Analysis of Mouse Urinary Proteins: A Combined Electron Transfer / Collision-Induced Dissociation Strategy with Linear Ion Trap Mass Spectrometry

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Overview

Purpose: To investigate potential approaches to intact protein analysis using Electron Transfer Dissociation (ETD) Linear Ion Trap mass spectrometry.

Methods: A Finnigan LTQ™ was modified to accept a chemical ionization (CI) source at the rear of the instrument. Anions of fluoranthene were generated in the chemical ionization source. Intact or digested Mouse Urine Proteins (MUPs) were analyzed using Collisionally-Induced Dissociation (CID) and ETD on the LTQ, and ECD on the LTQ FT™.

Results: On intact MUP which contains162 amino acids, ETD produced 33 c ions and 32 z ions from the precursor carrying twenty charges, corresponding to a sequence coverage of 40.1%. The total coverage improves to 55.5% if the results from ETD and CID are combined. ETD of a CID fragment, y45, produced c and z ions which are complementary to the c and z ions generated by ETD of intact protein at the same c-terminal region. Using ETD LC/MS/MS, digested MUPs were analyzed, and identification of all of the four MUPs' isoforms was achieved.

Introduction

The analysis of intact proteins can provide complete sequence coverage, including site-specific modifications or mutations, information which is difficult to obtain at the peptide level. ECD has been applied in 'top-down' proteomics, with successful applications reported for large peptides and intact proteins. However, intact protein ion dissociation efficiency is significantly lower than that of peptides, and implementation of ECD is limited to FT-ICR instruments. A novel fragmentation method, electron transfer dissociation (ETD) shows ECD-like polypeptide fragmentation patterns, and is implemented on the linear ion trap (LTQ) (1). In this study, an approach using both ETD and CID for protein ion activation was applied to the analysis of intact mouse urinary proteins (MUPs). MUPs are vitally important proteins in pheromone signal mediation in mouse urine. The polymorphism of MUPs act as a unique individuality signal ascribing ownership (2). Here, purified intact MUPs were fragmented using ETD or CID and improvement on sequence coverage was achieved when the results from both activation methods are combined. In addition to the analysis of intact MUPs, ETD was also applied to the digested MUPs for the identification of MUPs isoforms.

Methods

Instrumentation

A Finnigan LTQ was modified to accept a chemical ionization source at the rear of this instrument. Fluoranthene were generated in the chemical ionization source using CI gas. The anion target was adjusted from 1.5 e5 to 4e5 and the activation time was 100 msec. ECD experiments were performed using a linear ion trap FTICR mass spectrometer (Finnigan LTQ FT) equipped with an ECD ion source assembly (Thermo Electron Corporation). The resolving power of the FTICR was selected at 100,000 (FWHM). Individual charge states of the protein molecular ions were selected for isolation in the linear ion trap and subsequently fragmented by ECD in the ICR cell. The ECD parameters were adjusted for each sample to ensure maximum efficiency of fragmentation of the primary amino acid chain. In this experiment, the precursor ions were excited by ECD for 15 milliseconds duration. The MS/MS spectra were results of up to 3000 transients.

Sample Preparation and Analysis

Intact MUPs were purified, desalted as previously described (2) and reduced using DTT. The reduced protein was used for intact protein analysis. For online ETD LC-MS/MS the reduced protein was digested by enzyme into peptides.

For static infusion, the intact protein was dissolved in acetonitrile/water/formic acid (50:50:0.1) at a concentration of 5μM. Ten microliters of protein solution was transferred into the offline static nanospray PicoTip™ emitter with a 2 μm i.d. (New Objective Inc., Woburn, MA) using a gel-loader pipette tip (Brinkman Instruments Inc., Germany). The spray voltage was 1.1-1.2 kV resulting in a flow rate of 20-80 nL/min.

An Agilent® Technology 1100 capillary HPLC system was interfaced with the LTQ for online peptide separations using a C18 column. Spectra were acquired automatically on the LTQ using a Data Dependent™ alternating ETD/CID MS/MS instrument method (1 full MS plus 3 ETD and 3 CID MS/MS on 3 most intense peaks with dynamic exclusion) under the Xcalibur™ 2.0 software.

