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BIOCHEMISTRY

Faculty of medicine – JU2018

◉ Sheet ○ Slides

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Revision

In the previous lecture we mentioned the biochemical techniques that allowing us to study proteins or purify them according to some characteristics:

1-Charge. 2-Size. 3-Solubility. 4-Specific binding affinity.

Salting in and Salting out technique:

Depends on the solubility of proteins in a solution with certain ionic strength, so proteins aren't soluble in water and they are being soluble if we add salt(as ionic strength is increased/Salting in).

At very low or at very high con of salt the proteins prefer to interact with each other and become less soluble, if we add salt the proteins will interact with ions, but if we keep increasing the con of salt the proteins become aggregated and precipitated at the bottom and then we can separate them, okay if you ask yourself why they do precipitate, because the hydrophobic amino acids get exposed and interact with each other and the proteins precipitate.

Note: each protein has a specific tendency to precipitate or to get its hydrophobic amino acids exposed at different concentration of salts depending on no. of hydrophobic amino acids that contains.

⇨ In the precipitated protein there is a con of salt so if we need to study the protein furthermore we need to remove this salt and the protein was denatured so when we remove the salt the protein reforms its orginal shape this can happen by **Dialysis**.

Dialysis:

To remove certain molecules and keep others, we put the precipitate in a porous bag that prevent proteins from crossing and only salt ions can cross through the pores because they are small enough and we put this bag into a container filled with a buffer solution and this process is repeated many times to ensure that unwanted small molecules are removed but the disadvantage of the dialysis is the small proteins leak out and we lose them.

Chromatography

If we need to study proteins further or we need only single protein or a certain group of proteins we have no .of techniques known as chromatography(chroma=color , graphy= a graph, certain draw).

The concept is separating molecules that having different colors through a certain medium, which means Separation of molecules present in a liquid or gaseous environment (mobile phase) via passing through a column (long tube, with 2 openings from the top and the bottom) that contains an immobile phase (stationary phase).

You put the mixture of proteins that you want to separate it and they are separated from each other eventually they come out from other end as fractions one by one and you can collect the 1st fraction (a certain group of protein) and then the 2nd and so on.

3 types of chromatography techniques:

1-Gel filtration.(according to their size)

2- Ion-exchange chromatography.(according to the net charge)

3- Affinity chromatography.(according to specific ligand binding affinity)

Ion-exchange chromatography:

Basically separating proteins according to their **charge**.

PI: is the PH of protein when it is neutral(has no charge) and proteins have different PI's (and net charges at various pH's).

For example PI for a specific protein at PH=7 , If I lowering the PH (less than 7) the groups of protein become protonated, so COO– will lose its charge (COOH) and NH2 get protonated (NH3+) and **the protein mainly is positively charged and vice versa.**

So in this technique we take the advantage of this property of proteins whether they have + or – charge to separate these protein, so we have a column filled with beads differ from the beads that we used in Gel filtration they don't have tracks rather than they have on their surface groups (+ or – charge) **depending on what proteins are that we want to isolate.**

There is 2 types: Cationic or Anionic -exchange chromatography

In Cationic exchange we are separating/isolating proteins that are positively charged or proteins that have high PI value by using **negatively charged beads** so negative proteins can't bind to the beads and they **washed out.**

Now we are releasing proteins that are bound to the beads by **ELUTION,** by different means we can change the PH of the column **Gradually** or we can increase the con of salts(NaCl) **Gradually**.(like 0.1 then 0.2 and so on).

Why??!!!!

If more ions are added now you have a competition between proteins and the ions, in cationic between positive proteins and Na+.

