Lecture :..24.....

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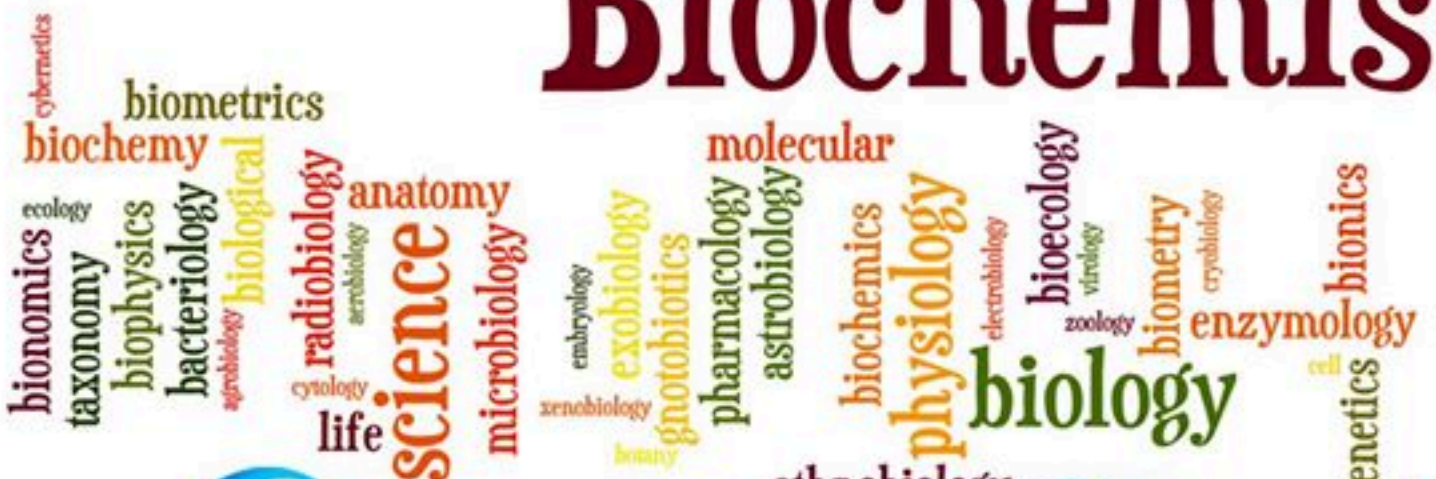
☐ Slide ■ Sheet

Medical Committee
The University of Jordan

Biochemistry



Protein Purification & Characterization Techniques

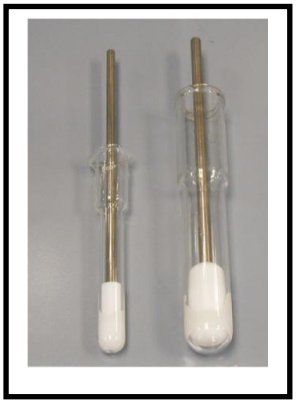
The goal of today's lecture is to know the different procedures that are being used in laboratories to detect the presence of proteins and to get the desired protein out of your sample as pure as you can.

So, what's the main source of proteins? No, it's not meat. We get them from breaking down the cells (the natural source of proteins) and get the protein out as a crude mixture (i.e. you get the protein of interest with other proteins that you don't need), then we use different techniques in order to purify it and only get the protein of interest.

❖ Homogenization:

The first step of purification is homogenization, as the name implies, in this step we want to make the sample homogenous. How is it done?

There are many ways to achieve homogenization:



1 - Potter Elvejhem homogenizer :

The simplest and most used method and it is done by using a special grinder similar to that used in grinding garlic in kitchens

It consists of a hard test tube and a hammer.

2 - Sonication:

It's done by using a sonicator which is a machine that produces sound waves, it can break down cells and even humans. If the sound waves were

high, it could damage the eardrum and even be fatal, as God implies in the Holy Quran, this is one of the ways used in torturing {الصيحة} .

When you put your sample near the sonicator, the cell's membrane starts to vibrate and eventually, it will break down releasing the contents of the cell.

