thermoscientific

Quantitative Top-Down Proteomics of Human Tears Reveals Proteoform Changes Related to Age

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ABSTRACT

Purpose: Apply newly developed Top-down Label Free Quantitation (LFQ).to measure proteoform level changes between age groups from human tear fluid.

Methods: Tear fluid was collected using simple non-invasive Schirmers test strips. Samples were then submitted to Top-down LC/MS analysis with (Ultraviolet Photodissociation at 213 nm) UVPD enabled fragmentation. Resulting data was analyzed via ProSightPD 4.0 (beta) for determination of quantitative changes in human tear proteoforms associated with patient age.

Results: Due to numerous crosslinked species observed in tear fluid, UVPD was selected for fragmentation of these proteoforms. A significant portion of the identified proteoforms were truncated species not known in UniProt, therefore Top-down MS with ProSightPD Subsequence analysis is the preferred method to accurately identify these species.

INTRODUCTION

Protein abundance measurements can be valuable tools for differentiating biological conditions, including health and disease. Following the trend towards minimally invasive samplings, we focus on tears, a unique body fluid, rich in proteins, which reflect the (health) condition of the eye and, by extension, of the individual as a whole.[1] In this study we perform quantitative top down proteomics on tear fluid from two different human donor groups (old and young) to study age-related proteoform differences. Additionally, multiple fragmentation methods are explored in an effort to maximize proteoform characterization by overcoming the limitations imposed by intact disulfide-bonds.

MATERIALS AND METHODS

Sample Preparation

Tears were collected via Schirmer strips from patients. Tears fluid was extracted from strips using an aqueous buffer. Samples were then frozen for storage. Prior to analysis samples were thawed and centrifuged.

Methods

Tear fluid supernatant was transferred to an injection vial and analyzed by LC/MS using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer modified with UVPD. MS/MS acquisition was performed using UVPD fragmentations at a resolution of 120,000 at m/z 200.

Data Analysis

Raw files were analyzed using ProSightPD™ 4.0 (beta) nodes in Proteome Discoverer for proteoform identification and quantification. Proteoform correlated abundances where further processed in Microsoft Excel.

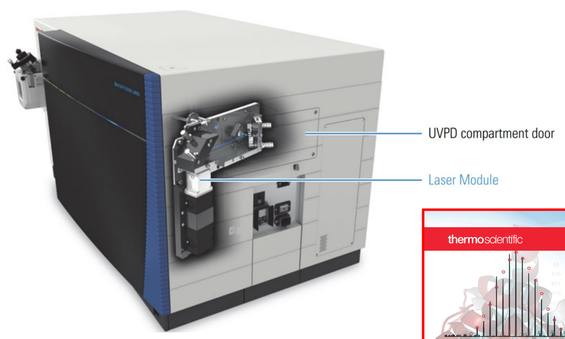


Figure 1. Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™ with UVPD module for MS analysis coupled to the ProSightPD 4.0 nodes for Proteome Discoverer for proteoform identification.

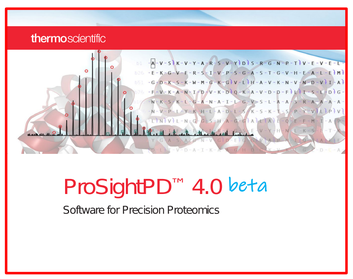


Figure 2. UVPD show superior fragmentation and sequence coverage for this heavily disulfide bound truncated proteoform of lysozyme. HCD and ETD fragmentation is restricted by the presence of intact disulfide bonds and require sample alkylation to improver coverage

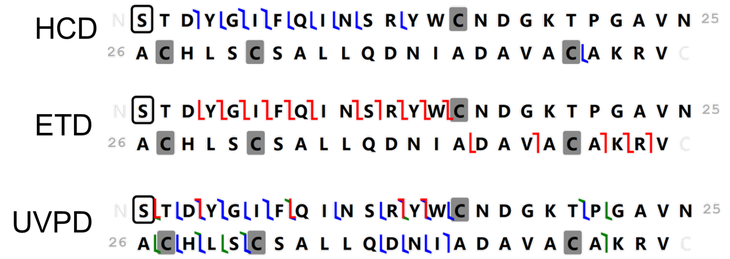
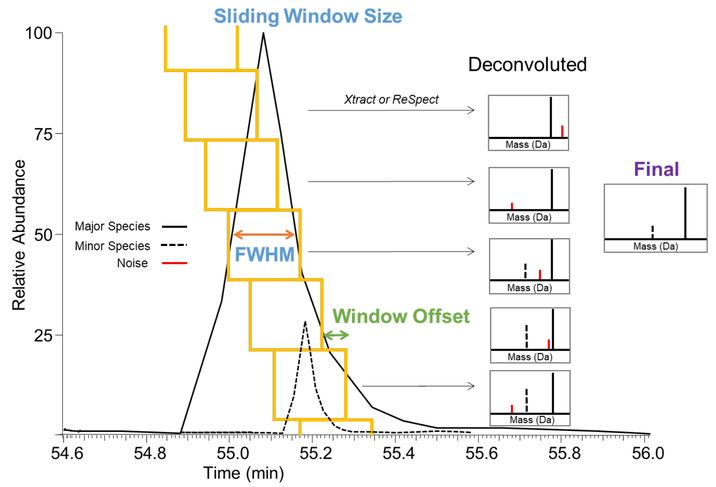


