Characterization of Intact Antibodies by Pre-Fractionation Using Gel Electrophoresis and ESI-MS

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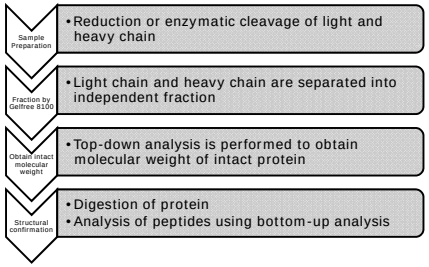
OVERVIEW

- Increased therapeutic applications of antibodies necessitates analytical methods for the rapid, sensitive characterization of antibody structure.
- Ideally, antibody characterization by mass spectrometry would involve a combination of both top-down and bottom-up analysis to confirm protein primary structure and characterize PTMs.
- Few high resolution separation/fractionation tools are available for intact proteins. Coupling 1D gels and LC/MS allow one to analyze antibodies using a bottom up approach. Intact mass measurements using LC/MS allow one to validate the bottom-up result. These two strategies require different approaches to sample preparation.
- This presentation introduces a single sample preparation strategy for monoclonal antibodies, enabling isolation of the light and heavy chain in a manner fully compatible with both top-down and bottom-up methods of analysis.

INTRODUCTION

Antibodies represent an important class of proteins due to their central role in the immune response. Moreover, there is an increasing interest in the use of recombinant antibodies as novel drug therapies. The structural analysis of antibodies is important for a variety of reasons ranging from understanding the structure of biologically active antibodies to monitoring antibody production to ensure a quality product. Post translational modifications play an important role in the biological activity of antibodies. Of the many modifications, glycosylation is highly variable depending on the method of antibody productions and the variations are highly correlated with variations in biological activity. This project presents a strategy for the isolation and characterization of antibodies that combines gel electrophoresis and LC/MS.

ANTIBODY CHARACTERIZATION WORKFLOW



Reagents
Monoclonal IgG antibody was purchased from Abcam. All other reagents were purchased from Sigma.

Sample preparation
A 1 mg/mL solution of antibody was prepared in 50 mM ammonium bicarbonate. Reduction was performed by adding 10uL of antibody solution to 88uL of 50mM ammonium bicarbonate and 2 µL of 0.5 M DTT. The solution was heated to 50°C for 30 minutes. 4.8 µL of IAA was added to the solution along with 15.2uL of 50mM ammonium bicarbonate and the reaction proceeded for 1 hr at room temperature in the dark. A volume of 30 µL of 5x Gelfree Sample Buffer was added to the sample, yielding 150 µL of volume containing 10 µg of total reduced antibody.

Gelfree 8100 fractionation
The reduced IgG was fractioned using the Gelfree 8100 as directed by the manufacturer's suggested method. The collection time for the antibody were between 53-62 minutes for the light chain and 65-92 minutes for the heavy chain.

SDS Removal
SDS was removed from the sample using Detergent Removal Spin Columns (Pierce).

Digestion Protocol (bottom-up only)
The Gelfree fractions containing the reduced and alkylated antibody fragments were digested by adding trypsin (1:100 enzyme/substrate) and heating for 1 hour at 37°C.

Mass Spectrometry

Intact Analysis
A volume of 30 µl of each acidified sample were loaded on a 2.1 x 50 mm C4 XBridge column with 3.0 µM particle size, 300 Å (Waters). LC-MS data were acquired on a LTQ Orbitrap (Thermo) coupled to a Agilent 1100 capillary LC system. A 25 minute step gradient was used for elution (A: 1% FA in H_2O; B: ACN).

Bottom-up
A volume of 8 µl of each peptide digest was loaded on a 0.075 x 150 mm C18 Chip LC column (Agilent). LC-MS data were acquired on an Agilent 6340 Ion Trap coupled to a Agilent 1200 nano LC system. A 45 minute gradient was used for elution (A: 1% FA in H_2O; B: 90% ACN).

