MOLECULAR WEIGHT DETERMINATION ELECTROPHORESIS and LC-MS & MALDI-TOF-MS

Introduction:

Electrophoresis is a commonly used technique in bioanalytical chemistry. In gel electrophoresis, molecules migrate through a gel medium according to their charge (applied by the electrodes). In the case of proteins, charge is proportional to mass and therefore molecular weight resolution is achieved. Mass separation is also achieved by the composition of the gel. These basic principles apply to all types of electrophoresis but there are small differences depending on the molecule of interest.

Electrophoresis is a powerful separation technique, however the technique only gives a crude estimate of molecular weight and identity. It is often used in sequence with other more precise methods. In proteomics, enzymatic digestion is often performed on specific separated protein bands excised from the gel to get a more accurate identification of the protein by mass spectrometry. The benefit of this technique is that the proteins are separated before digestion. The extraction however is a fairly long and laborious technique. You will perform digestion on a solution of a single protein which will also be used for electrophoresis.

MALDI – Matrix Assisted Laser Desorption Ionization is a form of mass spectrometry. Samples are crystalized within a matrix, spotted on a target and analyzed in a time of flight (TOF) mass spectrometer. The ionization is performed by a laser.

The first week you will perform the digestion of Lysozyme and run electrophoresis of this protein and of a number of proteins individually and as a mixture. The second week you will do sample clean-up, preparation and analyse the samples on the LC-MS and the MALDI.

Prelab:

1) Briefly describe the basic principles of LC-MS.
2) Briefly describe the principles of MALDI-TOF.
3) What are some common digestive enzymes used in analysis of proteins?
4) What is reverse pipetting? Why is it a benefit to use this pipetting technique for these applications?

Note: You will need to know the theoretical tryptic digestion peptide masses of B.S.A. You also will need the masses of B.S.A., myoglobin, lysozyme and IgG.
One data base that you can use is: http://web.expasy.org/peptide_mass/. When you are in this data base in the large box type P02769 . Select $[M+H]^+$, then average, and choose iodacetamide (IAA) in the box "cysteines treated with". Select trypsin as the enzyme and allow 1 missed cleavages. This should give both 0 and 1 missed cleavages.

Week 1: SDS-PAGE and In-Solution Digestion

Part A: Electrophoresis

1. Bring the water bath to a boil.
2. Prepare (dilute) 1x Tris-Glycine Running buffer from the 10x stock. You will need 800 mls. in total.
3. There is sample buffer and reducing agent supplied as 4x and 20x the concentration needed, respectively. These buffers are stored at room temp. They need to be diluted to 1x in the sample, (see table 1 below).
4. Use the multicolor 500 uL centrifuge tubes to prepare the dilutions in Table 1. **Add the beta-2-mecaptoethanol (B2mE) last and in the fume hood – it is very volatile and has a strong odor! You will need to use micropipette to pipette these volumes.**

Table 1. Concentrations and dilutions of proteins for electrophoresis.

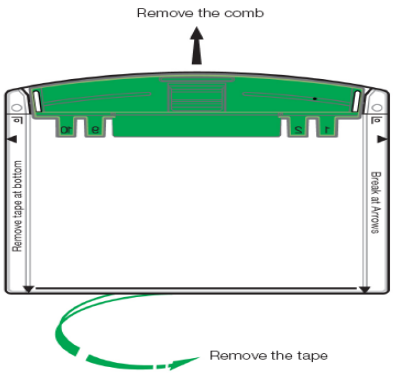
Sample	Concentration (mg/mL)	Volume Sample Required (uL)	Volume Sample Buffer (uL)	Volume B2mE* (uL)	Volume Milli-Q H2O (uL)	Final Volume (uL)
IgG	2	7	25	5	63	100
B.S.A.	2	7	25	5	63	100
Myoglobin	2	20	25	5	50	100
Lysozyme	2	7	25	5	63	100
Protein Mix	1.2 ug/mL	34	25	5	37	100
Protein Shake		5	25	5	65	100

* beta-2-mercaptoethanol.

5. The prestained standards (Precision Plus protein standard) **do not** need to be boiled. You will only need 10 uL and the standard which can be loaded directly onto the gel. Load 2 lanes if you choose.
6. Boil the proteins and mixtures (Table 1) for approximately 5 minutes using the floating rack in a beaker on the hot plate. This will denature the proteins.