3 -Freezing and Thawing:

With repeated and multiple freezing and thawing, water crystallizes on the membrane each time, breaking it down gradually.

4 -Detergents:

They can bind the membrane and eventually rapture it releasing the contents.

❖ Differential Centrifugation:

-The process of splitting the components of the mixture (sample) according to the molecular weight (mass) and density. Each material has a special molecular weight which makes it split at a specific speed. So you start separating different materials according to their molecular weight. It has its own calculations and laws, and its unit is Svedberg (a unit of time).

-So eventually if you know what protein you want and what its molecular weight, then you can get it when its certain speed is achieved.

-The problem is that the results are crude (not pure), because many proteins have the same or really close molecular weights that would

precipitate at the same speed. The unit of speed of centrifugation is (g) related to the gravity force.

~ There are examples for some materials' speeds in the slide, but you are not required to memorize them.

So , if the protein of interest was in the mitochondria , what will do to separate it?

- we open up the cells using **homogenization**, and then by **differential separation** we separate the components of the cells that have different molecular weights according to the speed of rotation.
- We get rid of nucleus, ribosome and other parts, and only keep mitochondria, since we want to study the proteins present in it.
- Since mitochondria contain a lot of proteins , this method is crude as all mitochondrial proteins will precipitate.
- In order to purify the proteins and only extract the protein of interest, we can use ***Salting in & out*** , by adding salts to the aqueous solution . Proteins are initially found in an aqueous medium since they are soluble.
- Keep in mind that Salts can strongly bind to water.

-In order to understand this mechanism better, let us divide it into two phases:

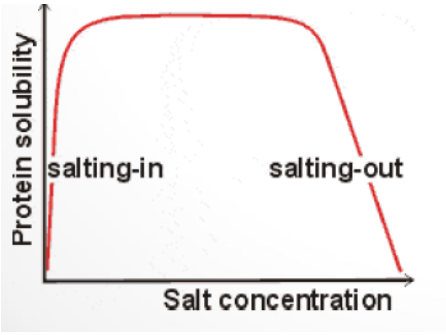
❖ Salting in:

when salts are initially added in low concentration to the mixture, they will ionize fast forming a shell of water around them ,since their ability to bind to water is higher than anything else. So, once the salt enters the solution, it ionizes to anions and cations enhancing the electricity of the solution → enhancing the partial charge of water

and certain amino acids of the proteins → binding between water and the protein becomes stronger.

-So, at the beginning when the concentration of salt is low , solubility of the protein increases .

(look to the figure)



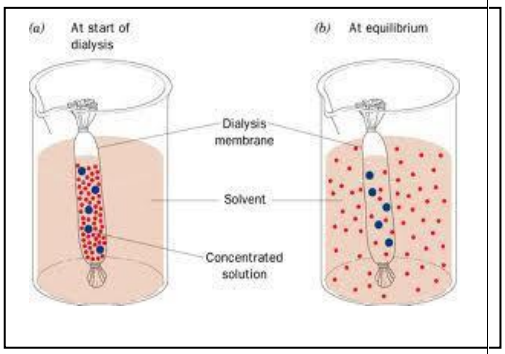
❖ Salting out:

As we add more salt, its concentration increases. And since the binding of water and salt is stronger than that of water and the protein, salt starts competing, causing the proteins' solubility to decrease , Then the hydrophobic parts of proteins in the mixture aggregate and precipitate giving us crude results. Note that as the salt's concentration increases, the precipitation also increases.

-This method mainly depends on the *solubility of proteins in water.*

-*Ammonium sulfate* is the most common reagent to use at this step

❖ Dialysis:



Another procedure is dialysis ,*which depends on diffusion*. It consists of a bag full of pores that's opened from both sides ,which are later closed by clippers.

- Then the bag filled with the sample is put in a beaker of hypotonic solvent (usually water) ,and also a stir bar is put in the beaker. Then the beaker is placed on a rotating magnet causing the stir bar to rotate, as a

result, the bag also rotates (by this rotating action , you make molecules in constant motion).

