بسم الله الرحمن الرحيم

BioChemistry | FINAL 16

Protein purification & Characterization Techniques

Last modified in the First Year

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Protein Purification and Characterization Techniques

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Extracting Pure Proteins from Cells

- We're now starting protein purification. Since enzymes are proteins, they go through the same purification steps we'll learn about today, which is why we're covering this after finishing enzymes.
- Purification means separating one specific protein of interest (POI), protein of interest (POI) is a specific protein that researchers want to study or analyze, often in the context of biological processes, disease, or therapeutic development, from a mixture that contains many protein; allowing us to study or use that protein alone.
- Protein purification is widely applied in research, diagnostic labs, and hospitals.

- Purification techniques focus mainly on **size & charge**

The samples used for protein purification can be either solid, such as a single cell or tissue, or liquid, such as body fluids (blood, saliva, urine, etc..), which may also contain cells

Extracting Pure Proteins from Cells

- The first step is **homogenization** :: to make the sample homogeneous, meaning the contents are evenly distributed. This is done by breaking open the cells to release their contents and mix them into a uniform solution. BY ::

شكلها زي مدقّة الثّوم☺

grinding, Potter–Elvejhem homogenizer –> Old method: it was once the only way to break cells, using a tool similar to a garlic crusher as a mechanical process. However, its efficiency was very low, but at that time it was the only available method.

sonication –> subjecting it to ultrasonic vibration (sound waves)

Repeated freezing and thawing –> when water freezes, it expands in size. Repeated freezing and thawing damages the cell membrane and breaks the cells. However, this method is not ideal because it can also damage proteins, which is something we want to avoid

detergents

- **Differential centrifugation (**600 *g:* unbroken cells & nuclei; 15,000 *g:*
- mitochondria; 100,000 *g:* ribosomes and membrane fragments

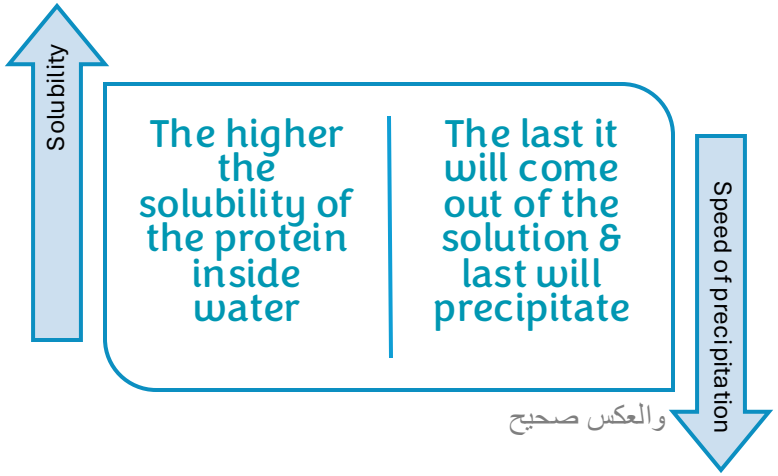
Salting in & out :

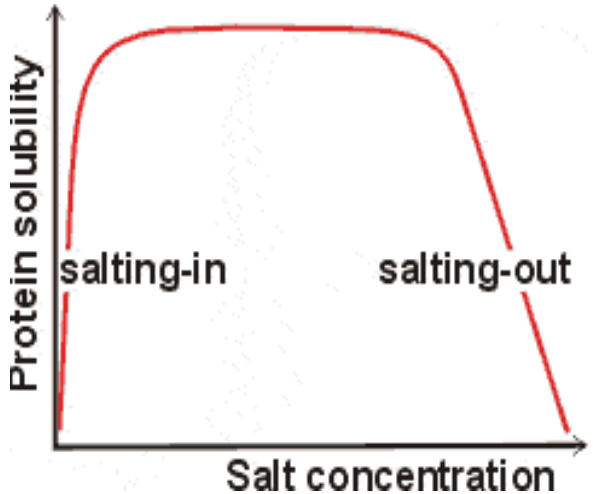
Solubility

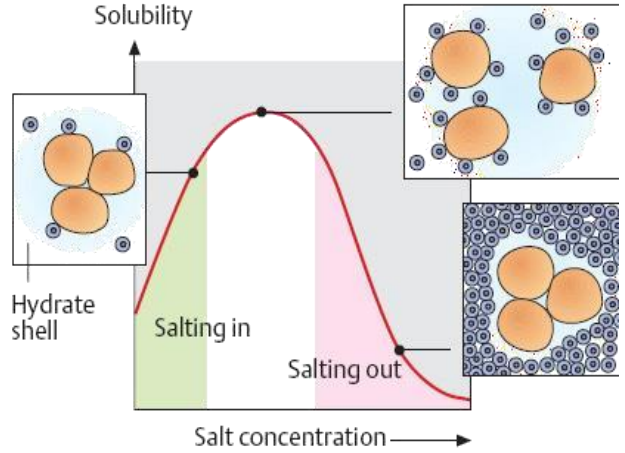
We did talk about *salting* in plasma proteins, this technique depends on **SOLUBILITY** concept

Salt is the cheapest thing u can deal with; so it's commonly used

- Are proteins soluble? If yes, to which limit?
- Salt stabilizes the various charged groups on a protein molecule and enhance the polarity of water and increases the ionic strength, thus attracting protein into the solution and enhancing the solubility of protein
- Ammonium sulfate is the most common reagent to use at this step
- This technique is important but results are crude

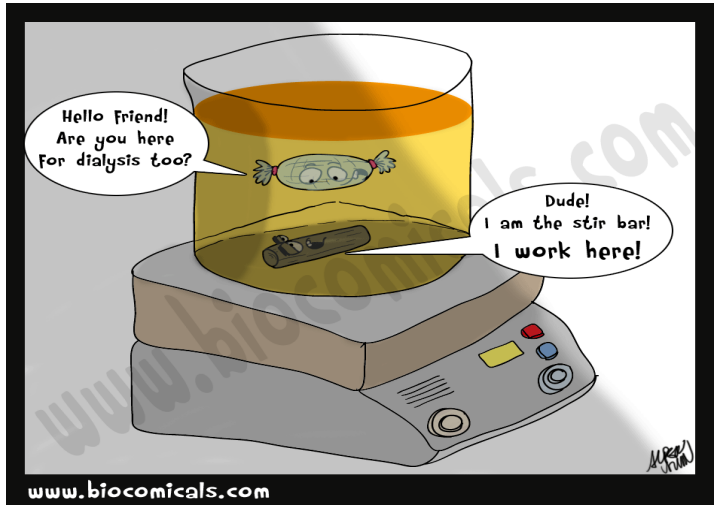




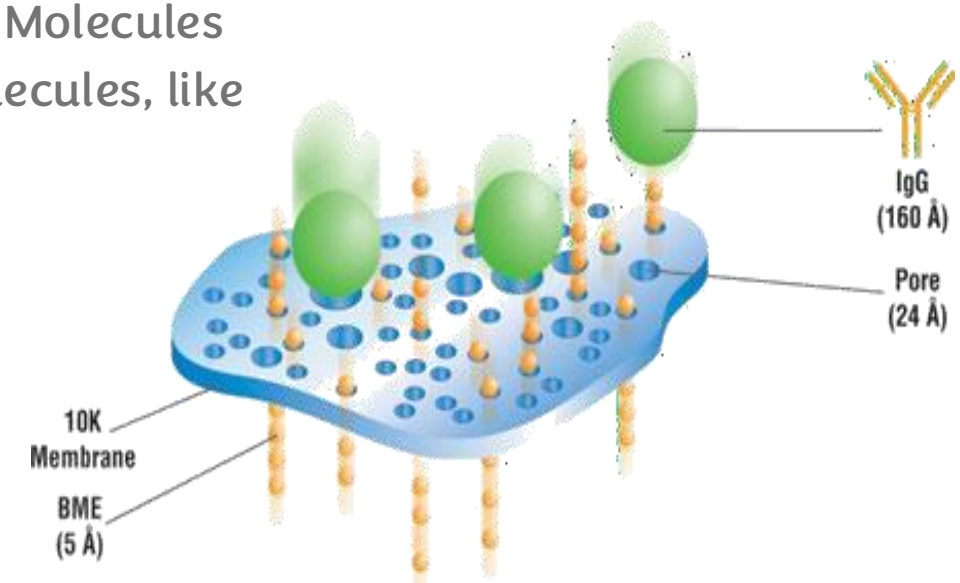


Dialysis

separation technique that uses semipermeable membranes (sheets)



- Principle of diffusion
  - ✓ These membranes have pores of a defined size
  - ✓ The "size" is usually specified as a molecular weight cut-off (MWCO) in kilodaltons (kDa)
- Concept of MW cut-off :: is the size limit of molecules that can pass through a dialysis membrane. Molecules smaller than the MWCO pass through, while larger molecules, like proteins, are retained.
- Pure vs. crude



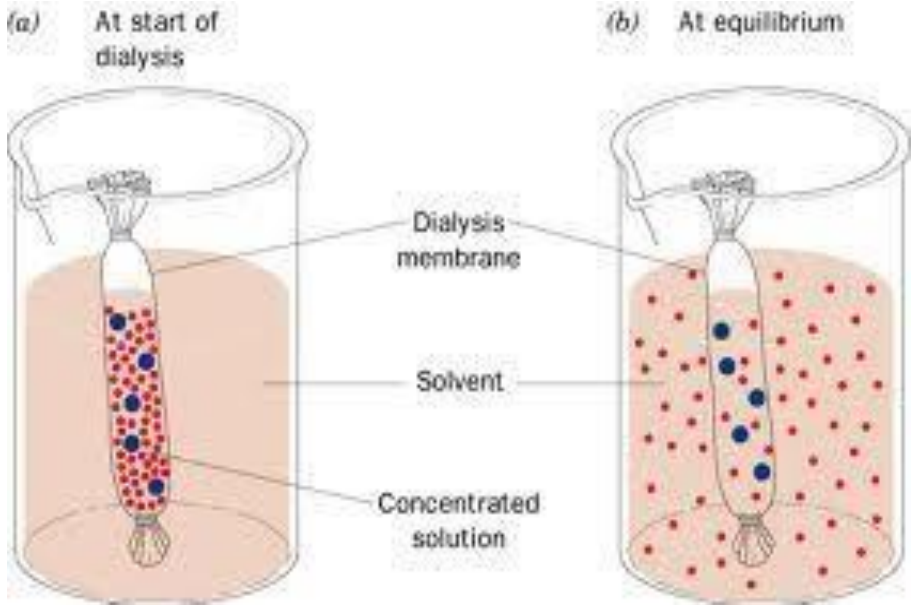
Dialysis

- Principle of diffusion
- Concept of MW cut-off

✓ it uses a special sheet (membrane) that is porous, allowing small molecules to pass through while retaining larger ones like proteins. The membrane can also fold and stick onto itself

✓ Separation occurs by diffusion, moving substances from an area of high concentration (inside the bag) to low concentration (outside solution/beaker)

