

Internal fragments generated by electron ionization dissociation enhances protein top-down mass spectrometry

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ABSTRACT: Top-down proteomics by mass spectrometry (MS) involves the mass measurement of an intact protein followed by subsequent activation of the protein to generate product ions. Electron-based fragmentation methods like electron capture dissociation (ECD) and electron transfer dissociation (ETD) are widely used for these types of analysis, however these fragmentation methods can be inefficient due to the low energy electrons fragmenting the protein without the dissociation products; that is no detection of fragments formed. Recently, electron ionization dissociation (EID), which utilizes higher energy electrons (> 20 eV) has been shown to be more efficient for top-down protein fragmentation compared to other electron-based dissociation methods. Here we demonstrate that the use of EID enhances protein fragmentation and subsequent detection of protein fragments. Protein product ions can form by either single cleavage events, resulting in terminal fragments containing the C-terminus or N-terminus of the protein, or by multiple cleavage events to give rise to internal fragments that do not contain the C-terminus or N-terminus of the protein. Conventionally, internal fragments have been disregarded as reliable assignments of these fragments were limited. Here, we demonstrate that internal fragments generated by EID can account for ~20-40% of the mass spectral signals detected by top-down EID-MS experiments. By including internal fragments, the extent of the protein sequence that can be explained from a single tandem mass spectrum increases from ~50% to ~99% for 29 kDa carbonic anhydrase II and 8.6 kDa ubiquitin. By including internal fragments in the data analysis, previously unassigned peaks can be readily and accurately assigned to enhance the efficiencies of top-down protein sequencing experiments.

INTRODUCTION

Top-down proteomics using mass spectrometry (MS) has emerged as a technique to analyze proteins and proteoforms [1, 2]. Typically, intact protein ions are generated using electrospray ionization (ESI), followed by dissociation of the intact protein ion within the mass spectrometer to generate product ions that can be used to return information about protein identification and primary structure, *i.e.*, sequence. Electron-based dissociation techniques such as electron capture dissociation (ECD) [3, 4] and electron transfer dissociation (ETD) [5] employ the use of a low-energy electrons to generate protein fragment ions. ECD/ETD for protein dissociation experiments confers many advantages over other dissociation techniques (*e.g.*, collision induced dissociation (CID) [6], surface induced dissociation (SID) [7], and ultraviolet photodissociation (UVPD) [8]), including but not limited to conserving post-translational modifications and non-selective fragmentation of the protein backbone [9, 10]. Due to non-specific cleavage during electron-based dissociation, fragmentation by electron-based methods has the potential to generate more protein fragments that allow for richer sequence information to be obtained [11, 12]. Despite the many advantages and prevalent use of ECD/ETD (ExD) [13], these fragmentation techniques can be limiting due to the reliance of generating protein ions in higher charge states [14], and proteins have low capture efficiencies, thus potentially limiting ExD efficiency [3, 15].

Electron ionization dissociation (EID) is a recently discovered alternative ExD fragmentation technique for peptide and protein characterization [16-18]. EID is a fast activation method that utilizes high energy electrons (> 20 eV) to induce protein fragmentation along the backbone. In EID, the interaction of a multiply charged protein ion with a high energy electron results in the formation of the oxidized species. Subsequent rearrangement of the oxidized species and/or capture of a second high energy electron promotes backbone fragmentation. Using EID, Zubarev and co-workers demonstrated that fragmentation efficiency for some proteins and peptides can be close to 100% [18]. This data suggests that EID can result in more efficient fragmentation of polypeptides compared to ECD, which would be especially beneficial for the analysis of large proteins. Recently, Loo and co-workers demonstrated that by using EID for native top-down MS, extensive fragmentation of apo-human superoxide dismutase 1 homodimer complex (32 kDa) could be achieved, whereas ECD resulted only in reduced precursors and no protein fragmentation [17]. Despite the potential advantages conferred by EID compared to ECD/ETD, the use of EID for protein top-down MS has not been extensively explored.

