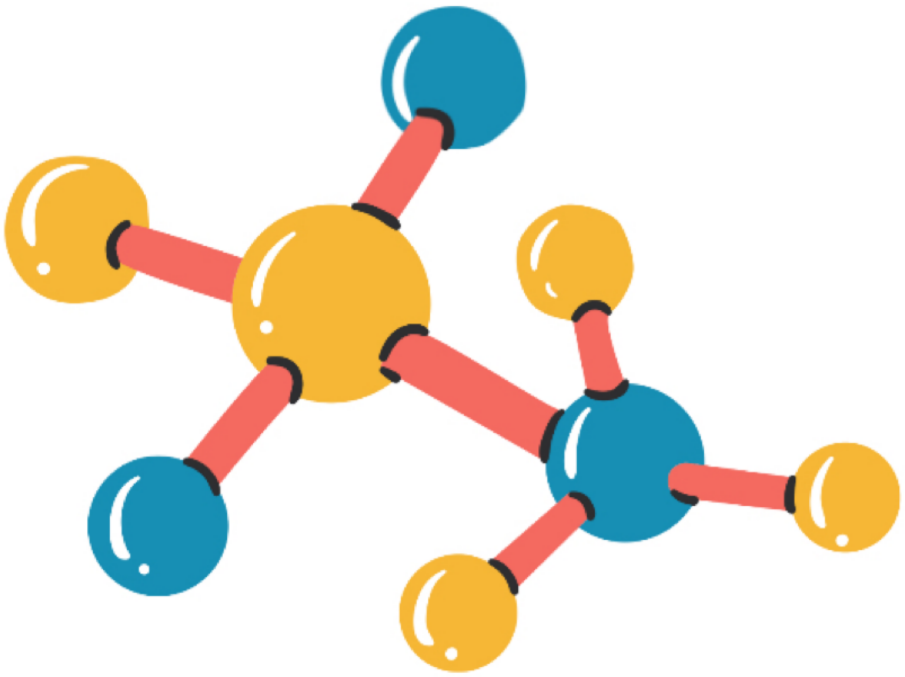
Biochemistry



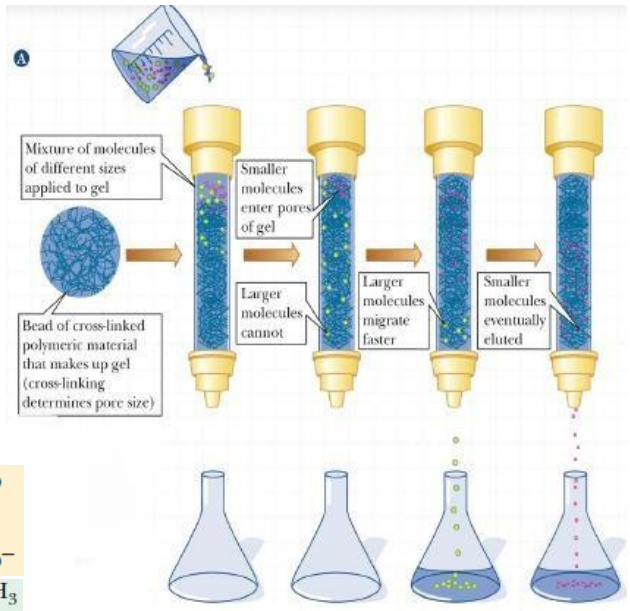
Summer 2022

Writer: Doctor 2020
Corrector: Doctor 2020
Doctor : Dr.Nafeth AbuTarboush

how do we get soluble protein outside the cell?
Homogenization (make the solution homogeneous) → differential centrifugation→ purification techniques of the protein in the solution (salt in& out "crude results depend on the concept of solubility", dialysis "crude results depend on the concept of MW", column chromatography)

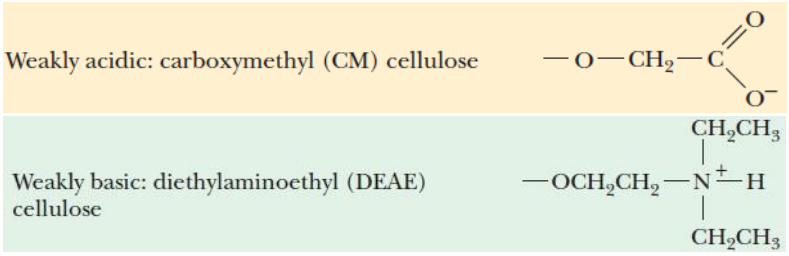
Size exclusion chromatography (Gel filtration chromatography):

Depends on the MW (size of the protein)
Stationary phase: (gel) "as gel electrophoresis (usually with DNA especially when you use agarose)" , consists of 2 polymers: the 1st is carbohydrate (dextran or agarose)& the 2nd is polyacrylamide
Mechanism: the gel is cross-linked& its pores are locating laterally, we put the sample from the top so the proteins will enter the pores depending on their MW, the bigger (size) proteins will instead keep moving throw out the void volume and separate first, the biggest proteins will remain at the top of the column (gel) and don't go inside.
Finally, we add buffer solution to mobilize the protein and get them out of the pores (they'll go out from the biggest (inside) to the smallest "order of elution).
Gives an estimation of MW, Each gel has range of sizes that separate linearly with the log of the molecular weight.
The 3rd synonym for this method is (Molecular sieve chromatography) which indicates the structure of the gel