The clippers:



Dialysis

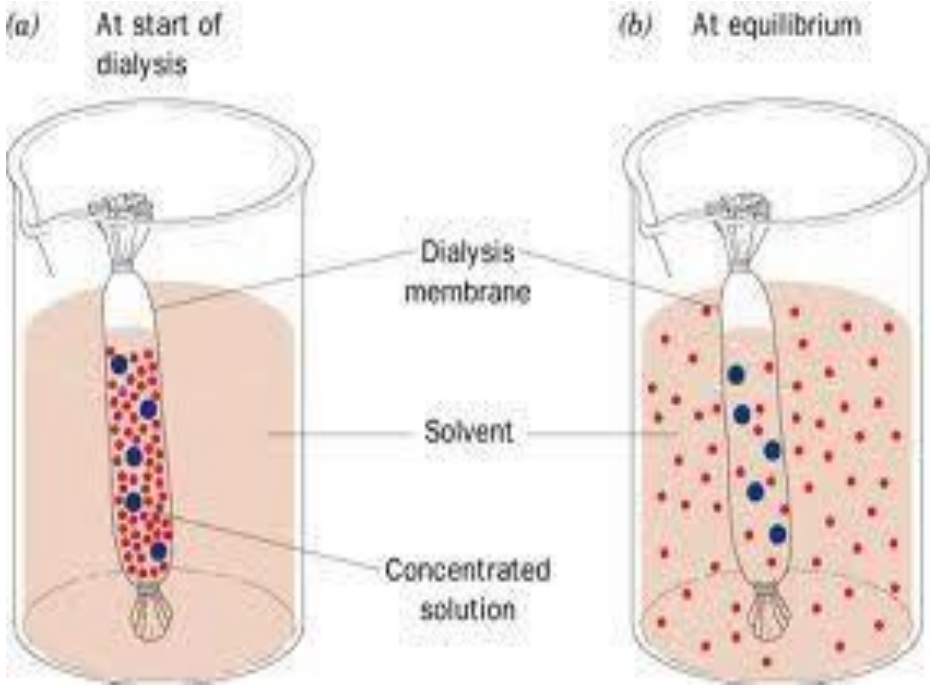
Practical Setup

1.Sheets of dialysis membrane are wrapped and clipped at one side.
2.The sample is loaded into the tubing.
3.The other side is clipped → the dialysis bag now looks like a حبّـة شوكولاتة ☺
4.The bag is placed in a beaker containing a large volume of buffer solution.

Example

•MWCO = 10 kDa
•Molecules <10 kDa (e.g. salts, amino acids, small peptides) → diffuse out of the bag.
•Molecules >10 kDa (e.g. proteins of interest) → are retained inside the bag.

👉 So, if you choose a 10 kDa cut-off, your protein of interest (POI) must be larger than 10 kDa.



Dialysis

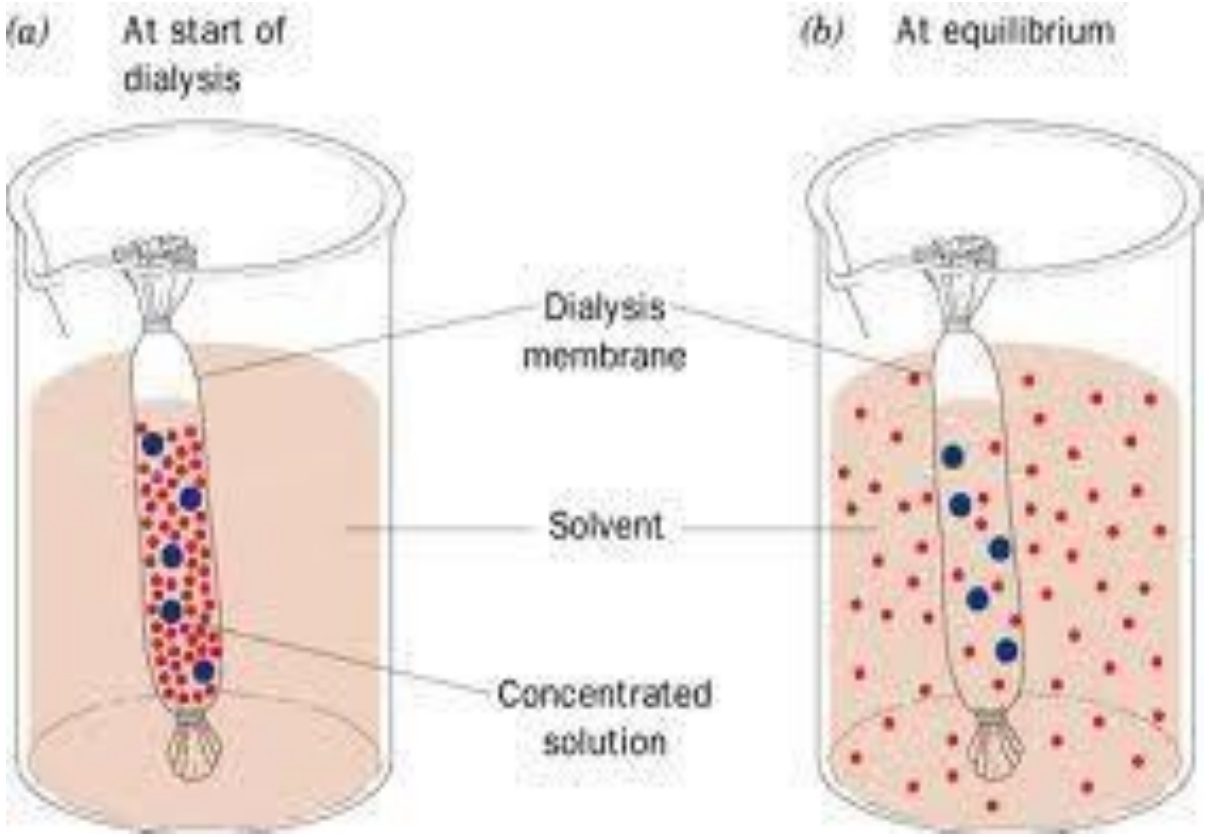
- Pure vs. crude

Outcome

- Useful for removing small contaminants (salts, urea, small proteins/peptides).

➢ Results are crude:

- All molecules larger than the MWCO are retained (even unwanted large proteins).
- Purification is based only on size, not on exact identity.



Column Chromatography

A little bit expensive
لذا بتيجي في المرتبة الثالثة بعد
Salting & Dialysis الـ

- Greek chroma, “color,” and graphein, “to write”

✓ One of the oldest biochemical techniques for protein separation.
✓ As the name implies, it involves the use of a column filled with material (stationary phase).

✓ The protein of interest (POI) had to be colored to follow its movement through the column.
✓ When the colored protein exited the column, fractions were collected.

✓ وهذا الحكي مربوط مع –> pencil on a chart recorder :
- The pencil moved up and down based on **absorbance** of the proteins leaving the column.
- This created a graph that showed protein peaks.
- ومن هُنا جاء الاسم ..

Column Chromatography

- ✓ Now, detection is done with software and computers, not pens.
- ✓ The protein does **not** need to be colored:
- ✓ Modern detectors can measure absorbance at different wavelengths, even outside the visible spectrum.
- ✓ Even though colors are no longer required, the technique is still called chromatography.

Column Chromatography

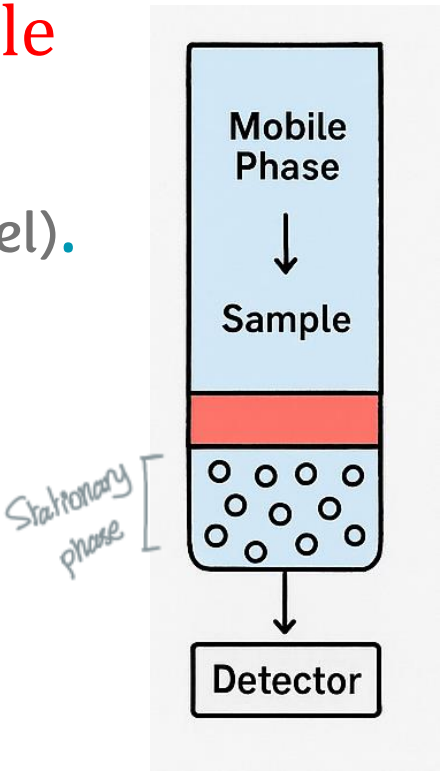
- Chromatography is based on two phases: stationary & mobile

➢ **Stationary Phase:**

- **The solid material packed inside the column** (resin, beads, or gel).
- Responsible for interacting with proteins and slowing some molecules more than others.

➢ **Mobile Phase:**

- **The liquid (buffer) poured on top of the column.**
- Carries proteins through the stationary phase.



Principle

•Proteins move at different speeds depending on their interactions with the stationary phase.

•This leads to separation into different bands/fractions, which can then be collected and analyzed.

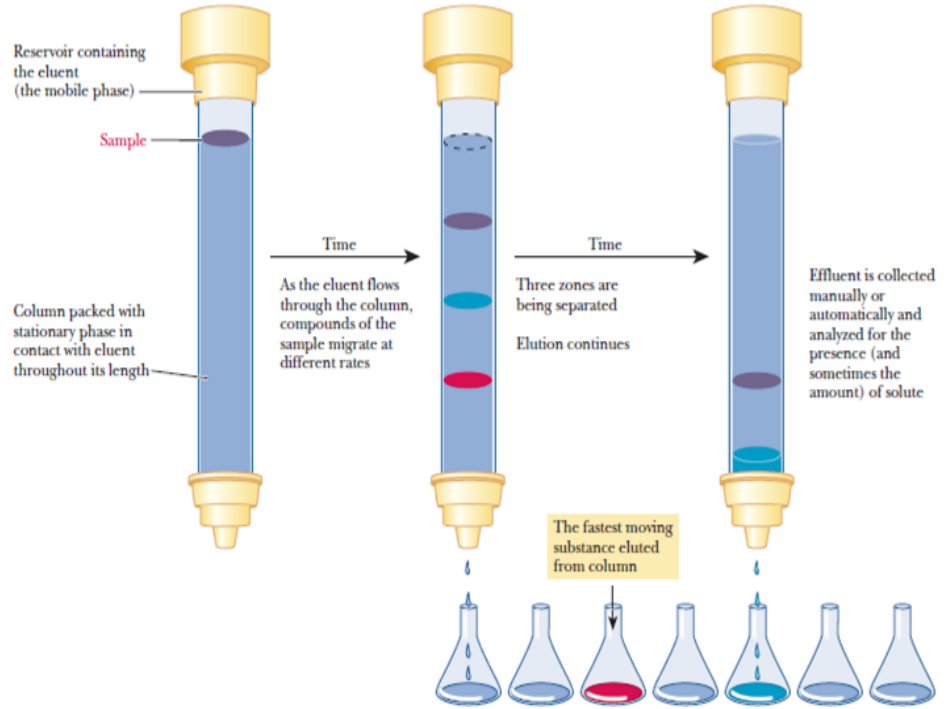
Column Chromatography

- Is it just for colourful proteins?
- Washing or Elution?
  - **Washing** is done by adding a buffer on top of the column to remove substances that are not bound to the column or are not of interest (contaminants)
  - **Elution** is the process of releasing the protein of interest (POI) from the column so it can be collected. Sometimes more than one protein may elute, depending on the conditions.
- What are the different kinds?