NOTE: Proteins are binding to the beads **Reversibly.**

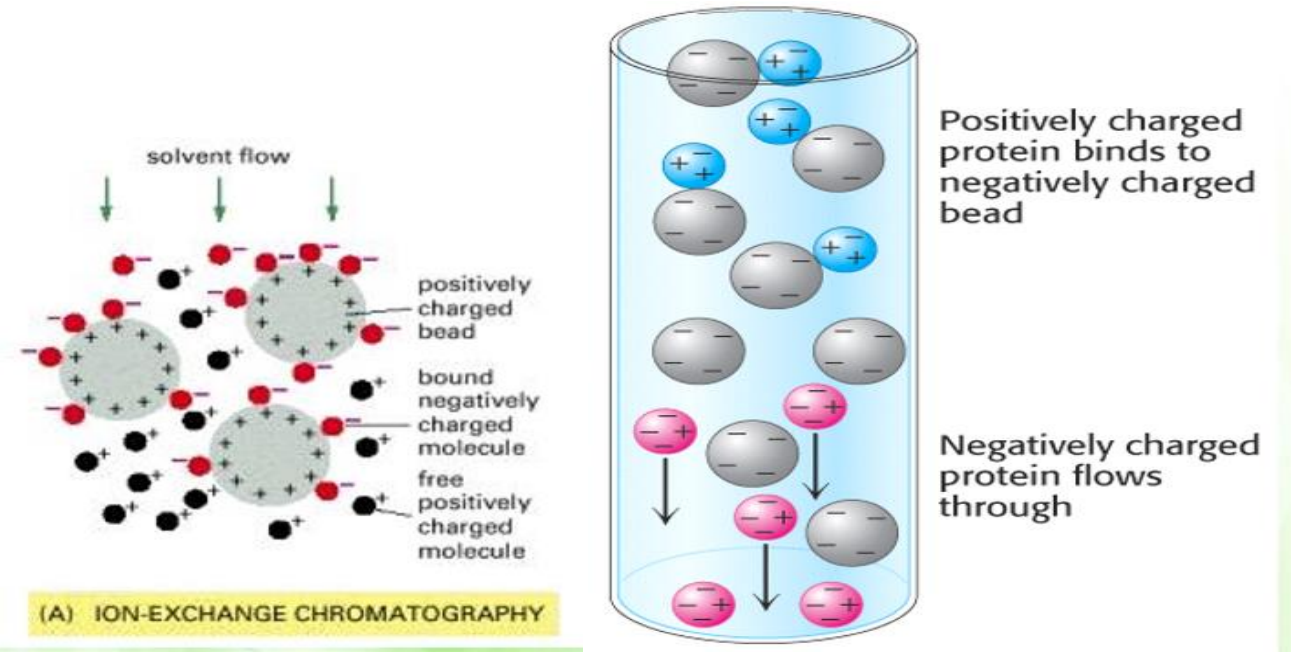
By adding more of salts concentration the Na+ will win this competition and + proteins will be dropped out or eluted, lower + net charge will come out firstly that they have lower PI.

OKAY Why?????!!!

Because proteins have different PI values and if PI values are increased the net + charges are also increased and the **Affinity** as well as get increased so with low PI values with low affinity with low net + charges and they will come out **Firstly**. ☺

Anionic-exchange chromatography: + charged proteins will be washed out and the – charge proteins will bind to the beads.

How I can elute – charge proteins by increasing negative ions concentration (cl-…..) there will be a competition between – charged proteins and Cl- so Cl- will win the competition so protein with lower net negative charge and with relatively higher PI will come out **Firstly**.



At Slide 15:

You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pI's). pI#5 = 2.3 pI#4 = 4.7 pI#1 = 7.2 pI#2 = 9.1 pI#3 = 12.1 Starting the column at pH 6.5, the sample is added and then washed to remove unbound molecules. What is the order of protein elution in a Cationic-exchange chromatography? An anionic exchange chromatography?

At the first we must recognize the proteins that will elute at cationic exchange and they are #1 and #2 and #3 to order them the proteins with lower PI value will come out firstly so the order is #1 then #2 and finally #3.

#4 and #5 will be eluted by anionic exchange chromatography, to order them the proteins with relatively higher PI value will come out firstly so #4 firstly and #5 at last.

If you ask yourself why I do need in cationic exchange Cl- ions or why I do need Na+ ions in anionic exchange?

The answer is that I need them to make proteins that I want to isolate more soluble and then I can separate them by Dialysis.

Affinity Chromatography:

Affinity: strength of binding between two molecules.

This is the best way of purifying proteins because it depends on the specific characteristic of protein that is **Affinity** of the protein to bind to other molecules whether they are proteins group or not it's really specific, so we take the advantage of this property, you have beads and on this beads there is a molecule that is bound to this beads and this molecule will bind to a specific protein

of course depends on the type of the molecule I use.

