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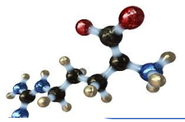

BioChemistry

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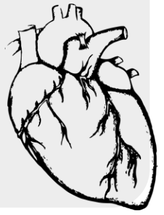
Final | Lecture 18

Protein Analysis

Written & Reviewed by :

Yaman Khalil

Yamen Aljarrah



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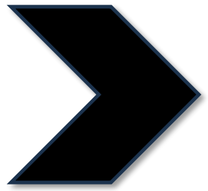
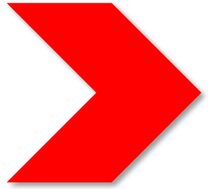


Black: the original slides

***Gold**: the doctor's explanation/words*

Gray: additional information and explanation



Red: important information

Protein analysis

Prof. Mamoun Ahram

PART I: PROTEIN PURIFICATION

Bases of protein separation

The most common technique of purification

- Proteins can be purified using chromatography techniques on the basis of:

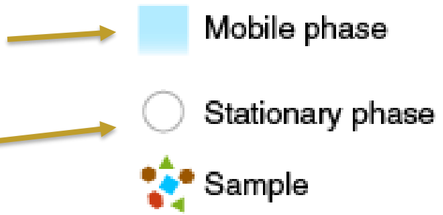
Size	Charge	Specific binding affinity	Hydrophobicity

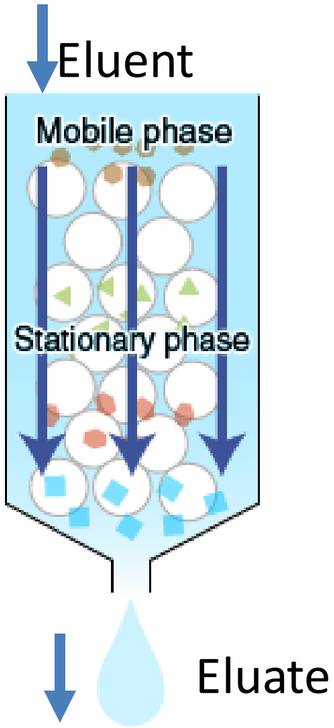
Isoelectric point

The pH when a protein (amino acid) is charged but has a net charge of 0 (zwitterion)

The moving solution (with gravity) containing the proteins

The beads





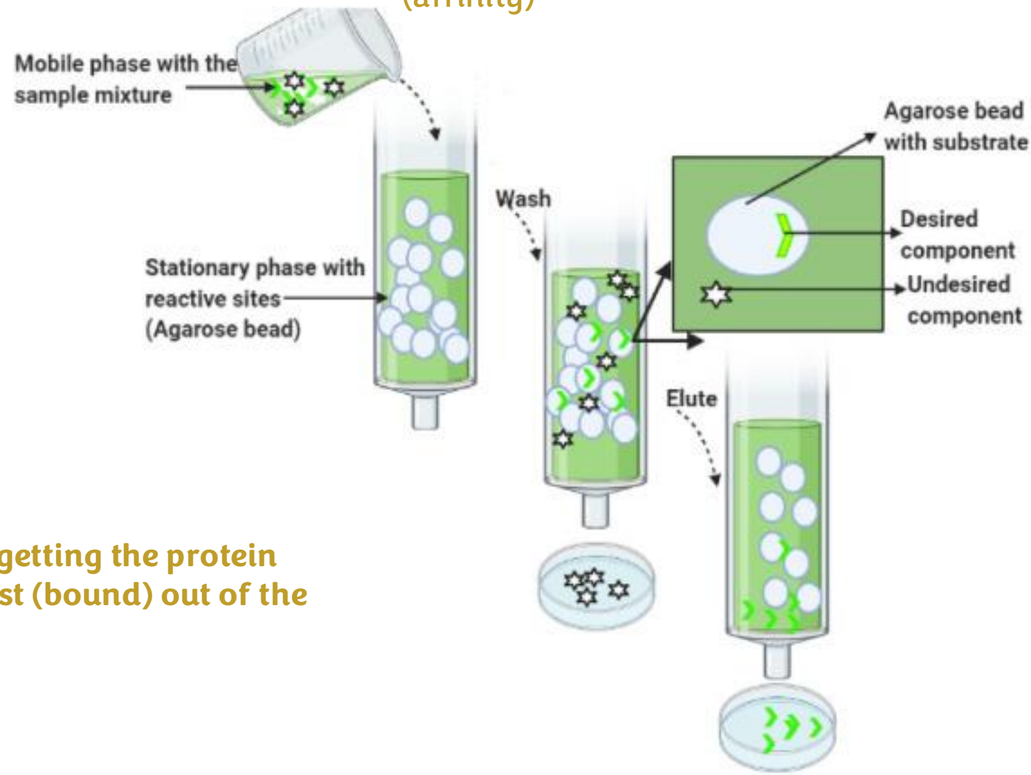
Basis of charomatography

The stationary phase is composed of beads, and those beads are the basis of the separation

For example, if the beads are negatively charged at pH=8, the positively charged proteins at that pH will bind to it and the negatively charged ones will pass (charge)

Or we put antibodies on the bead and some protein will bind to it (affinity)

- A chromatography column (tube) containing a stationary phase (usually beads with a specific or treated surface) is prepared.
- The sample containing mixed proteins, for example, is added.
- The proteins bind to the bead surface.
- **Unbound proteins are washed out of the column.**
- **Elution is carried out where an eluent (a solvent) is added to the column to elute (release) the proteins that are bound to the beads.**
- The eluate containing the proteins of interest is collected and analyzed further.



Elution: getting the protein of interest (bound) out of the column

We collect the proteins as fraction (for example each ml is a fraction) each fraction contain different proteins