Data Analysis

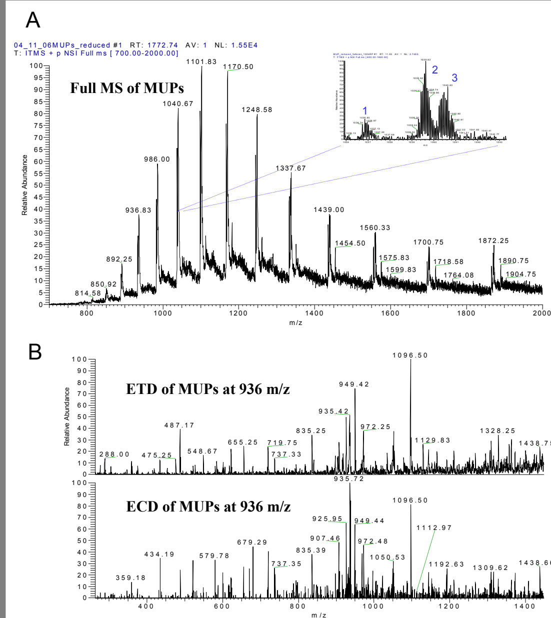
Intact protein data obtained using ETD or CID on LTQ was analyzed manually. BioWorks™ 3.2 (SEQUEST®) database search algorithm was used to identify the ETD peptides from protein digests. Each raw file was searched for different charge states from 2 to 5. Peptides identified by the software with significant Xcorr were evaluated manually to eliminate possible multiple assignments to any single spectrum which could be caused by performing multiple independent search events. ECD and CID files from LTQ FT were deconvoluted to monoisotopic mass lists using the Xtract™ program (Thermo Electron Corporation) that is part of the LTQ FT data system. The resulting Xtract output was submitted to ProSight PTM (https://prosightptm.scs.uiuc.edu) for analysis.

FIGURE 1. Electron Transfer Dissociation of Intact MUPs.

A: Full MS spectrum of intact MUPs. The inset shows the isoforms of MUPs

B: Comparison of ETD and ECD spectra

C: c and z ions identified by ETD of intact MUPs carrying twenty charges



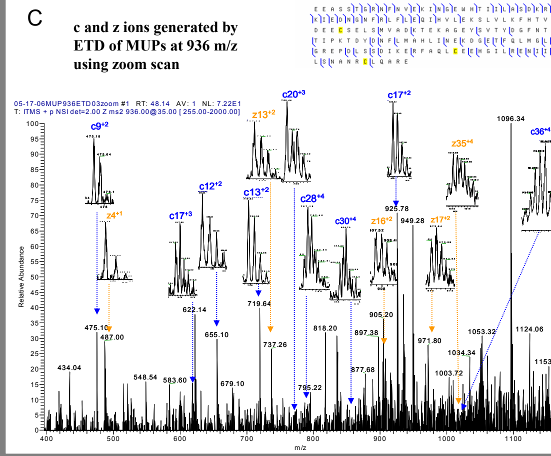
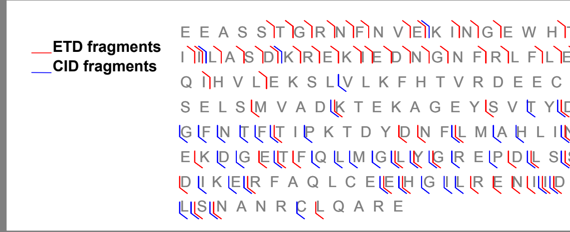


FIGURE 2. Sequence coverage of CID and ETD on intact MUP X00908

Fragments generated by LTQ with ETD are in red. Fragments generated by CID are in blue. Sequence coverage from ETD or CID are 40.1% and 28.4% respectively. The Combined sequence coverage from ETD and CID is 55.5%