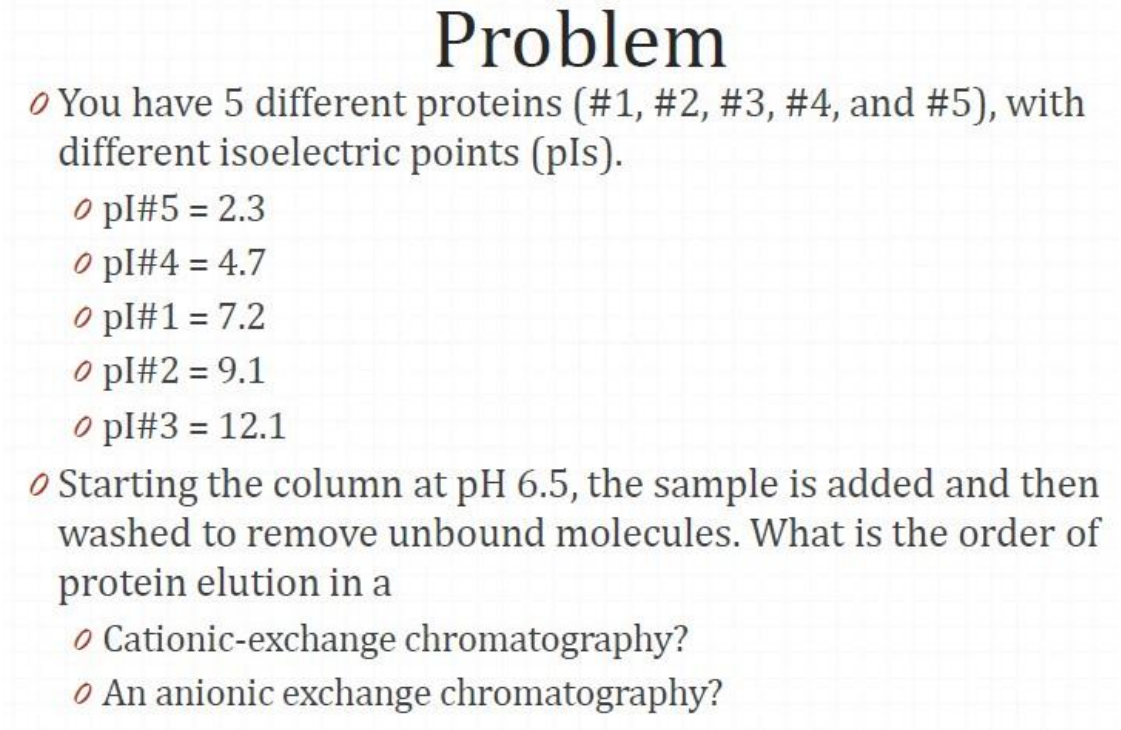
Ion exchange chromatography

The name indicates Interaction based on net charge. (less specific)
The stationary phase: polymer which is designed to be inside the column with positive or negative charge.
(the column needs to be positively charged in anion exchange chromatography)



I should know the net charge of the protein before doing this technique
(less important) I should know pI of it to know the charge at certain pH of the solution.
There will be multiple proteins which will bind in the column, how to separate the protein of interest? We add salts to do elution
(the salt will dissolve in water and compete the proteins for binding with the stationary phase, with more salt be added the proteins will start to separate, the least charged protein (positive or negative) will separate firstly).

Or we can change the pH of the solution to control the protonation state & charge of the proteins. The problem is: this may cause denaturation.
Note: most denaturation is irreversible.



How to solve it?
the protein with pI>pH is positively charged& vice versa.

So #5=(-), #4=(-), #1=(+), #2=(+)& #3=(+), the range between pI& pH determine the affinity (charging degree).

- Cationic exchange chromatography? The material is negatively charged
  Proteins with numbers 5&4 will be washed out
  Proteins with numbers 1, 2& 3 will be eluted (3→ 2→ 1)
- An anionic exchange chromatography? The material is positively charged
  Proteins with numbers 1, 2& 3 will be washed out
  Proteins with numbers 5&4 will be eluted (5→ 4)

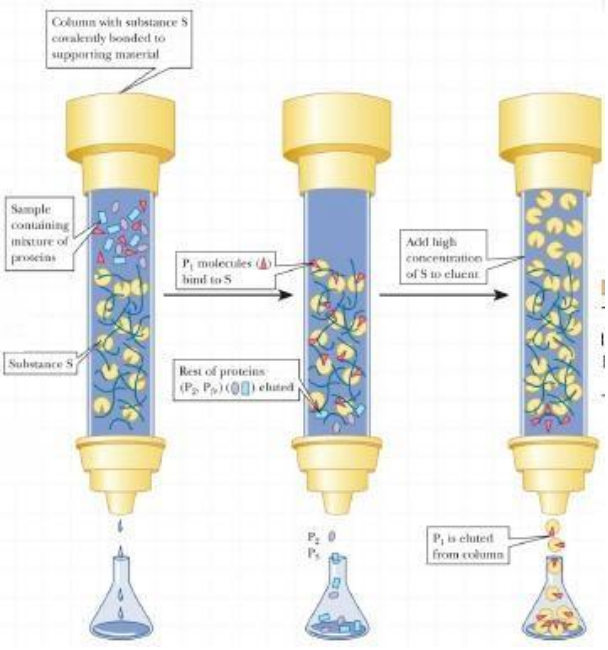
Affinity chromatography: (best technique)

It uses the concept of the affinity of protein to its substrate. (better results than Gel filtration chromatography), and it's so good in dealing with non-colored proteins.

The polymer (stationary) is covalently linked to a ligand that binds specifically to the desired

Protein. (The purist results)

Like using (Antigen antibody, His-tag& GST-Tag) His-tag has high affinity for Ni, so we use it as stationary phase



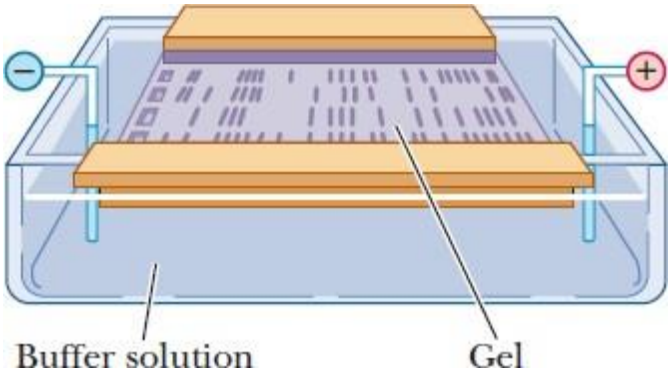
How to get the protein of interest out of the column? By using the concept of competition

For example we can add histidine or imidazole, Ni will bind to them and the protein of interest will get out. The problem with this method: very expensive.