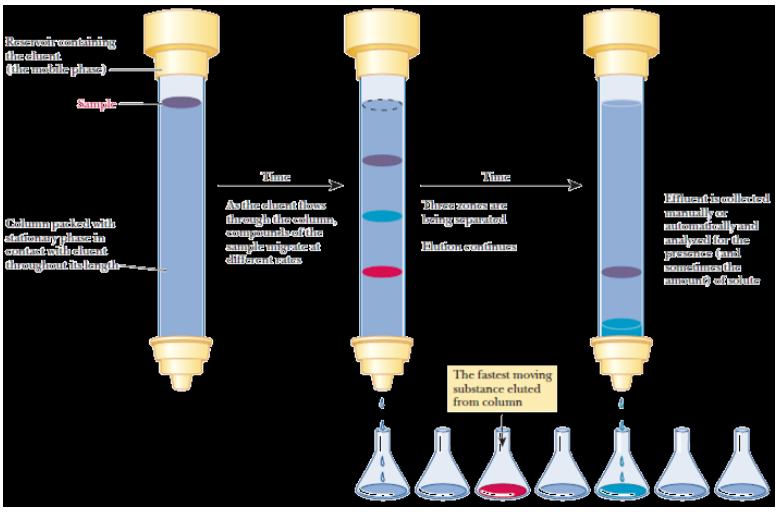
-Since the bag is porous, it acts as a molecular weight cut. The pores have a certain molecular weight capability that allows proteins to pass through them if they have that weight or less.

- Assume that a pore's capability is 60 KDa(kilo Daltons) ,any protein that weighs 60 KDa or less would pass , while those which weigh more would be retained.

- ❖ this mechanism is also crude. It will give us the protein of interest plus other proteins with matching molecular weight or above.

❖ Column Chromatography:

It's the most common procedure for purification. As the name implies, it involves columns*, chromatograph*y came from the Greek word "chroma" which means color, and *graphien* means "to write".



To understand its mechanism, we should be familiar with two definitions:

1 -Stationary phase: the already existing material in the column and able to do cross linking and polymerization and it's constant (it is always in the column and doesn't come out)

2 -Mobile phase: the proteins that we are adding to the column and eventually would come out, it's liquid and soluble, and we can add buffer or water to it.

*So we bring the column with the stationary phase and add to it the mobile phase (the protein that needs purification).Then the proteins (mobile phase) would bind to the existing material (stationary phase). Then, we follow the proteins as each one goes down the column and exits it to be collected in its own beaker . A Pencil is attached to the machine, which may draw curves (I am not sure what is its function)

-So it was important for the protein to be colored so that it can be easily observed during its movement down the column . But with new

technology, the pencil is replaced with special software, and sensors for UV and infrared light were added allowing us to also study invisible proteins. If the protein was colorless , the computer will tell you that the protein is now coming out to start collecting it.

Types of column chromatography :

- There are many types of column chromatography, each one has its own theories that depends on the interaction between the stationary and mobile phase. We will discuss each one thoroughly .

1-Size-exclusion chromatography:

A.K.A. **Gel-filtration chromatography** and **molecular sieve chromatography** (three names for the same procedure)

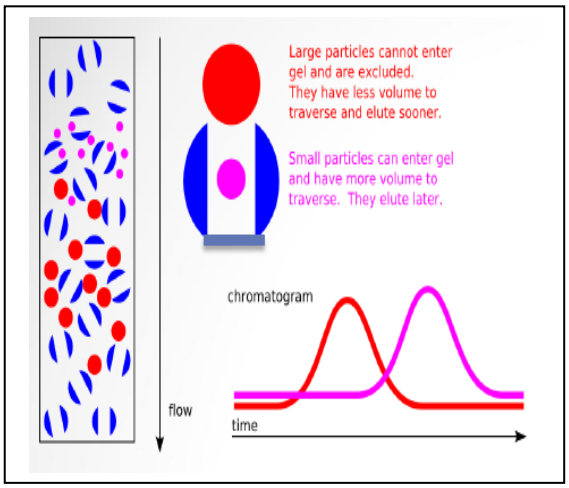
Why was it named like that?