Type of chromatography techniques

The last fraction contains the highest negative/ positive charge

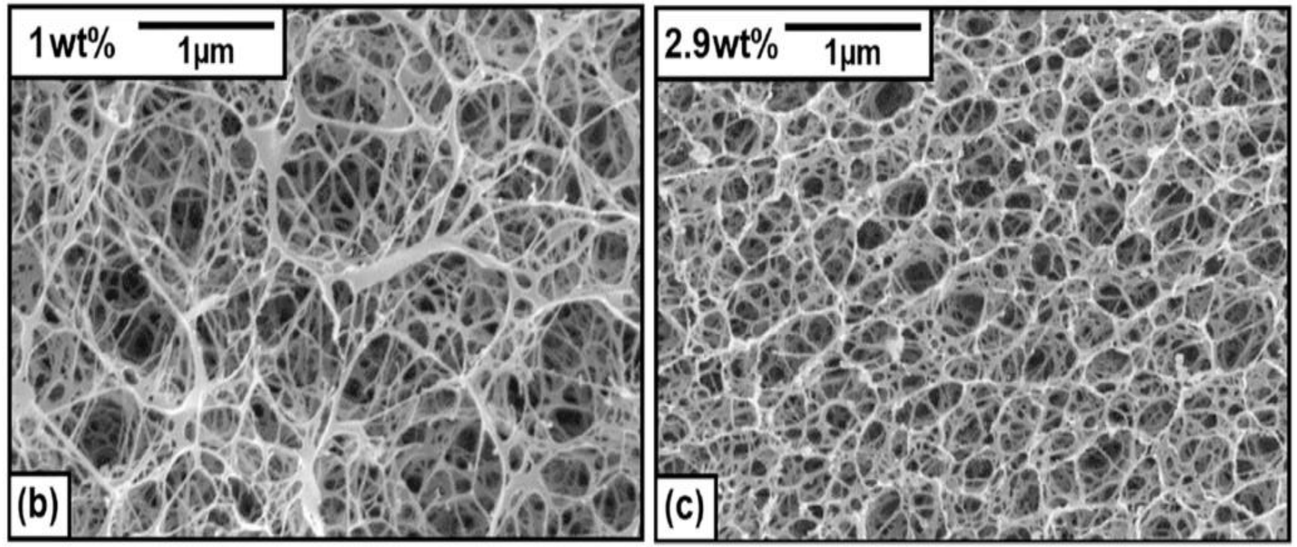
Type	Purpose
Size exclusion chromatography (AKA molecular sieve filtration chromatography/gel)	Separation of proteins based on size. The last fraction contains the smallest proteins. **Larger proteins come out first and smaller proteins later**
Ion-exchange chromatography	Separation of proteins based on isoelectric point
A. Anionic exchange chromatography. The protein of interest has low pI	A. Highly negatively charged proteins with lower PI come out later. Beads are positive
B. Cationic exchange chromatography. The protein of interest has high pI	B. Highly positively charged proteins with higher PI come out later. Beads are negative
Affinity chromatography. Usually using antibodies. Beads have antibodies on their surface	Separation of proteins based on their specific interaction. Bound proteins are eluted by **competitive binding of a free competitor** added to the eluent or **by reducing the non-covalent interactions**
Reverse phase chromatography. Beads are non-polar	Separation of proteins based on their **hydrophobicity**, where **non-polar proteins come out later**. The last fraction contains the most hydrophobic proteins

PART II: PROTEIN ANALYSIS

Gel electrophoresis

- A molecule (protein) with a net charge moves in an electric field
- This phenomenon, termed electrophoresis, offers a powerful means of separating proteins.
- In gel electrophoresis, proteins are separated as they move through a gel, which serves as a molecular sieve.

https://www.youtube.com/watch?v=i_6y6Z5UvwE



Gels differ in the size of pores depending on the type

- Smaller proteins moves faster through pores
- The order from anode to cathode (if proteins are similar in size):

High negative ---> low negative ---> low positive ---> high positive

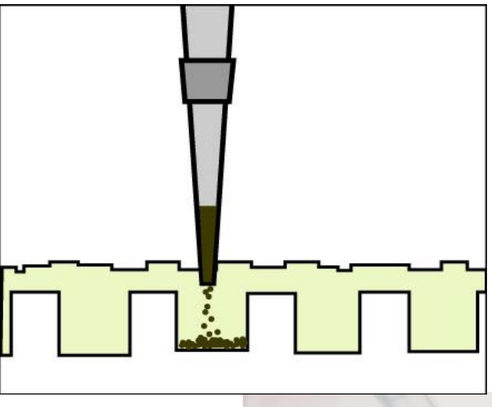
As a result, we can separate proteins based on size, charge, or both via gel electrophoresis

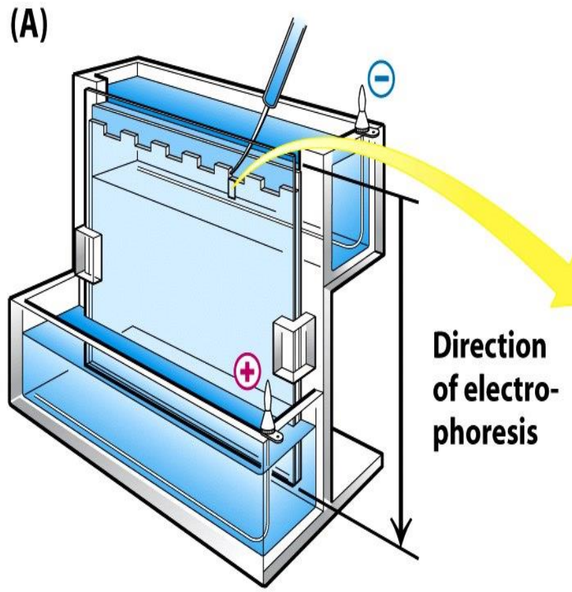
The process

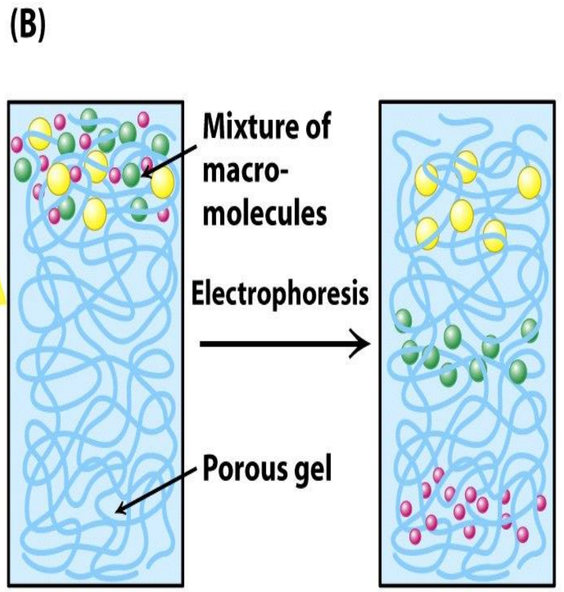
- The **most commonly used** protein electrophoresis technique is termed sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis **(SDS-PAGE)**.
- It is performed in a thin, vertical gel.
- The top of the gel consists of wells onto which samples are loaded.

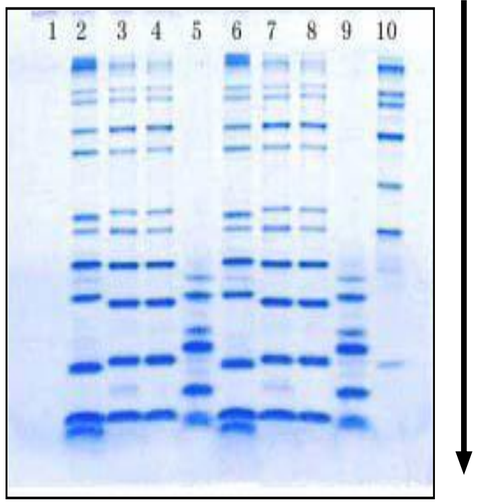
From the gel (after staining) we can know two pieces of info:
1- the size of the protein
(don't care about the charge see slide 12)

2- the amount (as much as the band is dark, it has much more amount)





