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Droplet Phase Ion-Ion Chemistry

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Derivatization of Peptides

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Introduction

Proteins are essential building blocks and components of cells and are responsible for a host of cellular functions. As such, protein purification and identification is of vast importance to many techniques in modern laboratories. There are currently many methods used for the digestion and subsequent identification of proteins but still a demand exists for improved techniques. In the research lab of Prof. Franco Basile at the University of Wyoming, the proteins are digested using microwave-assisted acid hydrolysis. This is a technique for rapid protein degradation and is followed by analysis via mass spectrometry for protein identification.[1]

Enzyme Digestion

A common technique used for digestion of proteins in modern laboratories involves the use of Trypsin, an enzyme that cleaves peptides into smaller surrogate peptides at the amino acids Arginine and Lysine.[2] While this method of digestion is very accurate and predictable, it takes a long period of time, typically 5 hours to as long as 24 hours. In the experiment performed in the article by Wu et al. the solutions had to be left overnight which is necessary for enzyme digestion. This method also is inconsistent when performed on proteins and peptides that have sequences of lysine-lysine, arginine-arginine, or a variety of others involving arginine and lysine.[3] This shows another

[1] Zhong, Marcus, and Li, "Microwave-Assisted Acid Hydrolysis of Proteins Combined with Liquid Chromatography MALDI MS/MS for Protein Identification."
[2] Wu et al., "A Strategy for Liquid Chromatography/tandem Mass Spectrometry Based Quantitation of Pegylated Protein Drugs in Plasma Using Plasma Protein Precipitation with Water-Miscible Organic Solvents and Subsequent Trypsin Digestion to Generate Surrogate Peptides F."
[3] Ibid.

disadvantage for Trypsin digestion, in addition to the need to develop a faster method with a larger variety of applications for protein sequencing.

Microwave-Assisted Acid Hydrolysis

Microwave-assisted acid hydrolysis (MAAH) is a technique that commonly involves the use of 25% trifluoroacetic acid (TFA) or 6M HCl and requires between 1-10 minutes to digest a sample.[4] However, the products of this digestion have many non-specific cleavage sites which lead to difficulty in reproducible identification of microorganisms. Many enhancements of this technique are currently being employed in an effort to increase the reproducibility of this technique to allow for better total sequencing. An example of this was performed in the paper by Swatkoski et al. that involved the use of a combination of 3% formic acid and microwave heating. This modification produced both aspartyl-specific cleavage, and non-specific cleavages.[5] MAAH has been found to work well for sequencing of hydrophilic proteins and some membrane proteins. It also produces larger peptides for sequencing. However, MAAH provides limited cleavage sites and can result in N-terminal formylation of

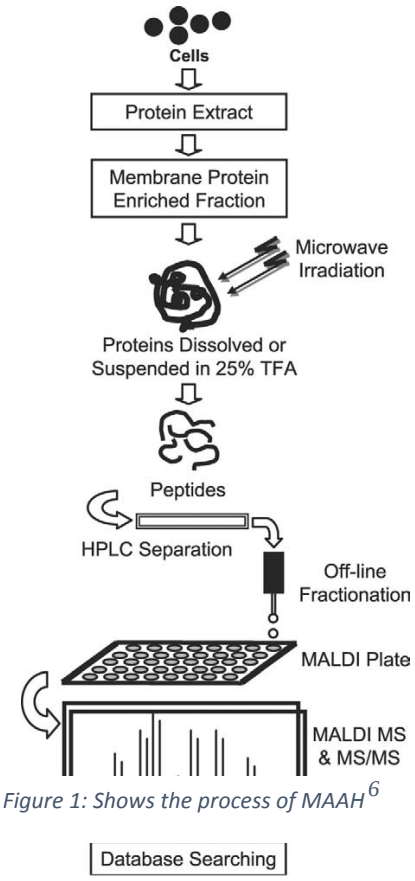

Figure 1: Shows the process of MAAH[6]

[4] Swatkoski et al., "Rapid Chemical Digestion of Small Acid-Soluble Spore Proteins for Analysis of *Bacillus* Spores."
[5] Ibid.

peptides.[6] The production of larger cleaved peptides with unknown cleavage points can have drawbacks such as an inability to provide complete sequencing of a protein. This drawback could potentially be mitigated via derivatization of the peptide with a label of some kind.