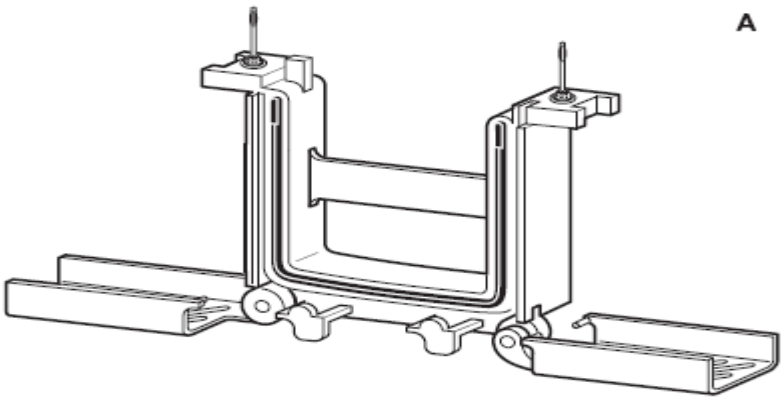
Assembling the Gel Apparatus:

1. Remove the gels from the pouch. Remove the tape at the bottom. Slide the comb out by GENTLY pulling up in one smooth motion. Be careful, since if you damage the stacking gel in this step, those lanes become useless. Rinse the wells with running buffer (1x made as above) using a Pasteur pipette about 4 times.



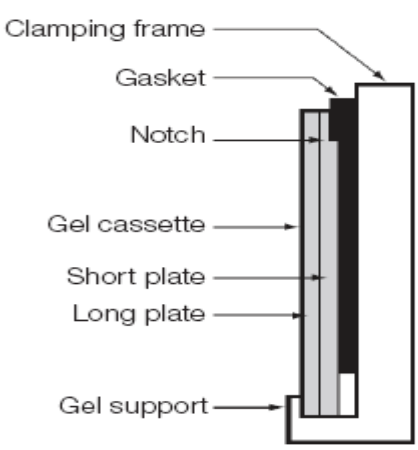
a.

2. Set the gel holder to the open position on a clean, flat surface



a.

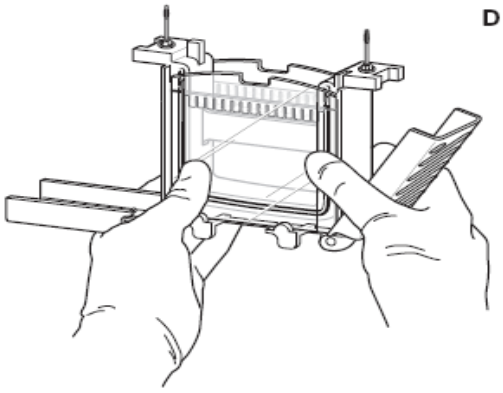
3. Place the cassettes with the wells of the gel facing inward. If using only one gel, the buffer dam will replace the second cassette. Make sure the assembly remains balanced and does not tip over. The cassettes will rest at a 30 degree angle. **(B)**

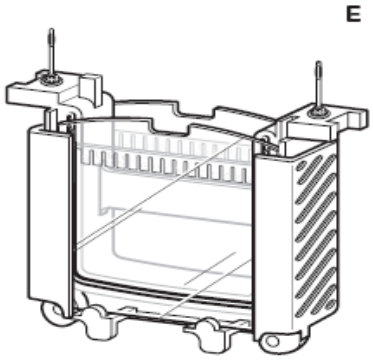


4. Gently push the cassettes together, making sure they rest firly and squarely against the green gasket at

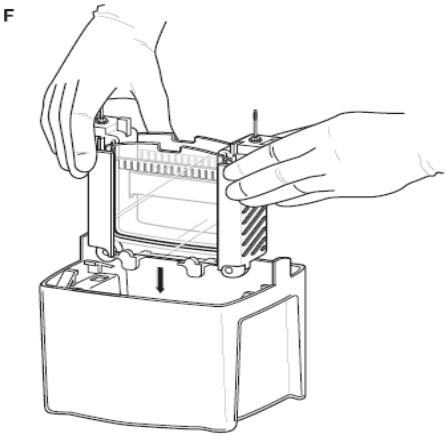
the bottom of the assembly. Align the short plates and check that they align just below the notch at the top of the green gasket. **(C)**

5. Maintain gentle pressure on each gel cassette. Slide the green arms of the clamping frame one at time over the gels, locking them in place. The wing clamps lift the gel cassettes into the notch, forming a tight seal. Check to ensure everything is square and sitting flat, then pour a small amount of running buffer into the inner chamber to ensure it is not leaking.