Figure 3. Top-down feature detection in ProSightPD 4.0 is performed using the Sliding Window Deconvolution method. Sliding Window Deconvolution captures co-eluting species while removing noise. The resulting feature list reports the intact mass of all proteoforms detected in the run.



RESULTS

Comparison of Activation Methods for Tear Proteoforms (HCD, ETD, UVPD)

Figure 2 shows a comparison of fragmentation methods for a disulfide bound proteoform of Lysozyme. UVPD is the only method capable of effectively sequencing the disulfide bound regions.

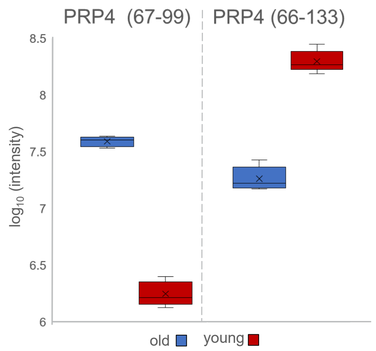
Implementing Top-down LFQ in ProSightPD

In a Top-down workflow proteoforms are measured directly without the burden of inference, therefore quantitation is performed at the proteoform level. To overcome the challenge of multiply charged proteoforms the raw data is first deconvoluted and quantitation is done at the intact mass level. Often, accurate deconvolution is hampered by poor signal to noise which can be exacerbated by signal splitting across multiple charge states. One solution to poor signal to noise is spectral averaging. **Figure 3** depicts the concept of sliding window deconvolution which utilizes multiple averaged windows across an entire LC/MS run to enhance proteoform signal to noise (improving detection) and remove noise signals (signals which do not persist for multiple windows are removed)[2]. ProSightPD 4.0 utilizes the sliding window deconvolution method for feature detection, ProSight search algorithms for proteoform identification and Proteome Discoverer for statistical analysis and result visualization.

Differentially expressed PRP4 proteoforms in tear fluid

Truncated proteoforms of Proline Rich Protein 4 (PRP4) were among the most differentially expressed. Clustering string analysis (**Figure 5**) shows that PRP4 is closely associated with other regulatory proteins (red cluster) from tear fluid (LACT, CST4) and may be associated with proteins active in the CNS (green cluster) and sulfate modifying enzymes (blue cluster). Interestingly certain truncated proteoforms of PRP4 are more abundant in the young group while others are more abundant in the old group (**Figure 4**). This indicates that peptide level quantitation will likely obscure the underlying biological dynamics observable when proteoforms are measured directly.

Figure 4. Abundance of PRP4(67-99) decreased in the young patient group while PRP4 (66-133) increased. Due to the similarity of these proteoforms this would be missed in a peptide based LFQ experiment



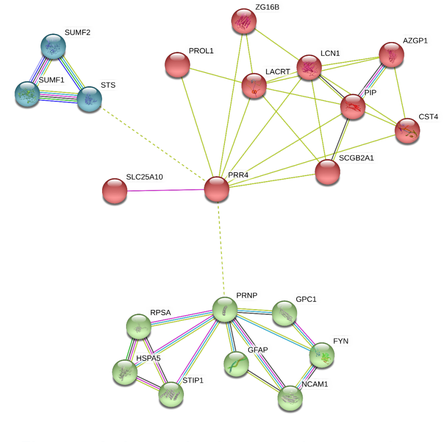


Figure 5. A clustered string analysis centered on PRP4 shows its close association with other identified tear proteins including modulators such as CST4 and LACRT

CONCLUSIONS

Novel activation techniques coupled to high performance mass spectrometers and advanced bioinformatics platforms offer streamlined quantitative analysis of biological samples such as tear fluid.

- UVPD effectively sequences previously intractable regions such as proline rich motifs and across disulfide bonds.
- A new top-down LFQ workflow for proteoform level quantitation is available in ProSightPD 4.0
- Proteoform level quantitation reveals biological dynamics obscured by proteolysis in traditional LFQ approaches.

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2. Moehring, F., Waas M., Keppel, T.R., Rathore, D., Cowie, A., Stucky C., & Gundry, R.L. Quantitative Top-Down Mass Spectrometry Identifies Proteoforms Differentially Released during Mechanical Stimulation of Mouse Skin J. Proteome Res. 8, 2635-2648 (2018)

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