High-Throughput Bioconjugation

A paper by Cotham et al. showed a reaction involving the labeling of the primary amine in a peptide cation using the compound 4-formyl-1,3-benzenedisulfonic acid (FBDSA) via Schiff base formation of the aldehyde moiety of the FBDSA anion. While this technique could provide a more complete sequencing of a protein, derivatization reactions frequently are the rate-limiting step of a sequencing procedure. This is due to the fact that derivatization usually requires additional time in off-line solution-phase chemistry prior to sequencing via mass spectrometric analysis. Cotham et al. employed a technique that utilized the mass spectrometer to facilitate rapid functional group derivatization.[7] Their technique involved the use of a dual-electrospray mass spectrometer (MS). The goal of their research was to "…modulate electrospray ionization in an online manner via fast mixing of droplets or

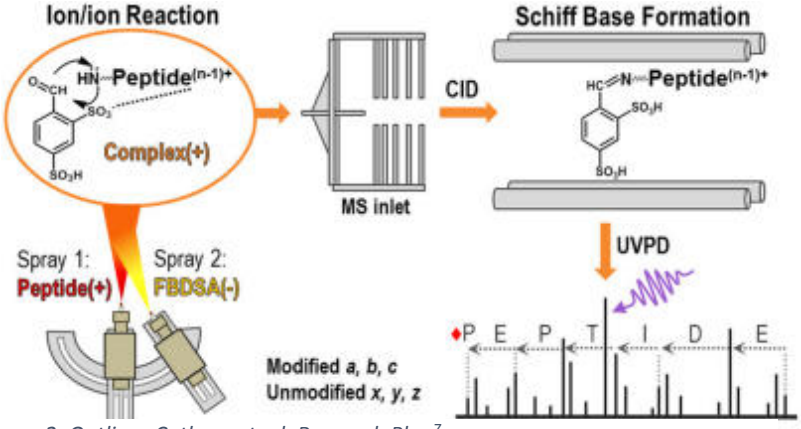

Figure 2: Outlines Cotham et. al. Research Plan[7]

[6] Zhong, Marcus, and Li, "Microwave-Assisted Acid Hydrolysis of Proteins Combined with Liquid Chromatography MALDI MS/MS for Protein Identification."
[7] Cotham, Shaw, and Brodbelt, "High-Throughput Bioconjugation for Enhanced 193 Nm Photodissociation via Droplet Phase Initiated Ion/Ion Chemistry Using a Front-End Dual Spray Reactor."

exposure of droplets to gaseous acids or bases."[8] The dual electrospray was used to facilitate the Schiff's base reaction between FBDSA and the labeled peptides via an online dual-spray to enable continuation of sequencing with bioconjugation directly out of the LC.

After reading through the paper by Cotham et al., and realizing the potential application of their proposed derivatization method combined with MAAS, we came up with a series of goals at the beginning of the semester. 1) Replicate the results of a study undertaken by Cotham et al. 2) Change the method used to improve the measurement of the amino acid sequence of a peptide by mass spectrometry. The proposed method used collision induced dissociation (CID) to initiate a droplet phase ion/ion Schiff's base reaction followed by photodissociation with UV light. The two solutions were brought together in the droplet phase via positive (peptide) and negative (FBDSA) electrospray to test whether collision induced dissociation was achieved via the quadrupole ion trap mass spectrometer's MS/MS/MS capability. This test was to assess whether MS/MS/MS can be used in place of the two step CID plus photodissociation with UV light process. This test involved the use of broad band MS to include the product peak with and without water adducts. 3) If the above worked: test the feasibility of FBDSA labeling of peptides digested by MAAS.

[8] Ibid.

