Protein cooking: Combining thermal-CID and EAD for intact protein sequencing

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INTRODUCTION

Top-down analysis is an emerging tool for intact protein characterization. Fragmentation techniques such as electron activated dissociation (EAD) and ultraviolet photodissociation (UVPD) have enabled detailed MS/MS analysis that provides high sequence coverage for small proteins (<30 kDa) . However, as protein mass increases, fragmentation efficiency typically decreases, which also reduces sequence coverage. An approach used to overcome these limitations is to rely on middle-down analysis in which the protein is first broken into smaller subunits, typically by using enzymes in solution, and each subunit is analyzed by CID and EAD to yield higher sequence coverage. Here, we propose using thermal collision induced dissociation (thermal-CID) fragmentation to generate protein subunits, followed by CID and EAD to increase protein sequence coverage.

MATERIALS AND METHODS

Samples
Insulin, ubiquitin (UBT) and carbonic anhydrase (CA) were obtained from Sigma-Aldrich (St-Louis, MO). Synthetic peptides GLEFSDPLK (GK9-3) and TTDWVDLR (TR8) were obtained from Neobiolab (Cambridge, MA) at >98% purity and used without further purification.

Mass spectrometry
Analyses were performed on a SCIEX ZenoTOF 7600 system equipped with an OptiFlow Pro ion source and microflow analytical probe (50–200 µL/min). The system was operated with nebulizing gas (G1) and the heating gas (G2) at 30 psi and 70 psi, respectively. The ion spray voltage (ISV) was set to 4500 V and the source temperature was varied between 150ºC and 650ºC. For EAD, the filament current was set to 2500 nA with an electron transmission coefficient (ETC) of 100% and electron kinetic energy (KE) of 0 eV.

Differential mobility separation (DMS) measurements were performed on a TripleTOF 6600 system equipped with SelexION device and an IonDrive TurboV ion source. The system was operated with nebulizing gas (G1) and heating gas (G2) at 35 psi and 70 psi, respectively. The ISV was set to 5000 V and the source temperature was varied between 150ºC and 650ºC. The DMS was either operated in transparent mode, with separation voltage (SV) set to 0 V, or under separation mode (SV = 3800 V) with the compensation voltage (CoV) ramped between 0 and 40 V using nitrogen as the transport gas.

LC-MS analysis
Chromatographic separations were performed with an ExionLC system (Sciex). Peptide separations were performed with Aeries Peptide XB-C18 (2.1 x1 00mm, 2.6 µm) column from Phenomenex (Torrance, CA) with a flow rate 350 µL/min and oven temperature of 45ºC. For denatured protein analysis, a Poroshell 300SB-C8 (1 x75 mm, 5 µm) from Agilent (Santa Clara, CA) was used with a flow rate of 300 µL /min and oven temperature of 40ºC. For C8 and C18 separation, a gradient of water and acetonitrile with 0.1% formic acid was used. For native-like protein analysis, an Acquity Protein BEH-SEC (2.1 x 150 mm, 1.7 µm) column from Waters (Milford, MA) was used. This column was operated under isocratic conditions with 5 mM ammonium acetate in water, a flow rate of 75 µL/min and oven temperature of 40ºC.

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RESULTS AND DISCUSSION

The term "in-source fragmentation" has frequently been used in the literature in a generic way to describe fragmentation that occurs prior to precursor mass selection in MS/MS analysis [1, 2]. In most publications using this terminology, fragmentation of the analyte is typically obtained by raising the declustering potential applied in the atmospheric-to-vacuum interface region. The high rate of collisions occurring in that region generate fragment ions akin to CID and has been used to perform "pseudo-MSMS" on single quadrupole instruments or pseudo-MS3 for instruments that offer a single stage of CID fragmentation (e.g., QqQ or QqTOF). For this reason, the term "up-front CID" has been proposed, as it describes more appropriately the region where fragmentation occurs [3]. In these approaches, the fragmentation occurs in the transfer region from atmospheric pressure to the first vacuum region. These approaches do not generate fragment ions on the atmospheric side of the source, or more particularly in the spray region of an ion source.

