy offered by the UHMR Q Exactive HF Orbitrap compared retrospectively. This proof-of-principle study demonstrates the potential to further develop the platform to increase the sensitivity and expand the UVPD to other proteoforms for MALDI-MSI applications.

METHODS:

An elevated pressure MALDI source from Spectroglyph (Kennewick, WA)[15] was mounted on a Thermo Scientific Q Exactive HF Orbitrap MS upgraded with UHMR boards and operated under custom privilege licenses, as described in great depth elsewhere.[2] Detailed notes for instrumental parameters and sample preparation are presented within Supplementary Information. Briefly, the spectrometer was operated under default parameters with several specifically optimized for MALDI-UVPD. For all experiments the source and instrument parameters remained the same; where 500 MALDI laser shots were acquired per microscan, with 5 microscans coadded prior to averaging of a total of 1500 microscans. UVPD implementation was accomplished with a 193 nm excimer laser (ExciStar XS 200, Coherent, Santa Clara, CA) triggered externally from 'IonGun' experiments,[16] and one excimer laser pulse was used per microscan. Optics for alignment were housed within a light tight enclosure (Supplementary Figure S1), and the measured attenuation of laser pulse energies was 73% (Supplementary Figure S2); pulse energies noted throughout the text are the set energies within the laser control software.

Raw spectral data resultant from 1500 microscan averages is located within Supplementary Information for further inspection. Here, spectra were annotated and visualized in LcMsSpectator (v1.1.7158.24217);[17] all ion types (a, b, c, x, y, z) and side chain fragment ions (d, v, w) were annotated with possible neutral losses of water or ammonia with a minimum signal-to-noise ratio (SNR) of 5, Pearson correlation of 0.8, and relative intensity threshold of 3. Heat maps of fragment ion types were plotted using results from Xtract deconvolution and UV-POSIT assignment.[18] ClipsMS was used to evaluate internal fragmentation after Xtract processing in Freestyle (v.1.8 SP1).[19] All fragment types were searched for a minimum of 20 amino acids. All analyses used a mass tolerance of 5 ppm.

RESULTS AND DISCUSSION:

Previously, MALDI-UVPD of singly charged ubiquitin was completed by MALDI TOF/TOF, but despite averaging 30000 experiments the spectrum was poorly annotated.[20] Indeed, high baseline from chemical noise is observed in the previous report,[20] likely a result of spectral congestion or metastable decay. Here, MALDI-UVPD allowed for dissociation of ubiquitin with a conservative annotation of sequence coverage at 86.7% (SNR of 5) as shown within Figure 1A for 3.0 mJ experiments (Figure 1A). As seen within the inset zooms (Figure 1B and 1C), the mass range (*m/z* 4000-8500) is dense in fragment ions, but the resolving power and increased time scale for detection afforded by UHMR HF Orbitrap permitted isotopic resolution throughout the spectrum (Supplementary Figure S3). Less conservative annotation of the spectrum using a reduced threshold (SNR of 3, Pearson correlation of 0.7) resulted in higher sequence coverage at 96% for the same 3.0 mJ experiment; but is likely to also annotate isotopic noise. In contrast, using higher-energy collisional dissociation (HCD) with collision voltages less than 200 V negligible fragmentation of the precursor was observed, and collisional energies greater than 200 V afforded up to 8% sequence coverage (Supplementary Figure S5). These current results demonstrate a breakthrough for intact protein MALDI-UVPD, with high levels of sequence coverage generated per unit time (Supplementary Figure S5 and S6), and high levels of coverage are obtained from only 100 averaged acquisitions (Supplementary Figure S6, analyzed area of 0.09 mm^2 in 51.2 seconds).

Furthermore, these high *m/z* regions (Figure 1B and 1C) contained nearly continuous series of isotopic peaks. Many cannot be matched to N-/C- terminal ions but are presumably overlapping internal fragments with variable neutral losses. Considering internal fragments, over-fragmentation is a concern (Figure 2), occurring readily above 4.5 mJ with an observable decrease in fragment ion counts. Inspection for internal fragments (Supplementary Figure S7) also showed complete coverage above 3.5 mJ, but given the large number of theoretical fragments, false positives are likely, and our current analysis solely relied on mass accuracy. Several high intensity fragment ions also formed hot spots (Figure 2), where b/y ions were at acidic or proline residues (y37 D/Q; y44 D/K; y39 P/D; y40 P/P; y55 D/T; y58 E/P; b52 D/G), which are well-recognized and consistent with recent statistical analysis of TDP datasets.[21, 22] Further investigation of the fragmentation mechanisms for singly charged protein ions may help us better annotate the unassigned fragments, where charge state dependent structures have been confirmed recently,[23] dependencies of MALDI-UVPD have not been explored for singly charged proteins. Regardless, we demonstrate UVPD can feasibly be accomplished for characterizing intact proteoforms desorbed and ionized by MALDI.