The best molecule that I can use it ⇨ Antibody ☺

Because it's really specific for certain Antigens or Proteins, so I can develop antibodies against human protein, how?

I take the human protein and I purify it and whatever technique I use and then I inject this protein into mice or rabbits or any animal these animals will immediately recognize the human protein as antigen because it has different amino acids, different structures that they used to, so they will produce antibodies, I collect this antibodies, but if I take single B cell producing one antibody I can develop (molecule-antibody) if I do this test today or tomorrow or after 20 years in the same conditions I get exactly the same results because it bind specifically to the protein.

So we have beads and antibodies attached to their surface and the proteins will pass through the column only one protein will bind to the beads and the other proteins washed away except one single protein and then I can elute it by changing the PH.

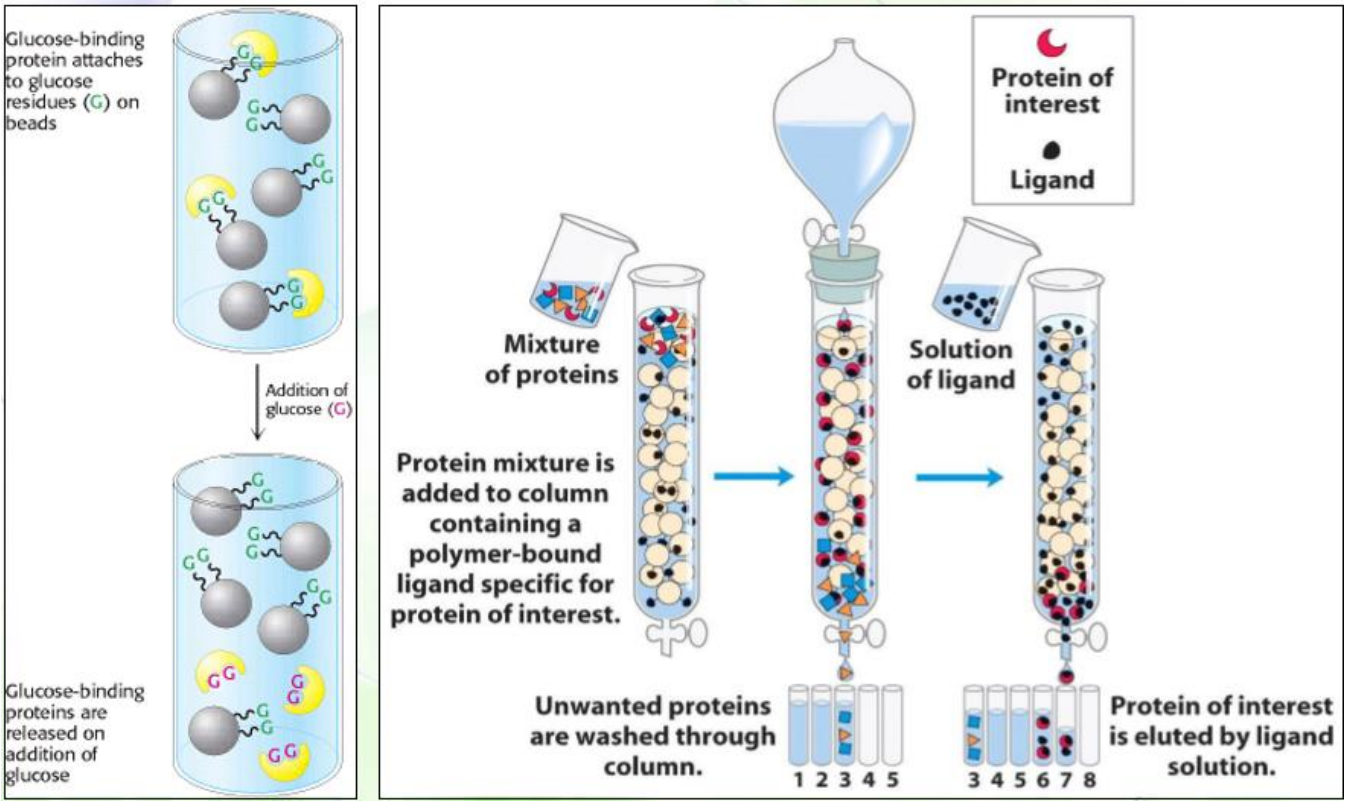
Example: there is a plant protein called Con A (concanavalin A) which binds to glucose with high affinity, can be purified by passing a protein mixture through a column of beads attached to glucose residues, Con A, but not other proteins, binds to the beads because it has higher affinity and specificity for binding to glucose.