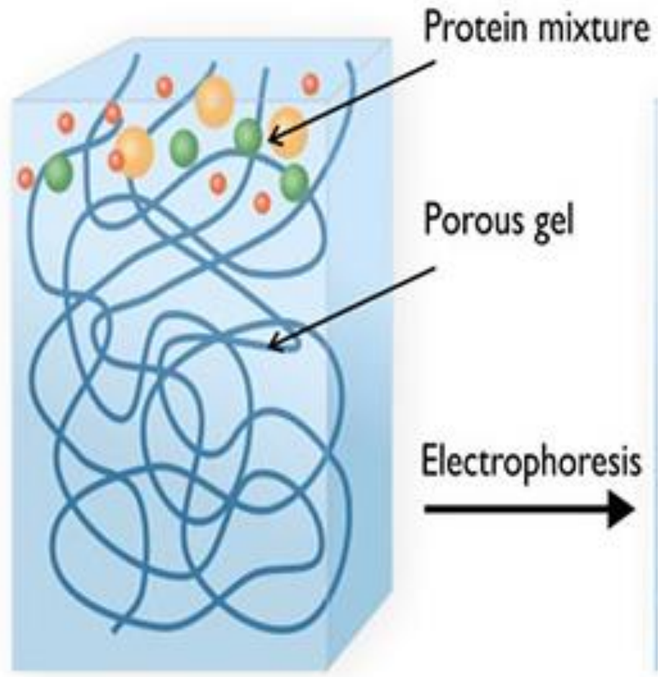


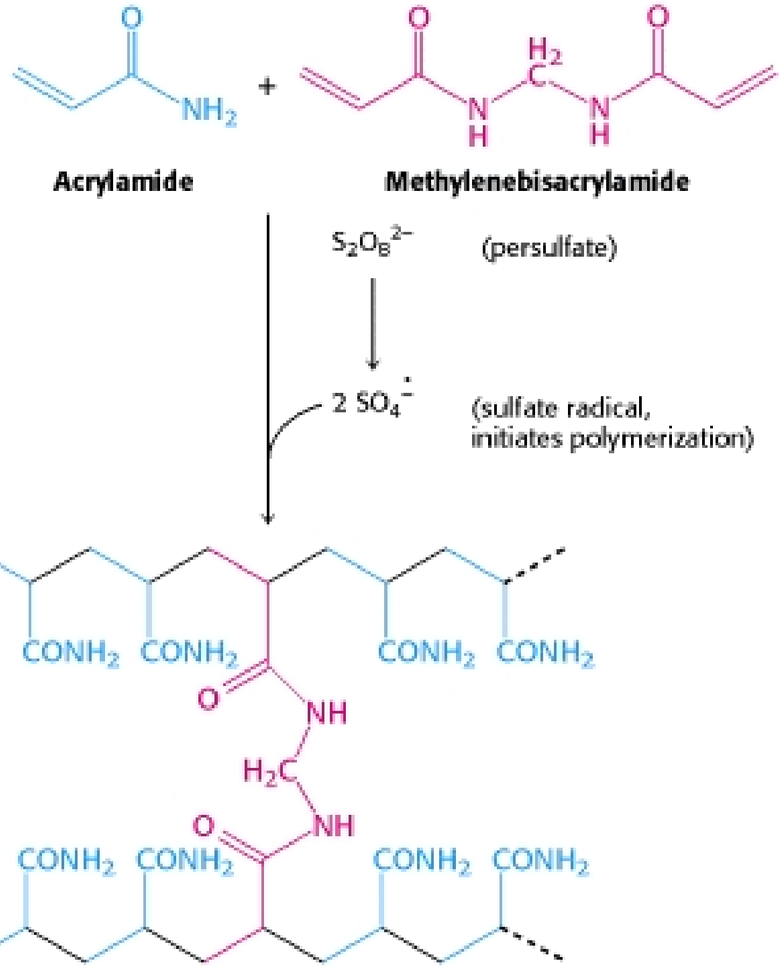


Formation of the gel

- The gel is made of a material known as **polyacrylamide**, which is formed by the **polymerization of acrylamide and cross-linked by methylene-bisacrylamide.**

Forming a network (because of that, there are pores)





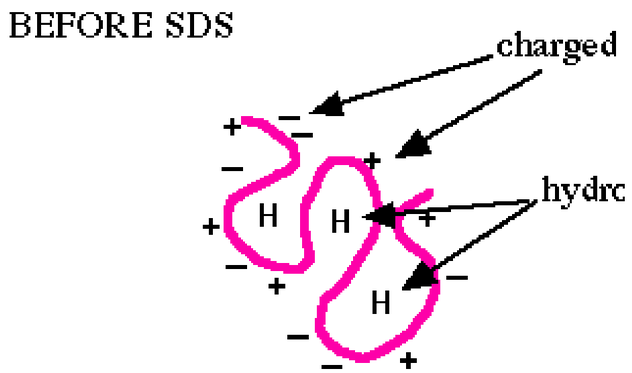
Purpose of SDS

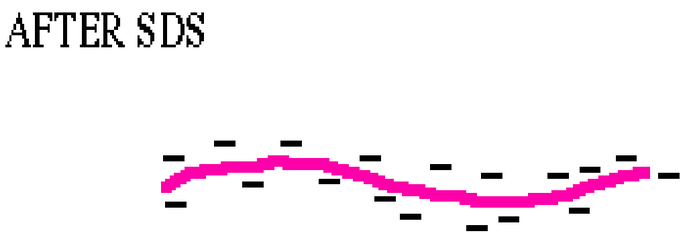
Sodium lauryl sulfate

- This technique utilizes a **negatively charged detergent** (sodium dodecyl sulfate) to **denature** and solubilize proteins (denaturing condition).
  - Otherwise, non-denaturing condition or native condition where proteins maintain their original structure and shape and are **separated based on charge, size, and shape.**
- SDS makes **proteins have a uniform negative charge.**

For that reason, all the proteins will move from the cathode to the anode

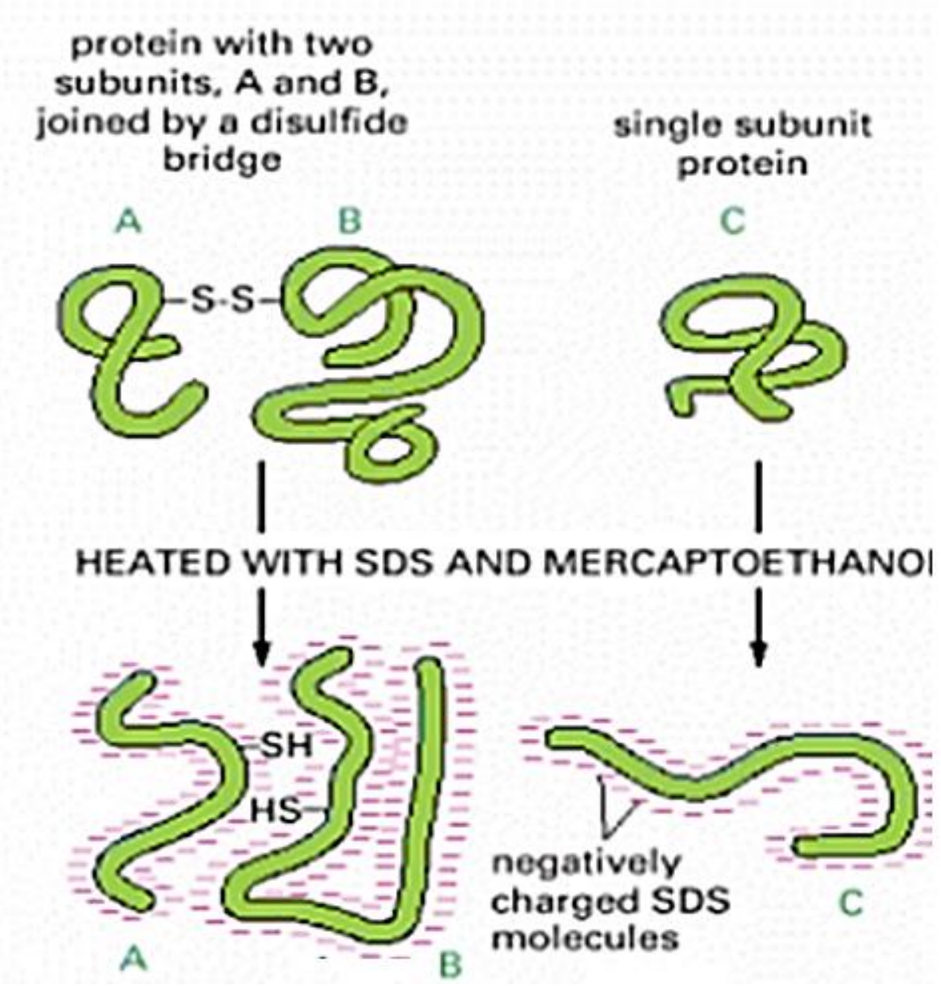
Important:
It binds uniformly, so all proteins have similar charge/mass ratio but different masses indeed