Distinct differences in proportions of all fragment ions produced from MALDI generated ions can also be noted (Figure 3) in comparison to 193 nm UVPD of low-charge state ubiquitin produced through electrospray methods.[24] As expected, for MALDI-UVPD above 3.0-4.0 mJ, decreases in ion type counts were observed for a/x and b/y fragment ions, and increased counts were noted for side chain fragment ions (d, v, w). Photodepletion of the precursor was also observed at only 32% for the 3.0 mJ experiments (Supplementary Figure S8), opening several avenues for advanced experiments such as inclusion of multiple laser shots per acquisition. However, advanced modulation of the ion packet may be needed as benefits can be limited.[25] Ultimately, the scope and upper bounds of MALDI-UVPD is not currently known, and to date our UHMR Q Exactive HF has not detected a proteoform larger than 17 kDa from tissue. Further development of the instrument, and MALDI-UVPD will offer both increased sequence coverage and reduced experimental time, yet in the most basic form broad utility for MALDI applications has been demonstrated.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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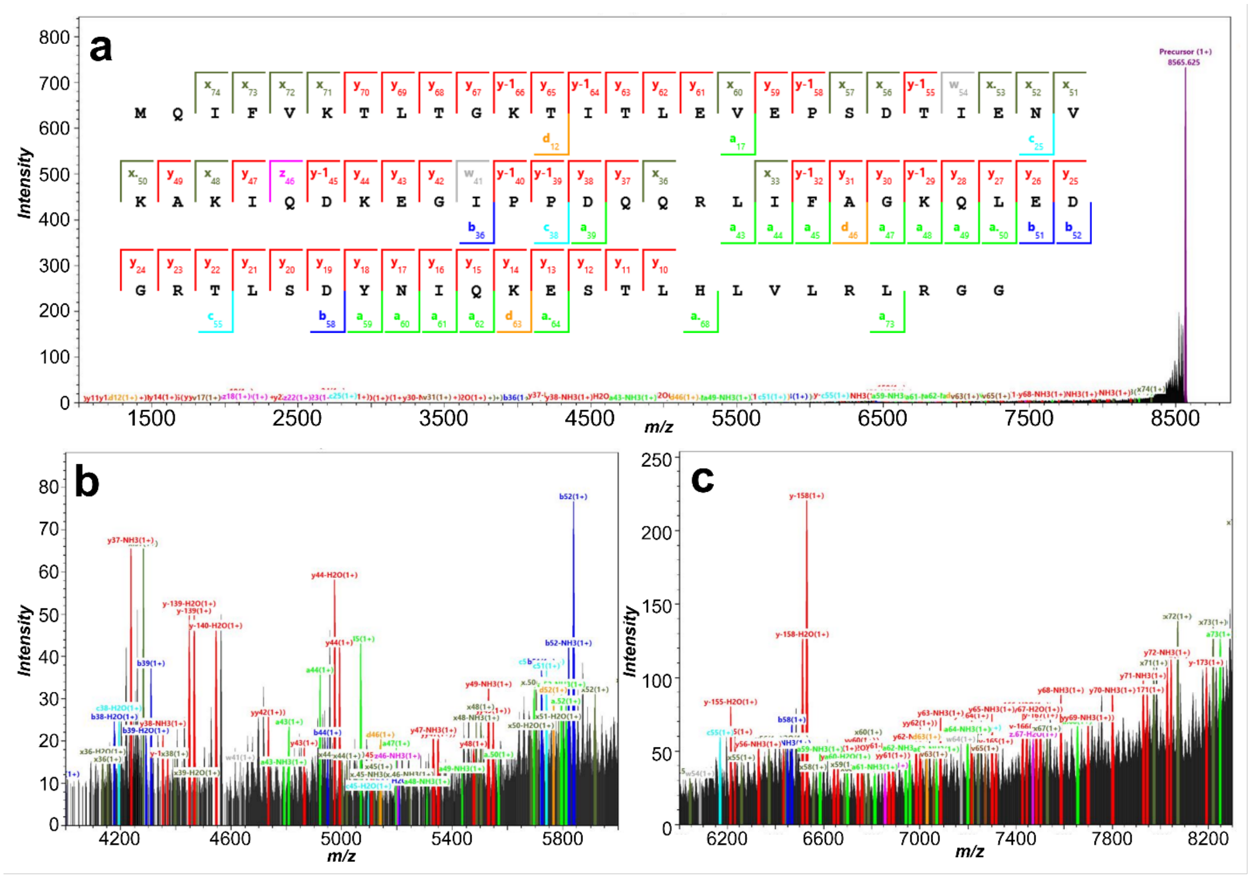