Note: you can use the previous methods to get the sample as small as possible to reduce the costs of this method

Electrophoresis:

Liquid material that turns into semi-solid (before it turns completely we put comb to make pores called “wells” to put the samples on them).



Then you’ll apply an electrical current and the samples start moving

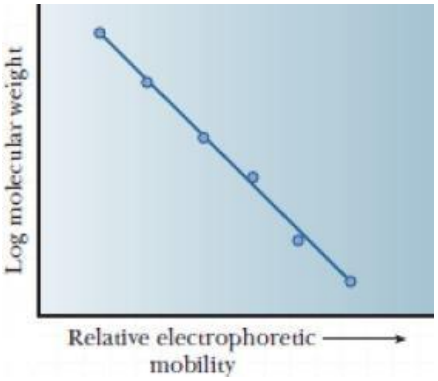
This technique depends on : **size** (determine whether the moving is fast or small), **charge**(the protein will move to electrode that have the opposite charge)& **shape**(monomer, dimer.. etc., determines moving through the pores).

Polyacrylamide is commonly used with protein samples (provides better resistance)

Agarose or PAGE? Agarose (nucleic acids), PAGE (proteins)

In PAGE: SDS or NO-SDS, detergent, $CH_3(CH_2)_{10}CH_2OSO_3Na^+$

SDS completely denatures proteins (multi subunit proteins)



Binds with almost all amino acids of the protein & cover them with negative charge.

By using SDS we neglect the effect of shape (it denatures) & charge (all have negative & the ratio of charge-size is almost the same), so the proteins will move (fast or slow) under the effect of MW (size) only.

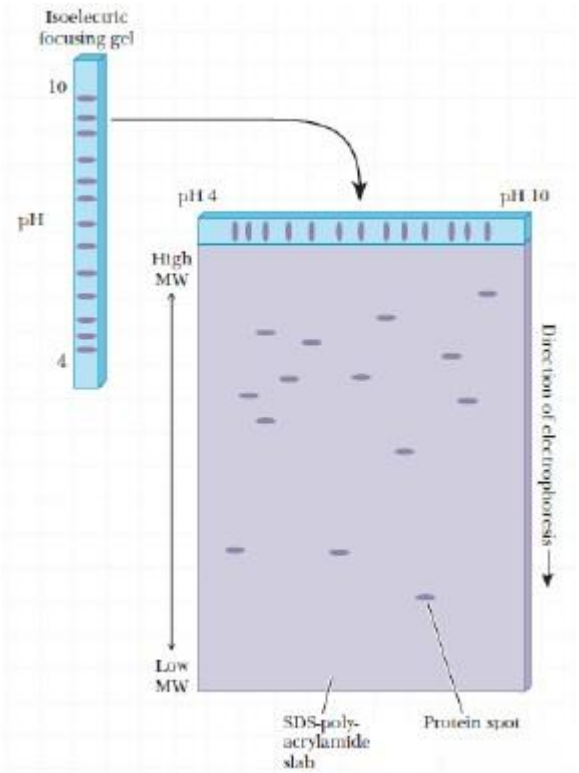
Native gel electrophoresis: doesn't use SDS. (proteins move according to 3 factors)

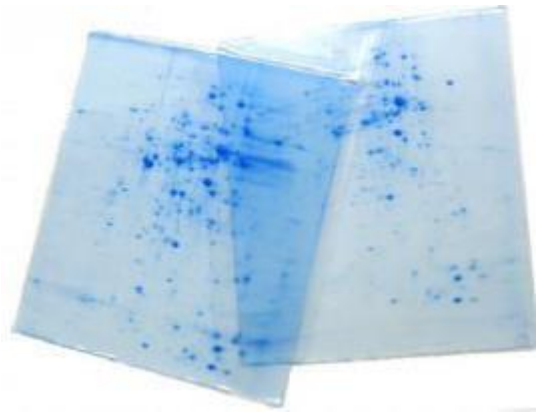
SDS can't denature proteins that have **disulfide bonds**, so we add materials that can reduce them (like Dithiothreitol and 2-mercaptoethanol (ME))and the technique is called (reducing SDS gel electrophoresis)

Isoelectric focusing:

Depends on the concept of isoelectric points, we use gel with gradient (scale) of pH , so the protein will keep moving until it reaches its pI , it will be neutral and won't move under the effect of electrical current and stop moving.

