Exercise 5: Polyacrylamide gel electrophoresis (PAGE)

1.- INTRODUCTION

In biochemistry, many important molecules, such as amino acids, peptides, proteins and nucleic acids possess ionizable groups that are found in the form of electrically charged species (negatively or positively) in solutions. Molecules having similar charges will possess different charge-to-mass ratios (Q/m) due to their differents molecular mass. These differences constitute the basis for the differential migration of such charged molecules when they are subjected to the action of an electric field.

Polyacrylamide gel electrophoresis (PAGE) is mainly used to separate proteins, although it can also be useful with nucleic acids. They are prepared in such a way that their pores are of comparable size to those of proteins so they produce a molecular filter effect; the electrophoretic separation then depends upon the charge density of the molecules and their size so that, two proteins with identical charge densities but different sizes can be separated because the support difficulties the movement of those which have larger sizes.

These gels are chemically inert against biological molecules, transparent and stable within a wide interval of pH values, temperature and ionic force; they are also resistant to denaturing agents (urea, detergents) and are mechanically stable, so they can be dehydrated and reduced to a fine film, facilitating their storage.

PAGE under non-denaturing conditions. PAGE develops under conditions where the native conformation of proteins is not altered, so that once the electrophoresis finishes, functionality studies of these proteins can be conducted (enzymatic activity, antibody binding capacity, binding to receptors, etc.). Under this situation, the proteins migrate in function of their charge, size and form.

PAGE under denaturing conditions. Proteins lose their native structure in the presence of some chemical compounds; such compounds, called denaturing agents, produce protein unfolding, which remains without the characteristic three-dimensional organization of its biological function.

The most common denaturing agents are:

- Urea, which acts upon the hydrogen bonds that stabilize the macromolecular structure.
- Detergents, which act upon the hydrophobic interactions in proteins that are substituted by detergent-protein interactions. Three principal types of detergents exist: a) **Non-ionic detergents** weakly denature and do not alter the charge of the proteins they bond to. Triton X-100 is an example of these; b) **Ionic-Detergents** may have a positive charge (cationic) that are used for the separation of very acidic or very basic proteins; of these, cetyltrimethylammonium (CTAB) is used most. Others are negatively charged and possess a strong denaturing character; here, the most employed is sodium dodecyl sulfate (SDS), and; c) **Amphoteric detergents** are weakly denaturing and do not affect the protein's charge. Some, like 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) is a good choice to solubilize membrane proteins.

In some proteins, there are disulfide bridges between the Cys residues (to form cysteines) of a same polypeptide chain (intrachain bridges) or different chains (interchain bridges) of some proteins with quaternary structure. These bridges can be broken by treatment with specific reducing agents, like for example ß-mercaptoethanol (ß-ME) or dithiothreitol (DTT).

SDS-PAGE permits the calculation of molecular parameters, while SDS-protein complexes are strictly separated depending upon their molecular size.

Proteins bond to an SDS molecule for each two amino acids, which implies that the protein`s charge remain masked or annulled; likewise, the SDS molecule supplies a negative charge so that the SDS-protein complexes are uniformly negatively charged (the charge per unit of mass is practically constant for all complexes).

As previously mentioned, the electrophoretic mobility in PAGE is a function of the size and the charge per unit of mass; as this is constant for all SDS-protein complexes, the mobility depends on the molecular mass, i.e., the smaller the protein's molecular mass the greater mobility will be, and vice versa.

SDS-PAGE is the most utilized electrophoresis for protein analysis due to:

- The vast majority of proteins are soluble in SDS.
- All SDS-protein complexes have a negative charge, and therefore migrate in the same direction.
- Its charge density is very high, and therefore its migration velocity is also, and electrophoresis is very fast.
- The separation depends upon the molecular mass, which can be calculated.
- SDS-protein complexes are easily stained.

SDS-PAGE can be performed under reducing or non-reducing conditions. The protein with an intrachain disulfide bridge denatures partially in the presence of SDS, although with the area corresponding on the disulfide remains unfolded. Treatment with SDS+DTT breaks the disulfide bridge and allows complete unfolding of the protein.