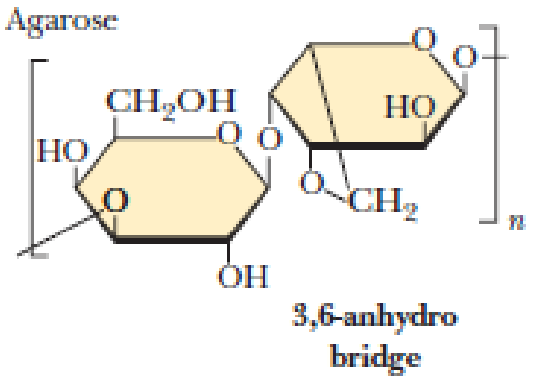
1. **Size-exclusion chromatography**
   Gel-filtration chromatography
   Molecular-sieve chromatography

   } 3 synonyms for the same technique

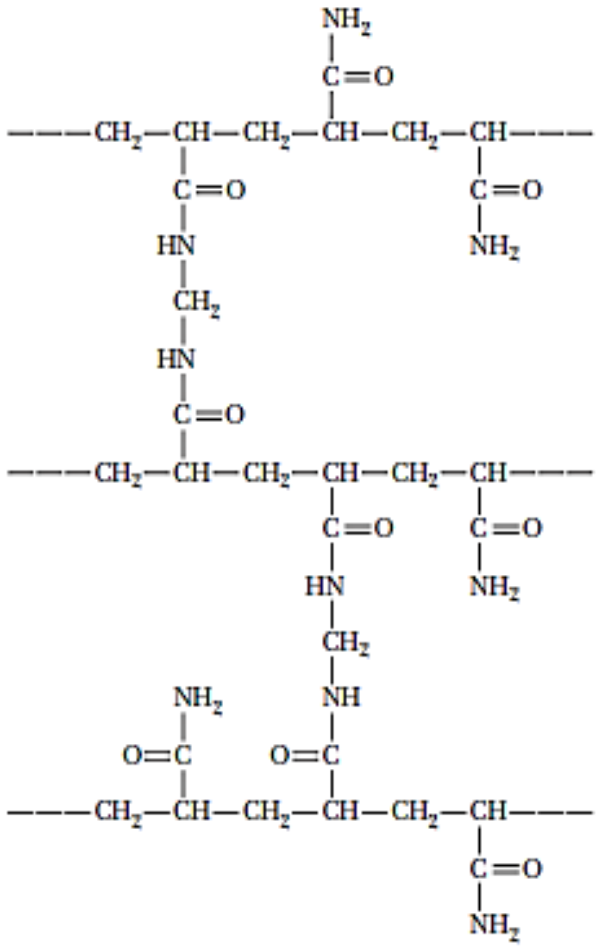
2. **Ion-exchange chromatography**
3. **Affinity chromatography**



Size-exclusion chromatography
Gel-filtration chromatography



- Separation on the basis of size (MW)
- Stationary (cross-linked gel particles): consist of one of two kinds of polymers; the 1st is a carb. polymer (ex. **dextran** or **agarose);** often referred to by Sephadex and Sepharose. The 2nd is based on **polyacrylamide** (Bio-Gel)
- Extent of crosslinking & pore size (exclusion limit)
- Convenient & MW estimate
- Each gel has range of sizes that separate linearly with the log of the molecular weight



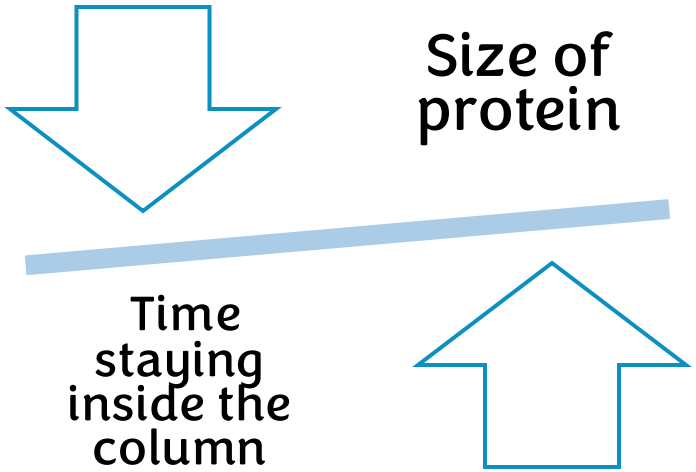
Size-exclusion chromatography
Gel-filtration chromatography

As the name implies, it adopts the concept of size ((molecular weight))

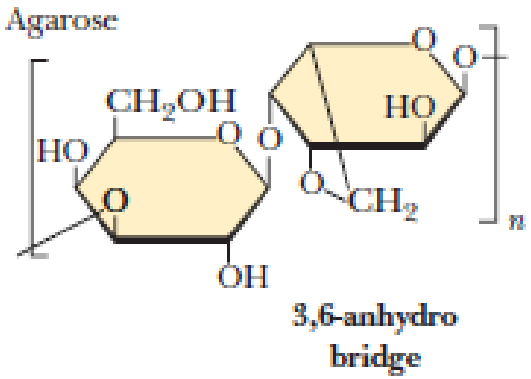
- It involves using gel inside the column
- uses a gel inside the column that acts like a sieve or screen. The gel is made of polymers that form a network of lines and pores.
- The pores are larger than the lines, and each column has a molecular weight cut-off (MWCO), which determines which molecules can enter the pores.
- Proteins with a higher molecular weight cannot enter the pores, so they pass around the gel and come out first. Smaller proteins, however, can enter the pores and get trapped temporarily, so they move more slowly through the column.

❖ **The smaller the protein, the longer it stays inside the column.**

✓ To push these smaller proteins out, more buffer must be added to the column.