#Size-exclusion→It separates proteins according to their molecular weight (size).

#Gel-filtration → the stationary phase is a gel-like material

#Molecular-sieve→ it separates proteins by passing through a sieve (منخل)

❖ what is the idea of this procedure?



-Imagine a sieve that has many spaces with really thin Strings and you are adding a mixture on of small and large particles on it .

- The small particles would be able to hold themselves on the strings, while large ones cannot.

Let's apply this on the procedure ☺

* The material of the stationary phase (which will form our sieve) is composed of two materials:

Carbohydrates (which could be dextran or agarose) that can form a polymer , and the other material is polyacrylamide (which can also form a polymer) .

* Both materials do cross_linkings that have pores and spaces (our sieve). The pores make retention for small molecules (strings in the sieve example) , and the large proteins pass through the spaces between the gels .

*So if we put a mixture of large proteins and small proteins ,large proteins would go through the spaces first. And if we added water or a buffer, the smaller particles would also pass, and the smallest would be the last to pass

Now we have two cases :

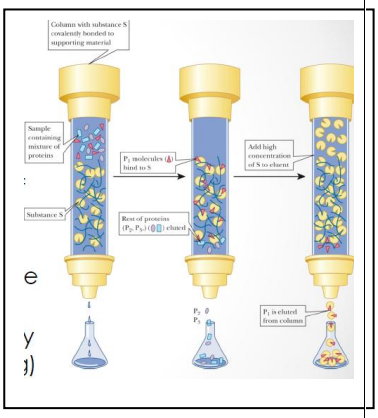
1- if we know the molecular weight (MW)of our protein of interest, we will know when to collect it ,as we know when it'll come out .

**You can make a graph of the molecular weight versus the volume you are adding during the mobile phase , and from it you can estimate when the protein will come out.

2- If we don't know the protein's molecular weight, we start adding the mobile phase and when it comes out, we can give an estimation of its molecular weight.

❖ The main purpose of this technique is to give us the convenient molecular weight or an estimation at least. Its results are pure but it's not specific (not 100% pure) as proteins with close molecular weights come out too.

❖ Affinity chromatography:



As the name implies it's a chromatography procedure that depends on the ***affinity*** between the mobile phase and the stationary phase. You create the stationary phase that you want based on the mobile phase that contains the protein of interest. Unlike the size exclusion chromatography, the affinity chromatography's results are specific (100% pure). It gives us the protein of interest without any other contaminants.

Mechanism:

-In the stationary phase, we use a ligand with *high affinity to the protein of interest*. Once we start adding the mobile phase, binding of protein of interest to the stationary phase will occur, and any other protein won't bind because binding depends on the affinity.

-We can put an enzyme and its substrate, and manipulate with the temperature or any other parameter ,so that the reaction won't occur, and we can extract the enzyme. Even if the reaction occurred, we still can extract the enzyme.

-Also , we can use an antibody and its antigen . (i.e. we can purify the protein using its antibody)

-Antibodies were synthesized for most common proteins like hemoglobin ,myoglobin and albumin. So the antibody can identify the protein and bind to it specifically .

-we can put an antibody in the stationary phase that is specific for hemoglobin for example , and when we add the mobile phase ,only hemoglobin would bind to it.

- So again, it depends on *the specific binding between stationary phase and mobile phase.*

- How do we get a protein of interest in large amounts and study it?

We bring the gene of that protein (that is found in the human DNA), and we add it to the genetic material of bacteria , the bacteria can grow rapidly and in large amounts .So, you induce that gene to produce large amounts of that protein .

This will help us to multiply the amount we have.

How can this help us in the affinity chromatography?

-We can add 6 histidines to the C terminus of a protein via using codons . So when the protein comes out, 6 histidines will be attached to its c terminus.

-The 6 histidines will form a ring structure around a nickel atom ,since histidine has a high affinity to nickel.

-So when nickel is added to the stationary phase, only proteins with 6 histidines at the end would bind to it, and even if the protein of interest was in the mobile phase and it doesn't have 6 histidines, it won't bind. This is known as **His-tag**.