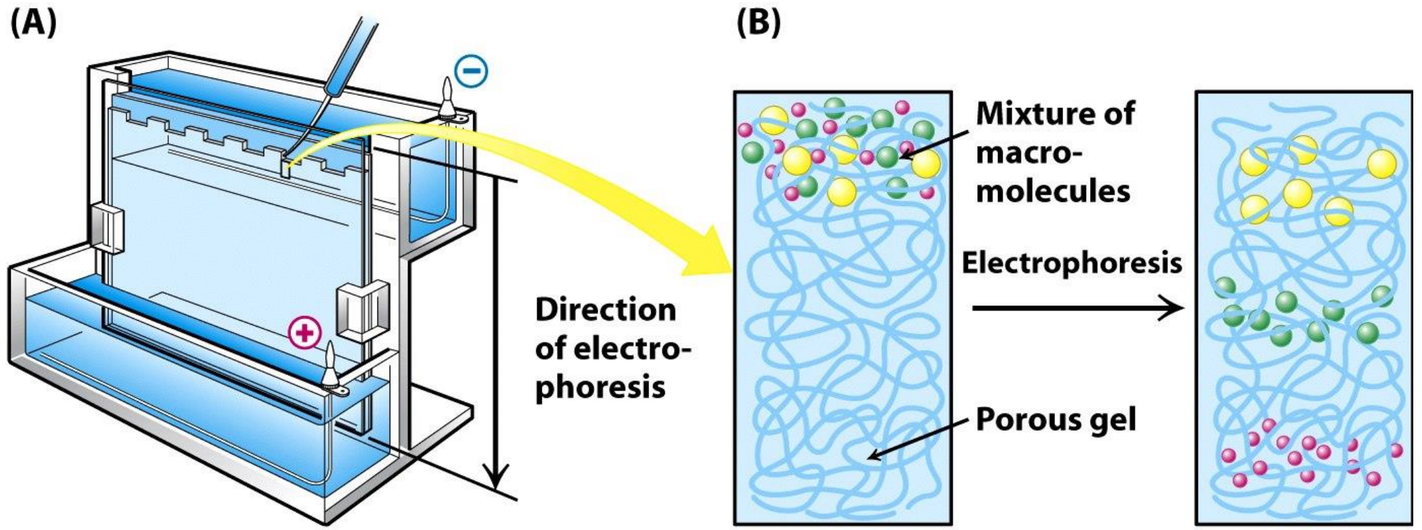
Purpose of reducing agents

- The mixture of proteins **is also treated with reducing agents** like **β-mercaptoethanol or dithiothreitol** to reduce disulfide bonds (reducing condition).
  - Otherwise, non-reducing condition



Migration of proteins

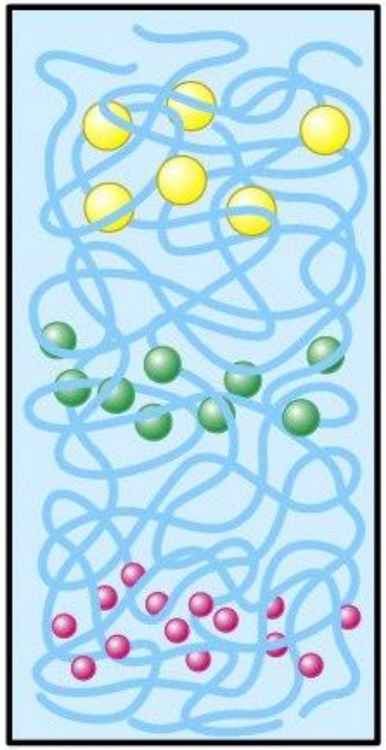
- When an electrical voltage is applied between the upper and lower ends of the gel, all proteins move in one direction towards the anode (positive) **according to size only.**
- The direction of movement is from **top to bottom**.
- Whereas smaller molecules move readily through the gel, larger molecules are slower.

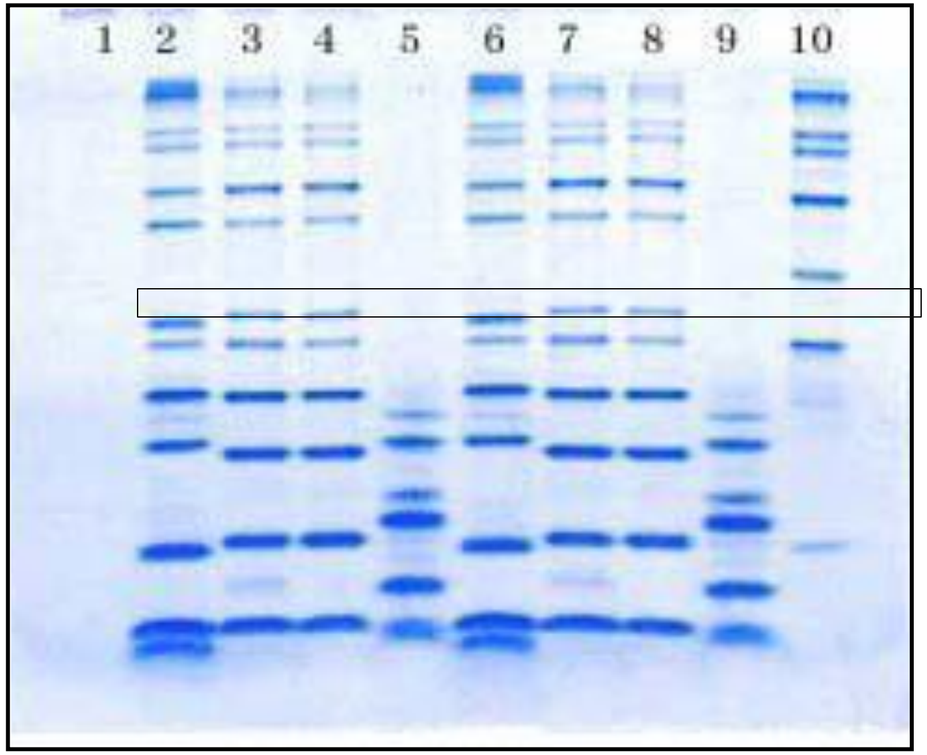


Then…

- Once a gel has been "run", the separated proteins are **stained** to reveal their positions and they appear as bands.

