Utility of EThcD and AI-ETD for the Differentiation of Leucine and Isoleucine Residues in Large-Scale Shotgun Proteomic Experiments

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Background

Discriminating between leucine (Leu) and isoleucine (Ile) by mass spectrometry is challenging due to their isomeric nature. Methods to differentiate these residues have been proposed for decades, typically relying on MS^n-based approaches where peptide fragments are further dissociated to produce fragments or neutral losses which are specific to either leucine or isoleucine. In recent years, EThcD, where a precursor is first activated by electron transfer dissociation (ETD) and secondarily activated by higher energy collisional dissociation, has been demonstrated to effectively differentiate between leucine and isoleucine in several small-scale, targeted investigations [1, 2]. With this method, $z^\cdot$ ions are first produced during the ETD reaction and diagnostic w ions are produced in the subsequent HCD activation step by the neutral loss of 43 Da for leucine and 29 Da for isoleucine (Figure 1).

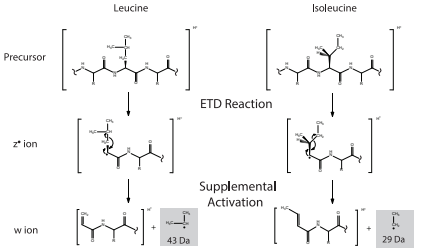

Figure 1. Fragmentation pathways for diagnostic w ion production for the discrimination between leucine and isoleucine residues.

As w ion production, and hence the ability to discriminate between leucine and isoleucine, simply requires the production of $z^\cdot$ ions and a secondary activation event, we hypothesized that w ions may also be produced using activated ion electron transfer dissociation (AI-ETD). During AI-ETD the ETD reaction is concurrently irradiated with infrared photons from a CO_2 laser (10.6 μm) [3]. Despite the utility of EThcD for the differentiation of leucine and isoleucine in small-scale analyses, the ability to differentiate between the residues in large, non-targeted proteomics experiments has not yet been investigated. Here, we investigate the possibility of using AI-ETD and EThcD in shotgun proteomics experiments where complex peptide mixtures are analyzed.

Methods

Samples: Purified bovine thyroglobulin (trypsin digested) and human K562 lysates (trypsin and chymotrypsin digested).

Instrumentation: All analysis was performed on a Thermo Fusion Lumos that has been retrofitted with a 10.6 μm continuous wave laser to allow for the implementation of AI-ETD [3].

Data Collection: Protein digests were analyzed using back-to-back scans where all precursors were fragmented with HCD first (25% NCE), followed by either AI-ETD (varying laser powers), or EThcD (varying NCEs). MS^1 scans were collected in the Orbitrap at 120k once every three seconds, and MS^2 scans were also collected in the orbitrap at 15k resolution.

Data Analysis: De novo sequencing and database searching was completed using a version of PEAKS Studio (Bioinformatics Solutions Inc.) that was modified to treat AI-ETD data as EThcD, as the fragmentation pathways of these two methods are similar (i.e. both produce c, $z^\cdot$, b and y ions). Calling of leucine and isoleucine residues was completed using in-house developed software tools.

Parameter Optimization

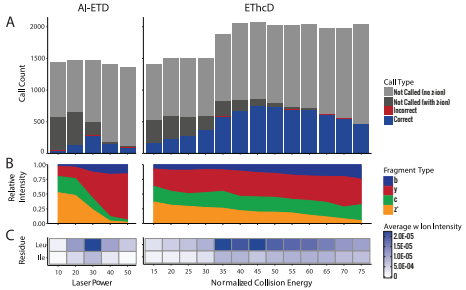

Figure 2. AI-ETD laser power and EThcD NCE optimization. Number of correct, incorrect, and not called leucine and isoleucine residues (A) as determined by comparison with database search results. Relative fragment ion intensities by type (B), and average w ion intensities (C).

The ability to discriminate between leucine and isoleucine is maximized at 30% of max laser power for AI-ETD and at 45% normalized collision energy (NCE) for EThcD. At these levels, $z^\cdot$ ion production is still significant (B), while average w ion intensity is also maximized (C). Interestingly, at an NCE of 45% for EThcD, essentially all residues (90%) for which a z ion was observed a w ion was also present, allowing for the residue to be called as either leucine or isoleucine.

Intensity Difference Cutoff

We investigated cases where w ions indicative of both leucine ($z^\cdot$ – 43 Da) and isoleucine ($z^\cdot$ – 29 Da) were observed for the same residue. We interrogated the data when imposing different intensity difference cutoffs such that one w ion must be a certain fold higher than the other w ion for the residue to be called as a leucine or isoleucine.