6. Place the assembly inside the module. Then fill the outer chamber with the rest of the running buffer, approximately to the line indicating 1 gel.



Loading Samples and Running the Gel:

1. Load 35 uL into each well (except standards – use only 10ul) using reverse pipetting technique and the gel-loading tips provided. Make sure you arrange your samples so that you will be able to tell protein location should the gel get turned a different way while being processed.

2. Place the lid onto the gel box. It will snap into place if done correctly

3. Connect the electrodes to the power supply and switch power on. Set the voltage to 200, then press run. There should be a steady stream of air bubbles coming from the solution, if not there is little or no conductivity. Observe the dye front to make sure it begins to migrate. **It should be relatively straight across all the lanes. If not, consult the instructor within 5 minutes of starting the run.**

Removing the cassette and staining the gel:

4 Once the dye front reaches the bottom, press Stop. Turn the power supply off and disconnect the electrodes. Remove the lid from the apparatus and remove the get cassette from the assembly.

5 To open the cassette, align the arrow on the opening lever with the arrow on the cassette. Apply downward pressure and the cassette will snap apart.

6 Pull the two plates apart from the top, and gently remove the gel and place in staining reservoir. **BE CAREFUL IT IS REALLY EASY TO RIP THE GEL.** There is a lip at the bottom which is thicker and can be used to lift off if necessary. The gel can be dislodged around the edges by carefully running a pipette tip around the edges between the gel and the cassette.

7 Rinse the gel 5 times with Milli- Q water. Pour in the Imperial protein stain, just enough to cover the gel and gently swirl for a few minutes. You might see bands after about 5-10 minutes, but it might take longer. Leave samples in the stain overnight.

8 Cover your gel with plastic (saran wrap) and label with your lab# and names. The lab instructor will decant stain the next morning and replace with Milli-Q water periodically throughout the next day, until the background is clear. A picture can be taken for your write-up during the following lab period when you are doing the next part of the lab.

Part B: Digestion

Digestion of lysozyme:

Solutions:

1) **100 mM NH_4HC0_3** (F.W. 79.056): Weigh 0.0395 g. and dissolve in volume of 10 mLs with milli Q H_20

2) **2 M Iodacetamide (I.A.A):** (F.W. 184.96): Weigh 0.362 g. Make volume to 1 mL with 100mM NH_4HC0_3
3) **1 M Dithiothreitol (D.T.T.) –** (F.W. 154.25) – Weigh 0.154g. Bring final volume to 1 mL with 100mM NH_4HC0_3
4) **100 mM D.T.T**. – Add 100 uL 1M D.T.T. to 900 uL NH_4HC0_3
5) **200 mM I.A.A** - Add 100 uL 2M D.T.T. to 900 uL NH_4HC0_3
5) **Trypsin** – Should be added to the samples at a ratio of 1:50. Dilute the stock solution to an appropriate concentration with NH_4HC0_3

Protocol:

1) Prepare 100 mM D.T.T. using the 1M stock provided to you. 1 mL is sufficient.
2) Aliquot 25 ug of the Lysozyme solution into 2 tubes.
3) Add D.T.T at a final concentration (in the samples) of 10mM (1/10)
4) Incubate for 45 min @37 degrees Celsius
5) During incubation, prepare 200 mM I.A.A from 2M stock solution.
6) Remove tube from 37 degrees and quickly place on ice to cool to room temperature.
7) Immediately add the IAA so the final concentration will be 20mM
8) Incubate 30 min @ r.t. in the dark (cover tube with foil).
9) Add 0.8 uL D.T.T to neutralize I.A.A and prevent trypsin alkylation. Incubate 20 min @ r.t.
10) Remove the trypsin from the freezer
11) Add trypsin at a ratio of 1:50 and incubate overnight @ 37 degrees.

The technician will look after putting your samples away the next day. They will be stored in the freezer until the following week.

WEEK 2: MALDI-TOF MS.

You will perform the sample clean up and LC-MS in the MCAL laboratory. The MALDI procedure will be performed in Dr. Perreault's lab on the 5th floor of Parker. with the assistance of the technician or TA.

