

An on-membrane Digestion Protocol for the Full Length Sequencing of Monoclonal Antibodies

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ABSTRACT

- A methodology for mapping the Complementarity Determining regions (CDR) and Fc region of an antibody is described.
- This approach applies a combination of chemical and enzymatic cleavages of antibody blotted onto PVDF-membrane, N-terminal sequencing, mass spectrometric analysis and database searching, to generate and analyze complimentary overlapping peptides.
- Utilizing this method, the entire sequence of one antibody was derived and the sequence of another antibody is currently 90% complete.

INTRODUCTION

- The past decade has welcomed the introduction of monoclonal antibodies as biotherapeutic agents for the treatment of malignancies, autoimmune disease and other maladies.
- The CDR and Fc regions play important roles in antigen-binding specificity and effector function of an antibody. Mutations of antibodies in these regions have been utilized to induce the desired specificity & immunogenicity.
- Protein-based sequencing of antibodies is necessary to verify translation products of specific mutations.
- Here we describe an approach to derive the antibody sequence using a combination of on-PVDF chemical or enzymatic digestions using various proteases with capLC reverse phase separation, N-terminal sequencing, MALDI-TOF & LC-MS/MS analysis, with database & *de novo* sequencing.

MATERIALS & METHOD

- The following enzymes Lys-N, Clostripain, Trypsin, Asp-N & Glu-C were obtained from Seikagaku, Cal-Biochem, Promega & Roche respectively. CNBr was purchased from Pierce.
- The antibody (Ab) was generated in-house against a known autoimmune related antigen (Figure 1). This was reduced (DTT) and alkylated (NIPIA) and heavy and light chains were separated by SDS-PAGE.
- Procise 494 N-terminal sequencer, Voyager DE-STAR MALDI-TOF & QSTAR XL were from Applied Biosystems. The LCQ Deca and capillary HPLC™ were from Thermo and Waters.

- CNBr cleavage was performed at RT overnight. Asp/Pro was carried out as previously described.¹
- All enzymatic on-PVDF digestions were performed in 0.5% Zwittergent 3-16 at 37 °C overnight.
- Peptides were sequenced on the Procise sequencer using a 20-min cycle.²
- Masses were analyzed by MALDI-TOF and peptide mass fingerprinting (PMF) was performed on the QSTAR. LC/MS/MS was used for sequence verification.
- Database searching was performed using FragFit and Mascot softwares.
- Method is summarized in Fig 1.

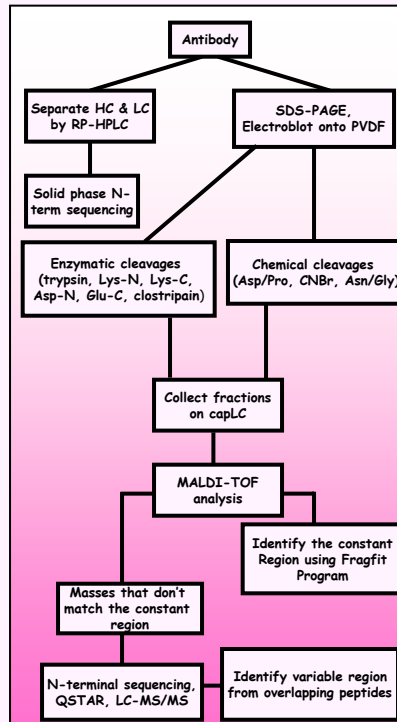


Figure 1: Flow Chart of Antibody Characterization.

RESULTS & DISCUSSION

- The antibody was raised against the auto-immune disease antigen of interest (Figure 2). Mutations were induced in the CDR and Fc regions for better antigen-binding specificity and stability.
- An ELISA was performed to evaluate the neutralization capabilities.
- Peptide mapping of the Ab with the highest neutralizing capacity was crucial to verify the points of induced mutation.
- On -PVDF membrane digestions were used as well as traditional solid-phase sequencing.
- Chemical cleavages such as CNBr, Asn/Gly & Asp/Pro often generate large peptides, which were subjected to database searches (high Homology is present in all the constant regions of the HC & LC).
- These constant regions were successfully identified in the first few chemical cleavage attempts.
- A variety of enzymatic digestions were employed to generate the overlapping peptides, which, eventually characterized the variable regions of the HC & LC.
- Sequence information was obtained using both MS/MS (QSTAR & LC-MS/MS) and N-terminal sequencing.
- Typically for the variable regions a combination of both techniques were employed for double sequence confirmation.
- Figure 3. shows the LC region of the fully sequenced anti-autoimmune disease in-house designed monoclonal antibody.

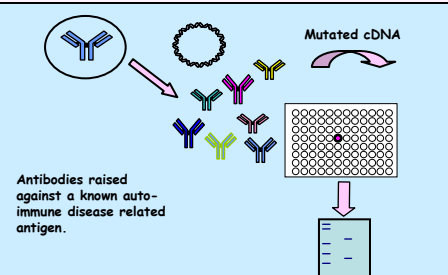


Figure 2: Flow Chart of Antibody generation and immunogenic characterization



Figure 3: Peptide map from Antibody proteolytic and chemical digestions.

CONCLUSION

- A technique for the proteomic characterization of the CDR and Fc region of an anti-auto-immune disease related antigen has been described.
- Using a combination of proteolytic techniques along with N-terminal sequencing and MS a full antibody sequence has been characterized.

REFERENCES

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