GELFREE 8100 FRACTIONATION SYSTEM

Figure 1. The Gelfree 8100 instrument (left) is an eight channel electrophoretic controller that supplies voltage independently to each of the eight channels in the pre-cast cartridge (right). To use the system, the user programs the sequence (or chooses from a pre-programmed sequence) for each of the eight electrophoretic channels and starts the experiment. The device automatically pauses the experiment when each time interval has expired, allowing the user to extract the molecular weight fraction of interest. Measurement information for the eight channels is displayed to the user in tabular and graphical format during the course of an experiment.

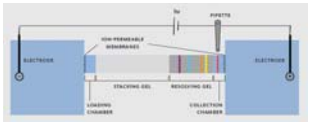


Figure 2: Schematic of the Gelfree device. The Gelfree 8100 uses SDS-PAGE using specialty pre-cast gels to separate analytes based on molecular weight. As molecular weight fractions elute from the end of the gel, they are entrapped in a 150 µL liquid layer defined by the end of the gel and a molecular weight cut-off membrane.

OPTIMIZATION OF FRACTIONATION

Optimization of Protein Fractionation: A Model Study
Gelfree 8100 can be programmed by the user to isolate the proteins of interest into a single fraction at high recovery.

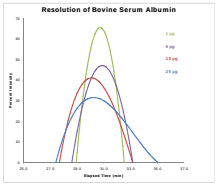


Figure 3: Resolution of separation as a function of protein load. Bovine serum albumin peak width as a function of protein load. Peak width varies between 2 and 7 minutes, FWHM. Width increases at higher loading. Fractions were collected at 90 second intervals to define the elution window.

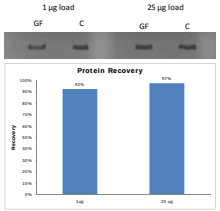
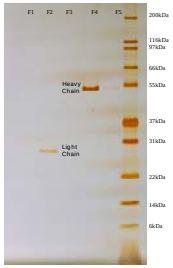


Figure 4: Optimization of recovery. Simple adjustment of the collection times allows for a protein to be collected in a single fraction at near quantitative recovery. Using the elution time data in Figure 3, collection times were optimized for maximum recovery. Gel image shows the Gelfree fraction (GF) containing albumin, 1 µg and 25 µg, compared to control.

Figure 5. Analysis of intact mass of light chain and heavy chain mAb. Gelfree collection times were optimized for collection of the light and heavy chain mAb in distinct fractions. The optimized recovery of the light and heavy chain are demonstrated using 1D gel analysis (a). Analysis of these fractions using ESI-MS is shown for the light chain (b-c) and the heavy chain (d-e). Heavy chain mass indicates presence of the G0F form of the mAb.



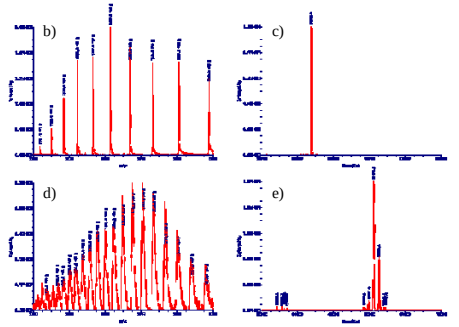
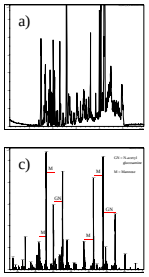


Figure 6. Further analysis of mAb heavy chain. Tryptic digestion of the heavy chain mAb (Fraction 4, Figure 5) allows for further characterization of the heavy chain. A glycopeptide found in the chromatogram (a) was selected for fragmentation (b). A selected range is highlighted and annotated to show fragmentation of the glycan (c).



SUMMARY

- Mass spectrometric characterization of antibodies requires multiple approaches, including both top-down and bottom-up approach.
- Gelfree 8100 Fractionation System provides a simplified, universally applicable, method for isolation of antibodies and antibody fragments.
- Gelfree 8100 fractionation can be optimized by the user to ensure high recovery of the protein of interest.
- Gelfree fractionation is compatible with the intact analysis by ESI; likewise, the sample can be analyzed using bottom-up techniques.

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