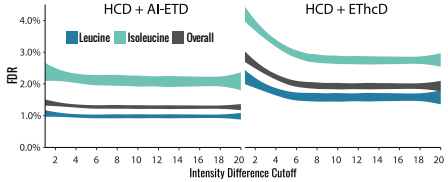

Figure 3. False discovery rates implementing different intensity difference cutoffs.

An intensity difference cutoff of 3 was chosen to minimize false discovery rate while balancing the loss of correct calls.

Local Confidence Cutoff

To limit the number of incorrect calls and incorrect identifications, we investigated implementing a local confidence cutoff such that differentiation between leucine and isoleucine is only attempted on residues with high de novo scores. False discover rates (FDRs) were calculated using different local confidence cutoffs.

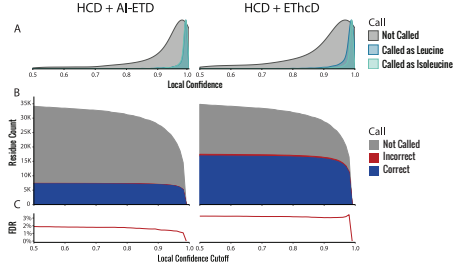

Figure 4. Implemention of local confidence cutoffs on de novo results prior to leucine/isoleucine differentiation. Distribution of local confidence scores if a local confidence cutoff of 0.5 is implemented (A). Response of the number of called residues in response to different local confidence cutoffs (B) and the corresponding false discovery rates (FDR) for those cutoffs (C).

A minimum local confidence filter of 0.9 was imposed for leucine/isoleucine discrimination as a minimal decrease in the number of correctly called residues was observed, but the FDR was decreased slightly with this filter.

De Novo Summary

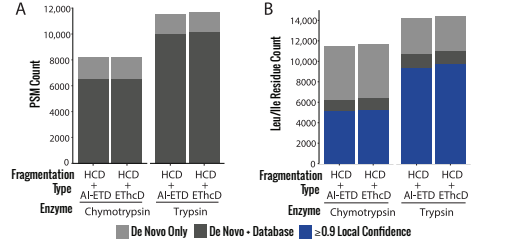

Figure 5. Summary of the de novo results from the human samples on the peptide spectral match (PSM) level (A), and the leucine/isoleucine level (B). Results were filtered using a minimum ALC score of 50% for the de novo data and a 1% FDR for the database search.

Final Leu/Ile Discrimination Performance

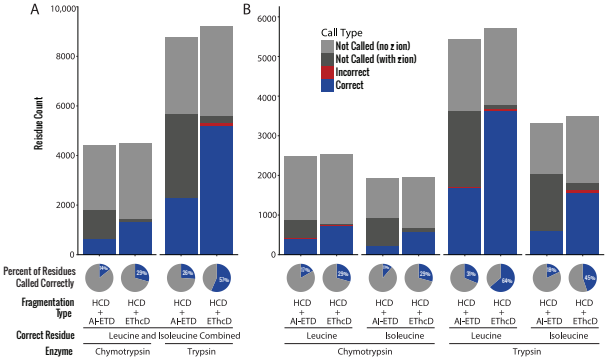

Figure 6. Success of discriminating between leucine and isoleucine in the human de novo experiment. Data is represented on the overall experiment level (A), as well as with leucine and isoleucine separated (B).

Conclusions

Previously, differentiating between leucine and isoleucine required the design of time-intensive, targeted experiments, making it impractical on a large scale. Here, we illustrate that it is possible to differentiate between leucine and isoleucine residues in de novo sequencing-based, non-targeted proteomics experiments where AI-ETD or EThcD dissociation is performed. EThcD significantly outperforms AI-ETD, but this is the first time that the production of w ions from leucine and isoleucine has been demonstrated by AI-ETD. As the use of electron-based dissociation is now commonly used to complement collisional methods such as CID or HCD in de novo sequencing experiments, leucine and isoleucine can be differentiated without any additional data collection and with no increase in instrument duty cycle. Approximately 50% of residues determined to be either leucine or isoleucine by de novo sequencing can be accurately (< 3% FDR) differentiated, substantially decreasing the time investment required for follow up experiments. The implementation of this ability in publicly available de novo sequencing software would aid researchers performing large, de novo sequencing experiments, where genomic information is not available.

References

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