Protein product ions can either be (i) a terminal fragment ion, where only one cleavage event occurs to generate N-terminal-containing *a*, *b*, *c* fragments or C-terminal-containing *x*, *y*, *z* fragments [19], or (ii) an internal fragment ion, where two cleavage events occur generating *ax*, *ay*, *az*, *bx*, *by*, *bz*, *cx*, *cy*, *cz* fragment ions depending on the type of cleavage occur-

ring [20-23]. The number of theoretical internal products that can be generated is significantly greater than the number of theoretical terminal fragments that can be generated [21], suggesting that many matching a mass spectral signals could be matched to an internal fragment. Despite this, traditionally internal fragments have been largely ignored due to the inability to reliably assign internal fragments [24]. Due to this, a plethora of information that can be accessed but has largely been ignored.

The analysis of internal fragments previously has been limited to peptides and small molecules [25, 26]. Assignment of internal fragments for intact proteins has been relatively limiting owing to the complexity of the fragmentation spectra. Kelleher and co-workers showed that internal fragments from CID fragmentation of the common test protein, ubiquitin (8.6 kDa), can be assigned to result in significantly greater protein sequence coverage [21]. Similarly, for other intact proteins, the inclusion of internal fragments that can be generated by the dissociation process could result in greater explanation of the protein sequence [27, 28]. Our laboratory demonstrated that internal product ions can be generated from top-down MS of large, native protein complexes [29]. These examples suggest that the inclusion of internal fragments in top-down protein sequencing experiments should significantly enhance the protein sequence coverage and the efficiency of top-down mass spectrometry experiments.

Here, we investigate the use of EID and the analysis of internal fragments for top-down protein sequencing experiments. By using EID for top-down MS of ubiquitin (Ubq) and 29 kDa carbonic anhydrase II (CAII), the number of product ions is significantly higher compared to ECD, and approximately 20-40% of the fragments in the mass spectra could be assigned to internal fragments. By including internal fragment ions in the analysis, nearly complete sequence coverage for CAII can be achieved from a single top-down mass spectrum.

EXPERIMENTAL

Materials. Proteins bovine carbonic anhydrase II and bovine ubiquitin were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. LC/MS grade water and methanol were obtained from Fisher Chemical (Hampton, NH, USA). For electrospray ionization, aqueous solutions containing 10-20 μM protein, 49.5% water, 49.5% methanol, and 1% formic acid (v/v) were prepared.

Mass spectrometry. All experiments were conducted on a 15-Tesla Bruker Solarix Fourier transform ion cyclotron resonance (FTICR)-MS equipped with an infinity ICR cell (Bruker Daltonics, Billerica, MA, USA). Protein solutions were loaded into metal-coated borosilicate capillaries (Au/Pd-coated, 1 μM inner diameter, Thermo Fisher Scientific, Waltham, MA, USA) and electrospray was initiated by applying a voltage between 0.9-1.4 kV on the ESI capillary. Charge states were isolated in the quadrupole, with an isolation window of 10 m/z , before EID/ECD fragmentation. For ECD fragmentation, the pulse length was set at 0.05s, with a lens voltage of 50 V, and an ECD bias voltage of 2 V. For EID fragmentation, the pulse length and lens voltage were kept constant and the bias voltage was altered between 20-30 V. For each spectrum, 200 scans were obtained.

Data analysis. Peak assignments. Deconvoluted mass lists were obtained from Bruker Data Analysis software, using the SNAP algorithm. Deconvoluted mass lists were uploaded into our in-house-developed python program that calculates all possible terminal and internal fragments written and compares them to the experimental deconvoluted masses. The error for matching was set at 1 ppm error, and 42.0105603 for the mass of acetylation was added to all the N-terminal fragments for carbonic anhydrase II.