How I can release Con A?

The bound Con A can be released by adding a concentrated solution of glucose (free glucose).

What kind of proteins would a concanavalin A-linked bead separate?

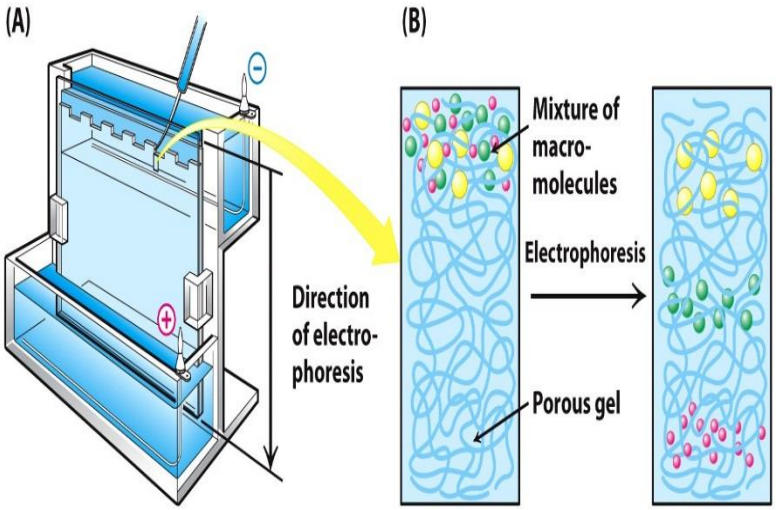
Definitely **Glycoproteins** specifically the protein with high con of glucose.



★Gel electrophoresis

◆In this technique, proteins are separated according to different properties (especially, their size) as they move in the gel through an electrical field.

◆Large proteins will move slowly (as they keep hitting the gel), while small proteins will move fast. So, larger proteins will be on the top of the gel (as in the following figure).

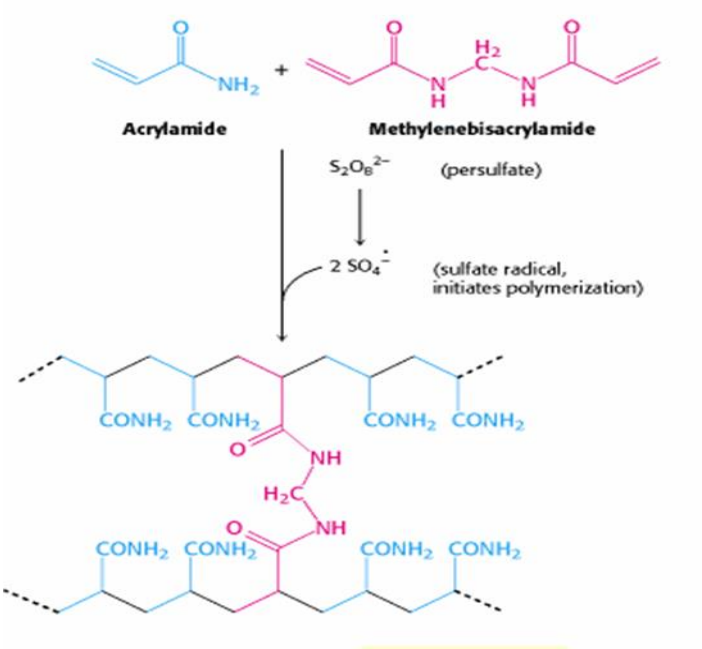


◆This technique is like the one used to separate DNA molecules, except that the gel here is vertical (vs horizontal in DNA electrophoresis) and is made of different material called polyacrylamide.

◆The most common used technique is called SDS polyacrylamide gel electrophoresis (SDS-PAGE), which we will discuss in detail now.

