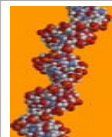


PROTEOMICS AND THE HIGH THROUGHPUT CHALLENGE



30,000 genes



400,000 proteins

"PROTEIn complement to a genome"

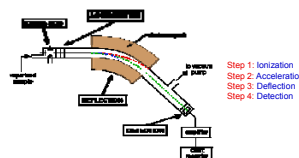
Proteomics is the study of the expression, function, and interaction of proteins that are expressed as a result of a genome in an organism. It is a cross-disciplinary field of study having many medical, dental, and biological applications.

Mass Spectrometry, specifically MALDI-MS, is commonly used to identify proteins.

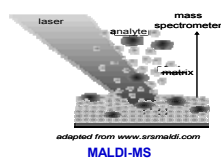
Currently, complete protein processing and analysis can take up to 2 days. The goal of using a droplet based device is to reduce total processing time to a few hours.

For this project, focus was put on the digestion step varying digestion time, enzyme, enzyme concentration, & pH.

MATRIX ASSISTED LASER DESORPTION/IONIZATION-MASS SPECTROMETRY



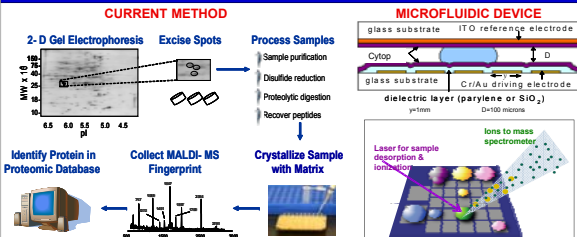
How Mass Spectrometry Works



MALDI-MS

A laser is used to vaporize and ionize a sample. The matrix is used to absorb the ranges of UV light that could harm the molecule and the sample can ionize successfully.

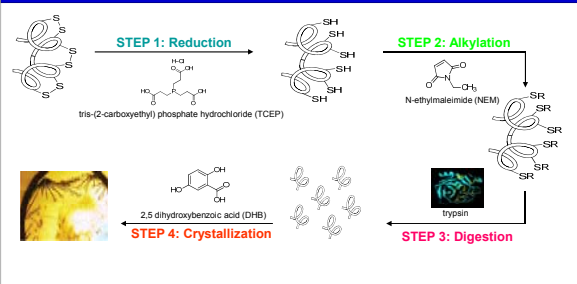
CURRENT PROTEIN ANALYSIS METHOD VS. MICROFLUIDIC DEVICE



KEY ADVANTAGES OF DROPLET-BASED MICROFLUIDICS:

- *Reconfigurable, programmable sequence of droplet movements.
- *Samples processed individually or in parallel
- *Samples processed identically or uniquely
- *Incorporated optical, thermal, electrochemical, or MS detection capabilities.
- *Platform inserted directly into mass spectrometer (no sample transfer losses)

BASIC PROCESSING STEPS FOR AN ISOLATED & PURIFIED PROTEIN



IN SOLUTION PROTEIN PROCESSING

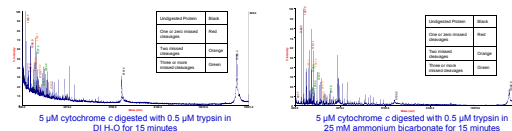
Protein	Concentration	Volume	Reagent	Concentration	Volume	Time
Cytochrome c	5 μ M in 50% acetonitrile (ACN) & 50% DI H ₂ O	25 μ L	Reducing Agent	TCEP 5 mM in 50 mM ammonium bicarbonate	25 μ L	15 min
Myoglobin	5 μ M in ACN	25 μ L	Alkylating Agent	NEM 5 mM in DI H ₂ O	25 μ L	15 min
Ubiquitin	5 μ M in ACN	25 μ L	Enzyme	Trypsin 0.5 μ M in DI H ₂ O	25 μ L	15 min, 30 min, 1h, 2h, 4h
PNase A	5 μ M in ACN	25 μ L	Enzyme	Lysyl endopeptidase (Lys-C) 0.5 μ M in 25 mM ammonium bicarbonate	25 μ L	15 min, 30 min, 1h, 2h, 4h
α -lactalbumin	5 μ M in ACN	25 μ L	Matrix	DHB 1% trifluoroacetic acid (TFA), 49% DI H ₂ O, & 50% ACN	2 μ L	Until crystallized
Protein Mixture	2 μ M/protein (10 μ M total) in ACN	25 μ L				

Digestions were carried out in 1.5 mL eppendorf tubes. Upon completion, 2 μ L of the digested peptides were spotted onto a stainless steel MALDI-MS plate followed by 2 μ L matrix.

IN SOLUTION RESULTS

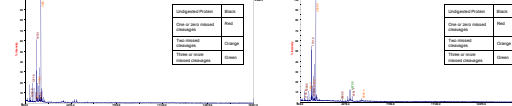
DIGESTIVE ENZYMES WORK BETTER IN BASIC pH (pH 7-8)

Current protein digestion methods using trypsin keep the pH between 7 & 8 because trypsin is most efficient at digesting proteins in this pH range. However, water is much easier to move on chip than ammonium bicarbonate, the normal reagent used to control the pH of the solution. Cytochrome c was digested with trypsin in a 25 mM ammonium bicarbonate solution and in DI H₂O in order to assess the need for a basic pH. The spectra on the left is a 15 minute digestion in water and the spectra on the right is a 15 minute digestion in 25 mM ammonium bicarbonate. The spectra on the right shows much less intact protein than the spectra on the left (peak in 13000 m/z range).