Part A: C_{18} Ziptip Peptide Clean-up Protocol for Maldi

Introduction:

Many buffers and compounds are not Mass Spectrometry compatible, such as those found in biological fluids or those used in the tryptic digestion process. (ie Urea, guanidine, DTT, etc.) The Ziptips contains a C18 sorbent to effectively bind the peptides, allowing to washout the contaminants, and then elute the peptides off the C18 sorbent in mass spectrometry compatible solvents.

Important Notes:

- **Sample must be free of organic solvents.**
- **Avoid introducing air into the membrane, this will dry it and effect binding**

- **Have everything prepared and ready before starting**
- **PLEASE DO NOT WASTE TIPS AS THEY ARE EXPENSIVE!**

Solutions:

0.1% FA
5% Acetonitrile, 0.1% FA
50% Acetonitrile, 0.1% F.A.

Wet and Equilibrate Tip

1. Prepare 1 ml of all the above solutions in 1.5 mL Eppendorf tubes.
2. **Wet tip**: Aspirate and Dispense tip (discard on towel or Kimwipe) with 10μL of 50% Acetonitrile. Repeat 2 times.
3. **Equilibrate**: Aspirate and Dispense with 10μL of 0.1% F.A. Repeat 2 times.
4. **Bind:** Aspirate 10μL of sample into the tip. Depress the plunger, but do not go all the way to the stop. Re-aspirate the sample, (release the plunger) and repeat this cycle for a total of 10 times.
5. **Wash:** Wash the tip by aspirating 10μL of 5% Acetonitrile/0.1% F.A. Repeat 2 times.
6. **Elute:** Aspirate 10μL 50% Acetonitrile/0.1% F.A. Dispense into a micro-centrifuge tube. Repeat with a fresh 10 μL Acetonitrile.
7. Lyopholize sample upstairs in speed vac while preparing solutions.

Part B: Maldi – TOF MS

Solutions:

1. **Matrix Solvent A:** 30% Acetonitrile in 0.1% formic Acid
2. **Matrix Solvent B:** Ethanol
3. **Matrix A:** 20 mg/mL CHCA (alpha-hyrdoxy cinammic acid) in Matrix Solvent A.
4. **Matrix B:** 20 mg/mL SPA (sinapinic acid) in Matrix Solvent B.

Protocol:

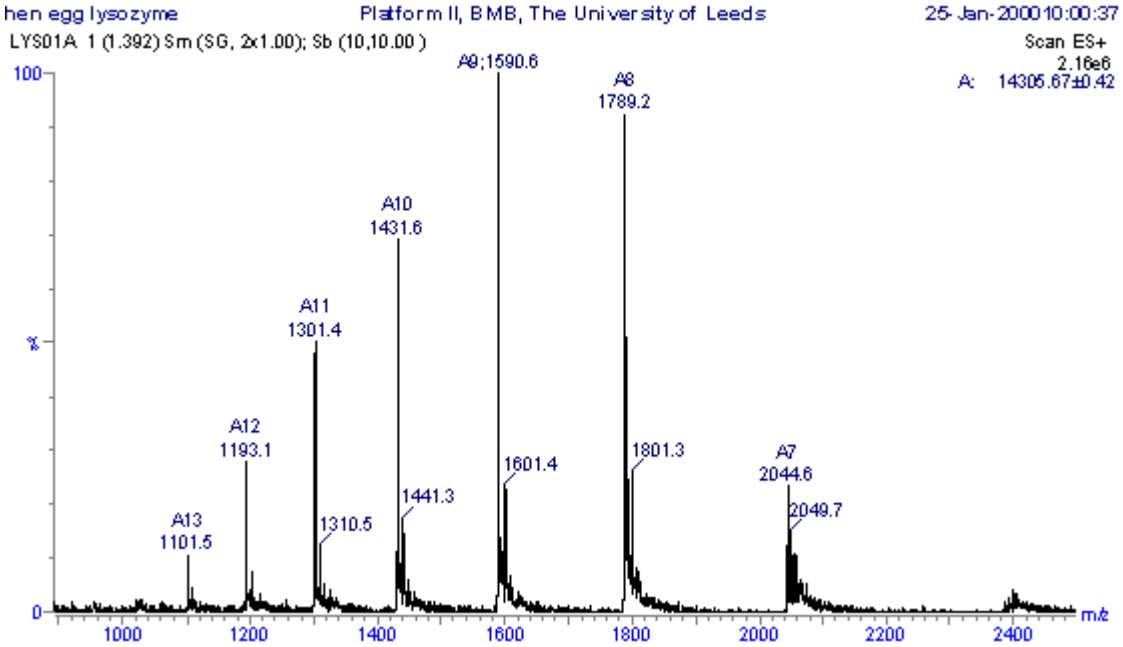
1. Prepare both matrix A and B in their respective solvents.
2. Mix sample and matrix supernatant in a 1:1 ratio.
3. Deposit 1 uL on target
4. Allow to dry.
5. Analzye on Maldi instrument.