The OptiFlow Pro ion source and IonDrive Turbo V ion source use similar heater configurations designed to provide maximum drying of the spray plume prior to sampling in the curtain-orifice region. For simplicity, only the OptiFlow Pro ion source is depicted in Figure 1. Under typical conditions, the heaters are operated between 150ºC and 400ºC, depending on the liquid flow rate used for LC separation (50 to 600 µL/min). These conditions ensure optimum sensitivity for the formation of the protonated form of the analytes over a wide range of chemical species. However, when the applied temperature exceeds 500ºC, fragmentation of peptides and proteins can be achieved in the spray region, prior to their sampling through the curtain gas region. Since fragmentation is achieved by applying heat in the spray region, we use the term "thermal-CID" to distinguish it from the more generic term "in-source fragmentation". The ability to fragment ions in the spray region implies that atmospheric pressure ion separation techniques (for example, ion mobility and DMS) can be used to separates precursor and fragment ions prior to mass analysis.

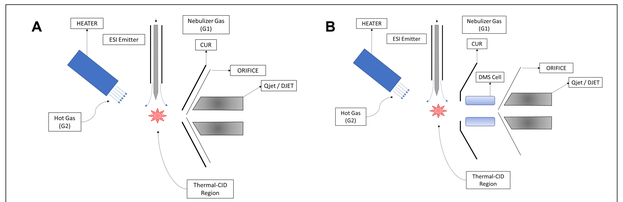


Figure 1. Region of thermal-CID with OptiFlow Pro ion source with regular interface (A) and with DMS (B)

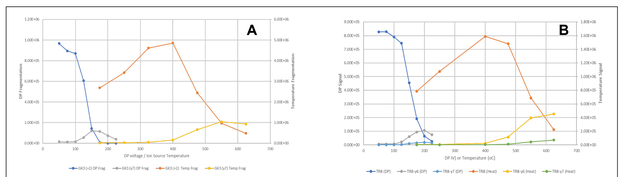


Figure 2. Precursor ion and major fragment ion signals as a function of applied DP for "up-front CID" and source temperature for "thermal-CID". Synthetic peptide GK9-3 (A) and TR8 (B) were used. All data were collected on TripleTOF 6600 system with the IonDrive Turbo V source.

Two synthetic peptides (GK3 and TR8) were used to compare "up-front CID" using the delustering potential (DP), and Thermal-CID (Figure 2). In both cases, when the precursor signal decreased, we observed the emergence of signal from fragment ions. Here, the fragment ions with the strongest signal were used to compare both approaches. When spectra at DP >200 V were compared to thermal-CID >550ºC, similar fragment ions were observed. In other words, thermal-CID did not produce unique fragments that differed from conventional CID, whether formed via up-front CID or collision cell CID.

Figure 3 shows the MS analysis for bovine insulin under regular source conditions and under Thermal-CID conditions. Residual precursor signal was observed in both cases. However, under thermal-CID conditions, fragment y_{11}^{+2} from the β-chain of insulin was easily observed. Other multiply charged species associated with thermal-CID are also highlighted. Insulin was evaluated as it contains 3 disulfide links (2 inter-chain and 1 intra-chain) which can create challenges for gas phase fragmentation techniques.

Figures 4 and 5 show the sequence coverage obtained for ubiquitin (UBT, ~8.5 kDa) and carbonic anhydrase (CA, ~29 kDa) using thermal-CID. Most of the observed fragments were *b*-type and *y*-type. In all cases, fragment ions generated were observed over several charge states, providing further confidence in their identifications including at the MS1 level. Figure 6 shows the thermal-CID profile for the depletion of intact UBT and CA. Similar to what is seen with the synthetic peptide, the appearance of fragment ions is typically observed at temperature exceeding 550ºC, even when native-like LC conditions are used (UB, grey trace). These results indicate that thermal-CID is a generic tool that could be applied over a wide range of peptide and protein analysis conditions.