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❖ Ion exchange chromatography:

As the name implies, it *depends on the ionic structure* for either the stationary or the mobile phase.

-We either bring a stationary phase with positive or negative charge m depending on the kind of the protein .

- If the protein of interest was negatively charged (*you measure the total net charge of the protein)* , we use a positive stationary phase. So if a positive protein was in the mobile phase ,it won't bind and it will come out, and vice versa.

-Most proteins have negative charge in nature.

-In the stationary phase: We can use carboxymethyl (CM) cellulose (-ve charged) or diethylaminoethyl cellulose (DEAE) (+ve charged).

*DEAE is commonly used , as most proteins are –ve charged.

**This is a crude procedure.

So we talked about how we can bind our protein of interest to the stationary phase, but the question is: How do we eventually extract it?

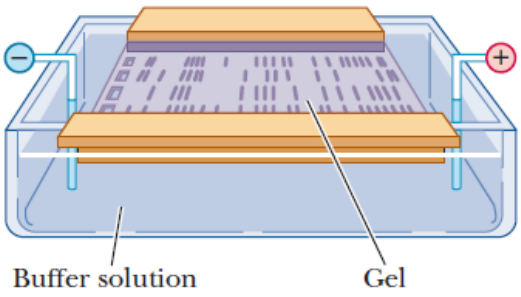
- Size exclusion chromatography -> add more mobile phase
- Affinity chromatography -> For example, in case of His tag , we add a buffer rich in histidine, so that the free histidine will compete with our protein for the nickel leaving our protein free.
  Or in case if we have the antigen- antibody … you add more free antigen .
- Ion exchange chromatography -> add salt, as salt is capable of binding to the existing charge more than the protein.

Weakly acidic: carboxymethyl (CM) cellulose	$-O-CH_2-C(=O)-O^-$
Weakly basic: diethylaminoethyl (DEAE) cellulose	$-OCH_2CH_2-N^+(CH_2CH_3)(CH_2CH_3)-H$

❖ Electrophoresis

We use a porous gel in which the

Protein moves.



What's the problem of Native Gel?

We have more than one factor that affects this movement. One of them is the shape (is it a monomeric or polymeric?)

-Also ,charge and molecular weight.(its shape may prevent it from moving as pores have specific shape)

-So, in case of native electrophoresis , motion is not an indicator of size .

-In order for us to be able to compare proteins, we must only have one variable (the molecular weight for example).

*What is the purpose behind this technique ?

Estimating the molecular weight of proteins .

*How to get rid of the other two variables?

 # shape -> we denature all the proteins, so they all become linear, and move according to the molecular weight.

*The higher the molecular eight, the lower the speed, and vice versa *

 # charge -> proteins with more negative charges would move faster to the positive electrode (anode).In order to overcome this variable, we saturate all the proteins with negative charges by adding SDS (sodium dodecyl sulfate) .

*Using SDS will denaturate proteins and also give them a negative charge .

-Gel electrophoresis can be native or denaturing (through using the SDS material).

-We can know the molecular weight depending on *the mobility on the gel.*

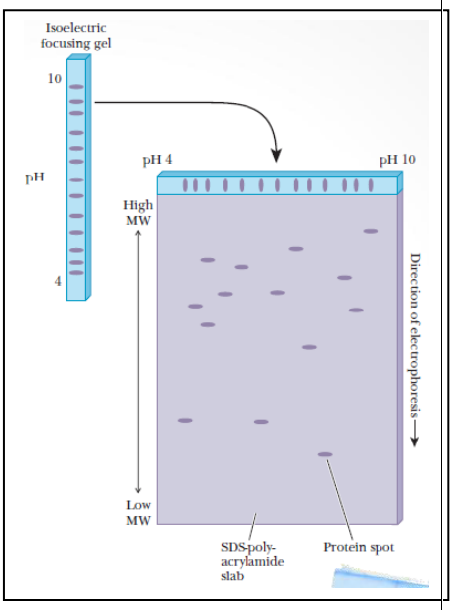
-The gel material can be either Agarose or Polyacrylamide (PAGE).

