Top-Down Sequencing Applications to Protein Characterization in Pharmaceutical Discovery with Nanoelectrospray-QqTof

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Gas-phase fragmentation of intact proteins, coupled with detection by mass spectrometry (MS), is an expanding area of research with multiple applications to protein characterization. To enable the analysis of small sample volumes for extended time periods, novel polyaniline (PANI) coated emitters were utilized for static nanoESI MS. These nano-emitters showed exceptional durability, routinely permitting analysis of 1-2 µl samples for several hours. The combination of nanoESI and top-down MS on commercial QqTof allowed localization of isotopic labels in recombinant protein targets, identification of differentially regulated protein from drug-dosing studies, and partial sequencing of monoclonal antibodies (mAbs).

Recombinant proteins were provided by the Protein Biochemistry Project Team at Abbott Laboratories. Plasma samples were obtained from Abbott Bioresearch Center (ABC, Worcester, MA) and was processed, spotted onto an H50 SELDI chip (Ciphergen Biosystems, Fremont CA) and analyzed with the Ciphergen PBSII instrument. The monoclonal antibody sample was obtained from ABC and was treated with dithiothreitol (DTT) for 30 minutes at 37^{o}C. After incubation, the sample was dialyzed and diluted to approximately 0.05 mg/ml. The samples were analyzed on a Q-Star Pulsar-I (Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray interface (Protana, Odense, Denmark). The polyaniline-coated nano-emitters were obtained from Nanogenesys (Amherst, NY) and provided extreme durability for extended analysis times.

To evaluate the ability of the Qq-Tof to fragment intact proteins, cytochrome *c* was initially tested. When properly tuned, the instrument was capable of resolving charge states as high as +17 for the most intense peaks. Figure 1 revealed that isolation and CID of the +17 peak at m/z ~ 728 produced extensive fragmentation to form a, b, and y ions. An examination of the spectrum revealed 4 a ions, 21 b ions, and 28 y ions. A similar study utilizing myoglobin revealed multiple a, b, and y ions, in addition to apparent fragmentation ions whose charge state could not be determined by isotopic spacing (Figure 2). Our experience has shown that, for peaks of low intensity, it is difficult to determine charge states higher than ~ +10. Presumably, these fragment ions possessed charge states greater than +10 and the peak intensity was insufficient to achieve isotopic resolution.

C-Jun NH_2-terminal kinase-1 (JNK-1) has been shown to express well in insect cells and was chosen as a model system for developing ^{13}C-methyl labeling protocols for protein production. Labeling the hydrophobic residues Met, Leu, Ile, and Val is important since they are frequently present in ligand binding sites. Taking a stepwise approach to completely labeling these residues, efforts were first focused on ^{13}C-labeling the methyl carbon of methionine. Initial method development produced protein that showed a mass difference of 13 Da vs. unlabeled material. However, 14 methionine residues are actually present in the molecule. The single, intense peak observed in the ESI spectrum suggested that complete labeling had taken place, but the mass uncertainty of +/- 1 Da did not allow us to conclude this with certainty. Bottom-up sequencing revealed the presence of 13 labeled Met residues. However, peptides containing Met-367 were not detected, despite digestion with a variety of different enzymes. Partial tryptic digestion, followed by MS analysis, revealed a peptide of 6860.4 Da. This mass was 1 Da higher than expected for the tryptic peptide Ile-316 to Arg-372. To confirm labeling at Met-367, MS/MS was performed on the +7 peak of the 6860.4 Da species. 11 b-ions and 15 y-ions were formed by fragmentation. All observed b-ions (in this case, all b-ions were formed by cleavage on the N-

terminal side of Met-367), and the y-ions formed by cleavage C-terminal to the labeled Met, showed the expected mass. Cleavage on the N-terminal side of Met formed y-ions that were 1 mass unit higher than expected, indicating that ^{13}C labeling occurred between residues 47 and 53. These data suggested that ^{13}C labeling had occurred at Met-367.