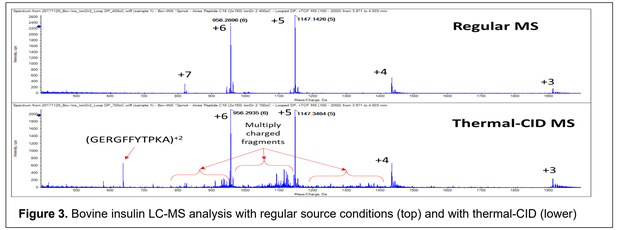


Figure 3. Bovine insulin LC-MS analysis with regular source conditions (top) and with thermal-CID (lower)

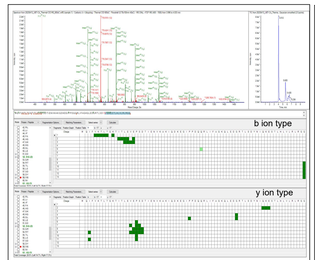


Figure 4. Sequence coverage for thermal-CID of UBT.

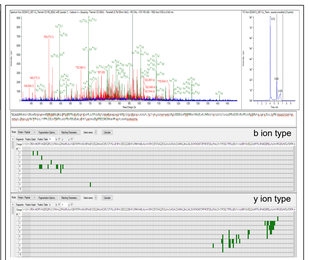


Figure 5. Sequence coverage for thermal-CID of CA.

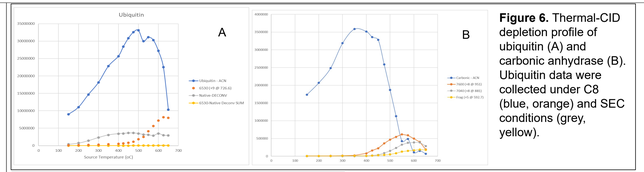


Figure 6. Thermal-CID depletion profile of ubiquitin (A) and carbonic anhydrase (B). Ubiquitin data were collected under C8 (blue, orange) and SEC conditions (grey, yellow).

Figure 7. CID and EAD MSMS of y_{58}^{+9} ion of ubiquitin generated via thermal-CID. For image clarity, only z-ions are labels and considered for sequence coverage.

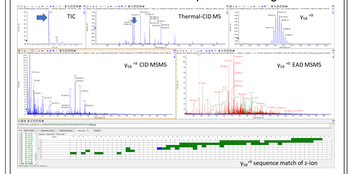
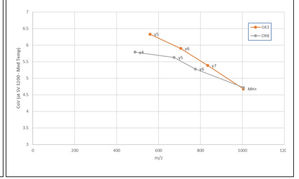


Figure 8. CoV optimum value for thermal-CID generated fragments of 2 synthetic peptides



The y_{58}^{+9} ion of UBT was generated with thermal-CID and subsequent MS/MS (both CID and EAD) were acquired to obtain complimentary sequence coverage for the protein (Figure 7). In addition to terminal fragments, thermal-CID can generate internal fragment ions. This can confound the MS signal interpretation and increase the possibility of precursor co-selection. However, the inherent production of fragment ions over several charge states increased the probability of an interference free ion to use as a precursor. The insertion of DMS as a means to separate the fragments ions generated by thermal-CID opens an additional dimension of separation to reduce the spectral complexity. Preliminary results obtained with synthetic peptides are shown in Figure 8 and will be the subject of future work for thermal-CID of peptide and protein ions.

CONCLUSIONS

Thermal-CID can be used to gain additional sequence information about peptides and proteins. It is not meant to replace conventional middle-down techniques but thermal-CID can provide supplemental data in these workflows. Thermal-CID provides additional information without additional sample preparation steps thus maintianing the same elution conditions (same RT) as the analyte in its native form. The technique is generically applicable to reversed-phase LC and SEC, and can be combined with atmospheric pressure separation techniques such as differential mobility separation (DMS).

REFERENCES

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