Top-down & middle-down sequencing of Immunoglobulin using Electron Capture Dissociation – Time of Flight Mass Spectrometer combined with Online Disulfide Bond Reduction

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ABSTRACT

NIST-mAb (humanized monoclonal antibody [immunoglobulin gamma 2] provided by NIST [National Institute of Standards and Technologies]) was analyzed by an ion trap based ECD device installed in a Quadrupole-TOF mass spectrometer. In top down and *de novo* approach, the variable parts in the heavy chain and the light chain were partially sequenced to identify the combination of the two chains. ECD did not provide sequence information in the disulfide bonded rings appeared in the chains. To obtain more complete amino acid sequences, IdeS enzyme was applied to the intact mAb as a middle down approach. In addition to the digestion, fast and facile disulfide-bond reduction was applied to the digests trapped on an LC-column. As a result, sequence coverage of 76.5% for heavy chain and 84.7% for the light chain was obtained.

INTRODUCTION

Intact antibody sequencing is one of the goals in current mass spectrometry. Such analysis was often tried when new high-resolution mass spectrometers and new dissociation techniques were developed (FT-ICR, TOF, Orbi-Trap, etc. with ECD, ETD, UVPD, etc.). Electron capture dissociation (ECD) provides unique features such as top-down sequencing, *de novo* sequencing, glycosylation analysis, and informative disulfide bond cleavage, which can be an ideal tool to analyze intact antibodies. We have developed a small and high throughput ECD device based on an RF ion trap (ref 1). This technology was applied to an intact monoclonal antibody in this work. We also implement a novel online disulfide bond reduction technique (ref 2).

MATERIALS AND METHODS

The ECD cell (ref. 1) was installed between Q1 and Q2 in a research grade quadrupole-TOF system (Sciex). Simultaneous trapping ECD mode (ref. 1) was used for high throughput analysis, which is a simultaneous injection of the electron beam and precursor ions into the ECD device. Typical electron beam irradiation time was 10 ms, and the electron beam intensity was tuned to obtain appropriate dissociation efficiency. The mass resolution of the TOF-MS is 35,000-47,000, which resolved isotope patterns of fragments up to Z~30+. A desalting LC column (Waters) was used for desalting, online reduction (ref 2), and LC separation. Humanized monoclonal IgG (NIST-mAb) was obtained from NIST. IdeS enzyme was purchased from Genovis.

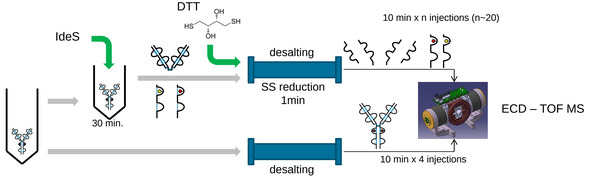

Figure 1. Sample preparation and LC-ECD MS/MS work flow for top down and middle down analysis.

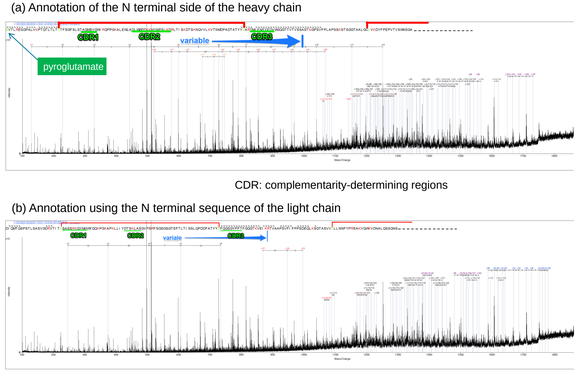

Figure 2. Top down ECD spectrum of intact NIST-mAb. All charge states of precursor ions, which appeared over m/z = 2000, were subjected to be dissociated by ECD. The spectrum contains N terminal fragments (z• fragments) of the light chain and the heavy chain and the C terminal fragments (c' fragments) of the heavy chain. (a) Annotation of the N terminal sequence of the heavy chain, (b) Annotation of the N terminal sequence of the light chain. The two spectra (a) and (b) are identical.

RESULTS

To obtain the best sequence coverage in top-down analysis using the ECD-TOF system, we found following tips. (1) A lower charged precursor may be selected to obtain lower fragment charge state distribution. (2) ECD with electron energy of 0-3eV and precursor consumption of 30~50% was optimal to detect large fragments in highly charged states. Longer electron irradiation (or stronger electron beam) induced secondly dissociation of primary ECD fragments, which loses the large fragments as well produces internal fragments, which are not informative for sequencing.