Results

1. **Electron Transfer Dissociation of Intact MUPs**
   MUPs were purified and reduced as described. The reduced MUPs were infused into the LTQ or LTQ FT. Fig.1A shows the Full MS spectrum of MUPs acquired on the LTQ. The inset in Fig1A shows the isoforms of MUPs containing eighteen charges. In this inset, peak one contains a single protein of 18636 Da, peak two contains two proteins of 18699 Da and peak three contains a protein of 18684 Da.
   Peak 2 and 3 at charge state twenty were isolated for fragmentation using ETD on the LTQ or ECD on the LTQ FT. Fig.1B shows the comparison of ETD and ECD spectra. The results indicates that ETD on the LTQ produced very similar fragmentation profiles as ECD on the LTQ FT. Every identified fragment generated by ECD was also observed in ETD spectrum (data not show). The ETD spectrum was manually analyzed and the peaks of some of the identified fragment ions are shown in the insets of Fig.1C. The ECD spectrum obtained using the LTQ FT was deconvoluted using Xtract software and fragment ions were identified using ProSight PTM®. The identification of ETD fragments shown in Fig.1C were confirmed by the same ECD fragments obtained using LTQ FT. Fragmentation of intact MUPs at this charge state was extensive using ETD. As shown in the top right inset of Fig1C, a total of 33 c ions and 32 z ions were generated by ETD, which covers 40.1% of the sequence of the intact protein of 18699 Da.
2. **Top-down sequencing of MUPs – improved sequence coverage using ETD and CID.**
   Analysis of intact MUPs was also performed using conventional CID both on the LTQ and LTQ FT. The CID spectra was analyzed manually or by using Xtract and ProSight PTM. Fig 2, shows the sequence coverage from ETD and CID. While ETD fragmented this protein extensively from both N-terminus and C-terminus and generated 33 c ions and 32 z ions, which covers 40.1% of the sequence, CID fragmented the same protein mostly at the C-terminus and generated 3 b ions and 42 y ions, which covers 28.4% of the sequence. The total sequence coverage from combined ETD and CID is 55.5%.
3. **ETD following CID – a combined approach on intact protein**
   To evaluate other approaches using a combination of ETD and CID, we have selected the MUP's precursor which carries twenty one charges for fragmentation using CID. The most intense CID fragment, y45, was then selected for ETD at MS^3 (Fig.3). ETD generated c and z ions on y45. The purple marks in Fig.3 indicate the ions generated by ETD on fragment ion y45. The red marks indicate c-terminal c and z ion generated by ETD on intact MUPs. Some of the ions produced by the two different approaches are unique. Thus, additional sequence information was obtained using ETD following CID approach.
4. **Identification of MUPs isoforms using 'bottom-up' ETD**
   Purified and reduced MUPs were proteolyzed. 500 fmol of the digest was loaded on a C18 column and analyzed by ETD LC/MS/MS as described . All of the four isoforms of MUPs were identified using BioWorks at charges between 2 to 5. A total sequence coverage between 82% and 85% was obtained for each isoforms (data not shown). Fig.4A shows the amino acid sequence of each isoform. The peptides which contained mutations and which were identified by ETD are in black with the mutations shown in red while the rest of the sequence is in gray. ETD spectra of all the peptides containing polymorphisms are shown in Fig. 4B, 4C and 4D. All the amino acid mutations in the isoforms were identified except for the K/Q exchange, which are of similar molecular mass. The identified mutations were also located precisely as show in the series of identified c and z ions in each spectrum.