Once PAGE is completed, the proteins are visualized with a dye; the most use is Coomassie Blue, with which 0.1-0.5 micrograms of protein can be visualized per band. Another staining method is silver salts, whose principal advantage is its high sensitivity (to 0.1 ng of protein per band), although it also has disadvantages - it is very laborious and expensive, presents a high background, has poor reproducibility, some proteins do not stain, and above all, it is not totally specific to proteins, as some lipopolysaccharides, nucleic acids and polysaccharides are also stained.

One method with a sensitivity comparable to staining with silver salts employs fluorescent compounds that specifically bond to proteins (the bond can occur before or after electrophoresis), like dansyl chloride (detects up to 10 ng of protein per band), fluorescamine (detecting up to 3-5 ng of protein per band) and MDPF (2-methoxy-2,4-diphenyl-3(2H)-furanone), which detects up to 1 ng of protein per band.

Once the polyacrylamide gel has been stained, the image obtained is a set of colored bands upon a transparent support. Its analysis allows identifying the minimum number of components (bands) from each sample. Each band is characterized by its relative electrophoretic mobility (R_f).

$$R_f = \frac{Distance\ migrated\ by\ a\ specific\ protein}{Distance\ migrated\ by\ the\ dye\ front}$$

Under SDS-PAGE conditions, if the logarithm values of the molecular weight (logMW) are represented versus the electrophoretic mobility (R_f) of a set of proteins, a linear relationship is obtained (Figure 1). Normally, a set of proteins with a known molecular weight is used (molecular weight markers) that are subjected to SDS-PAGE in the same gel used to analyze the proteins whose molecular weight we want to learn.

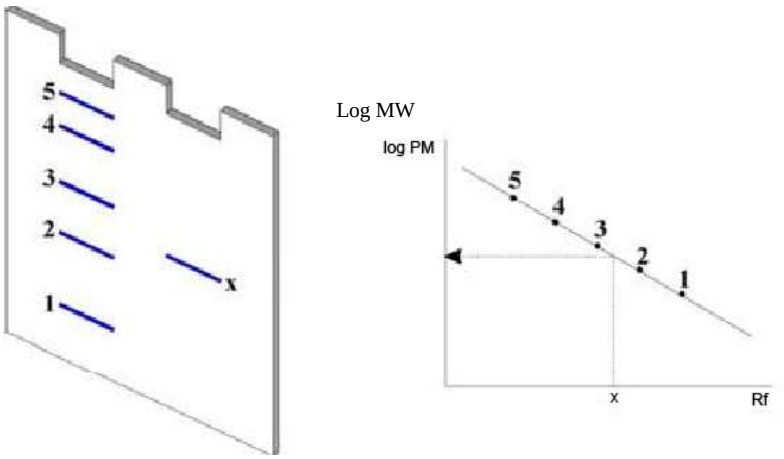
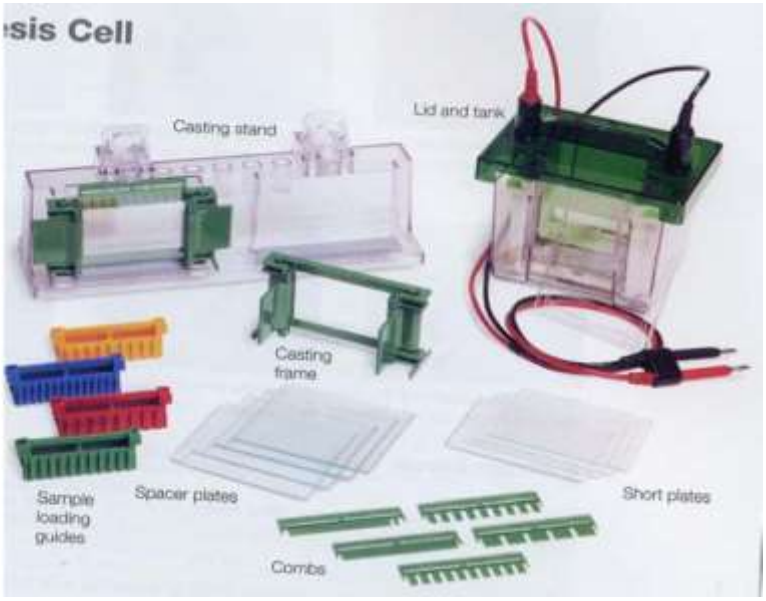


Figure 1. Calculation of molecular weights of proteins by SDS-PAGE.