Each band contain a large number of proteins (millions or even more)





Cathode (largest are here)

Each horizontal line contain proteins with similar size (mass)

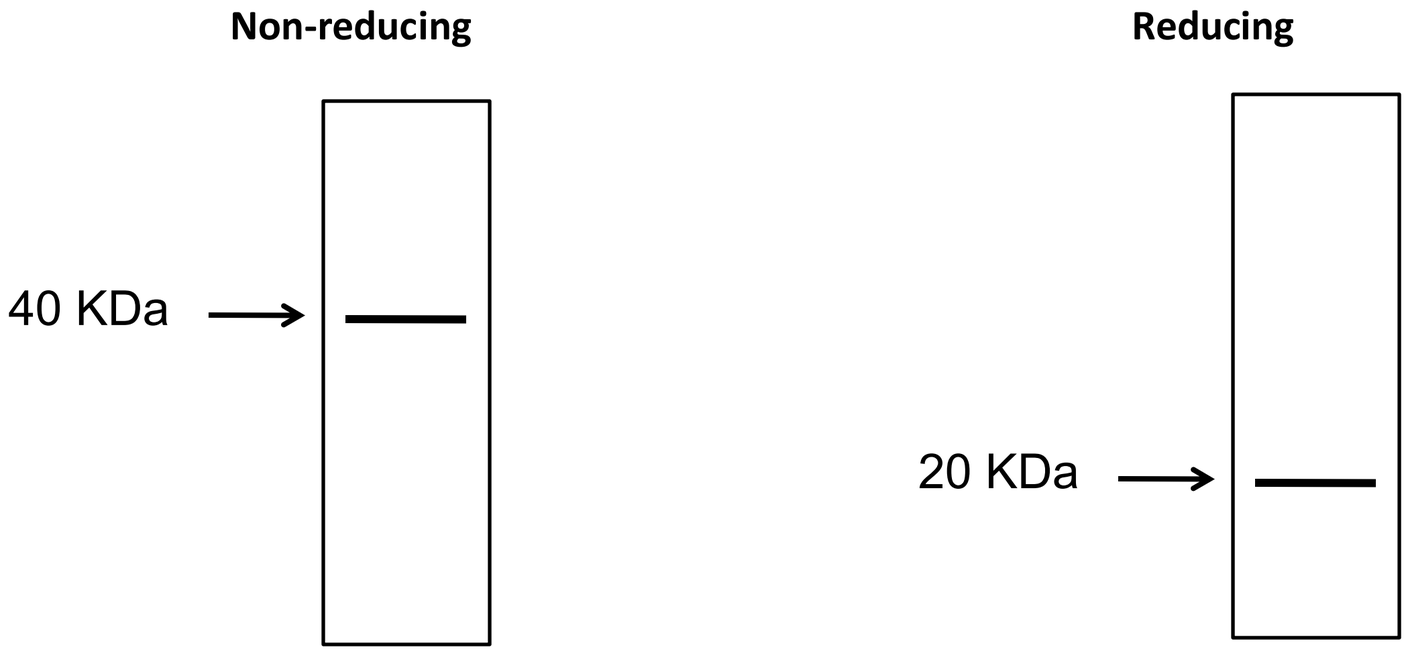
Anode (smallest are here)

Questions

- Describe the protein's structure based on the following results of SDS-PAGE:
  - Under non-reducing conditions, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as a one 20-KDa band.
  - Under non-reducing conditions, 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.
  - Under non-reducing conditions, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as a one band of 20 KDa.

When using affinity chromatography, you may purify a single protein (not one molecule but one protein).
However, other types gives you a range of something. For example, we use size exclusion chromatography and take the first fraction (1 ml for example), it gives us a range of size (mass) from 120KDa to 100KDA

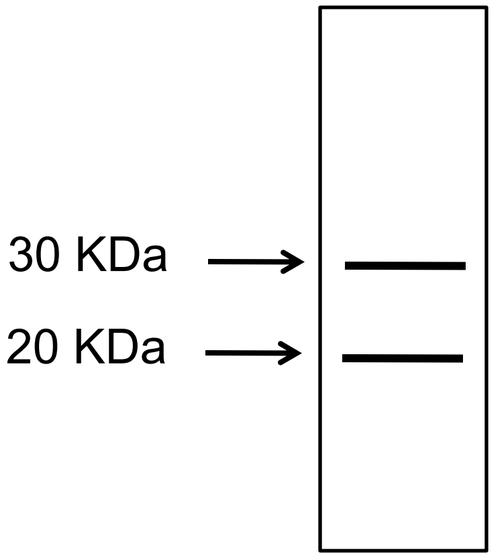
Under non-reducing, denaturing conditions, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as a one 20-KDa band.



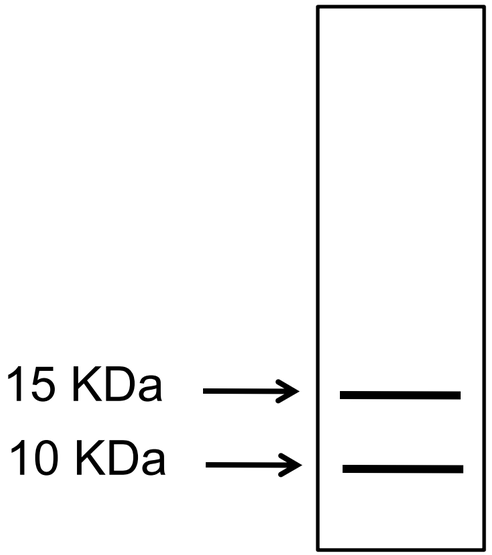
Ans: Homodimer (each subunit 20KDa and bound together via disulfide bonds)

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

Non-reducing



Reducing

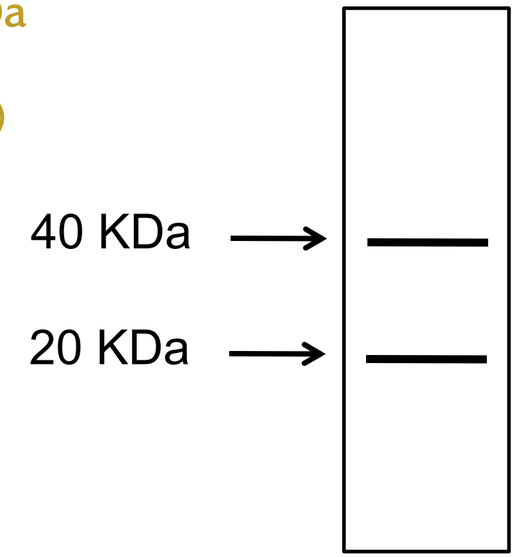


Ans: Heterotetramer (two subunits 10KDa each and bound together via disulfide bonds and other two 15KDa also bound together via disulfide bonds) but the two dimers are bound together via non-covalent bonds

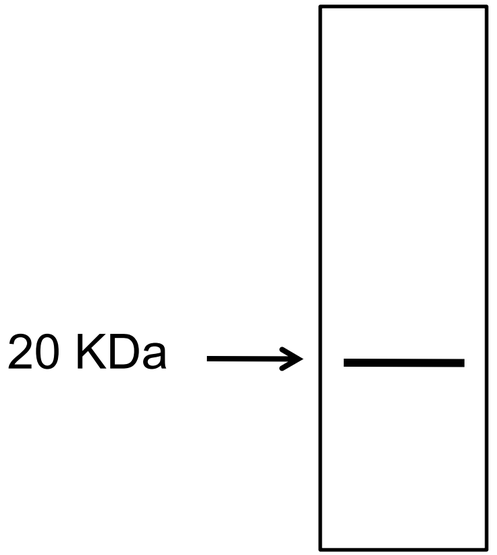
Under non-reducing, denaturing conditions, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as a one band of 20 KDa.

Ans: heterotrimer (two subunits 20KDA each are bound together via disulfide bonds and one 20KDa binds to them via non-covalent bonds)

Non-reducing



Reducing



Think!!