Figure 1: (a) The annotated coverage map within the average spectrum of 1500 single 3.0 mJ pulse MALDI-UVPD acquisitions for ubiquitin (SNR of 5, Pearson correlation of 0.8); where the precursor was identified at -1.48 ppm and ion types were manually validated. The overall sequence coverage was observed at 86.7%, however, this does not account for internal fragmentation pathways and annotation was conservative with higher signal to noise and isotope correlation filters. A zoomed inset from *m/z* 4000 to 6000 is shown in **(b)** and an inset from m/z 6000 to 8300 is shown in **(c)** from the overall average spectrum. Non annotated internal fragments can be seen within both zoomed views.

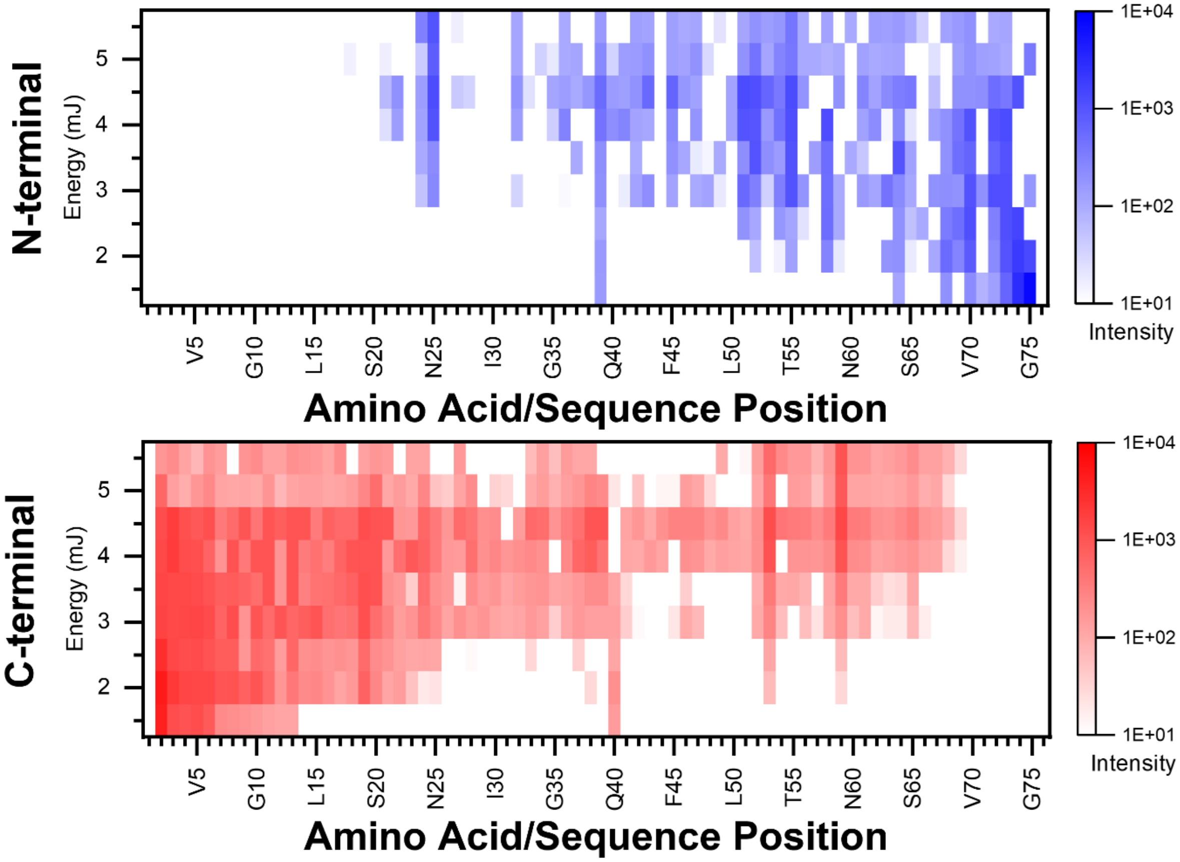

Figure 2. Heat map of fragment ion coverage where possible neutral losses of water or ammonia were investigated and plotted against the position within the sequence of ubiquitin. Fragment ion types (a, b, c or x, y, z) from the N- or C-terminus, respectively, were summed for all MALDI-UVPD experiments with 1500 averages from 1.5 to 5.5 mJ pulse energy. Distinct areas of hotspots can be identified within the heatmap signifying the relative intensity of the fragment ions within the UVPD experiments.