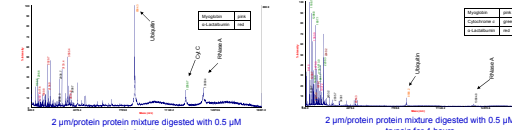
PROTEIN DIGESTION CAN BE DONE WELL AT ROOM TEMPERATURE

On bench protein digestion with trypsin is usually done for four hours at 37°C. For optimization on chip it would be ideal to perform protein digestion at room temperature and for much less than four hours. As is evident, though, digestion is not complete, there is no intact protein after 15 minutes of digestion. At four hours α -lactalbumin peptides have been further digested.



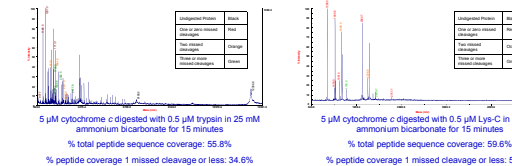
OPTIMAL DIGESTION TIME VARIES FROM PROTEIN TO PROTEIN

The enzyme trypsin cleaves a protein at lysine and arginine residues. Lys-C cleaves a protein at lysine residues only. Given the complexity of the protein and the ease with which the enzyme can reach the cleavage sites, the time it takes to break up a protein to complete digestion will vary from protein to protein. The ease or difficulty with which a protein is digested must be taken into account when optimizing the time it takes to digest on chip. Here you can see 5 different proteins after 15 minute digestion and 4 hours digestion. 2 proteins show no intact protein after 15 minutes while 2 other proteins show some still intact protein after 4 hours.



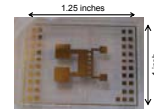
TRYPsin VERSUS Lys-C

Trypsin is one of the most commonly used enzymes in protein digestion. However, trypsin works best at an elevated temperature and has a tendency to not only break up proteins but to also digest itself. As well, trypsin has in the past been difficult to move on chip. Therefore it was desirable to find a more stable enzyme that would more easily move on chip. It was decided that Lys-C would be used to digest a number of proteins to assess its proficiency at room temperature digestion. The total percent sequence coverage as well as the percent sequence coverage of identifiable peaks with one missed cleavage or less would suggest that Lys-C is the better enzyme for room temperature digestion.



PROTEIN PROCESSING ON A DROPLET BASED MICROFLUIDIC DEVICE

Protein	Concentration	Volume
Cytochrome c	5 μ M in 50% ACN & 50% DI H ₂ O	0.3 μ L
α -lactalbumin	5 μ M in ACN	0.3 μ L



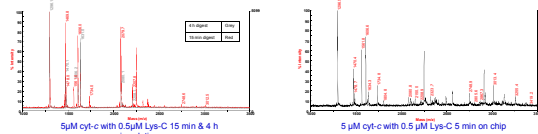
Digestions were carried out on 3% Cytop coated microfluidic device or a 1.2% Teflon coated ITO electrode (cover glass) and a 100 micron gap between the device and the cover glass.

Reagent	Concentration	Volume	Time
Reducing Agent	TCEP 5 mM in 50 mM ammonium bicarbonate	0.3 μ L	5 min
Alkylating Agent	NEM 5 mM in DI H ₂ O	0.3 μ L	5 min
Enzyme	Trypsin 0.5 μ M in 25 mM ammonium bicarbonate	0.3 μ L	5 min, 15 min
	Lys-C 0.5 μ M in DI H ₂ O		15 min
Matrix	DHB 0.5 μ M in 25 mM ammonium bicarbonate	0.3 μ L	5 min, 15 min
	DHB 1% TFA, 49% DI H ₂ O, & 50% ACN		Until crystallized

ON CHIP RESULTS

CYTOCHROME C DIGESTED WITH LYSYL ENDOPEPTIDASE

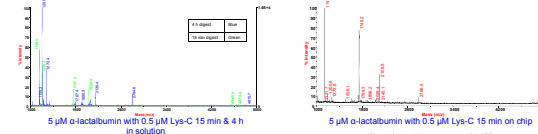
Cytochrome c digested on chip shows less complete digestion than in solution digestion. However, there was no intact protein remaining at the end of the 5 min digestion, and there was 87.5% total percent sequence coverage indicating an excellent digestion.



% peptide coverage 1 missed cleavage or less: 87.5%

α -LACTALBUMIN DIGESTED WITH LYSYL ENDOPEPTIDASE

Though, α -lactalbumin did not digest as completely on chip as in solution, there remained no intact protein at the end of the 15 min digestion and 61.0% total percent sequence coverage indicates adequate digestion.



% peptide coverage 1 missed cleavage or less: 61.0%

Only 5 min cyt-c and 15 min α -lactalbumin with 0.5 μ M Lys-C were successful digests on chip.

The other attempted digests failed because of poor fluid movement causing inefficient mixing.

As well, the Cytop coated cover glasses were either more hydrophilic than the Teflon coated microfluidic devices or the Cytop coated microfluidic devices were equally hydrophobic to the cover glasses causing sample loss when the cover glasses were lifted off after matrix crystallization.

SUMMARY

This project used in solution digestion to test protein processing parameters: pH, temperature, and enzyme to find optimal conditions. A pH of 7-8 was optimal for digestion, and Lys-C may be a better enzyme than trypsin for room temperature digestion.

Using these optimized conditions, a 5 minute on chip digestion of cyt-c with Lys-C resulted in 87% total peptide coverage with no intact protein remaining. These results show potential for high throughput proteomic analysis.

Additional work includes further studies to determine the optimal enzyme for room temperature digestion.

Future devices will be Cytop coated with a Teflon coated ITO electrode (cover glass). Teflon is a more hydrophobic material than Cytop, leading to less sample loss when the cover glass is lifted off for mass spec analysis.

Also, the other steps in protein processing (reduction, alkylation, crystallization) must be optimized for on chip conditions.

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