- How would the globular proteins that we studied appear in SDS-PAGE gels under reducing and non-reducing conditions?

Myoglobin (Monomer): Because it is a single polypeptide chain, it does not have a quaternary structure or inter-chain disulfide bonds. It will migrate based on its standard molecular weight and appear as a single band (around 17 kDa) under both reducing and non-reducing conditions.

Hemoglobin (Tetramer): Hemoglobin (2 alpha/2 beta) has a quaternary structure, but its four subunits are held together entirely by non-covalent interactions (hydrophobic, ionic, and hydrogen bonds). Therefore, the SDS detergent alone is strong enough to separate the tetramer into individual monomer subunits. Since there are no disulfide bonds connecting these subunits, Hemoglobin will look identical under both reducing and non-reducing conditions—typically appearing as one thick band (because the \alpha and \beta subunits are nearly identical in weight, ~16 kDa).

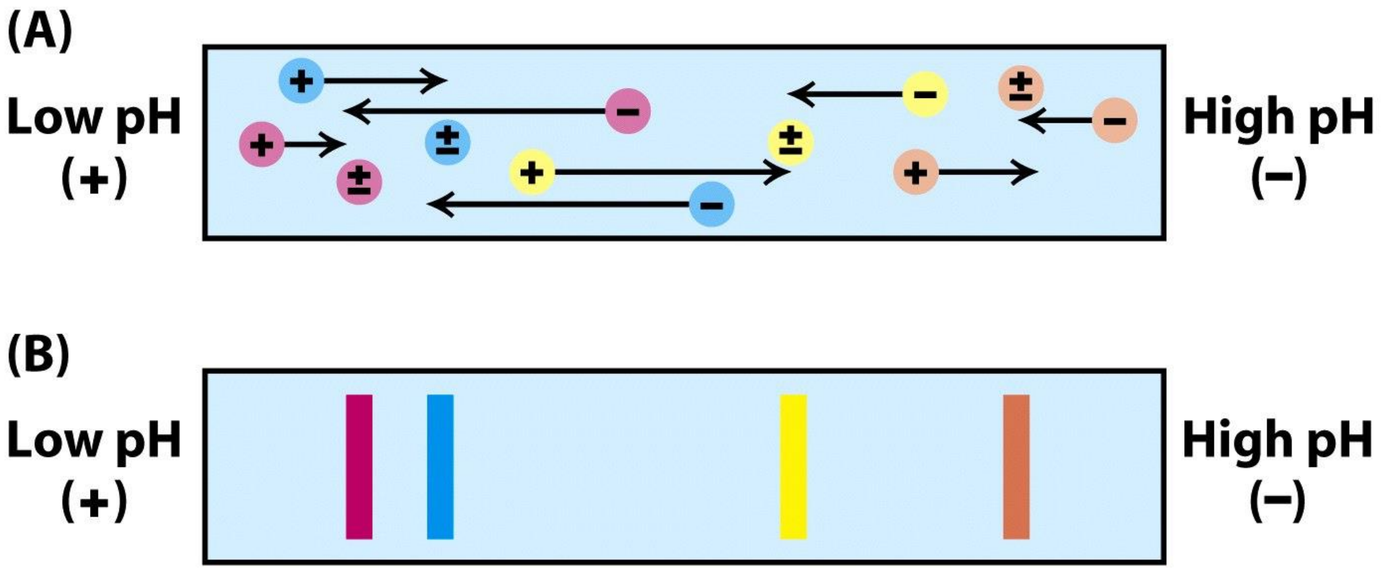
Immunoglobulins (e.g., IgG)
Unlike hemoglobin, which relies on non-covalent bonds, antibodies rely heavily on covalent intermolecular disulfide bonds. An IgG molecule is a tetramer made of two identical heavy chains (~50 kDa each) and two identical light chains (~25 kDa each).
Non-reducing conditions: SDS destroys the non-covalent interactions and coats the protein in a negative charge, but it cannot break the disulfide bonds holding the heavy and light chains together. Therefore, the antibody remains fully intact and migrates as a single, large band at ~150 kDa.
Reducing conditions: Adding a reducing agent (like \beta-mercaptoethanol or DTT) cleaves all the disulfide bonds. The antibody falls apart into its constituent chains. On the gel, you will see two distinct bands: one at ~50 kDa (representing the heavy chains) and one at ~25 kDa (representing the light chains).

Isoelectric focusing

Separates proteins based on isoelectric point.
(This method helps finding the isoelectric point of proteins.)

- A gel is prepared with a pH gradient. We use small charged molecules to prepare the pH gradient.
- When we close the electric current (use electricity), proteins migrate through the gel, they encounter regions of different pH, so the charge on the protein changes.
- Eventually each protein reaches the point at which it has no net charge—its isoelectric point—and no longer migrates.
- Each protein remains at the position on the gel corresponding to its pI, allowing for separation of proteins.

Proteins stop moving when reaching their isoelectric point because the become neutral, meaning that they won't be attracted to the positive or negative ends.



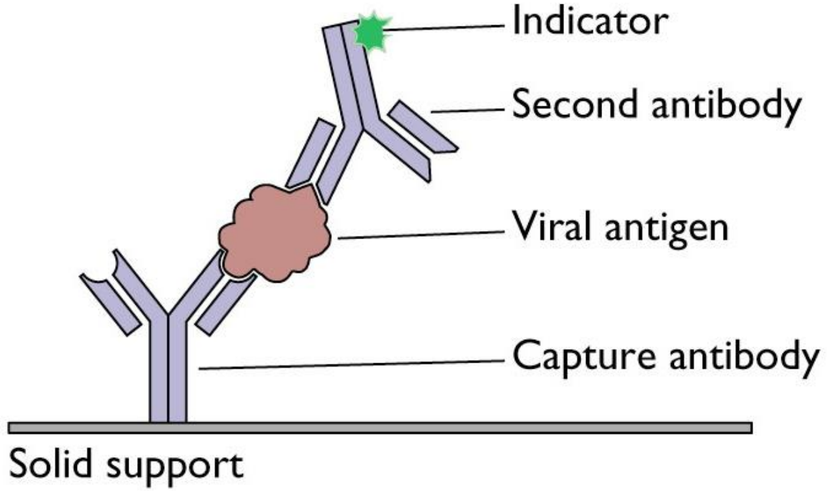
ELISA

- Enzyme-linked immunosorbent assay (ELISA)
- Same concept as immunoblotting but rapid, convenient, and sensitive (less than nanogram (10^{-9} g) of a protein)
- http://www.genscript.com/gsfiles/flash/protein_a_elisa_protocol.swf

This technique is used for:

1. Identify if a specific protein is found in the sample.
2. Calculate the amount of the specific specific protein in the sample.

This technique is used in labs to calculate hormone levels (eg. Estrogen) in our bodies. Pregnancy tests also rely on antibodies and uses the same concept of ELISA.