FIGURE 3. ETD following CID – a combined approach for intact protein analysis

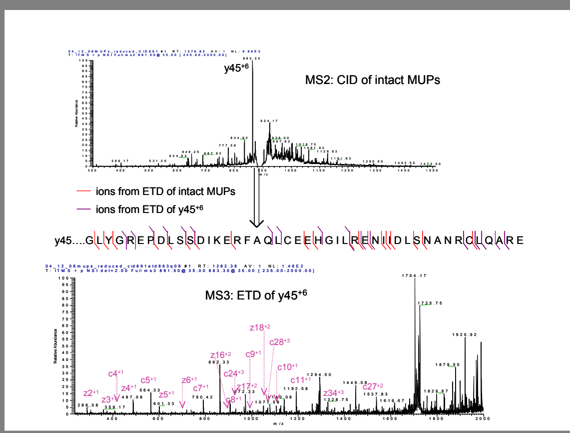
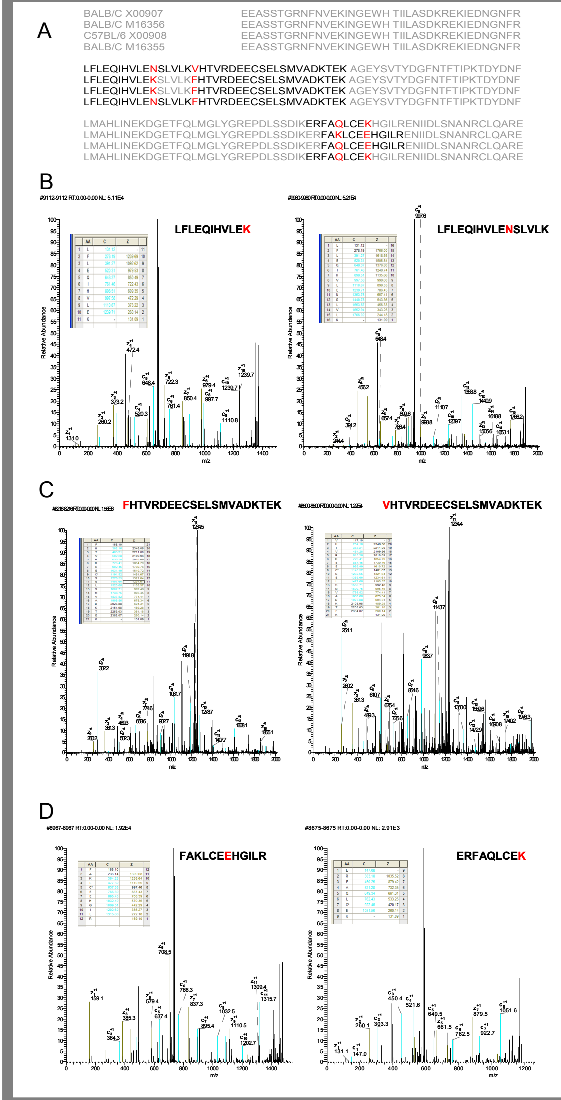


FIGURE 4. Identification of MUPS isoforms using 'bottom-up' ETD

A: Amino acid sequence of the four MUPs isoforms. The peptides identified by ETD which contain mutations are in black, those with mutations are in red. Other peptides are in gray.

B,C,D: ETD spectra of the identified peptides which contain mutations



Conclusions

Electron Transfer Dissociation (ETD) on a linear ion trap mass spectrometer is a very effective tool for the analysis of large peptides and moderate-size intact proteins (in this case, MUPs, an 162 amino acid, 18.6kD protein). We made several important observations:

- **ETD on the LTQ linear ion trap generated spectra and ion series which were qualitatively very similar to ECD spectra generated on an LTQ FT hybrid ICR instrument.**
- **ETD and CID are complementary dissociation techniques. ETD produced 33 c and 32 z fragment ions, corresponding to a protein sequence coverage of 40.1%. CID generated a complementary ion series which covers 28.1% of the sequence. Improved sequence coverage of 55.5% is obtained if the results from ETD and CID are combined.**
- **Additional sequence information was obtained by using a combined approach – ETD on a CID fragment. This combined approach produced c and z ions which are complementary to the c and z ions generated by ETD of the intact protein from the same sequence region.**
- **Bottom up analysis with ETD LC/MS/MS allowed identification of all four MUPs' isoforms with the mutations precisely located.**

References

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2. Armstrong SD, Duncan HLR, Cheetham SA, Hurst JL,Beynon RJ. 2005. Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. *Biochem. J.* 391:343-350.

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