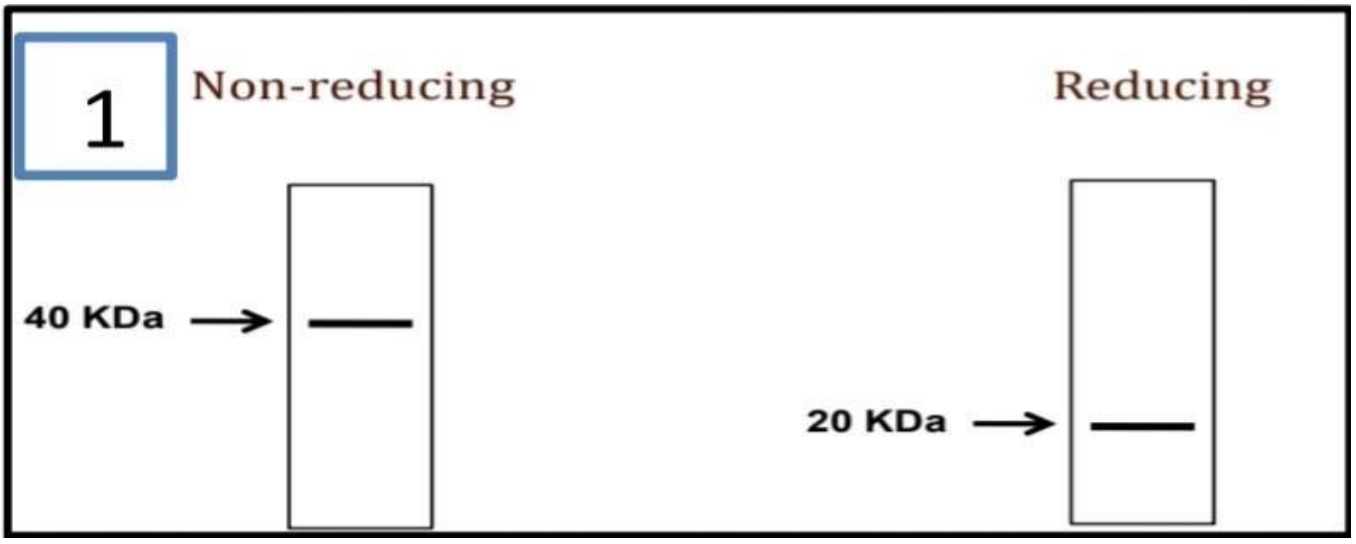
Another technique Two dimensional gel electrophoresis (2-D gels), uses the concepts of regular gel electrophoresis & Isoelectric focusing (put 2 layers of gel above each other, proteins will move according to their pI in the 1st layer, once they stopped you'll add SDS to each well to regain the negative charge for all proteins then you'll apply electrical current again, but in the other dimension), so you'll know both: the pI & MW of the proteins



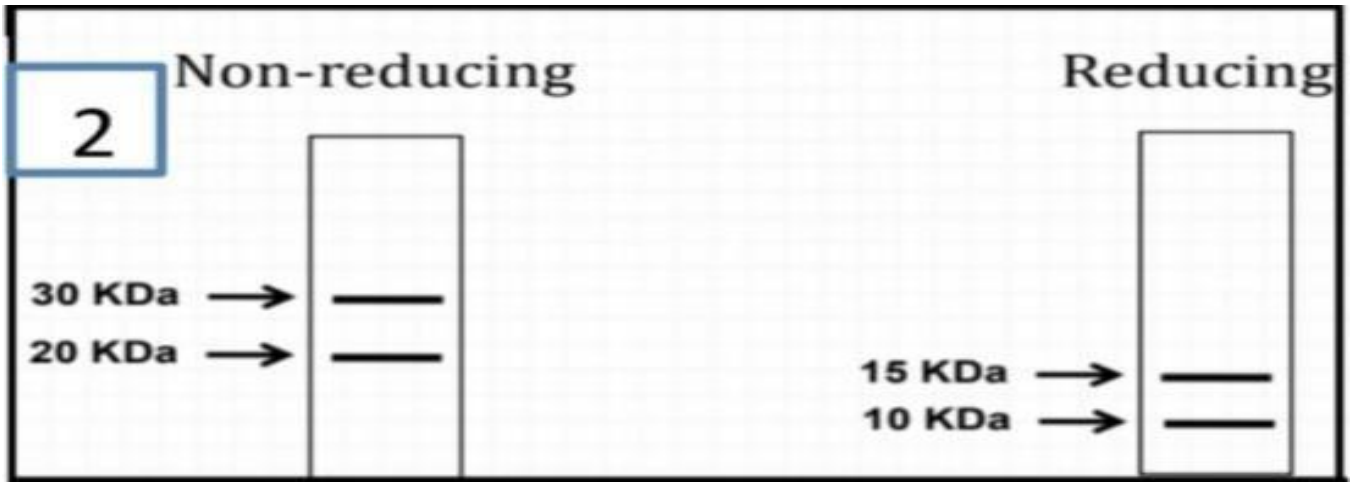


❖ Qusetions:

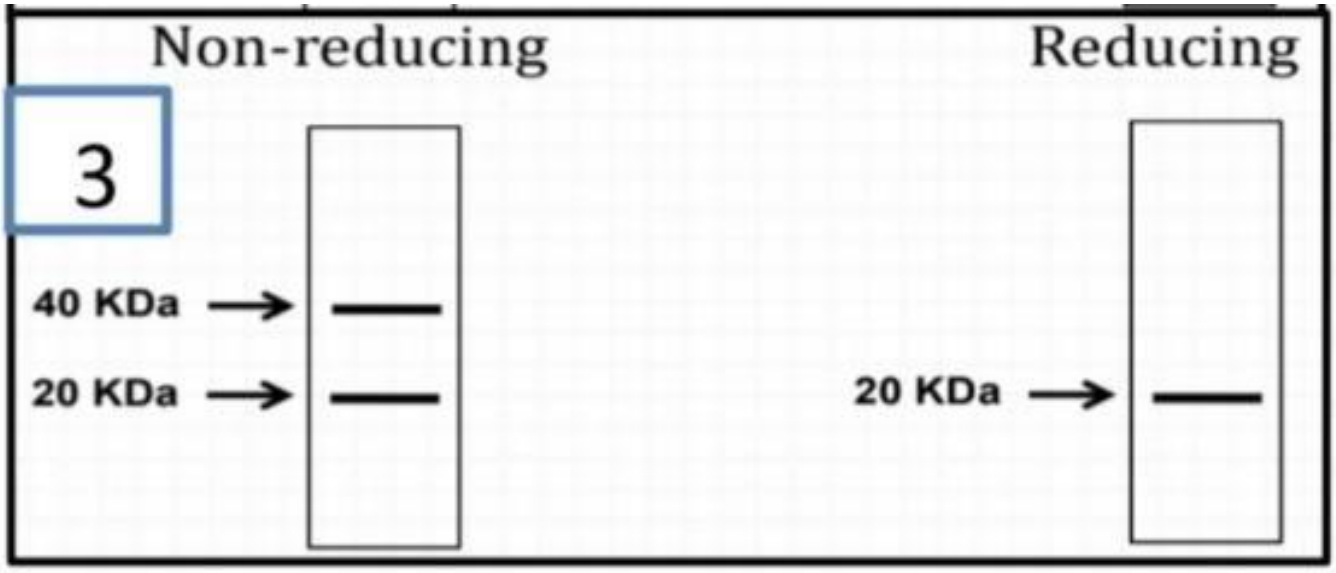
Describe the protein's structure based on the following results of SDS-PAGE:



- ✓ Notic that ander SDS non-reducing conditions which distrups all interactions except of the covalent ones ,a protein exists as one 40-Kda band ,but under SDS reducing conditions which distrupts the covalent bonds (disulfide bonds here) ,the protein exists as two 20-Kda bands which implies that the structure of the protein is **homodimer .**



- ✓ Under SDS non-reducing conditions ,a prortein exists as one 40-Kda band, but under SDS reducing conditions ,the protein exists as two 20-Kda bands which implies that the structure of the protein is **heterotetramer** each two **homodimer** are linked by **disulfide bonds** and the pair of the two **different homodimers** are linked via **non-covalent** interactions .



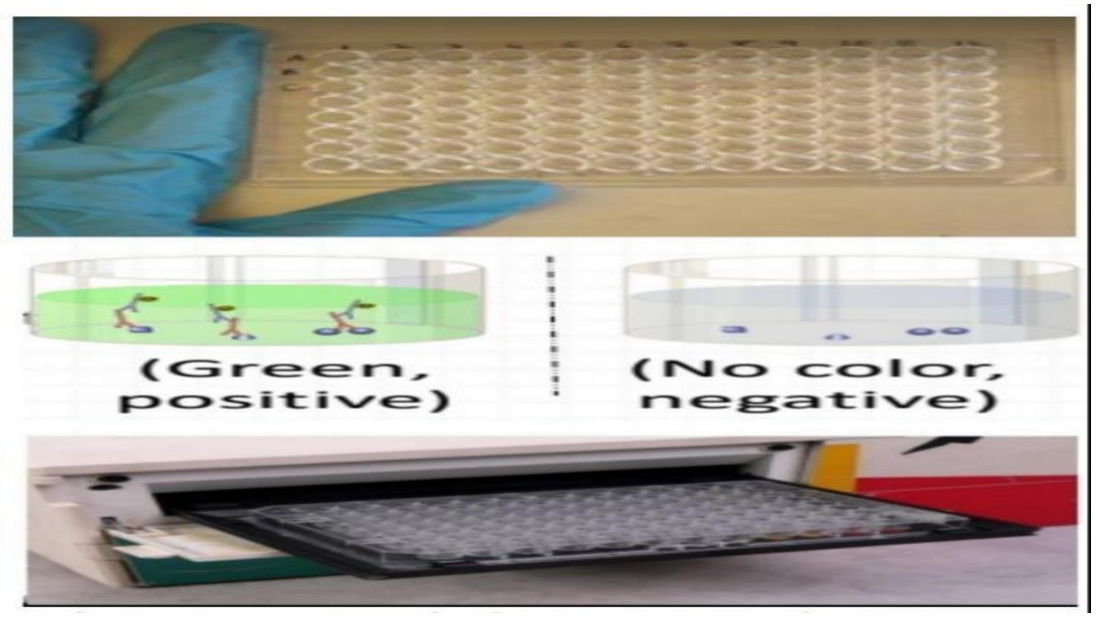
- ✓ In the last case ,under SDS non-reducing condition, a protein exists as two bands 40 Kda and 20 Kda , but under SDS reducing conditions, the protein exists as one band of 20 Kda, which implies that the protein is **homotrimer**; a **homodimer** 40 Kda linked together via **disulfide bonds** that is why they are not broken under reducing conditions and this **homodimer** is attached to a **momomer** of 20 Kda via **non- covalent interactions.**
- ✓

❖ Immunoassay – ELISA:

- Enzyme -linked immunosorbent assay.
- Detect & quantify substances (peptides,proteins,antibodies & hormones)
- Usually done in 96-well plates.
- Rapid, convenient, and sensitive (10 power to -9g)

- **Application:**

    - Screening (HIV, Hepatitis B&C)
    - Detecting food allergens, such as milk, peanuts, walnuts, almonds and eggs.
    - Hormones (HCG, LH, TSH, T3 ,T4)



So, this technique is widely used in research because it is cheap, the plate that is used is called **96 well plate** that it has **96 wells** within each well there is an antibody specific for certain antigen ,then we apply our sample and our protein of interest which is designed to bind with an antibody of interest will bind to it , then we do a washing so all other proteins that are unbound to the well will be washed away keeping only the protein of interest .

Now we have a binding but the question is , how to detect for the antigen that is bound to the anti body ?

Actually there are more than one way , the ELISA is one of them which is called **the sandwich technique** where we have an application for a **second antibody** that will bind to the **antigen** from the other side of the usual side (above the antigen instead of being down of it) (**Remember: we apply the use of the secondary antibody for the sake of detection**) and this secondary antibody has an enzyme attached to it ,which is able to change the color of a certain reactant that we design for this enzyme to catalyze ,once it is activated ,so after the addition of the secondary antibody to the plate we add the reactant and if the color changes this means that the antigen exists, but if the color did not change this means that there is no antigen in the sample .

An application of this technique is the pregnancy test (sold on the pharmacies only by 1 JD if you want)

Before explaining the mechanism of work of this test let us know at the first some facts about the urine of the pregnant woman.

After the fertilization occurs between the sperm and the ovum then we will a fertilized egg which will divide many times forming what we call a blastocyst which produces **Human chronic gonadotropin (HCG)** hormone which is secreted on the urine and the blood of pregnant woman and this is the antigen ion which our test depends.