Protein sequence coverage. Protein sequence coverages were calculated by equation 1:

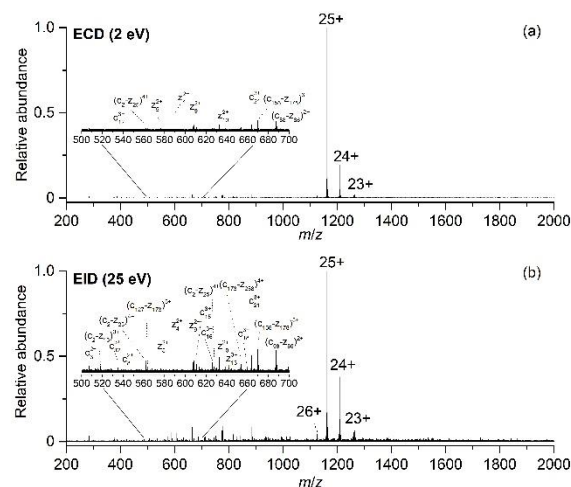
$$\text{Seq. coverage (\%)} = (\text{AA}_{\text{det}}/\text{AA}_{\text{tot}}) \times 100 \quad [\text{Eq. 1}]$$

where AA_{det} is the number of times an amino acid residue was detected in at least 5 different fragments, AA_{tot} is the total number of amino acids in the protein. The sequence coverage should give an indication of how much of the protein sequence can be explained by the fragments assigned.

Protein fragment coverage. Protein fragment coverages were calculated by identifying the number of observed inter-residue sites divided by the total number of inter-residue cleavages on the protein backbone. For example, Ubq and CAII have 75 and 259 inter-residue cleavage sites respectively.

RESULTS AND DISCUSSION

EID for top-down protein sequencing experiments can result in greater fragmentation compared to ECD (**Figure 1**). Isolation and EID (25 eV) of $[\text{CAII}, 25\text{H}]^{25+}$ resulted in the formation of the $[\text{CAII}, 26\text{H}]^{26+}$ ion with significant abundance (**Figure 1b**), which is in good agreement with Zubarev and co-workers observation that EID results in the formation of the oxidized species [30]. Representative mass spectral signals for some product ions identifiable within the mass m/z 500-700 range are shown within the insets of **Figure 1**.



fragment within the EID spectrum is ~ 30% higher in signal to noise compare to than the fragments detected in the ECD spectrum. On average, there is ~ 30-40% increase in signal to noise of fragments formed by EID compared to ECD, suggesting that EID is a more efficient fragmentation method.

The number of fragment ions that are generated and detected by EID are also greater in number than those generated by ECD. For example, EID of [CAII, 25H]²⁵⁺ resulted in the formation of 145 unique fragments, however ECD of [CAII, 25H]²⁵⁺ resulted in the formation of 100 unique fragments (Figure 2a). Similarly, for [Ubq, 10H]¹⁰⁺, more fragments are formed by EID (159 unique fragments) compared to ECD (102 unique fragments) (Figure S1). Previous data from our group demonstrated that using EID generated more fragments compared to ECD for native SOD-1 protein [17].

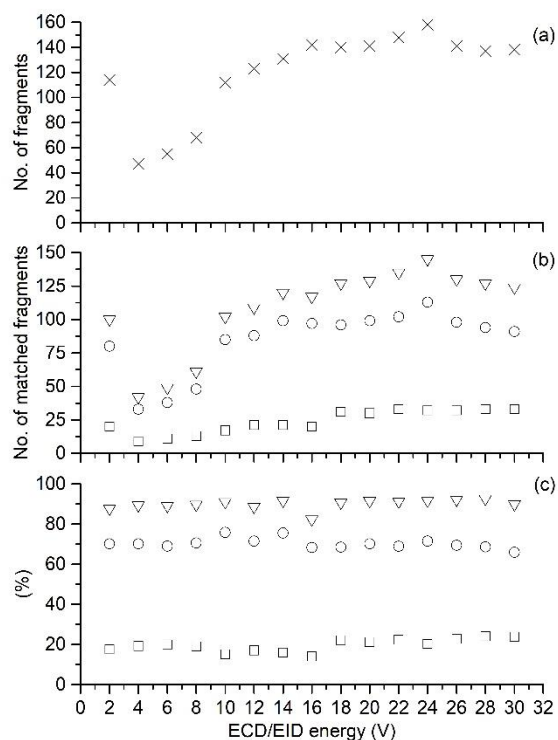


Figure 2. The effect of ECD/EID energy for isolated [CAII, 25H]²⁵⁺, where (a) is the number of fragments automatically deconvoluted, (b) is the total number of fragments identified (open triangles), total number of terminal fragments identified (open circles), and the total number of internal fragments identified (open squares). The percentage of the fragments matched are shown in (c) where the percentage of all fragments identified (open triangles), percentage of terminal fragments identified (open circles), and the percentage of internal fragments identified (open squares).