In this exercise, an SDS-PAGE variant will be used that is known as *discontinuous electrophoresis*, in which the gel is formed by two sections with different pore sizes and different pH. In discontinuous systems, the first gel (stacking gel) assures the concentration of all proteins at the migration front. The separation actually begins the moment the migration front reaches the edge of the second gel (resolving gel). The stacking gel has larger pores (a smaller percentage of acrylamide + bisacrylamide) and a more acidic pH than the resolving gel.

Numerous devices are available for performing electrophoresis in acrylamide gels. The following image shows an example of an extensively used commercial system from the Bio-Rad Corporation.

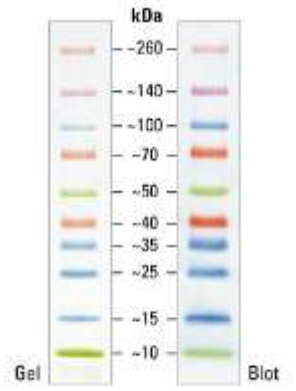


2.- OBJECTIVES

1. To learn about the protein electrophoresis technique in SDS-PAGE gels.
2. Protein separation under reducing and non-reducing conditions.
3. Determination of the molecular weight of the protein being studied.
4. Presentation and discussion of the results obtained.

3.- MATERIAL AND REAGENTS

- Lid and tank.
- Casting stand.
- Gel development stand for the cuvette.
- Plate stand.
- Plates of 2 different sizes.
- Spacers.
- Comb.
- Samples.
- Electrophoresis reagents.
- 50 ml conical plastic tube.
- 20 µl, 200 µl and 1000 µl micropipettes.



Images are from a 4-12% Tris-glycine gel (SDS-PAGE) and subsequent transfer to membrane.

Acrylamide is a chemical product catalogued as a carcinogen. WEAR GLOVES DURING THE EXERCISE.

4.- METHODS

4.1.- Work to do on the first day.

These are the steps to follow in an orderly fashion:

1. Clean the plates with water and soap, then with abundant distilled water. Finally, apply ethanol to them to accelerate drying.
2. Place the plates in the stand and introduce the 0.75 mm spacers between the 2 plates. To confirm that this has been correctly assembled, fill the space between the plates with distilled water.
3. Make a line with a marker on the glass to learn the point you need to fill with resolving gel. For this, place the comb and leave the mark 1 cm below it.
4. To a conical plastic tube add:

10% resolving gel	1 gel
Distilled water	2.1 ml
1.5 M Tris buffer; pH=8.8	1.25 ml
Acrylamide:bisacrylamide 30:0.8	1.6 ml
SDS 10%	50 µl

5. Mix well but prevent bubbles from forming as the oxygen inhibits polymerization. Add 40 µl of 10% ammonium persulfate (APS) and 5 µl TEMED. Keep in mind that

from the moment these two reagents are added the polymerization process will begin.

6. Gently deposit the resolving gel solution between the two plates with a Pasteur pipette.
7. Immediately add water to the gel surface. Do this very gently, making sure the gel surface distorts as little as possible.
8. Allow to polymerize for at least 20 min. Do not move the gel during the polymerization process.
9. To confirm the gel polymerization, look at the solution that remained in the conical plastic tube. If the resolving gel has polymerized, remove the water from the surface with filter paper.
10. To a conical plastic tube add:

4% stacking gel	**1 gel**
Distilled water	1.5 ml
0.5 M Tris-HCl; pH=6.8	0.625 ml
Acrylamide:bisacrylamide 30:0.8	0.325 ml
SDS 10%	25 µl

11. Mix well but prevent bubbles from forming as the oxygen inhibits polymerization. Add 20 µl of 10% ammonium persulfate (APS) and 4 µl TEMED. Keep in mind that from the moment these two reagents are added the polymerization process will begin.
12. Gently deposit the stacking gel solution between the two plates with a Pasteur pipette.
13. Introduce the comb to form the wells.
14. Allow to polymerize for 20 min. and gently remove the comb. Fill the wells with distilled water. Store at 4 ºC until using.