☆First: Formation of the gel.

◆The gel is made of 2 molecules, one is acrylamide and the other is methylenebisacrylamide. Acrylamide molecules are connected to each other to form an acrylamide polymer, this polymer is connected to another acrylamide polymer by methylenebisacrylamide forming the gel.

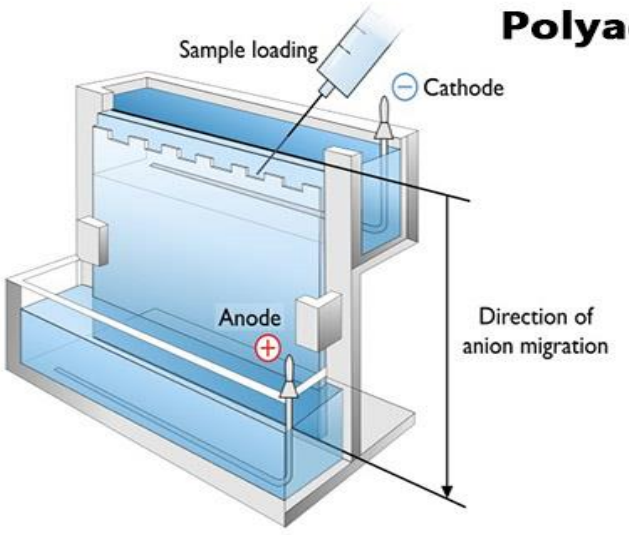


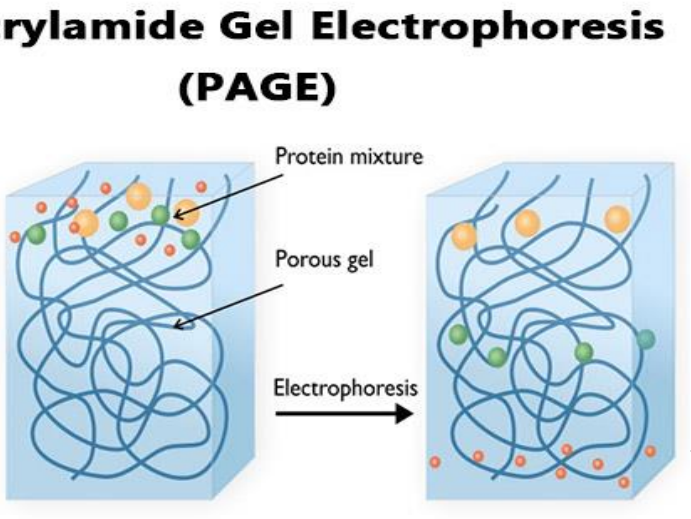
◆Therefore, this electrophoresis technique is called "polyacrylamide gel electrophoresis" or as abbreviated "PAGE".

☆Process of PAGE.

◆We add our protein sample onto the wells on the top of the gel, then and electrical voltage is applied between the upper and lower ends of the gel.

◆All proteins move from the negative cathode on the top to the positive anode in the bottom with different velocities according to their size. So, we will find larger proteins at the top and smaller ones down below them.