Fragment ions generated by electron dissociation methods can be classified as either terminal fragments (fragments containing the N-terminus or C-terminus), or internal fragments (fragments that contain neither the N-terminus nor C-terminus). The data shown here suggests that internal fragments can account for many of the mass spectral signals with-

in a mass spectrum regardless of fragmentation method. Interestingly, the ion abundances of internal fragments are not significantly lower compared to low abundant terminal fragments formed. For example, the fragment assigned to the internal fragment (c58-z86)²⁺ is similar in ion abundance to low abundant c and z fragment ions detected within the spectrum.

Considering that the abundances of internal fragments are not significantly lower than the ion abundances of low abundant terminal fragments, this suggests that these fragments are identifiable and can be assigned. Therefore, the inclusion of these internal fragments could result in greater sequence information for the protein compared to terminal fragments alone.

Conventionally, internal fragments have been ignored, owing to the inability to reliably assign them due to instrument limitations and computational limitations [24, 31]. However, many ion signals within tandem mass spectra can potentially be explained by internal fragments (Figure S2b). Here, we attempt to assign mass spectral signals that were previously unassigned to internal fragments by calculating internal fragment masses using an in-house written program (see Materials and Methods) which can be uniquely assigned if MS2 mass calibration is achieved to ≤ 1 ppm error to limit false positives. For ECD of [CAII, 25H]²⁵⁺, there were 100 unique fragments formed, of which 80 (80%) are assigned to terminal fragments and 20 (20%) are assigned to internal fragments. For EID, there were 145 unique fragments formed for [CAII, 25H]²⁵⁺; 113 terminal (78%) and 32 internal fragments (22%). On average, the number of internal fragment ions that are formed can account for 20-40% of the ion signals within a mass spectrum (Figure 2b).

The effect of electron energy on the ECD/EID efficiency of [CAII, 25H]²⁵⁺ was investigated (Figure 2). At conventional ECD energy (2 eV), 101 unique fragments were identified. However, at greater than 20 eV EID energies there are more fragments generated (> 125 unique fragments), with an energy of 24 eV being the optimal for generating the largest number of fragments (Figure 2a). Interestingly, as electron energy increases, the number of internal fragments formed also increases (Figure 2b); the number of internal fragments formed at 24 eV was 33 compared to only 20 at 2 eV. However, the percentage of fragments that are assigned as internal fragments do not increase significantly at 24 eV compared to 2 eV (18% to 24%, respectively). Similarly, for ubiquitin the percentage of fragments that can be explained by internal fragments formed at ECD energies is similar to the percentage of internal fragments formed at EID energies (Figure S1). The formation of internal fragments from EID of Ubq is in good agreement with previous data from Kelleher and co-workers who demonstrated that CID of Ubq results in the formation of internal fragments, with approximately 30% of the mass spectral signals attributed to internal fragments [21]. As internal fragments are generated within conventional dissociation experiments (e.g., CID, SID, ECD/ETD), the assignment of internal fragments should be beneficial for characterizing the protein sequence as more of the mass spectral signals can be assigned to protein fragments.

The efficiency of ECD fragmentation has been shown to correlate with the charge state of the precursor ion [14, 32-34]. Similarly, for EID the fragmentation efficiency increases with

charge (Figure 3a). For CAII, as the precursor charge increases from 10+ to 38+, the number of fragments increases from 105 to 250, respectively. A similar trend is observed for Ubq; the number of fragments increases from 69 (7+ precursor ion charge) to 175 (13+) (Figure S1). EID's greater fragmentation efficiency for higher charge states could be due to lowering the barriers for dissociation and larger reaction cross sections.