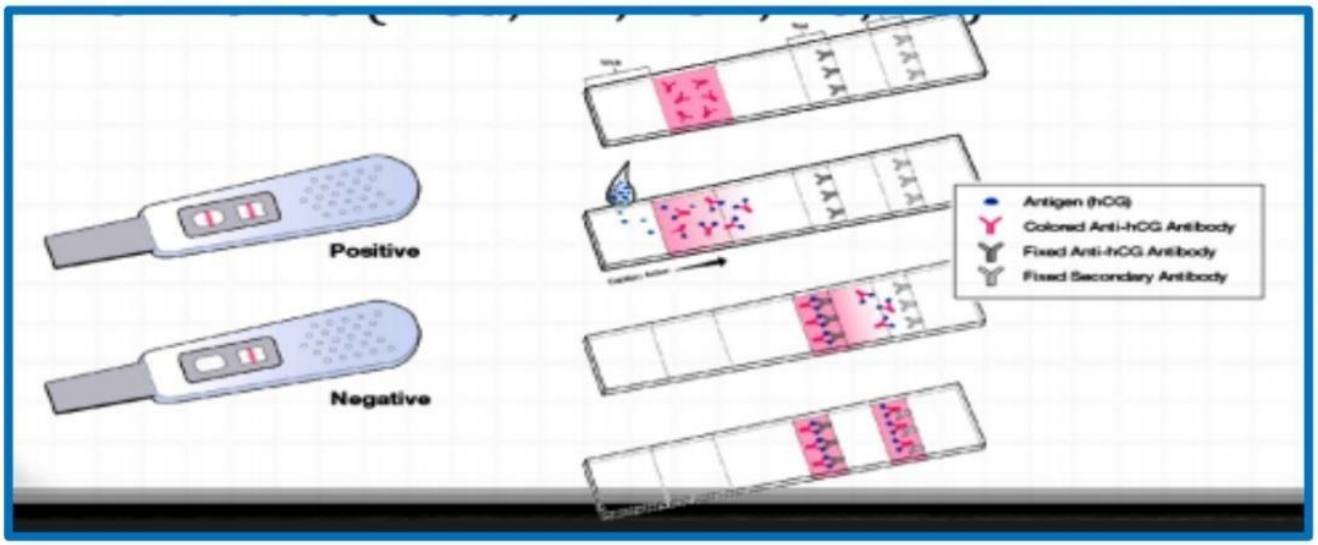
So our test is about a strip which has **3 types** of the antibodies and **two zones** ; **the control and test zone ,**so as we apply a drop of urine of a pregnant woman the **HCG** antigen will bind the first tyoe of antibodies which is called **colored anti -HCG antibodies** then the bound colored antibodies will pass to the first zone (**the test zone)** in which we have the second type of antibodies (**Fixed Anti-HCG Antibody**) this type depends mainly on the presence of the **HCG in order for it to bind (on a sandwich technique) thus the color will be produced** otherwise, it will not bind and there will no be a color produced at this zone (that what happens If we apply a drop of urine from a **non- pregnant woman) .**

At the second zone which is designed mainly in order to make sure that the test is working well , the case is different , here we have a **third type of the antibodies (Fixed secondary Antibody)** and this type don't care on about the presence of **HCG** antibody ,it bind to the **FC portion** of the **colored antibody** thus producing a color .

To sum up:

2 lines = pregnant (positive).

1line=not pregnant (negative) .



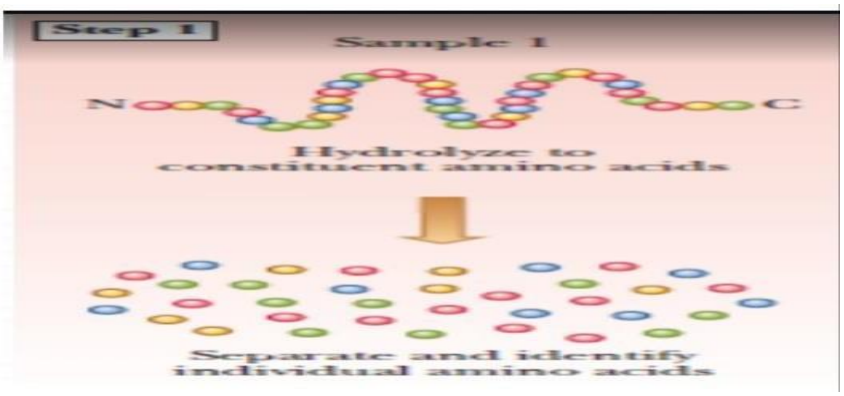
❖ Protein sequencing:

How can we know the sequence of amino acids of a new protein if we don't know neither mature mRNA nor the DNA sequence?!

- Protein sequencing is basically the process of knowing the amino acid sequence of a protein or a peptide.
- One technique in known as **Edman degradation.**
- this procedure involves a **step -by-a step** cleavage of the N-terminal residue of a peptide, allowing for the identification of each cleaved residue.