Intact NIST-mAb was analyzed by the LC-ECD-TOF mass spectrometer (figure 2). *De novo* sequencing on the intact ECD spectrum obtained by a single LC run indicated three sequences, and two of them were matched to N terminal partial sequences of the variable parts in a light chain and a heavy chain appeared in the human genome. Another one was matched to C terminal partial sequences of the fixed part in the heavy chain. To obtain candidates of the full sequences of the heavy chain and light chain, homology search (Protein Prospector) was applied to the partial sequence obtained by the *de novo* sequencing results. The intact ECD spectrum was further analyzed in top-down manner using the suggested full sequences (the full sequence is provided by NIST), where the data covered the variable parts of the light chain and the heavy chain in the mAb. ECD at electron energy of 3 eV did not cleave the disulfide-bonded rings in the protein.

To obtain nearly complete sequence coverages, offline IdeS digestion was applied (Figure 1) before LC injection. For the online reduction of disulfide bonds (ref. 2), the IdeS digest trapped on the desalting column for 1 min with DTT that was injected after the sample injection. The reduced digests were eluted separately by changing the concentration of the organic mobile phase (acetonitrile).

By this reduction, we obtained sequence coverages of 84.7 % for the light chain, 78.3 % for the variable part of the heavy chain (Fd'), and 84.7% for the fixed part of the heavy chain (scFc) (Figure 3). Further, ECD indicated the glycosylation site and its mass in scFc and CID informed the glycan composition.

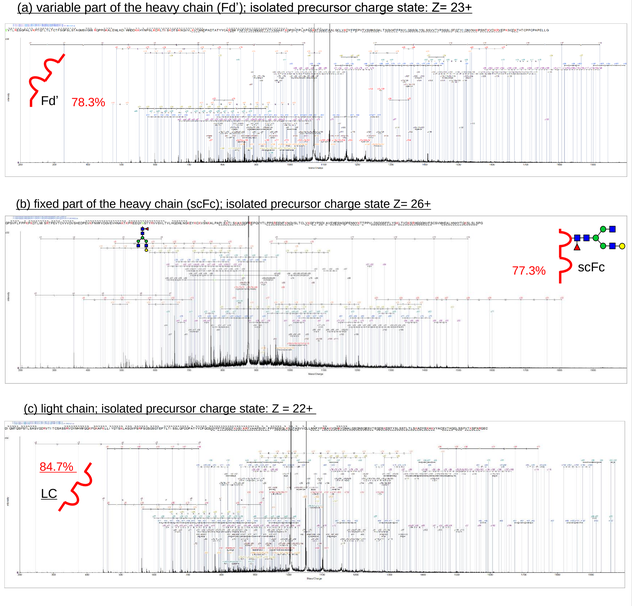

Figure 3. ECD spectra of online SS reduced IdeS products. ECD is applied on LC separated precursors that were reduced online (DTT was applied to the trapped IdeS digests on the desalting column). The sequence coverages were (a) 78.3% (Fd'), (b) 77.3% (ScFc), and (c) 84.7% (light chain).

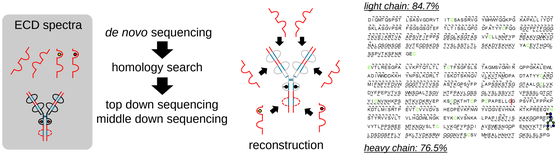

Figure 4. Data analysis and reconstruction of the structure of mAb using the top down and middle down sequencing by ECD

ECD provided amino acid sequences shown in the red lines in figure 4 in top-down and middle-down approaches, where the middle-down analysis of the reduced sample give better sequence coverages and the top-down analysis of the intact sample informs the combination of specific light chain and the heavy chain. Using the middle-down sequences, the complete sequence of the intact mAb is reconstructed by comparison to the top down results.

The technique will be applied to the mixed antibodies as well as other types of immunoglobulins.

CONCLUSIONS

NIST-mAb was analyzed by an ion trap based ECD device installed in a Quadrupole-TOF mass spectrometer. Using both top-down and middle-down approaches, sequence coverage of 76.5% for heavy chain and 84.7% for the light chain was obtained. Mass resolution of TOF-mass spectrometers (35,000-47,000) are good enough to cover the entire sequences of lengths of IdeS products of antibodies (typically 200~250 amino acid residues). Sequencing of the intact heavy chain (450 amino acid resudues), which was provided by disulfide bond reduction of intact mAb, was not promising because contribution of internal fragmentation is too strong.

REFERENCES

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