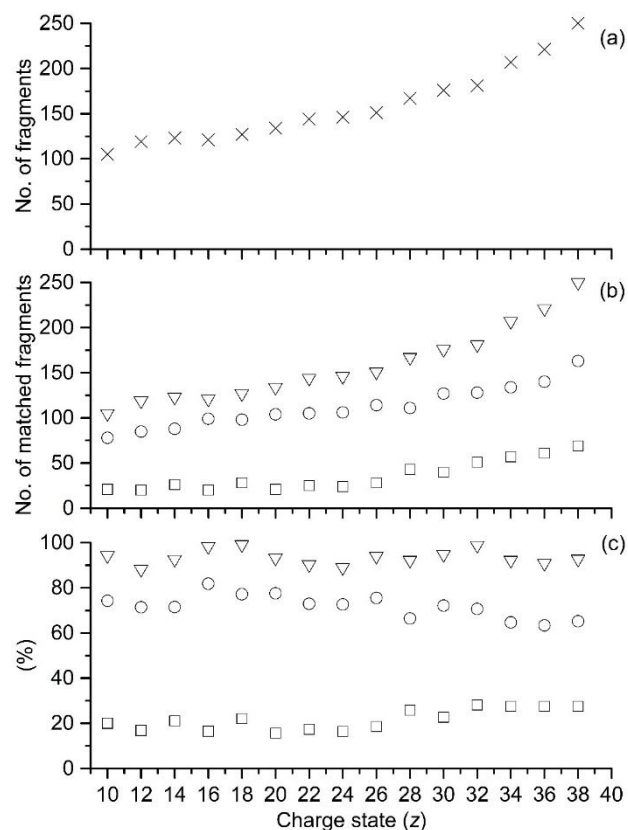


Figure 3. Performance of EID-MS for isolated $[CAII, zH]^{2+}$ ($z = 10+$ to $38+$), where (a) is the number of fragments automatically deconvoluted, (b) is the total number of fragments identified (open triangles), total number of terminal fragments identified (open circles), and the total number of internal fragments identified (open squares). The percentage of the fragments matched are shown in (c) where the percentage of all fragments identified (open triangles), percentage of terminal fragments identified (open circles), and the percentage of internal fragments identified (open squares).

The theoretical total number of fragments formed by dissociation scales to the power of 2, when compared to amino acid sequence length and when including internal fragments (Figure S2). For example, the number of fragments that can be generated theoretically by fragmentation between each amino acid residue, based on cleavages of the $C\alpha-C\beta$, $N-C\alpha$, and $N-C\beta$ bonds, ranges from a single amino acid residue to one less than the entire protein length. For Ubq (76 amino acid residues), the total is 25,429 compared to 299,929 for CAII (259 amino acid residues), with the vast majority originating from internal fragments; for Ubq and CAII, the number of theoretical internal fragments are 24,975 and 298,377, respectively.

This is in close agreement with previous calculations by Kelleher and co-workers who demonstrated that the number of internal fragments that can theoretically be generated outnumber the number of terminal fragments generated [21]. In addition, the formation of internal fragments should be especially beneficial for larger proteins because internal fragments are generally lighter than terminal fragments (Figure S2). For example, the average mass of a terminal fragment for CAII is 14.4 kDa, whereas the average mass of an internal fragment is 9.5 kDa (Figure S2b). This trend becomes significantly more important for larger proteins; the difference between the average mass of terminal fragments and internal fragments increases as protein size increases. By generating internal fragments that have smaller masses than terminal fragments, these internal fragments should fall within the dynamic range of most mass spectrometers, which should allow for greater identifications of these regions within the protein. As top-down MS analysis suffers from an upper mass limit, with one possible reason due to larger protein ion fragments not being detected by MS detectors [35, 36], the inclusion of internal fragments could potentially help to overcome this limitation.

We have found that internal fragment ions can be readily calculated using an in-house written program (see Materials and Methods) and can be uniquely assigned if MS2 mass calibration is achieved to ≤ 1 ppm error. As an example, to test the validity of these assignments, the theoretical fragment isotopic distribution was fitted over the corresponding fragment peak in the mass spectrum (Figure S3). For both terminal and internal fragments, the theoretical isotopic distribution of the corresponding fragment are in good agreement with the peaks observed in the mass spectrum (Figure S3). For example, the internal fragment $(c157-z70)3+$ isotopic distribution shows similar isotopic abundances and distribution as the corresponding theoretical distribution, suggesting that our peak matching algorithm can reliably assign both terminal and internal fragment ions.