ELISA

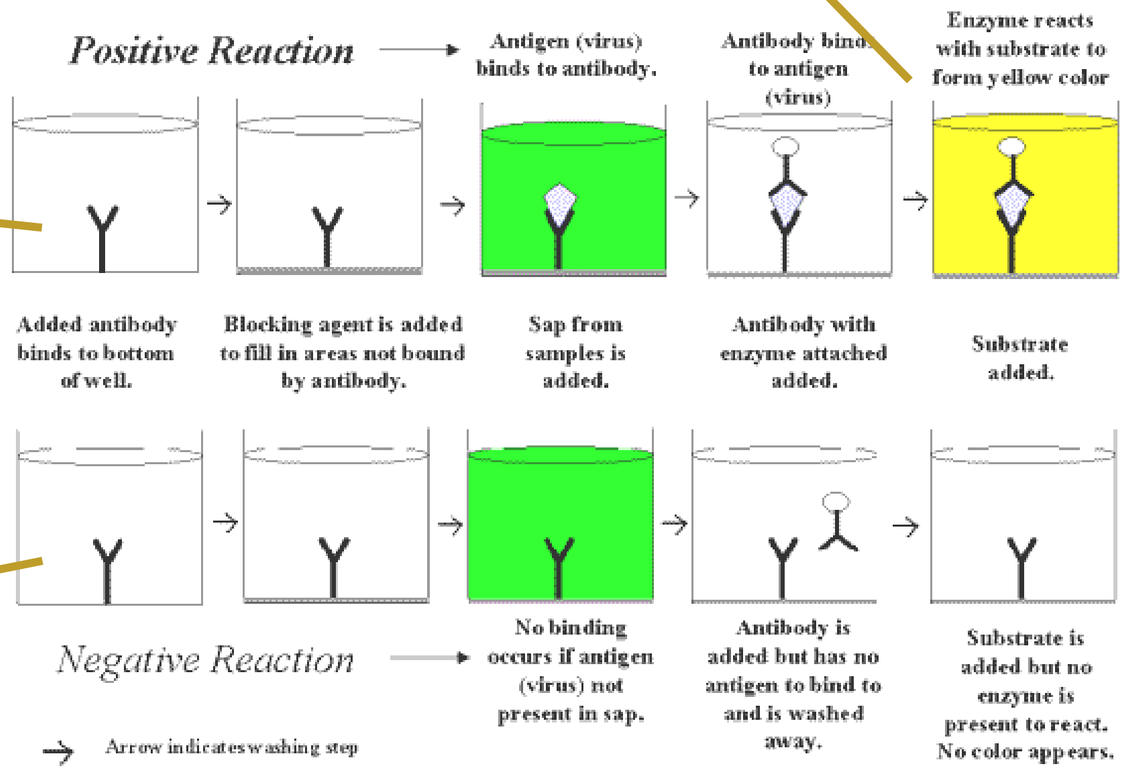
Remember: antibodies binding is very specific!

96 well-plate (a plate which has 96 wells)

The technique works by adding an antibody for a specific protein to a surface, and then the sample is added. If the sample has the specific protein that is being looked for, it will bind to the antibody and it will be captured, then we wash out the surface (all other proteins in the sample that didn't bind to the antibodies will be removed). After that, another antibody is added to bind to the protein from a different place, this second antibody (for the same antigen as the first one of course) has an indicatior which helps us get a signal that confirms that the protein we're looking for is in the sample. A stronger signal means we got more of the antigen or the protein we're looking for.

If the antigen is not found in the sample, there will be no color because the second antigen won't bind to anything and the indicator wouldn't work .

The more intense the color in this plate, the more antigens we have.



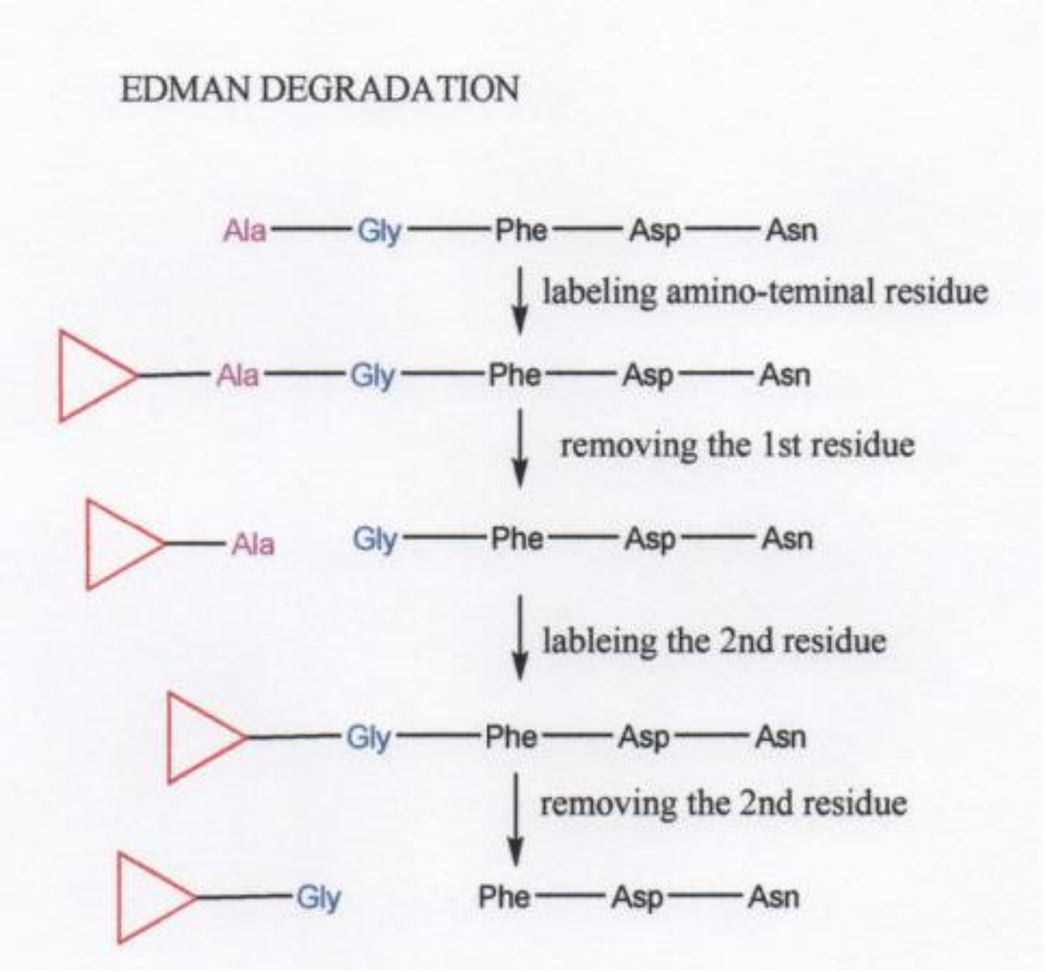
Protein sequencing

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

Procedure Edman Degradation

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

This technique works by adding PITC to the peptide, and it reacts with the amino acid on the N-terminal and it is seperated from the peptide chain. Then we do chromatography to identify the amino acid. This procedure is done again and again until we identify every single amino acid in the peptide.



Advantage

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

- The Edman degradation technique does not allow peptides more than 50 residues to be sequenced.