-Acrylamide offers higher resistance to large molecules .

*Aragose gel electrophoresis →DNA &RNA

*Polyacrylamide gel electrophoresis → Proteins

❖ Isoelectric Focusing:



-It detects the presence and purity of the proteins.

*A gel material with variance of pH is used (you have areas with PH = 4…5…6….etc).

*The charge of the protein will drive it along the gel until it reaches the isoelectric point, when it stops. That's why is called isoelectric focusing .

*Remember that the isoelectric point represents the pH at which the net charge of the protein is zero.

-then they used a 2D gel, when the protein stops horizontally, its total charge is zero. When SDS is added to all proteins and electrodes were used perpendicularly, they will move vertically according to their molecular weight.

-So, by using the 2D gel technique , the horizontal motion will depend on PI , and the vertical motion will depend on MW .

-each point on the 2D gel represents a protein with a specific MW and PI .

❖ Protein Sequencing Method- Edman Method

-It is used with proteins which we know absolutely nothing about.

Edman gave us an idea ☺

* Edman first made the protein linear (by denaturing it) , then he started degrading it by chipping from the **N terminus** of the polypeptide. Every time we chip off one amino acid by certain chemicals and analyze it. And by chipping amino acid by amino acid we will know the entire structure.

- It's main problem is that it doesn't work on long polypeptides, we need short ones (about 30 to 50 amino acids , but in reality its only true for 30).

-So from the first step (chipping) we get to know the number and type of the amino acid (you know the quantity and quality). In the next step, we bring another copy of the protein and see how many N terminals we have and how many C terminals we have .**why ?!**

- This let us know how many subunits our protein consists of; if it was composed of one subunit, we'll have one C terminal and one N terminal.

-Then we bring different enzymes and start chipping, each enzyme has a specific action site.

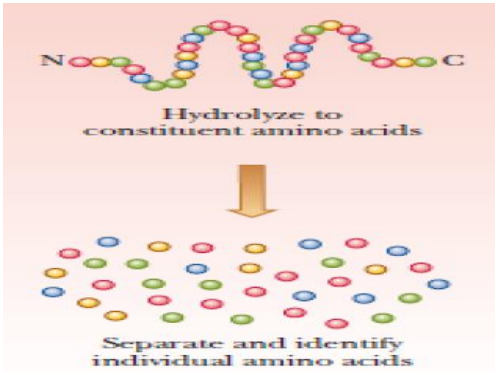
-If we add **trypsin** for example, it would identify **Arginine and lysine** (+ve charged Amino Acids) and breaks the bond after each towards the C terminus.

-If **chymotrypsin** was added, it would identify the aromatic amino acids (**tyrosine, tryptophan or phenylalanine).**

-Chemical compounds other than enzymes like **cyanogen bromide** (CNBr) identify **methionine.**

-We add these enzymes one by one and see where every enzyme acted and identify each piece then we do matching to know the sequence of the whole protein.

Read it's technique from the slides but you are not required to memorize the definitions and the materials' names. Just have an idea about how it works at the end.



❖ Protein Sequencing prediction from DNA & RNA

-It's a very simple way and done to know the sequence of the protein's genes (codons).

-If the sequence of the gene is known, this is very easy.

You already know the codon for each A.A and the gene tells you the sequence .

- If we don't know the sequence of the gene, we can bring a little piece from the protein and do sequencing to it to identify its codons and know the amino acids, then we make a complementary piece for the mRNA, then we add the complementary piece to the cell, its mRNA would bind to it and precipitate, then we study the precipitation to know the sequence of the genes and proteins.

Note ☺

Any title found in the slides and not written here is not required. But the things that are written about any technique in the sheet and the slide is required .

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"*An Arrow can only be shot by pulling it backward*" so in case you didn't do well in your mid exam, you still have a chance tomorrow (assuming that you will reach this sheet on Tuesday. if you reached it any time sooner then I send you my regards, Abu Alia.) may the odds be forever in your favor and see you next year.

لاتنسونا من صالح دعائكم ..... واعذرونا عن أي أخطاء سقطت سهوا

つづく