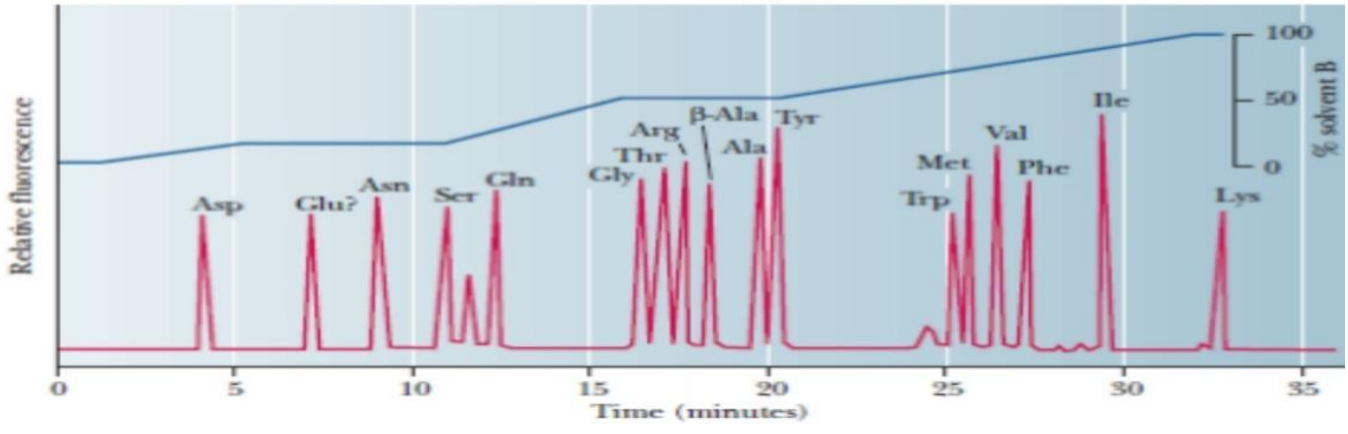
- This method of degradation should be **controlled** which means to be applied step by step otherwise, you will got the number of each amino acid involved in the peptide without determining its sequence.

NOTE: the simplest way of determining the peptide/protein sequence is by knowing the sequence of the mature mRNA.



❖ Edman method – protein sequencing:

- Divided into 3 stages **Hydrolysis, separation and hight performance liquid chromatography .**
- How much and which amino acid are involved .
- **Hydrolysis is for all amino acid of the protein (** heating +HCL) & **separation of amino acids** (ion-exchange chromatography or **HPLC)**
- **In order to know the sequence of the amino acid we make hydrolysis for the all-amino acids at the first then we use different chromatography methods that each amino acid has specific wave length.**



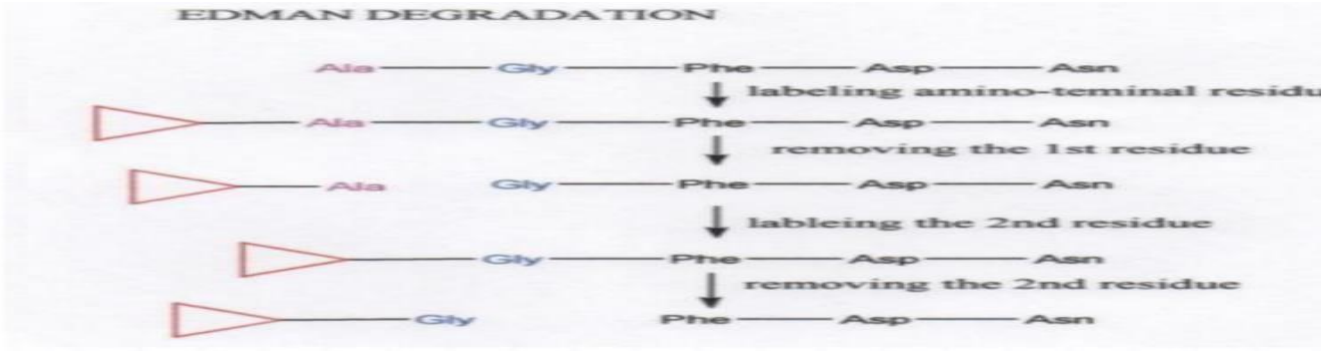
In thus figure:

- **The more the fluorescence absorbance the higher is the concentration of each amino acid.**
- **Each amino acid has a specific wave length.**

❖ procedure:

- This method utilizes **phenylisothiocyanate (PITC)**
- **(PITC)** to react with the **N-terminal** residue (only on **N-terminus** of each protein of each peptide).
- The resultant amino acid is hydrolyzed, liberated from the peptide, and identified by chromatographic procedures.

There is one N-terminus in each polypeptide or protein, if it has more than one subunit, there will be more than (PITC) reacted with the N-terminal residue . at the same time, we bind to PITC. we label the first amino acid and remove it , then we label the second amino acid and remove it and so on . So the sequence of peptide will be known one by one from the N-terminus toward the C-terminus .



○ Advantages :

- since the remainder of the peptide is intact, the entire sequence of reactions can be repeated over and obtain the sequence of the peptide .

○ disadvantage:

- The **Edman degradation** technique does not allow peptides more than 50 residues ,otherwise it will return back and bind other amino acid in the different region of the peptide thus, disrupt the ability of the enzyme to bind to N-terminus .

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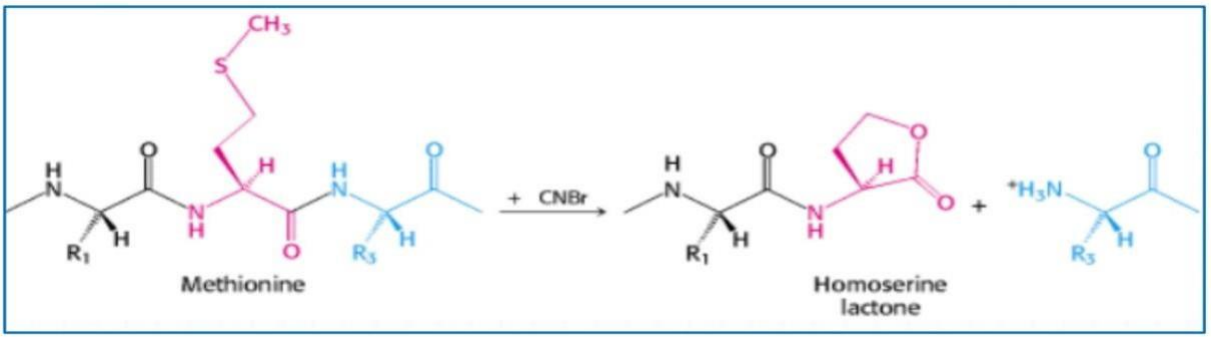
❖ Cleavage methods:

Which depends on the breakdown the big proteins into Fragments, which applies the use of enzymes to cut at **certain points** inside/outside the amino acid sequences.