Mass Determination of lysozyme LC-MS-TOF:

Electrospray MS: Pure lysozyme (Sigma) is analysed in a solution of 1:1 (*v/v*) acetonitrile : 0.1% aqueous formic acid and diluted to 1 ug/ml. and injected into the MS by use of the syringe pump. The instrument is used in full scan, positive mode with a wide scan range of 1000 to 3000 Daltons.

Samples with molecular weights greater than 1200 Da give rise to multiply charged molecular-related ions such as **$(M+nH)^{n+}$ in positive ionisation mode** and **$(M-nH)^{n-}$ in negative ionisation mode**. Proteins have many suitable sites for protonation as all of the backbone amide nitrogen atoms could be protonated theoretically, as well as certain amino acid side chains such as lysine and arginine which contain primary amine functionalities.

An example of multiple charging, which is practically unique to electrospray ionisation, is presented in the positive ionisation m/z spectrum of the protein hen egg white lysozyme.



Positive ESI-MS m/z spectrum of the protien hen egg white lysozyme.

The m/z spectrum shows a Gaussian-type distribution of multiply charged ions ranging from m/z 1101.5 to 2044.6. Each peak represents the intact protein molecule carrying a different number of charges (protons). The individual peaks in the multiply charged series become closer together at lower m/z values and, because the molecular weight is the same for all of the peaks, those with more charges appear at lower m/z values than do those with fewer charges (M. Mann, C. K. Meng, J. B. Fenn, *Anal. Chem*., 1989, **61**, 1702). The chromatogram and explanation are from an article by: Dr Alison E. Ashcroft, Mass Spectrometry Facility Manager, Astbury Centre for Structural Molecular Biology, Astbury Building , The University of Leeds.
(http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)

The m/z values can be expressed as follows:

$m/z = (MW + nH^+)/n$

where m/z = the mass-to-charge ratio marked on the abscissa of the spectrum;
MW = the molecular mass of the sample
n = the integer number of charges on the ions
H = the mass of a proton = 1.008 Da.

If the number of charges on an ion is known, then it is simply a matter of reading the m/z value from the spectrum and solving the above equation to determine the molecular weight of the sample. Usually the number of charges is not known, but can be calculated if the assumption is made that any two adjacent members in the series of multiply charged ions differ by one charge.

For example, if the ions appearing at m/z 1431.6 in the lysozyme spectrum have "n" charges, then the ions at m/z 1301.4 will have "n+1" charges, and the above equation can be written again for these two ions:

$$1431.6 = (MW + nH^+)/n \text{ and } 1301.4 = [MW + (n+1)H^+] /(n+1)$$

These simultaneous equations can be rearranged to exclude the MW term:

$$n(1431.6) - nH^+ = (n+1)1301.4 - (n+1)H^+$$
and so:
$$n(1431.6) = n(1301.4) +1301.4 - H^+$$
therefore:
$$n(1431.6 - 1301.4) = 1301.4 - H^+$$
and so:
$$n = (1301.4 - H^+) / (1431.6 - 1301.4)$$

hence the number of charges on the ions at m/z 1431.6 = 1300.4/130.2 = 10.

Putting the value of n back into the equation:

$1431.6 = (MW + nH^+)\ n$
gives $1431.6 \times 10 = MW + (10 \times 1.008)$
and so MW = 14,316 - 10.08
therefore **MW = 14,305.9**

You will be given instruction on the software associated with the LC-MS and get a spectrum of the lysozyme analysed in full scan mode. An explanation of the various software settings will be demonstrated. Using the information of the full scan run you will determine the MW of the lysozyme sample and compare this with the MW determined by electrophoresis.

Questions:

1. What are the components of the gel in Chem 4590 gel electrophoresis experiment?
2. Why do proteins separate in gel electrophoresis?
3. Does MW affect separation of proteins? Explain.
4. What are some advantages of the TOF verses Quadrapole MW determination?