Experimental

Angiotensin

The first step to our research was to view the protein chosen and FBDSA on their own in the MS .The protein chosen for derivatization was Angiotensin II due to availability, common use in labs, and possessing a molecular weight that was similar to those we expected of peptides upon MAAS fragmentation.[9] We attained strong signal from the MS of Angiotensin II as seen above. This spectrum was produced in the positive mode using a 1mM solution. This solution was produced by massing 0.0050g of Angiotensin, dissolving it in 2.5mL of CAN, adding 2.5mL H_2O, and then adding 5µL of formic acid. The MS was run in positive mode with a voltage of 1.75kV, a flow rate of 1.5µL/min, and a mass range from 200-2000m/z.

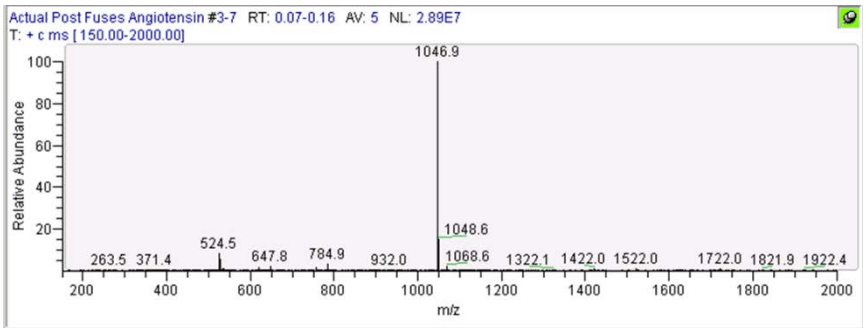

Figure 3: Shows spectrum of Angiotensin II via Front Spray positive mode MS. Main Peak at 1047 m/z is that of Angiotensin II.

[9] Zhong, Marcus, and Li, "Microwave-Assisted Acid Hydrolysis of Proteins Combined with Liquid Chromatography MALDI MS/MS for Protein Identification."

4-formyl-1,3-benzenedisulfonic acid (FBDSA)

The molecule used for derivatization of the protein was FBDSA. This molecule was chosen by Cotham et al. because it was found to increase the informative fragmentation of proteins to which it was covalently bound. FBDSA was used in the negative mode electrospray. A 1mM solution of FBDSA was prepared via mashing 0.0030g of FBDSA, dissolving it in 2.5mL of H_2O and then adding 2.5mL of methanol. The results obtained were using a mass range from 200-2000 *m/z* with a spray voltage between -2.5 and -3.0kV. The flow rate was maintained at 3.0 µL/min. The spectrum shown above is a good example of our results for the negative mode spectra. The peaks are those of the background noise and the calibration standards used for the LCQ that persist at low intensity levels in the background. Our negative mode spectra did not produce satisfactory results

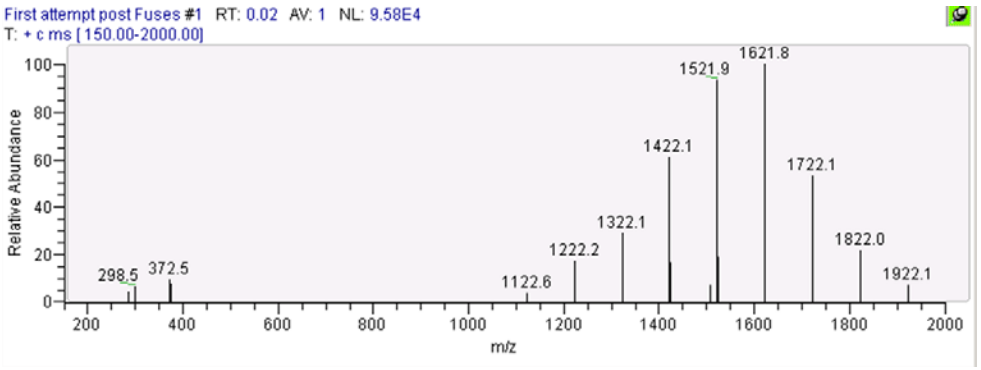

Figure 4: Shows spectrum attained when attempting negative mode MS of FBDSA. Peaks are of calibration compounds in background noise.