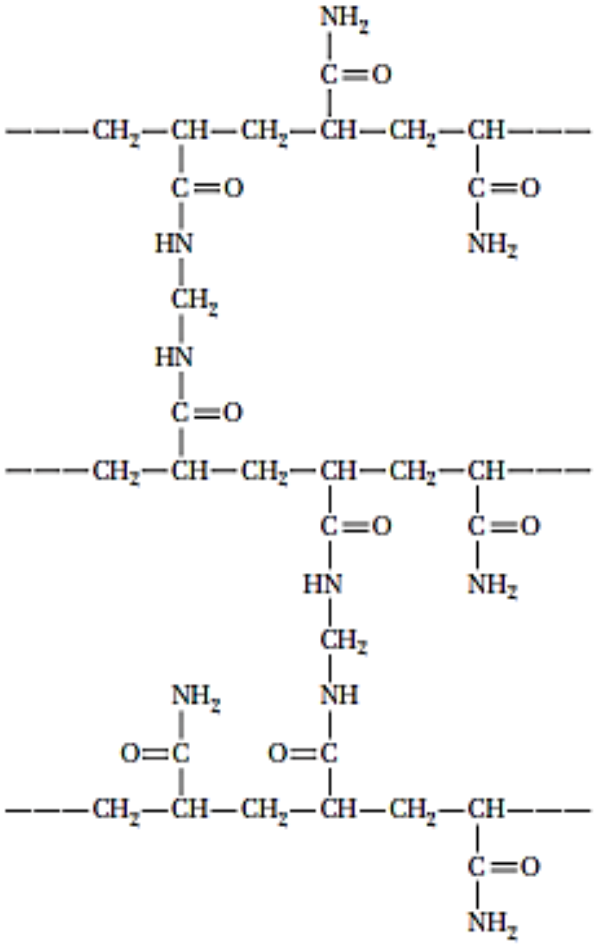


Size-exclusion chromatography
Gel-filtration chromatography

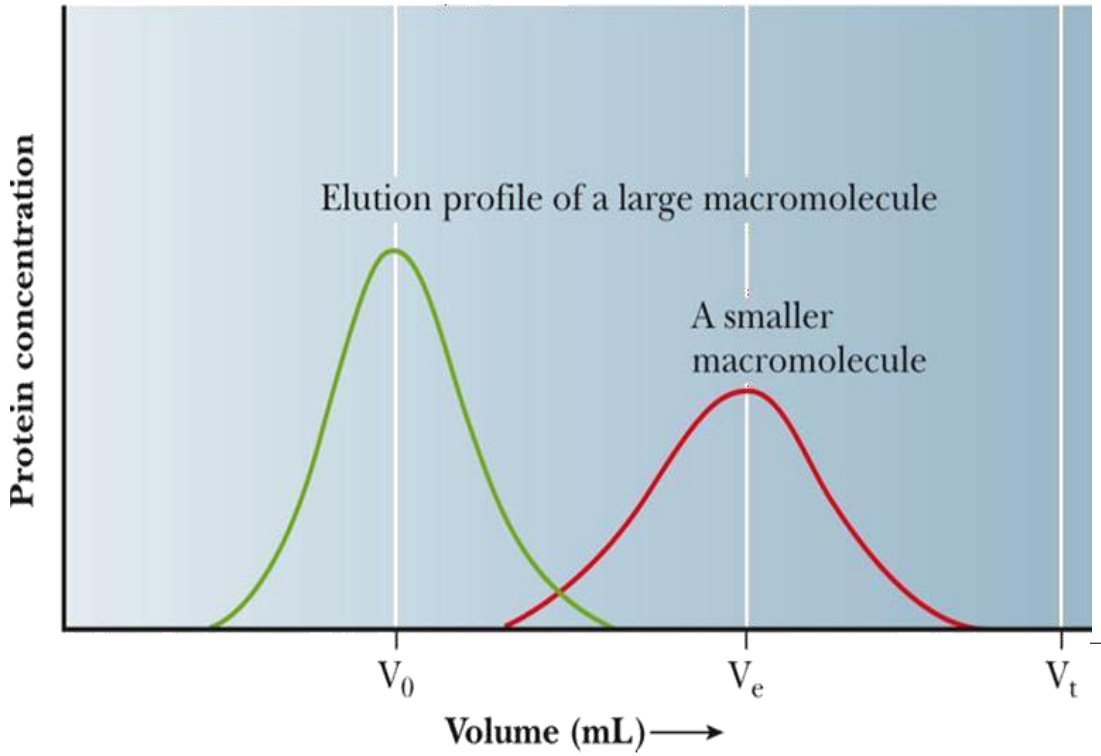


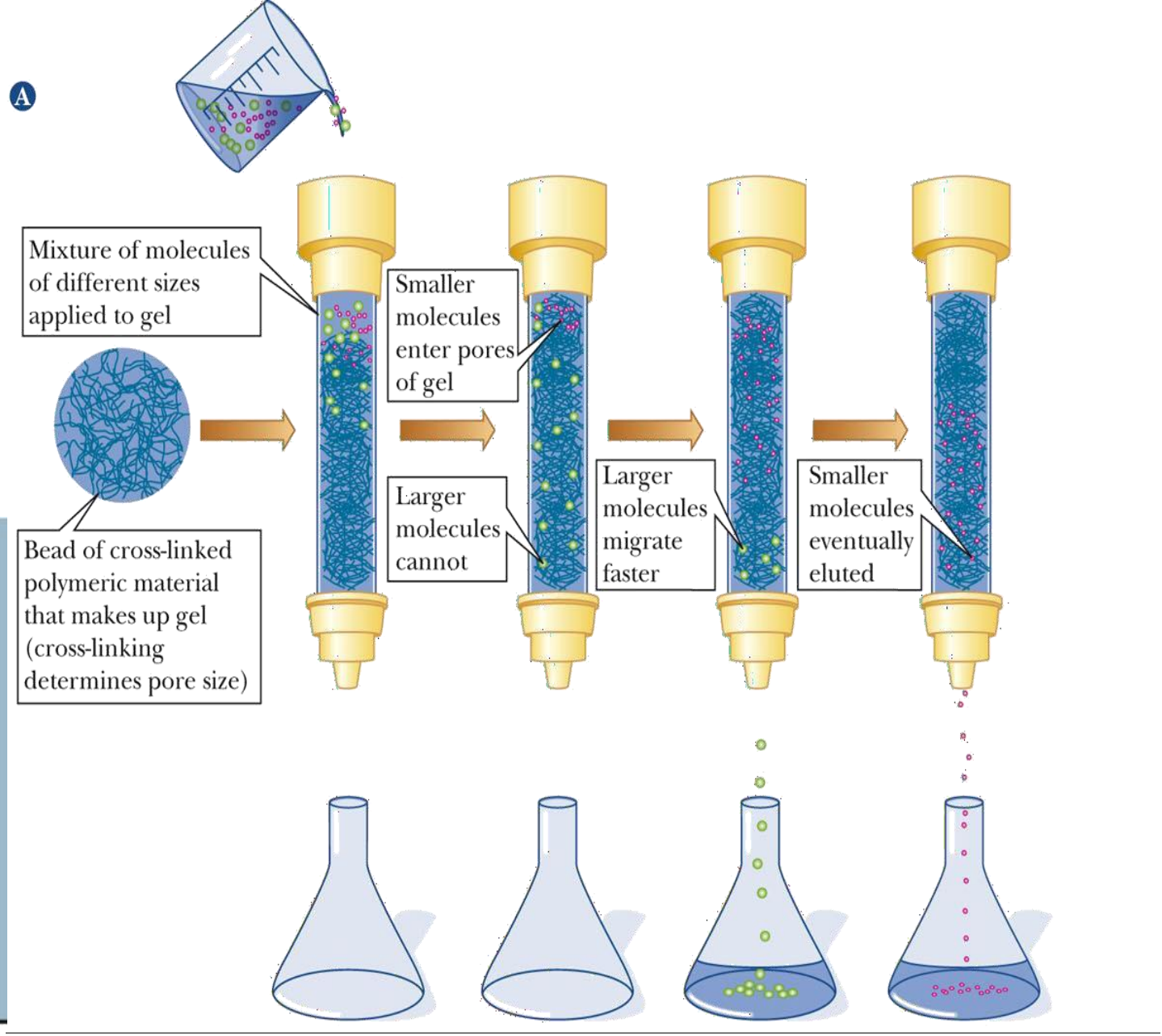
- **In size exclusion chromatography, how can I isolate my protein of interest?**

The separation depends on the size of the protein. When I load (pour) my sample onto the column, proteins pass through differently. To make my protein come out of the column, I keep adding more solution (buffer). Each protein leaves the column according to its size. In this way, proteins are separated physically, and I obtain them by washing the column with buffer.



Molecular-sieve chromatography

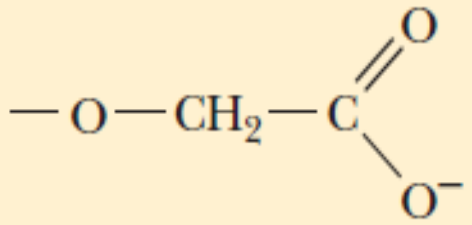




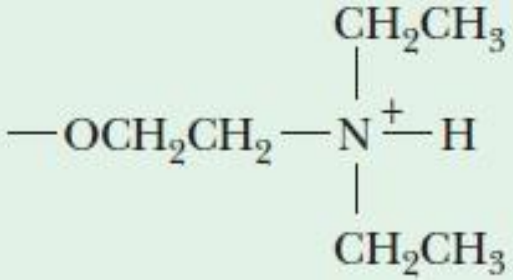
Ion-exchange chromatography

- Interaction based on net charge & is less specific
- Resin is either negatively charged (**cation exchanger) or** positively charged (**anion exchanger)**
- Buffer equilibration, exchange resin is bound to counter-ions. A cation-exchange resin is usually bound to Na+ or K+ ions, and an anion exchanger is usually bound to Cl^- ions
- Proteins mixture loading
- Elution (higher salt concentration)

Weakly acidic: carboxymethyl (CM) cellulose



Weakly basic: diethylaminoethyl (DEAE) cellulose



As the name implies.. Involves the concept of **CHARGE**

Ion-exchange chromatography

In ion-exchange chromatography, how can I release (exclude) my protein of interest?

One of the simplest and cheapest ways is by using salt. For example, if I have a negatively charged protein, it will bind to DEAE, which is a positively charged molecule present in the stationary phase of the column. Since the protein and DEAE are bound together, how can I separate them?

One method is to add a buffer with a different pH, but this carries a risk: proteins may denature under altered pH conditions. The easier and safer method is to add salt. By gradually increasing the salt concentration, the salt ions start to compete with the protein.

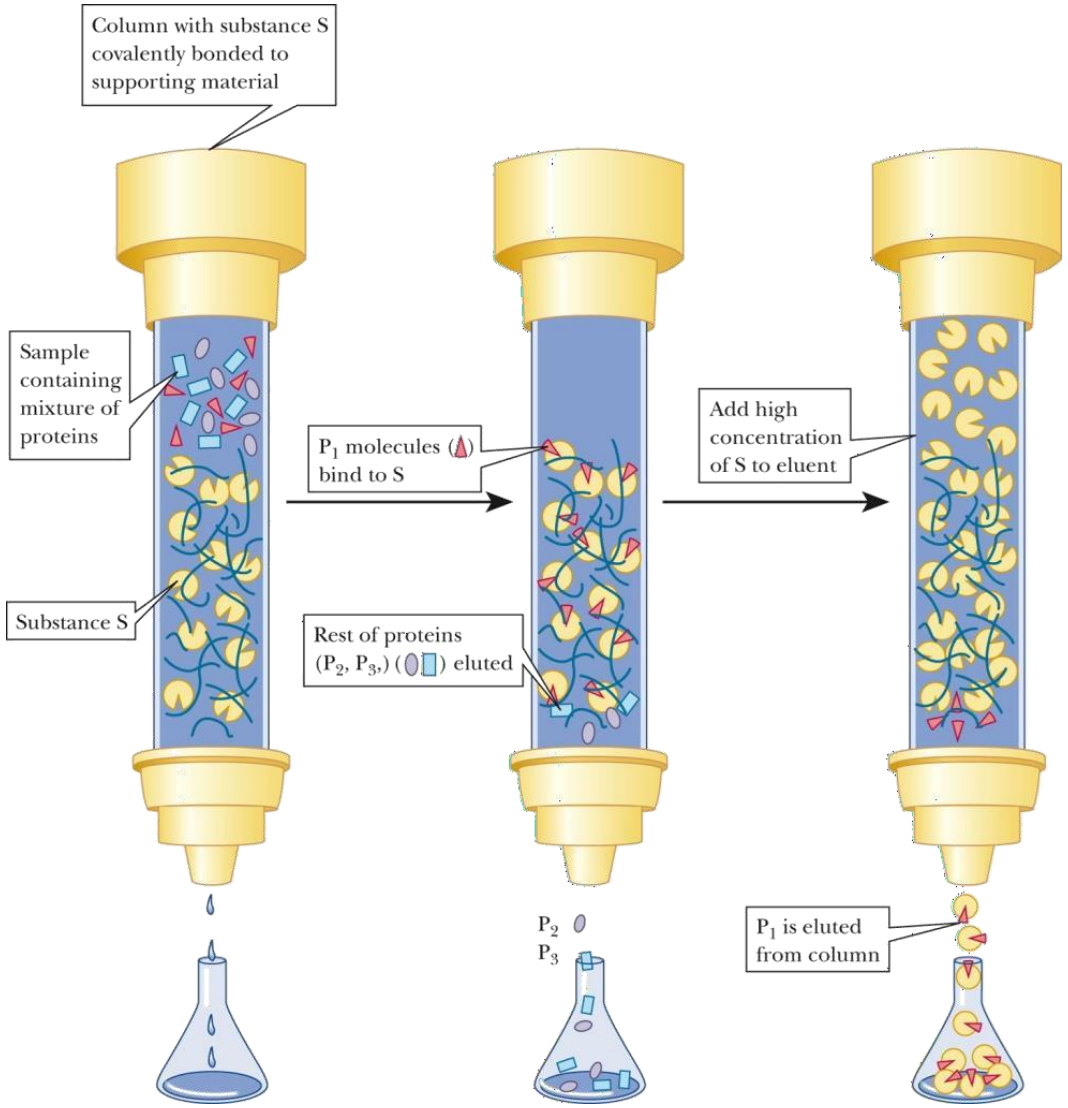
Why does this work? Salt is more soluble in water than proteins, and it contains both negative and positive charges. Because of its higher concentration and smaller size, salt binds more effectively to the charged groups on the column than the protein does. As a result, the protein is displaced and eluted from the column.

Problem

- You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pIs).
  - 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?

Affinity chromatography

- It has specific binding properties
- The polymer (stationary) is covalently linked to a *ligand* that binds specifically to the desired protein
- The bound protein can be eluted by adding high conc. of the soluble ligand
- Protein–ligand interaction can also be disrupted with a change in pH or ionic strength
- Convenient & products are very pure (Antigen-antibody, His-tag, GST-Tag)



Affinity chromatography

This technique is considered the gold standard because it provides the purest results. However, its main limitation is that **it is very expensive**.

As the name suggests, affinity chromatography is based on the principle of affinity. The stationary phase must have a very high affinity for the target molecule. For example, the stationary phase may contain an antibody designed to capture an antigen, or the opposite situation: an antigen in the stationary phase to purify an antibody. In general, **any two molecules with strong binding affinity can be used.**

This can also be done artificially by introducing tags to the protein of interest, such as a His-tag or GST-tag. For example, nickel is known to have very high affinity toward histidine. To use this, the protein or enzyme of interest is expressed in bacteria. The bacterial system is genetically engineered to add codons for several histidine residues (e.g., 6 or 8) at the end of the gene, forming a "tail" on the protein. This results in a 6 His-tag (if 6 histidine codons are added) or an 8 His-tag (if 8 codons are added). **A His-tag**: is simply a histidine sequence attached to the protein.