Top-down MS has the ability to systematically isolate and characterize individual components of protein mixtures without prior separation. To show the feasibility of using this technology for antibody characterization, a mAb sample was reduced with DTT, dialyzed, and analyzed by nanoESI. The mass spectrum of the light chain revealed the expected molecular weight of 23412 Da. Isolation and MS/MS fragmentation of the +23 peak at *m/z* 1019 produced 15 b-ions ($b_{4\text{-}7,\ 28,\ 38,\ 42,\ 50,\ 55\text{-}58,\ 115,\ 118,\ 142}$) and 32-y ions ($y_{2,\ 9,\ 11,\ 15,\ 27,\ 35\text{-}42,\ 48\text{-}60,\ 79,\ 96,\ 97,\ 125\text{-}6,\ 135,\ 139,\ 140}$) all of which confirmed the expected sequence. The mass resolution of the Qq-Tof permitted direct determination of fragment mass (via isotopic spacing) for species as large as 15525 Da (y_{140}). These data suggest that similar results could be obtained on the heavy chain hypervariable region after isolation and reduction of the $(Fab')_2$ species.

Top-down analysis can also be used in biomarker identification. As an example, plasma samples from control mice treated with an anti-inflammatory agent, dexamethasone, exhibited increased levels of a 9.4 kDa protein. This 9.4 kDa species was enriched using RP-HPLC, the eluted fractions were screened by MALDI-MS, and the candidate sample was profiled to confirm the previously observed regulation. An aliquot containing the 9.4kDa protein was concentrated, diluted in 49/49/2 methanol/water/acetic acid and loaded into a PANI coated nano-emitter for top-down MS analysis. Isolation and MS/MS fragmentation of the +8 peak at *m/z* ~1180 is shown in Figure 3. A Mascot search of the MS/MS data, using the mouse International Protein Database, suggested that the up-regulated protein was a fragment of serum-albumin precursor. Manual interpretation of the data confirmed this assignment.

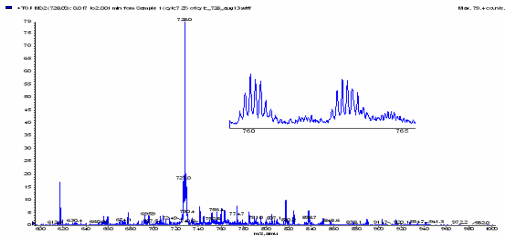

Figure 1: Top-down analysis of Cytochrome C.

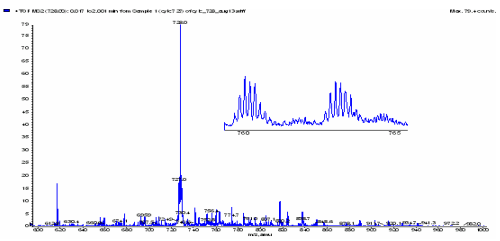

Figure 2: Top-down analysis of Myoglobin.

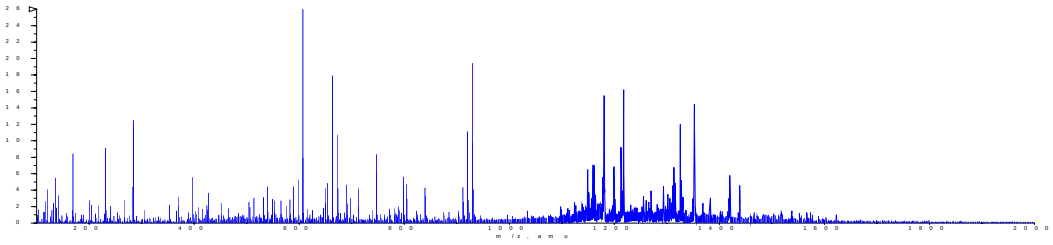

Figure 3: Top-down analysis of 9.4 KDa biomarker.

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