Dual Spray

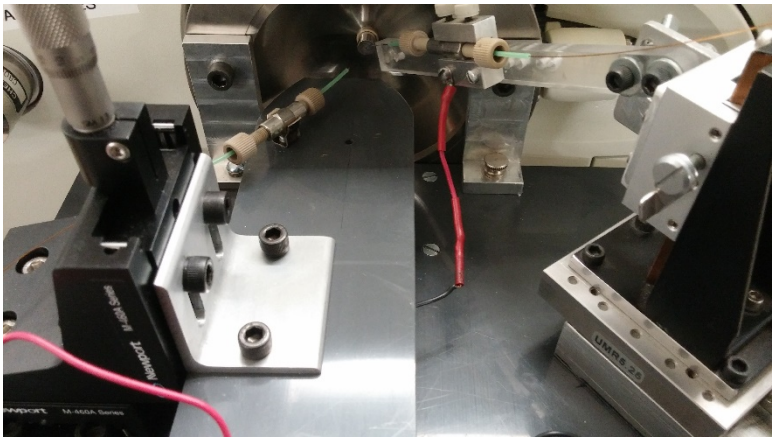

Figure 5: Shows the custom dual-electrospray board synthesized in Prof. Basile's Lab.

In an attempt to continue our research, we then constructed a dual-electrospray apparatus for our MS. The mass spectrometer that we used was an LCQ Deca XP. It is a single quad ion-trap mass spectrometer that came with a single spray. This instrument did not come with the ability to perform dual-electro-spray, so we used a standard infuse/withdraw PHD 22/2000 syringe pump along with a Bertan Associates, series 230, high voltage power supply to facilitate the negative mode spray of our dual-spray apparatus shown to the left. The tip on the left was used for our positive mode solution and the tip on the right was used for our negative mode solution. We were able to facilitate the angles between the tips by moving the boards as seen fit to attempt to maintain a 45° angle and strong signal intensity. The solutions prepared for this trial were the same as those used for the individual spectra as well as the same voltages and flow rates. The only exception being that we slowly increased the magnitude of the negative voltage as we attempted the dual spray. The result was severe arcing between the tips as shown to the right. This created a short circuit in the instrument and blew a few fuses that had to be replaced. A second trial run had the same results but the

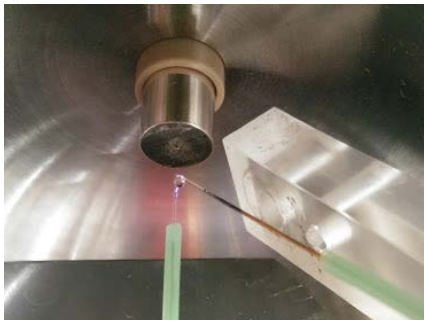

Figure 6: Shows the arcing between the positive tip (left) and negative tip (right) that occurred when dual-electrospray was attempted.

instrument was shut down immediately upon sight of arcing to avoid a second short circuit.

Conclusion and Future Work

While the results of the work in the lab this semester were disappointing, we gained insight into how to proceed over the summer. The main issue that we currently have revolves around the arcing that occurs due to the close proximity of the high voltage sources. This could possibly be alleviated via insuring that the high voltage sources were properly grounded while we were synthesizing the board used for our dual-electrospray.. Another option is that the use of a dry nebulizing sheath gas could help provide an atmosphere that is not conducive to arcing as well as helping the strength of the negative mode spray.

If successful with our research goals, there are many applications for a more accurate and complete sequencing of peptides via MAAH with droplet phase, online, derivatization with FBDSA .The main goal is to apply this technique to proteome analysis. The largest application of proteomics is around drug synthesis and targeting to fight disease. This is two-fold in first synthesizing antibiotics and other drugs to target a particular disease. Secondly, if a protein that is a marker for a particular disease is detected quickly in the lab via proteomics, a patient can be given a possibly life-saving drug on a more amenable time frame with quick identification via a streamlined proteomic analysis.