When the protein is synthesized, it now has this histidine tail. Since histidine has very high affinity for nickel (the stationary phase), only the tagged protein binds to the column, while everything else is washed away. **The strong binding is due to the imidazole ring in histidine, which specifically interacts with nickel**. (The rest of histidine's structure is just the common amino acid backbone.)

Affinity chromatography

Now, how do we elute the protein of interest from the column when the binding is so strong??

❌ Wrong approach #1: Adding a molecule with higher affinity.

This is incorrect because if such a molecule existed, we would have used it from the start, as it would give even better purification.

❌ Wrong approach #2: Changing the pH.

This is also wrong in this case, because the affinity here is not pH-dependent. (This method may apply only if the affinity interaction itself were based on pH.)

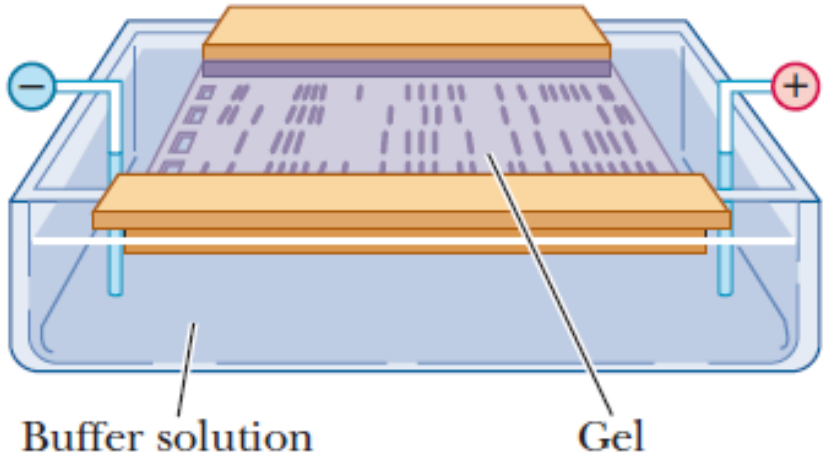
✅ Correct approach: Add the same molecule involved in the interaction, but at a much higher concentration.

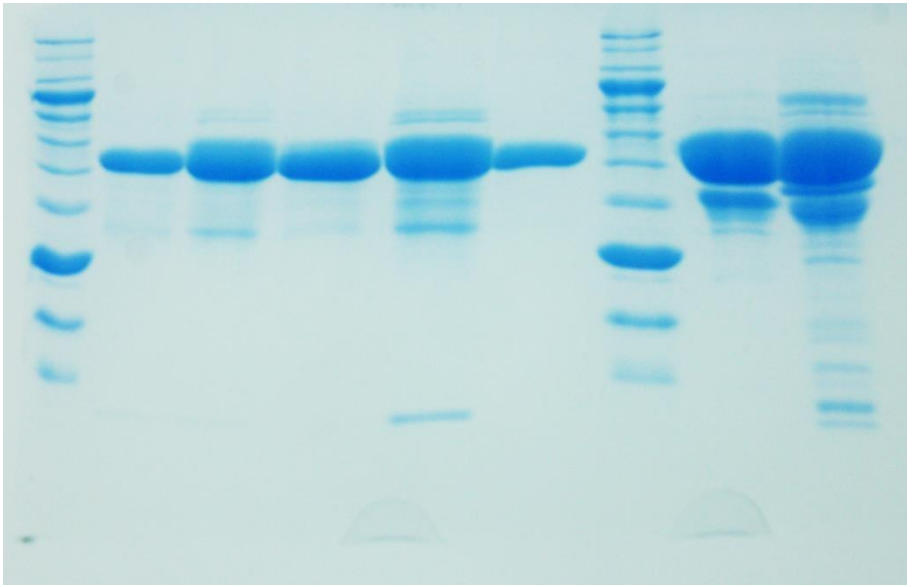
For example, the imidazole ring (responsible for binding) is commercially available. By adding a high concentration of free imidazole in solution, it competes with the histidine residues for binding to nickel. Because it is present in high concentration, it displaces the protein from the stationary phase, releasing the protein of interest.

The same principle applies in other affinity systems, such as antigen-antibody interactions.

Electrophoresis

- Based on the motion of charged particles in an electric field
- Macromolecules have differing mobilities based on their charge, shape, and size
- The most common medium is a polymer of agarose or acrylamide





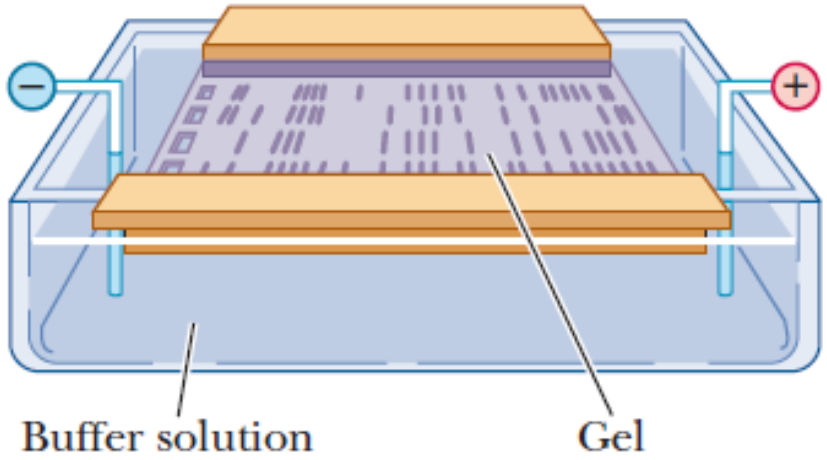
Electrophoresis

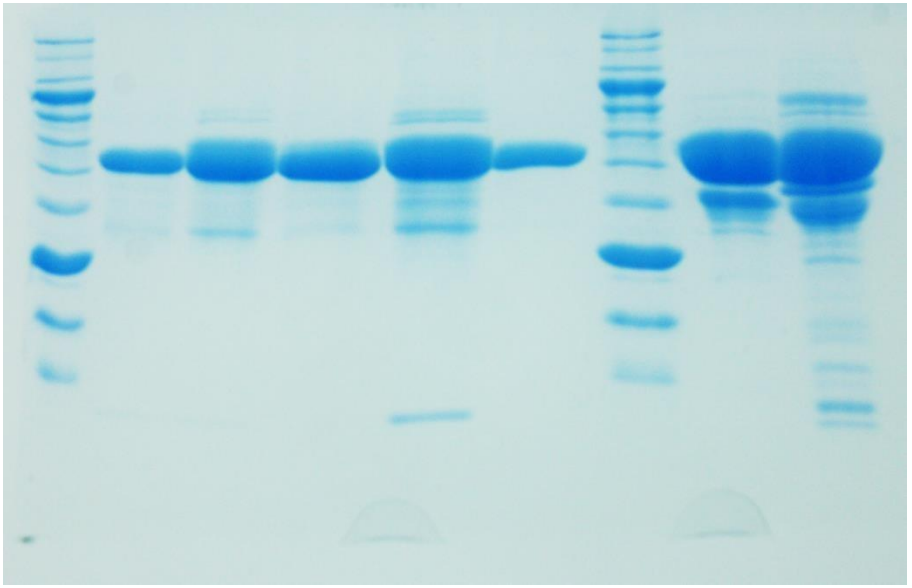
Electrophoresis is divided into two types depending on the gel used:

1. Agarose gel electrophoresis
2. Polyacrylamide gel electrophoresis (PAGE)

In general, agarose is usually used for DNA, while polyacrylamide is usually used for proteins, because polyacrylamide provides greater resistance. Technically, both gels can be used for either, but these are the most common applications.

Proteins move through the pores of gels depending on: **their charge, size, and shape.**





Electrophoresis

1. Native Gel Electrophoresis:

•In this method, proteins are placed in the gel in their native state.

•Here, movement depends on three variables simultaneously: **charge, size, and shape**.

•Because of this, it is difficult to obtain specific information about the protein (you cannot clearly determine its size, shape, or charge).

•Thus, native electrophoresis has limited applications, although it can be useful in some specific cases.

Electrophoresis

2. Non-Native (Denaturing) Gel Electrophoresis:

•To overcome the limitations of native electrophoresis, proteins can be denatured into their primary sequence.
•The most commonly used method for protein electrophoresis is **SDS-PAGE**.

•SDS (Sodium dodecyl sulfate):
•A hydrophobic molecule with a negative charge.
•Binds to proteins in a relatively constant ratio:
•Example: 10 amino acids → ~5 SDS, 100 amino acids → ~50 SDS, 200 amino acids → ~100 SDS.

•This nearly constant SDS-to-amino acid ratio eliminates differences in charge and shape, leaving only protein length (molecular weight) as the factor influencing movement.

•Therefore, in SDS-PAGE:
•All proteins are negatively charged.
•Movement occurs only according to molecular weight.
•Smaller proteins move faster through the gel toward the positive electrode, while larger proteins move more slowly. (Because smaller proteins pass more easily through the gel pores)

Electrophoresis

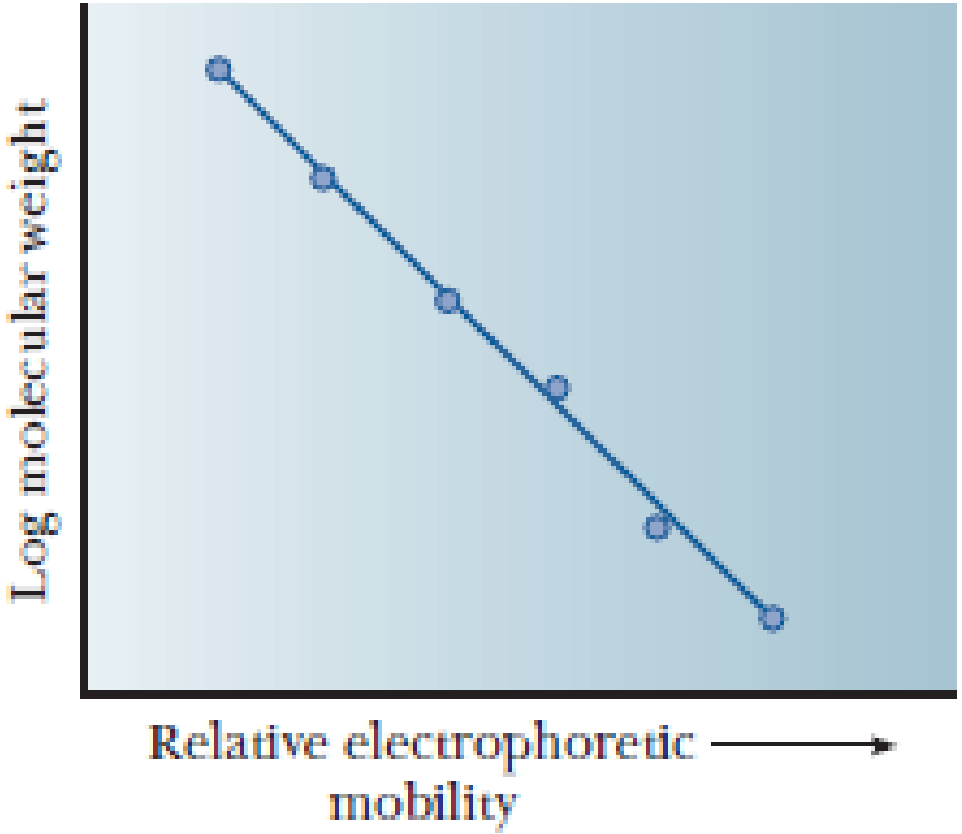
3. Reducing vs. Non-Reducing SDS-PAGE:

- SDS cannot disrupt disulfide bonds.
- To break these bonds, a reducing agent is added.
- For this reason, denaturing gel electrophoresis is classified into:

1. Reducing SDS-PAGE (with reducing agent).
2. Non-reducing SDS-PAGE (without reducing agent).

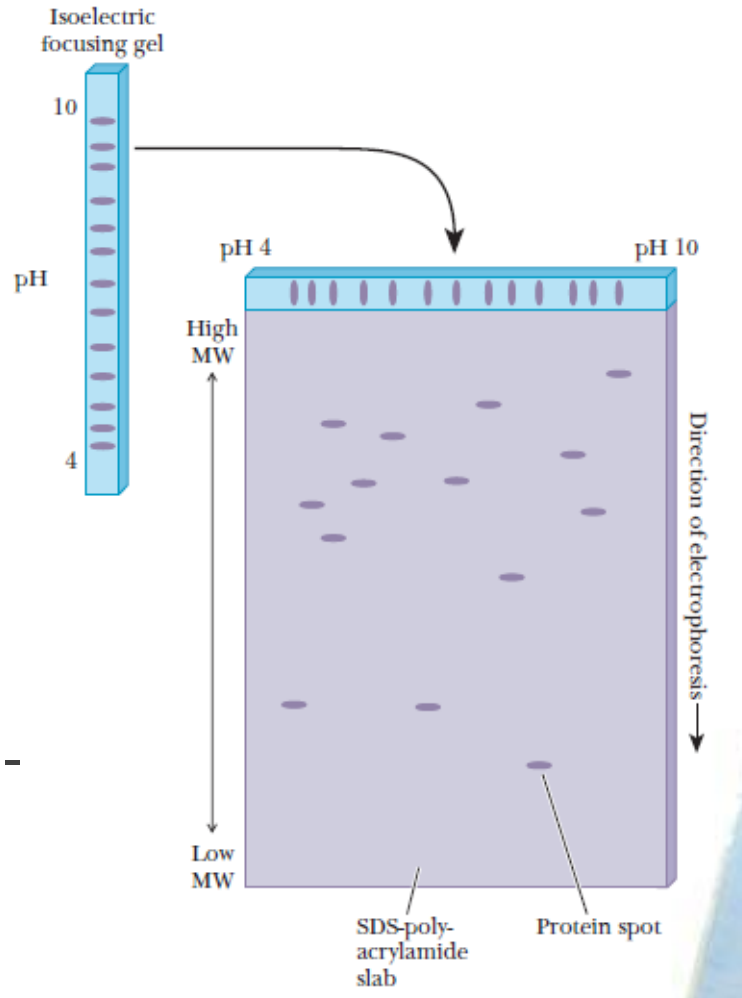
Agarose or PAGE?

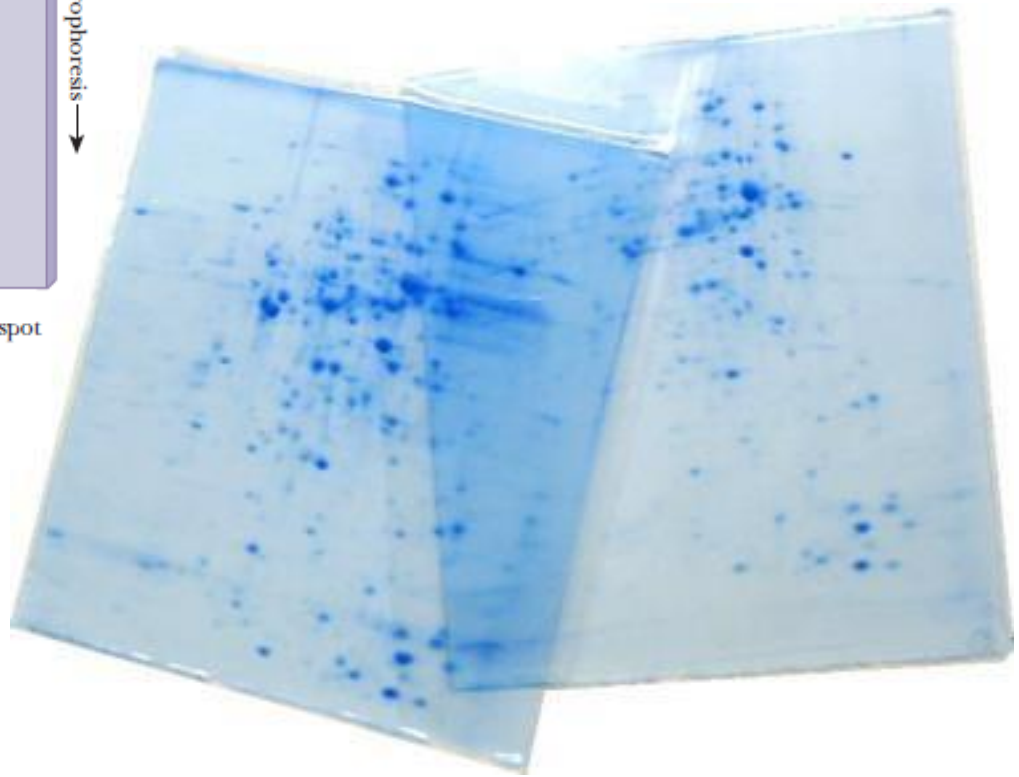
- Agarose (nucleic acids), PAGE (proteins)
- In PAGE: SDS or NO-SDS, detergent, $CH_3(CH_2)_{10}CH_2OSO_3Na^+$
- SDS completely denatures proteins (multi-subunit proteins)
- Acrylamide offers higher resistance to large molecules
- Shape and charge are approximately the same (sizes is the determining factor)
- Acrylamide without the SDS (**native gel):** study proteins in their native conformation (mobility is not an indication of size)



Isoelectric focusing

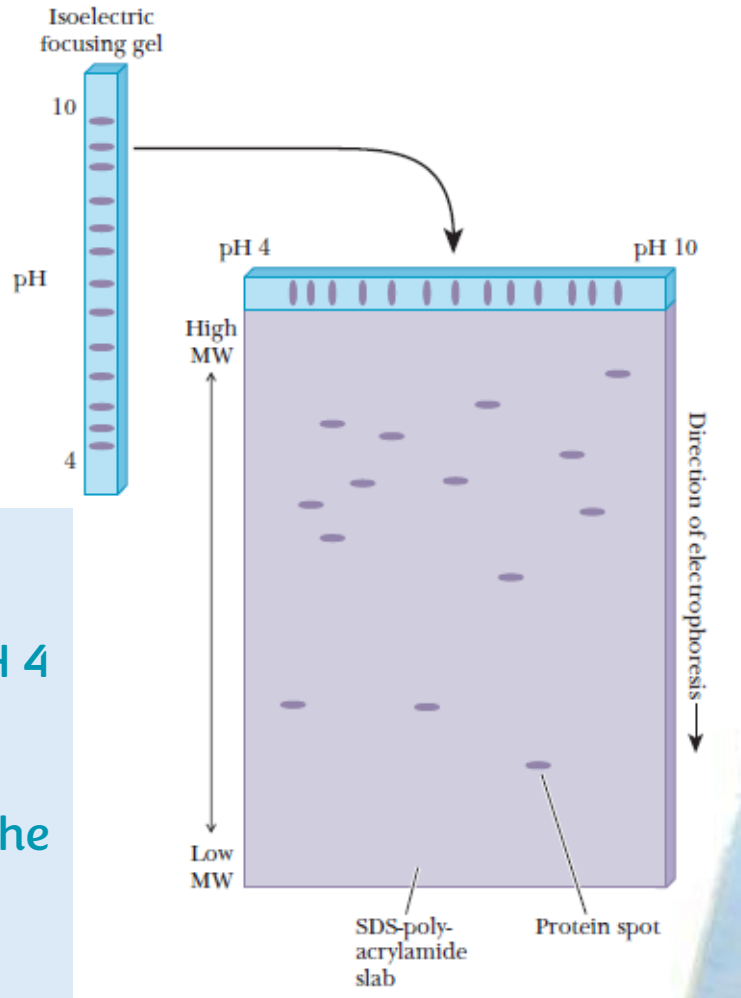
- Proteins have different isoelectric points
- Gel prepared with a pH gradient parallel to electric-field gradient
- Two-dimensional gel electrophoresis (2-D gels)