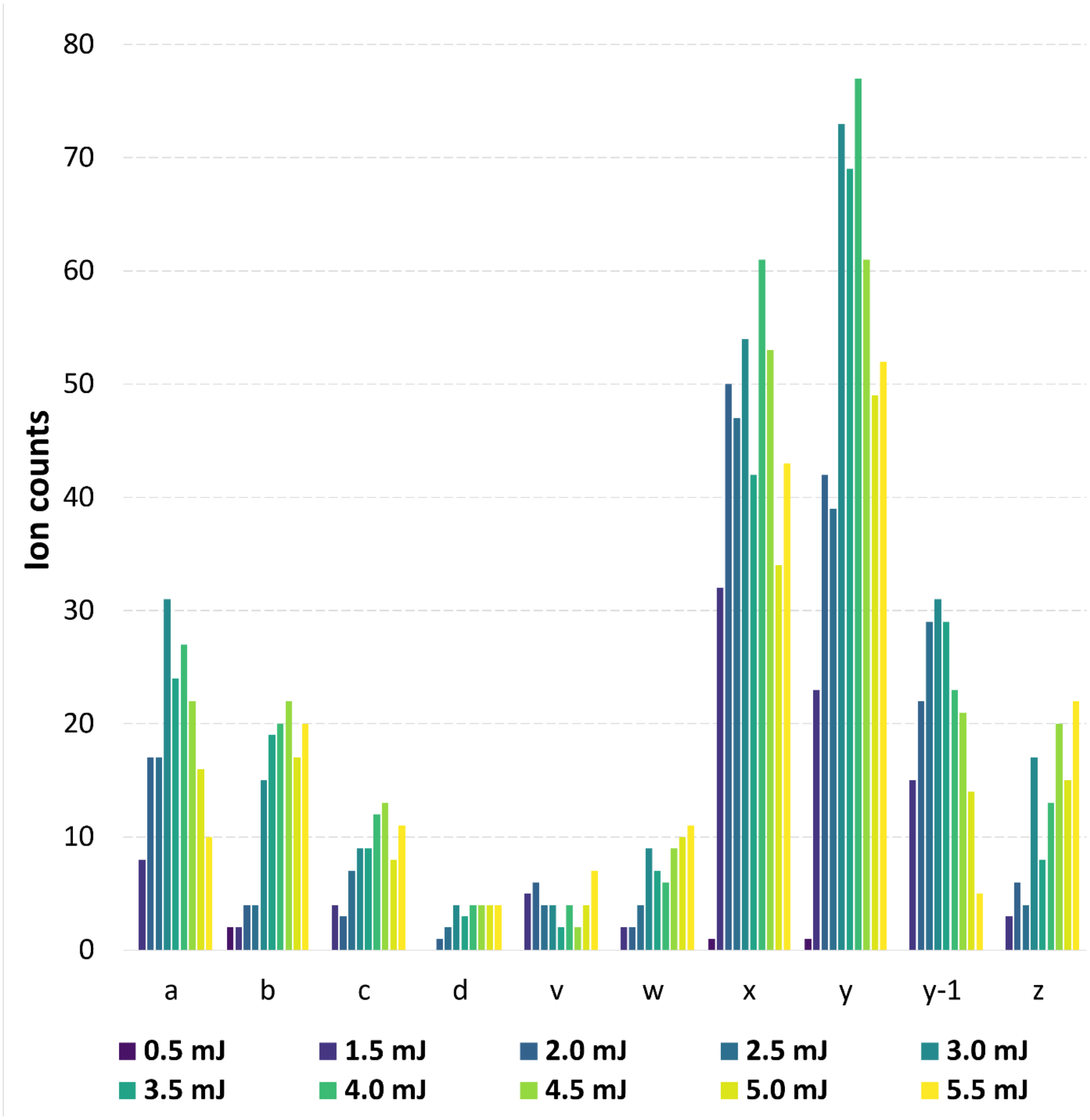

Figure 3. Annotation of spectra for each laser pulse energy used for MALDI-UVPD experiments with 1500 microscan averages. The distribution of the ubiquitin fragment type ions annotated were inspected and plotted. While side chain fragments (d, v, and w) increase with laser pulse energy and the same trend is observed for c/z type ions, a/x fragment ion counts reduce with higher fluence.