To measure the false discovery rate of the internal fragment assignments, the fragments that were assigned to internal fragments of $[CAII, 25H]25+$ were shifted with a given ppm shift from -500 to 500 and the percentage of fragments assigned to a different fragment are shown (Figure S4). On average, the percentage of internal fragments that were reassigned to a different internal fragment was $\sim 20\%$. However, considering that ExD fragmentation was utilized, internal fragments formed should be cz fragments that stem from multiple fragmentation events. When only cz internal fragments were considered, on average only 3.4% of the internal fragments were assigned to other cz internal fragments. By assigning only cz internal fragments, our false discovery rate should be sufficiently low ensuring that the internal fragments assigned here are as accurate as possible.

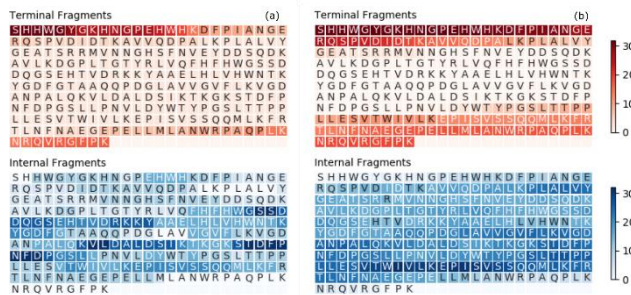


Figure 4. Heatmap depicting the number of times each residue is covered by a terminal fragment (top) and/or an internal fragment (bottom) for ECD of [CAII, 25H]²⁵⁺ (a), and EID of [CAII, 25H]²⁵⁺ (b). Darker colours indicate greater coverage.

By including both internal fragments and terminal fragments, a larger fraction of the protein sequence can be explained (Figure 4). Heatmaps of the number of times a residue is represented by a product ion are plotted to give an indication of hotspots within the polypeptide amino acid sequence corresponding to where fragments are formed and detected. For ECD of CAII (Figure 4a), terminal fragments only account for a small fraction of the protein sequence (28%). Interestingly, the inclusion of internal fragments yields nearly complete sequence coverage of carbonic anhydrase II (~90%). For EID, a larger percentage of the protein sequence can be explained by terminal fragments (47%), and similarly, the inclusion of internal fragments resulted in near complete protein sequence coverage (~99%). The inclusion of internal fragments was also beneficial for the percentage of the inter-residue cleavages that were observed. For ECD, inclusion of the internal fragments showed 200 of the 259 inter-residue cleavage sites were fragmented, and for EID, 234 of the 259 inter-residue cleavage sites were fragmented indicating that a majority of the protein backbone was cleaved to form fragment ions (Figure S5). For Ubq, similar trends are observed with many internal fragments assigned to fragments that contain the center of the protein backbone (Figure S6). Further, the majority of inter-residue cleavage sites were cleaved, resulting in fragments being detected and assigned to protein fragments (Figure S7). The heatmaps suggest that the amino acids covered by the internal fragments detected complement the amino acids covered by the terminal fragments.

CONCLUSIONS

EID for top-down MS can significantly enhance the efficiency of protein fragmentation. EID outperforms ECD, with larger numbers of fragments generated, as well as higher fragment ion abundances. Most significantly, the use of internal fragment assignments resulted in the explanation of a larger percentage of the protein sequence. As the internal fragments formed contain amino acid sequences that are complementary to the terminal fragments, these internal fragments should be useful for localizing post-translational modifications, and protein-ligand binding sites with native top-down MS. In general, incorporating the previously unassigned internal product ions generated by all activation/dissociation techniques, especially with EID, should greatly enhance the ability top-down MS for protein sequence analysis to larger proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is included as a pdf file titled: ‘Supporting Information for Internal fragments generated by electron ionization dissociation enhances protein top-down mass spectrometry’

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