On-Plate Digestion and Analysis of Immunoglobulins using MALDI-TOF-MS

Francine Yanchik-Slade[1]; Milaan Thirukumaran[1]; Nazim Boutaghou[1]

[1] Shimadzu Scientific Instruments, Columbia, MD

1. Overview

Samples containing immunoglobulins and proteins were analyzed by MALDI-TOF-MS utilizing on-plate enzymatic digestion to distinguish between differences in the variable region of the immunoglobulins. Method development determined that digestion could take place on non-reduced and non-alkylated samples, increasing the speed of analysis. On-plate digestion of immunoglobulins resulted in unique fingerprint spectra that could be sorted into groups using principal component analysis (PCA).

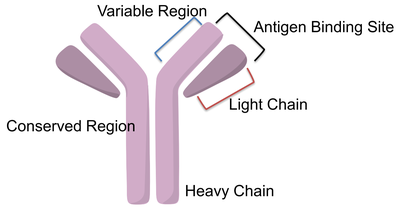

Figure 1. General structure of immunoglobulins, labeling key areas of interest

2. Introduction

Antibody-based therapeutics and research applications have become increasingly important in the treatment of challenging diseases and disorders. Antibody-drug conjugates (ADCs) and other antibody-based therapeutics have become critical for cancer treatment, are becoming top selling drugs and as such are subject to counterfeiting. Fast and accurate verification through different analytical methods are needed to ensure the quality of drugs available to customers. Mass spectrometry and MALDI-TOF-MS are techniques of choice and are amenable for direct on-site verification. Typical enzymatic digestion requires various pretreatment steps such as reduction and alkylation, followed by a long digestion period (~8-12 hrs). Removing these steps allows for a faster sample preparation and resulting identification. In this work a rapid on-plate digestion workflow was utilized to aid in characterization using MALDI-TOF-MS. A statistical analysis software was utilized to identify key peaks from different antibody samples to create groups to assist in sample verification.

System: MALDI 8030
Polarity: Positive Mode
Mass Range: *m/z* 500-7000
Acquisition: 995 shots @ 200Hz
Ion Gate (Blanking): 450
Pulsed Extraction: 3000

Figure 2. Analysis Conditions for MALDI-8030 analysis of on-plate digestions.

Table 1. Protein standards and abbreviations

Standard	Abbreviation
NIST Monoclonal Antibody Reference Material 8671	mAB
IgG from Human Serum	IgG
SILu Lite SigmaMAb Universal Antibody Standard Human (MSQC4-1MG)	IgX
Bovine Serum Albumin	BSA

3. Methods

On plate trypsin digest: Trypsin/Lys-C was spotted and dried onto a 48 well stainless-steel target before addition of immunoglobulin sample. Samples were briefly mixed with trypsin/Lys-C before a 25-minute incubation period. Incubation took place at either 40°C in a chamber humidified with KNO_3 , or on the benchtop at ambient temperature (~20 °C). Digestion was terminated by addition of 1% TFA. For analysis samples were mixed with CHCA (5 mg/mL) and analyzed in positive ionization mode. This method was adapted from a previous experiment.[1]

Method Development

Preliminary experiments included samples treated for reduction and alkylation, it was determined that the additional salts necessary for theses steps reduced the quality of the resulting MALDI-TOF-MS spectra and were not used for further testing.

Digestion was analyzed after incubation at room temperature and heated to evaluate spectral quality. **Figure 3** shows the results of room temperature digestion, resulting in an inconsistent number of peaks across all samples. **Figure 4** shows digestion at 40°C in a humidified environment, this resulted in a more consistent number of peaks across all samples.

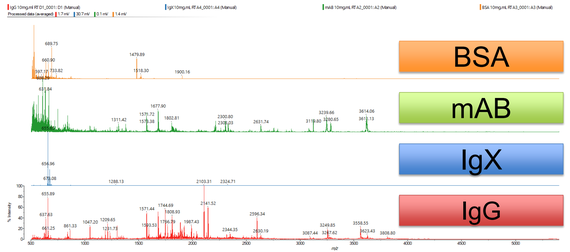

Figure 3. MALDI-TOF-MS Spectra of on plate digestions ambient temperature incubation.