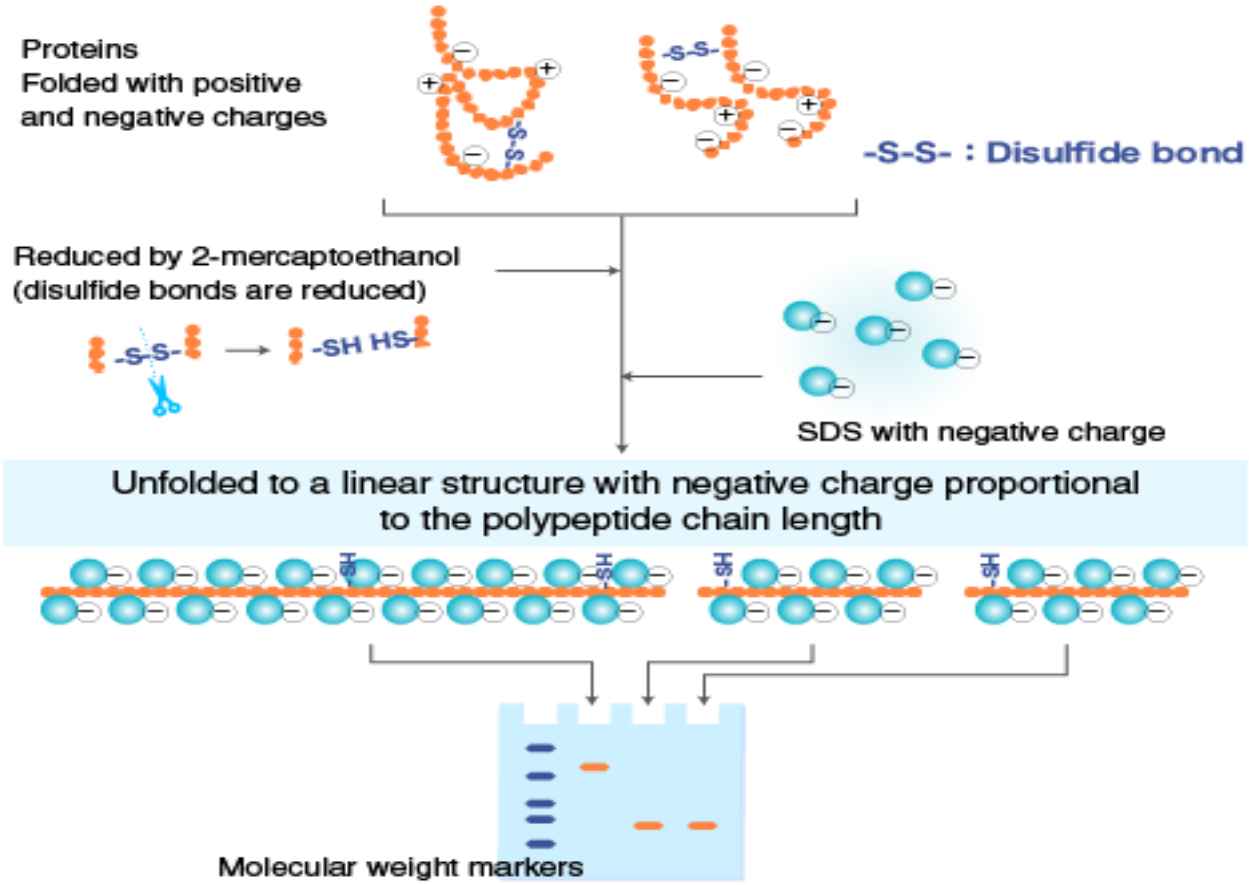
Different proteins have different charges; some are positive, others are negative, and some are neutral. But as you see in the figure, they all move from the negative end to the positive end. How does this happen? (answer below)

☆*Denaturation & unifying charges.*

◆To make all proteins move away from the negative cathode, all proteins must ①carry a negative charge,②be linear so they can move through the porous gel. For this purpose, we use an ionic detergent known as Sodium Dodecyl Sulfate (SDS), as well as a reducing agent like β-mercaptoethanol or dithiothreitol (DTT).

Sodium dodecyl sulfate (SDS)
MW 288

-SDS breaks hydrophobic interactions in proteins and coats them, its negative sulfate head makes proteins have a uniform negative charge. (see next page)



-What's left after adding SDS are disulfide bond, so we add a reducing agent to break

these bonds. Now we have a linear protein with a uniform negative charge surrounding it (which is now very similar to DNA)and is ready to use for SDS-PAGE.

★Now to put all what we have said together★

-SDS-PAGE is a technique used to separate proteins according to their size by moving them through a gel.

-We first make the gel by polymerizing acrylamide molecules and cross-linking many acrylamide polymers by methylenebisacrylamide.

-We then use SDS & a reducing agent. SDS to break all hydrophobic interactions and surround the protein with a negative charge, and the reducing agent to reduce disulfide bonds. Now we have a linear protein that is negatively charged.

-we place our protein sample in a well on the top of the gel and apply electrical voltage, proteins move from the negative end down to the positive end with different velocities.

◆Finally, once proteins are separated in the gel, we stain them to reveal their positions, they appear as bands, each band indicates that these are protein molecules of the same or different type but they have the same size.