- It is possible to sequence whole proteins (even the ones with more than 50 amino acids) by cleaving them into smaller peptides.
- This is facilitated by three methods:
  1. Chemical digestion
  2. Endopeptidases
  3. Exopeptidases

1. Chemical digestion

- The most commonly utilized chemical reagent that cleaves peptide bonds by recognition of specific amino acid residues is **cyanogen bromide** (CNBr) .
- This reagent causes specific cleavage at the C- terminal side of the methionine.
- A protein that has 10 met residues will usually yield 11 peptides on cleavage with CNBr.
- CNBr: has the ability ti cleave a peptide bond of a met residue whenever it sees it from it C – terminus.

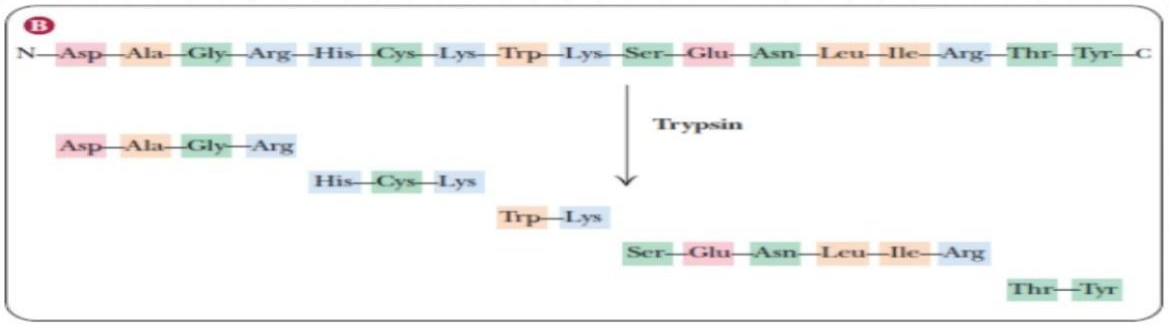


2 . Endopeotidases:

- These are enzymes tgat cleave at specific sites within the primary sequence of proteins.
- The resultant smaller peptides can be chromatographically separated and subjected to **Edman degradation** sequencing reactions.

- **Examples**:
- Trypsin cleaves polypeptide chains on the carboxyl side (c-terminus) of **Arg** and **lys** residues.
- A protein that contains 9 **lys** and 7 **arg** residues will usually yield 17 peptides on digestion with try.

Note: pepsine is a peptidase / protease which cleaves proteins inside our intestine, it goes to the c terminus of either lys or arg and cleaves after that .



Other examples

Enzyme	Specificity
Trypsin	peptide bond C-terminal to R, K, but not if next to P
Chymotrypsin	peptide bond C-terminal to F, Y, W but not if next to P Aromatic amino acids
Elastase	peptide bond C-terminal to A, G, S, V, but not if next to P
Pepsin	peptide bond N-terminal to L, F, W, Y, but not when next to P

Except if it was close to proline because it creates kinks that will prevent the peptide from fitting into the active site

Note: doctor says that all of them stop their clevage once there is a proline AFTER the specific amino acid residue, so forget about what is written above about the pro.

3.Exopeptidase:

- These are enzymes that cleave amino acide starting at the end of the peptide.
- There are two types:
  1. Aminopeptidase: that cleave at the N- terminus.
  2. Carboxypeptidase: that cleave at the C- terminus.

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Protein sequencing – prediction from DNA & RNA

- If the sequence of the gene is known, this is very easy
- If the sequence of the gene is unknown (newly isolated protein) then we sequence a short segment, complementary RNA, isolate mRNA, PCR or we use gene sequencing.

❖ Determination of 3°Structure

1.x-ray crystallography

- uses a perfect crystal; that is, one in which all individual protein molecules have the same 3D structure and orientation.
- Exposure to a beam of x-rays gives a series diffraction patterns.
- Information on molecula coordinates is extracted by a mathematical analysis called a fourier series.

The mechanism is as following:

We have a plate as (the 96 well plate) that contains a well and we cover it with a cover slip such as plastic then we put a drop of a solution that contains the protein which **has to be very pure** (if you apply gel electrophoresis you will get only one band)on the cover , the water will evaporate from the solution with time while we are conserving the proteins 3D structure as a crystal then we expose it to X- Ray beam, the different structures of the protein will give us different patterns of diffraction as the protein is composed of amino acids which in turn is composed from atoms each atom diffract the beam in a certain way which helps the computer to determine its identity (it takes days to do so) also it takes the distance between the atoms into consideration to predict the type of bonds between them so that predicting the whole structure of the protein ,and that is how we distinguish our organs and bone for ex. in an X- Ray image ,that each organ's cell has a specific characteristics that are similar to each other while different from other organ's cells in the contents thus in the

behavior, it is a magical technique.(if you have enough time return to this concept for better understanding)

The problem in this technique is that proteins or enzymes are tested under static conditions as crystals which does not mimic 100% its real conditions because proteins are dynamic in solution, it studies the prosperities of certain protein in certain posture ,also for enzymes for ex. They lose there catalytic activity in crystal phase which means that they become non functional under this conditions with some exceptions according to the latest researches .

2. 2-D Nuclear magnetic resonance (NMR) :

Can be done on protein samples in aqueous solution then apply. As the name implies , it involves the use of magnetic field , that you will put your protein of interest in a magnetic field accordingly the magnetic field will affect all the atoms inside any structure to align in a certain way , the same thing applies with (MRI) .
The principle is that the conc. of molecules is constant in the cells of the same type thus in same tissue, thus this tissue will align differently than the other tissues ,so that it will appear differently on the image and you can distinguish between different types of tissues accordingly , it is so much clear and some what magical but still not at that magical as the X-Ray crystallography , but it gives the benefit of studying the protein while in solution so it gives you a more representative method of knowing the protein structure while in solution (**remember**: we had to let the water to vaporize in the previous method)