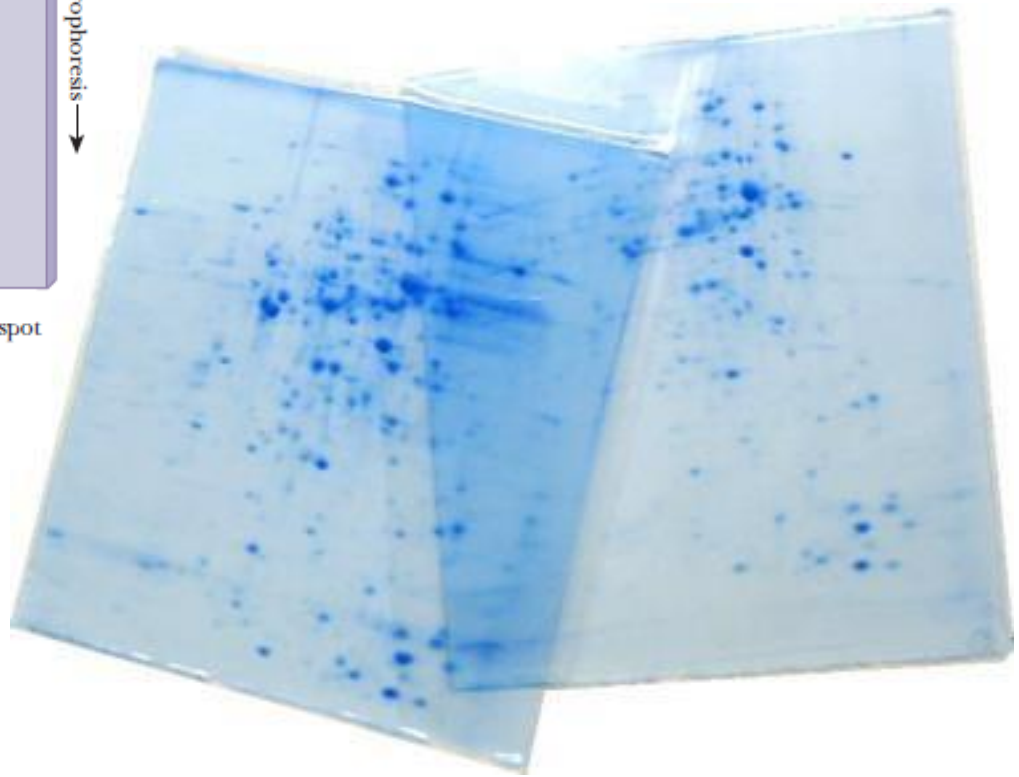




Isoelectric focusing

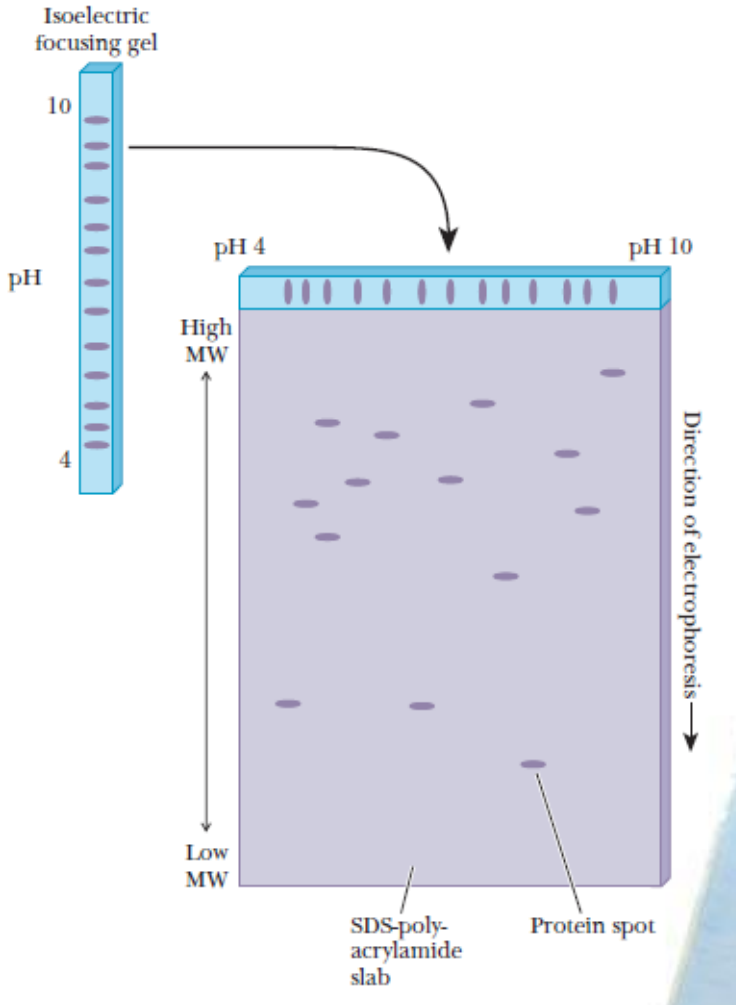
- This technique uses a gel with a pH gradient.
- Example: the gel can range from pH 4 at one end to pH 10 at the other (or even from 1–14 if desired).
- The protein of interest is placed in the gel.
- The protein moves according to the pH gradient and its net charge.
- It continues migrating until it reaches the point where its net charge = 0.
- This specific pH value is called the isoelectric point (pI) of the protein.





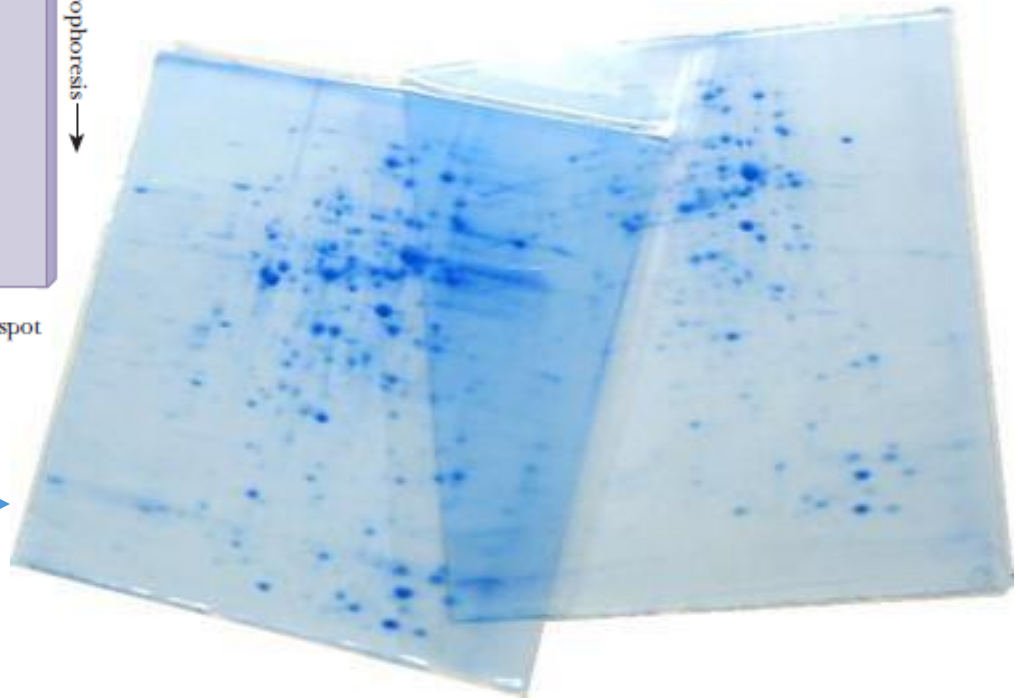
Isoelectric focusing

Isoelectric Focusing Gel Electrophoresis (IEF)
•Proteins move in the gel under the influence of their charge.
•A protein stops moving when it loses its net charge (reaches its isoelectric point, pI).
•In a sample with many proteins, each protein migrates until it reaches its own pI and then stops.
•After IEF, SDS is added to each protein band.
•This denatures the proteins.
•Shape and charge no longer affect migration.



Two-Dimensional (2-D) Gel Electrophoresis
•The gel is placed in an electric field.
•Proteins first move according to their isoelectric point (charge) (from IEF).
•Then, the same gel is run in a regular SDS-PAGE to separate proteins according to molecular weight.
•This method is called two-dimensional gel electrophoresis because proteins are separated in two dimensions:
•Charge (isoelectric point)
•Size (molecular weight)

This is how the real gel of 2-D gel electrophoresis looks →



Questions

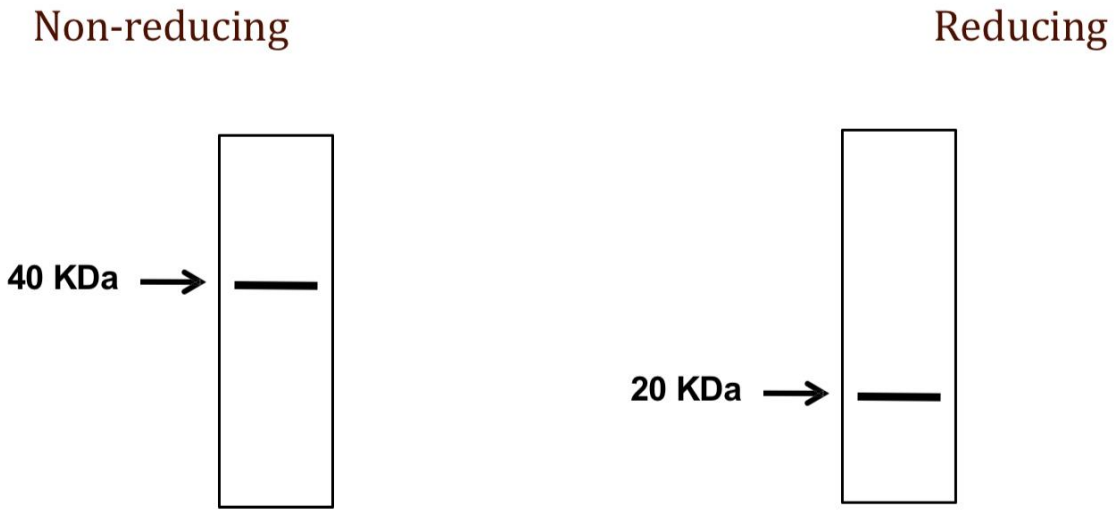
- Describe the protein's structure based on the following results of SDS-PAGE:

1. Under non-reducing condition, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands.

2. Under non- reducing condition, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein also exists as two bands, 15 KDa and 10 KDa.

3. Under non- reducing condition, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as one bands of 20 KDa.

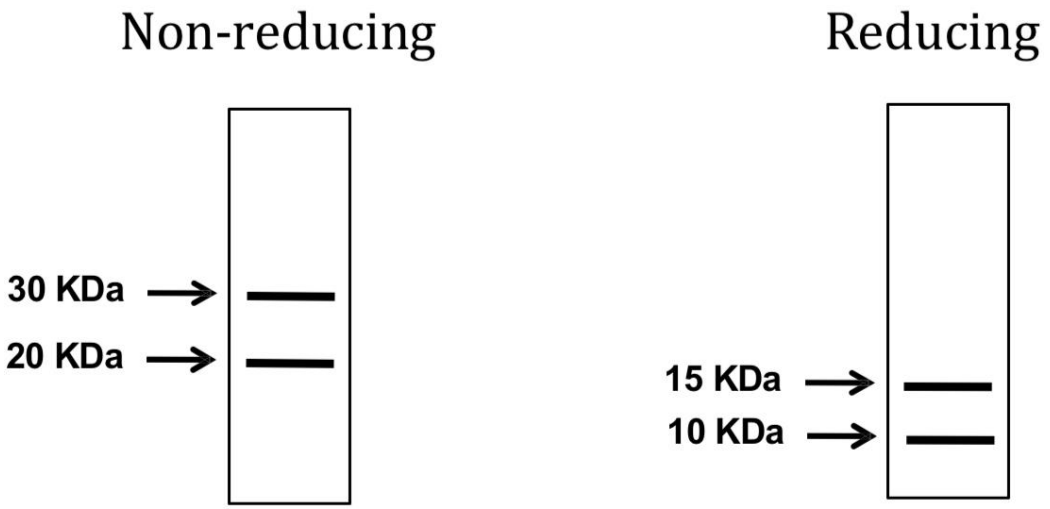
Under non-reducing condition, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands.



Because the two polypeptide chains are identical, they co-migrate in SDS-PAGE after reduction, appearing as a single 20 kDa band.