Sample preparation.

Take 10 µl of the fractions containing greater quantities of proteins from the chromatography experiment and place them in an Eppendorf flask. Add 2,5 µl of the 5X sample buffer. Prepare the sample with the two buffers, one with a reducer and other a non-reducer, and shake for 1 min. in a vortex.

Prepare the other sample (AcChR) with the two buffers also, adding 10 µl of sample and 2,5 µl of the 5X sample buffer

Sample buffer	**Reducer (5X)**	**Non-reducer (5X)**
H_2O	--------	5 ml
1.5 M Tris-HCl pH 6.8	4 ml	4 ml
Glycerol 100%	10 ml	10 ml
SDS	2 g	2 g
2-mercaptoethanol	5 ml	---------
Bromophenol Blue 0.1%	1 ml	1 ml

4.2.- Work to do on the second day.

1. Place the gel in the stand with the electrodes. Fill the interior reservoir with electrophoresis buffer (60 ml 5X + 240 ml water). Place the remaining buffer in the lower reservoir. Electrophoresis buffer pH 8.3 (5X), (Volume 1 L: Tris 15 g; glycine 72 g; SDS 5 g).
2. Load the samples with the help of a **micropipette**, depositing each sample in its corresponding well.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	ane 8	Lane 9	Lane 10
------	Hb NoR.	Sample noR.	---	Standard MW	--	--	Hb Red.	Sample Red.	------

3. The electrophoresis will be performed at a constant voltage of 200V (for approximately 45 min.). It is finished when the Bromophenol Blue line arrives at the lower portion of the gel. Disconnect the power source when the electrophoresis is completed. Remove the upper lid.
4. Take out the casting frame and discard the buffer. Take out the gel plates and loosen the screws. Carefully remove out the plates from the gel. Laterally separate one of the spacers without removing it completely. Gently force it to separate the plates and free the gel.
5. Deposit the gel in a cuvette with the corresponding staining solution. Hot in the microwave for 20 sec and shaking for 15 min.
6. Distain with the distaining solution for 15 min. and observe the results.

Staining solution 1 L	
Coomassie Blue	0.5 g
Acetic acid	90 ml
Water	450 ml
Methanol	450 ml

Distaining solution 1 L	
Acetic acid	90 ml
Water	450 ml
Methanol	450 ml

5.- EVALUATION

5.1.- Each student have to prepare a report with the results obtained in sections 4.1. and 4.2. of this protocol.

5.2.- The report has to include the answers to the following questions:

1. What type of gel is being used? Are there other types of stands for electrophoresis?
2. How is the polymerization of the gel produced?
3. How is the velocity at which the electrophoresis develops controlled?
4. Why don't you have to touch the acrylamide without polymerization with your hands?
5. What differences are observed in the gels between the samples treated with ß-mercaptoethanol and those without?
6. An SDS-polyacrylamide gel (SDS-PAGE) was produced from proteins *X* and *Y*. Estimate the MW for each one of them. Distance the front covers = 6.5 cm

Proteins	MW (kDa)	Migration (mm)
Bovine albumin	67	11
Ovalbumin	45	23
Carbonic anhydrase	32	34
Trypsin inhibitor	21.5	46
Lysozyme	14.4	59
Protein X	?	28
Protein Y	?	54

7. To purify an enzyme which catalyzes the reaction P $\rightarrow$ S, 5 g of liver mouse and 25 ml buffer pH 7 were used to make a homogenate. It was filtered and centrifuged at 26000 x g. With an aliquot of the supernatant (final volume 27 ml) protein determinations and enzymatic activity were made (table 1).

   Protein content was estimated according Lowry method, the final volume was 1 ml. BSA (2 mg / ml) was used as standard.