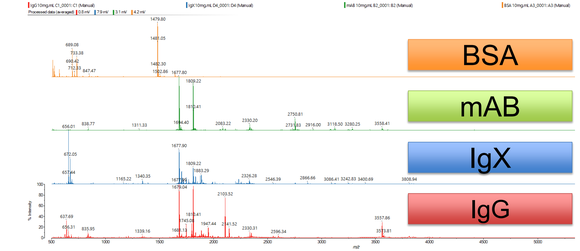

Figure 4. MALDI-TOF-MS Spectra of on plate digestions with 40 ° C incubation.

4. Results

Statistical Analysis

Antibodies are arranged in three globular regions; these are comprised of two identical heavy chains and two identical light chains that roughly form a Y shape. Both the heavy and light chains contain one variable domain that comprises the antigen binding site. Determining the difference between antibodies relies heavily on detection of the variable domains. Typically for antibody characterization on a linear TOF, intact mass analysis will not be enough to distinguish between two samples. Enzymatic digestion of the antibody allows for a more detailed analysis of the sample and can show more nuanced differences between samples. The data generated resulted in unique fingerprints for each sample, uploading the data into eMSTAT Solution™ allows for identification of key peaks. PCA plots (**Fig. 6**) show distinct groupings for each sample, indicating even with peak redundancy in the conserved region the peaks generated from the variable region could be identified to sort samples. Identifying peaks are labeled within the software and can be displayed as box plots (**Fig. 7**).

Figure 5. Sample and data analysis workflow

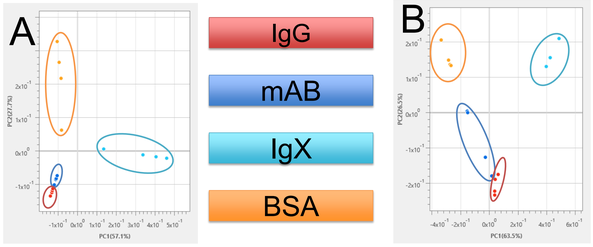

Figure 6. Principal Component Analysis plot of 40°C digestion (A) and ambient temperature digestion (B).

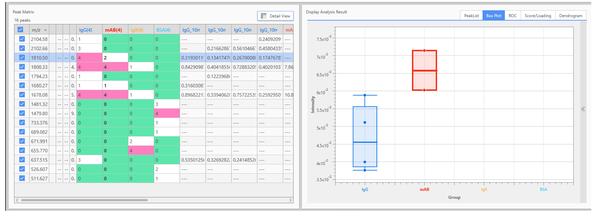

Figure 7. Box plots and peak scoring within eMSTAT Solution™. Selected peak at 1808 *m/z* shows a high score for IgG and mAB samples and no presence in IgX or BSA.

eMSTAT Solution™ takes multivariate data and converts it into discriminant analysis to create models to predict grouping for unknown samples. Models were generated for both data sets, both models could take in data from previous runs and experiments and sort them into their corresponding groups. Models generated from both digestion temperatures were able to correctly sort “unknown” samples based on key peaks.

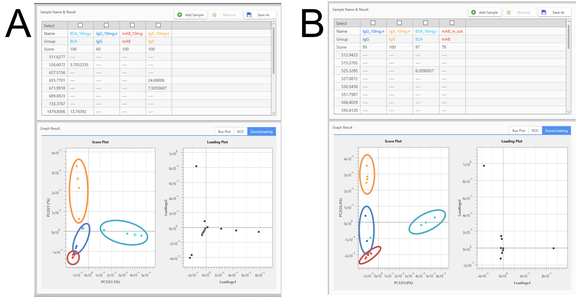

Figure 7. Discriminant analysis model of 40°C digestion (A) and ambient temperature digestion (B).

5. Conclusion

We have shown that a rapid on-plate enzymatic digestion can generate unique fingerprints for a range of immunoglobulins. When combined with a statistical analysis software, these fingerprints can be utilized to generate PCA plots that can assist with the formation of a discriminant analysis model to identify unknown samples. Digestion at ambient temperature and elevated temperature produced unique peaks that could be used to group samples.

This technique can be applied to pharmaceuticals that utilize immunoglobulins or other protein-based components to provide rapid identification of genuine or counterfeit products. Through the creation of standard libraries and discriminant models from genuine and counterfeit products, new samples can be quickly identified for further review.

6. References

1. Shimadzu Application News MALDI-2301

The authors declare no competing financial interest.
Disclaimer: Research Use Only (RUO). Not for use in diagnostic procedures.