- From these results, we can conclude the following:
- The protein contains disulfide bonds, because under reducing conditions its molecular weight decreased.
- The protein consists of two polypeptide chains.
- Under non-reducing conditions, it appears as a 40 kDa band.
- After breaking disulfide bonds with a reducing agent, it separates into two 20 kDa bands.
- If it had been a single polypeptide chain, its molecular weight would have remained 40 kDa even after reduction.
- Therefore, the protein is made of two identical polypeptide chains linked by disulfide bonds.
- This structure defines it as a **homodimer**, which is a type of quaternary structure protein.

Under non- reducing condition, a protein exists as two bands, 30 KDa and 20 KDa.Under reducing conditions, the protein also exists as two bands, 15 KDa and 10 Kda.



From these results, we can conclude the following:

•The protein is a **heterotetramer**.

WHY??

•Under reducing conditions, there are two bands (15 kDa and 10 kDa), indicating two different types of polypeptide chains.

•Each band represents a homodimer (two identical chains), based on the same principle discussed previously.

Homodimer + Homodimer = Heterotetramer

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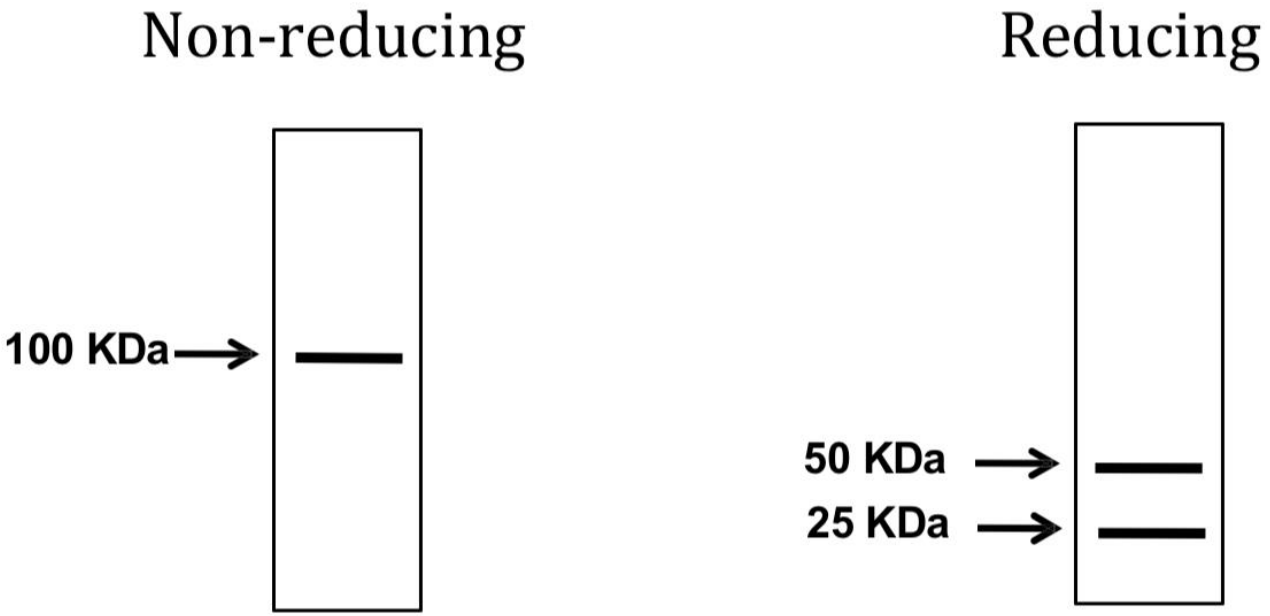
Non-covalent interactions

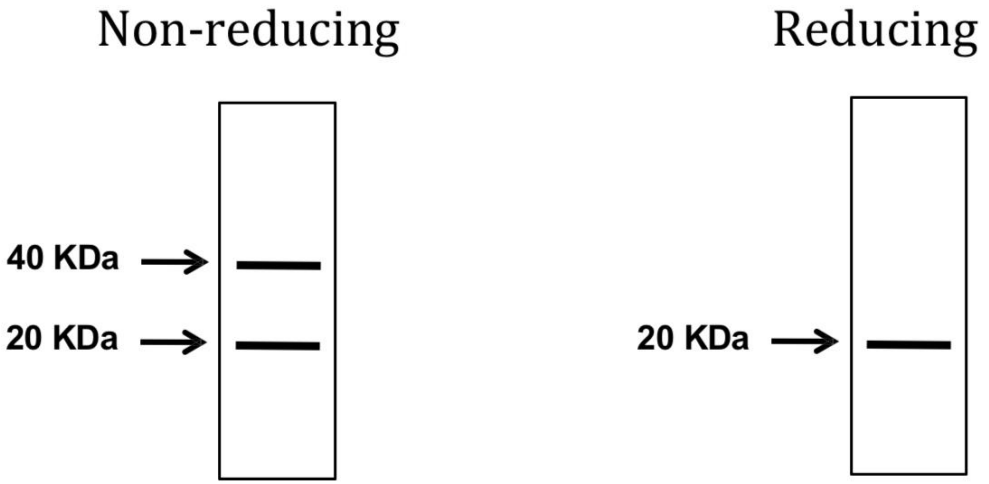
Under non- reducing condition, a protein exists as one band of 100 KDa.Under reducing conditions, the protein also exists as two bands, 50 KDa and 25 Kda.



- The protein is a **heterotrimer**.
- It is composed of 3 polypeptide chains: 2 identical chains and 1 different chain.
- All chains are connected by disulfide bonds.
- Under non-reducing conditions, denaturation without reduction produces only one band, because the disulfide bonds keep the chains linked together.

- After reduction, the disulfide bonds are broken, and the chains separate into two bands (15 kDa and 10 kDa), confirming the heterotrimeric structure.

Under non- reducing condition, a protein exists as two bands, 40 KDa and 20 KDa.Under reducing conditions, the protein exists as one bands of 20 KDa.



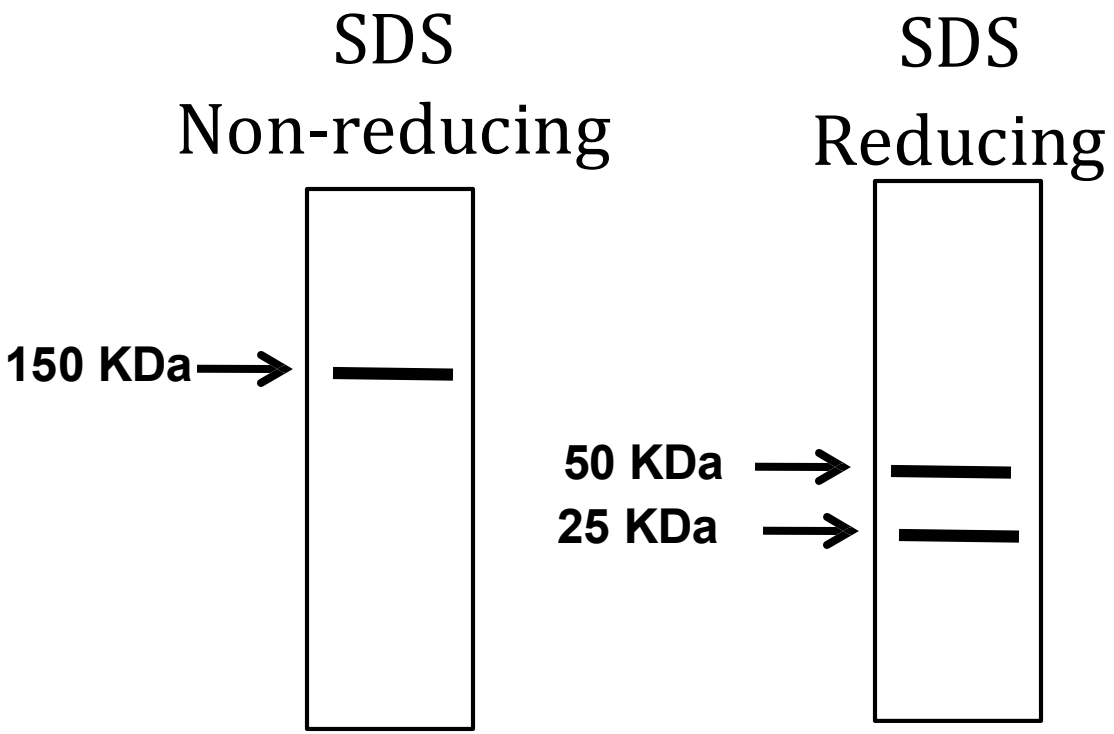
- The protein is a **homotrimer**.
- It consists of 3 identical polypeptide chains, each 20 kDa.
- 2 of the chains are linked by disulfide bonds forming a dimer, while the 3rd chain is a monomer.
- Under non-reducing conditions, the protein appears as 2 bands (40 kDa and 20 kDa).
- After reduction, the disulfide bonds are broken, and all chains separate into 1 band of 20 kDa, confirming the homotrimeric structure.

For more understanding: Since mass is neither created nor destroyed but only transformed from one form to another, we initially had a total of 60 kDa. After reduction, we obtained 3 chains, each 20 kDa.

- Initially, there was a single 20 kDa chain, representing a monomer.
- The 40 kDa band, after reduction and cleavage of the disulfide bond, split into 2 equal chains, indicating it originally represented a homodimer.

As a result, we have three identical polypeptide chains, so the protein is a homotrimer.

Under non- reducing condition, a protein exists as one band, 150 KDa.Under reducing conditions, the protein exists as two bands of 50 Kda and 25 Kda.



Homodimer + Homodimer= Heterotetramer
2(50) + 2(25) =150 KDa

For any feedback, scan the code or click on it.

Corrections from previous versions:

Versions	Slide # and Place of Error	Before Correction	After Correction
V0 → V1			
V1 → V2			

آخر رسالة من الفريق العلمي للسنة الأولى :

الحمد لك يا ذا الجود

ها نحن -بفضل الله- نطوي صفحة السنة الأولى في كلّيّة الطبّ

مُباركٌ لنا جميعًا اجتيازُ سُدسِ الطّريق.. كان فيه الله معنا يُيسّر لنا، يهدينا ويقوّينا

طريقٌ بُدِء بخوفٍ ورجاء صاحبَهُ يقينٌ بأنّ (**وَمَنْ يَتَوَكَّلْ عَلَى اللَّهِ فَهُوَ حَسْبُهُ**)

نحمد الله على سنةٍ مرّت بحُلوِها ومُرّها لنغلقَ اليومَ فصلًا من ستّةٍ أو يزيد

ما أجمل أن يُكتب في صحائفنا أنّنا صبرنا واحتسبنا، وأن كلّ خطوةٍ كانت في سبيل عبادة الله بالعلم، فلنشكر الله على فضله، ولنرفع أكفَّنا قائلين:

"اللّهمّ اجعل هذا العِلم حجّةً لنا، وزادًا لنا إلى جنّتك، ووسيلةً لنفع عبادك، واغفر لنا تقصيرَنا فيما مضى، وثبّت قلوبَنا لما هو آت"

دُمتُم في أمان الله وجميل رعايتِهِ دكتور 024

- **فريقُكُم العلمي**