   Table 1

Tube	BSA(µl)	Sample(µl)	A500nm
1	--	--	0.07
2	20	--	0.15
3	40	--	0.25
4	80	--	0.41
5	--	30	0.09
6	--	60	0.30
7	--	90	0.55

   Enzymatic activity: The activity was 124 units per ml of supernatant.
   Then, 20 ml of the supernatant were subjected to an affinity chromatography column that has S covalently bound to Sepharose. It was washed with 20 ml of buffer used in the homogenization and eluted with 15 ml of 5 mM S. The determinations of the activity and protein concentration from the fractions eluted from the affinity cromatography are in table 2. Fractions were collected of 3 mL / tube.

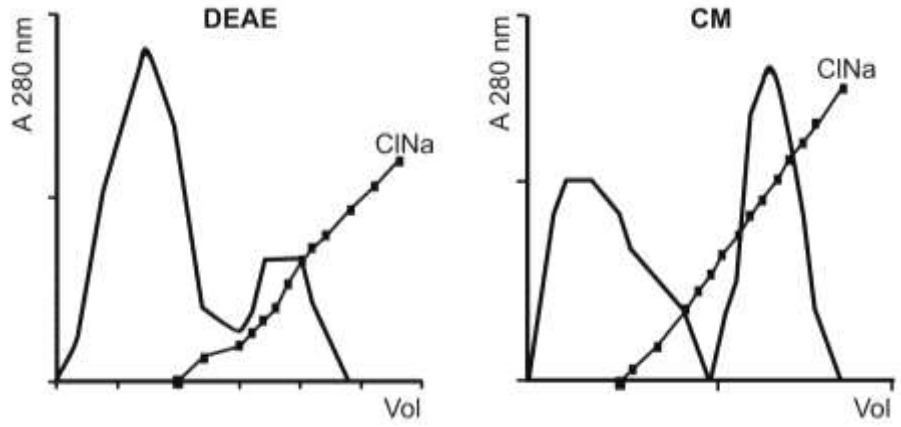
	A_{280}	Units of activity /tube
1	0.02	0
2	2.8	5.1
3	1	42
4	3.2	18
5	0.4	0.5
6	0.04	0
7	0.02	0
8	0.04	0.5
9	0.38	15
10*	0.98	823
11*	0.52	158
12	0.05	0.4

* The tubes 10 and 11 were pooled.

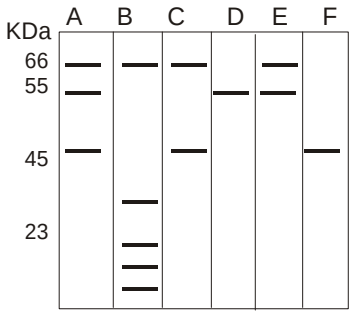
¿How many mg of the enzyme are in 5 g of liver mouse? What is the concentration of protein in the final volume (27 ml)?

PART B

3 aliquots were taken from the pool. The first was treated with cyanogen bromide (BrCN)(Cyanogen bromide hydrolyzes peptide bonds at the C-terminus of methionine residues) and the enzyme activity remained unchanged. The second was subjected to an anion exchange chromatography at pH 7 (DEAE) and the latter was subjected to a cation exchange at pH 7 (CM). In the last two cases, the elution profiles are shown below:



Finally, a SDS-PAGE under denaturing conditions (2-mercaptoetanol and boiling at 100 ºC for 5 min.) was run:
Lane A: pool 10 + 11, eluted from affinity cromatoghaphy
Lane B: aliquot treated with BrCN.
Lane C: protein not retained in DEAE.
Lane D: protein retained in DEAE.
Lane E: protein not retained in CM.
Lane F: protein retained in CM.



a- What is the PM and the pI of the protein under study?
b- Sort according to their pI proteins present in the pool
c- Why are two protein peaks in both DEAE and CM chromatography?
d- Why are 5 bands after BrCn treatment?
e- Design a protocol to purify the enzyme.

6.- HANDING IN WORK

Each student must make:

A Word file with the results responding to that asked for in epigraph **5.EVALUATION**.

To name in a systematic manner, you must follow the following example: I am student **Emilio Moreno Serrano**, and I am turning in the following file:

4_MorenoSerrano_Emilio. This file will be handed in **(AS A SINGLE PACKAGE)** via the **HOMEWORK** system the students have personal access to on the course webpage.

EMAILED DOCUMENTS WILL NOT BE ACCEPTED.