Cleavage methods

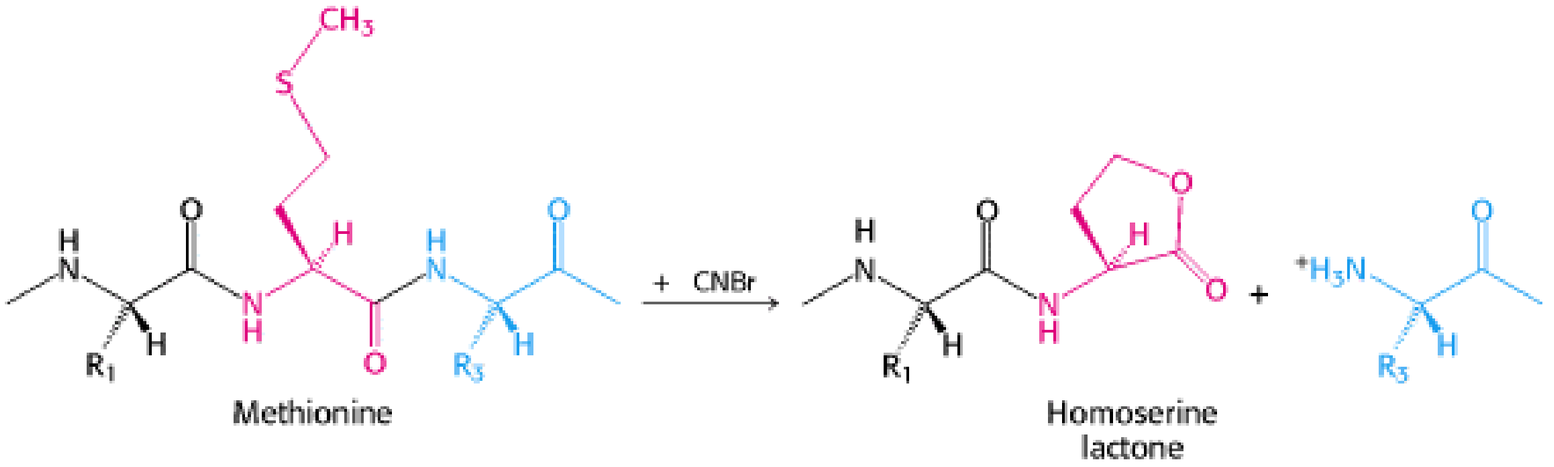
Specific cleavage of the peptide (cleaves the peptide from a specific place)

- It is possible to sequence whole proteins by cleaving them into smaller peptides.
- This is facilitated by three methods:
  - Chemical digestion
  - Endopeptidases
  - Exopeptidases

Chemical digestion

Breaks the peptide bond between methionine (from the C-terminal) and any other amino acid.

- 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 methionine residues.
- A protein that has 10 methionine residues will usually yield 11 peptides on cleavage with CNBr.



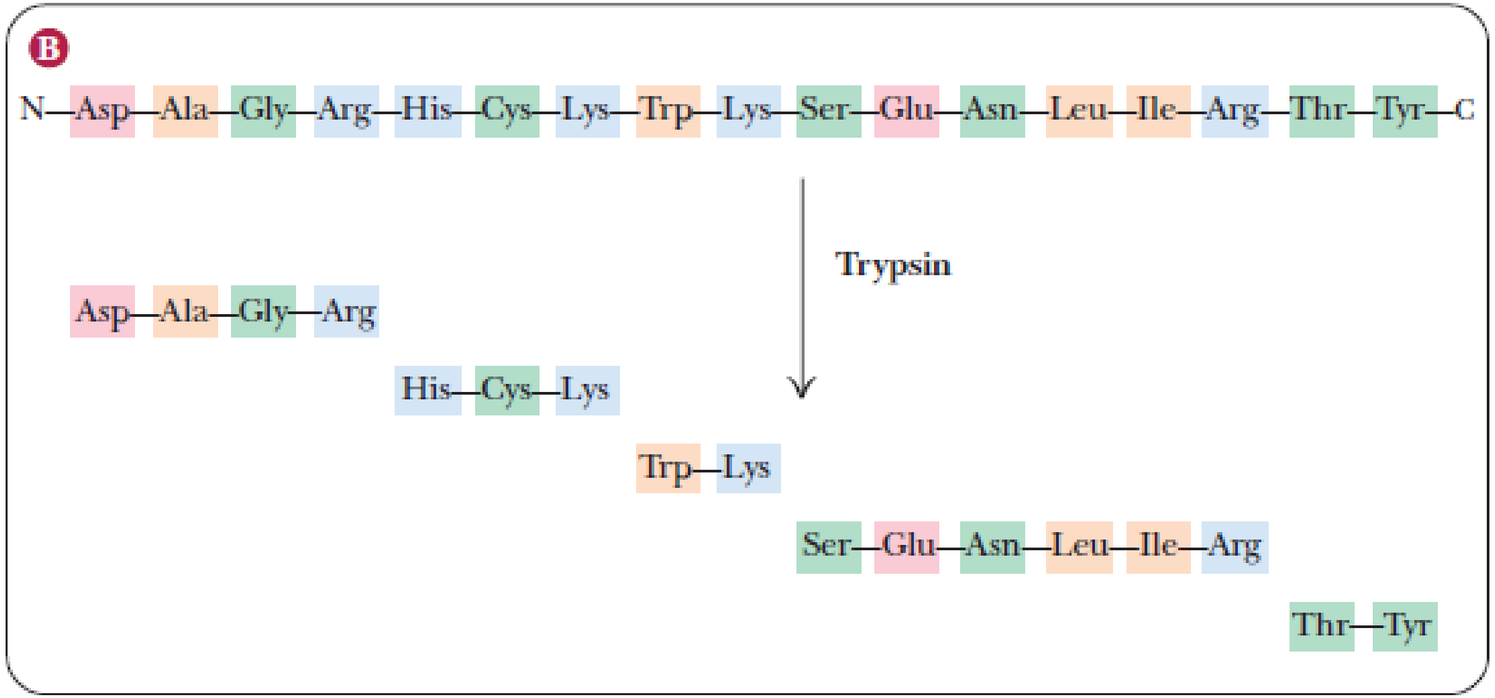
Endopeptidases Endo= Cleaves peptide bonds within the peptide (example: trypsin)

- These are enzymes that 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. (Endopeptidases is very useful to know the sequence of an amino acid which is longer than 50 amino acids, as it cleaves the peptides to amino acids sequences that are less than 50 amino acids long ,which is very important to be able to use Edman Degradation. Then we use Edman degradation on every part to know the sequence of the whole protein).

Example

- Trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues.
- A protein that contains 9 lysine and 7 arginine residues will usually yield 17 peptides on digestion with trypsin.

Another example



Other examples

Enzyme	Specificity
Trypsin	peptide bond C-terminal to Arg or Lys, but not if next to Pro
Chymotrypsin	peptide bond C-terminal to Phe, Tyr, or Trp, but not if next to Pro
Elastase	peptide bond C-terminal to Ala, Gly, Ser, or Val, but not if next to Pro
Pepsin	peptide bond **N-terminal** to Leu, Phe, Trp, or Tyr, but not if it follows Pro (if proline is before these amino acids, pepsin won't work)

Exopeptidases

Exo = Cleaves amino acids from the N and C terminus

- These are enzymes that cleave amino acids starting at the end of the peptide.
- There are two types:
  - Aminopeptidases that cleave at the N-terminus
  - Carboxypeptidases that cleave at the C-terminus

Final notes from the prof:

1. A question could come in the exam which says : you have a penta-peptide, it was cleaved using CNBr, and we got 2 peptides (one of them has 3 amino acids and the other has 2), the peptide with the 2 amino acids is positively is charged , and the other peptide is neutral for example. The question will ask you to give the amino acid order based on these information.

2. It is advised to revise the isolelectric point for amino acids concept (last lecture of midterm material but we put the pI table here to help you study and memorize).

Memorize the pI from the last lecture in the mid term in case it is not given in the exam (at least compare them if you won't memorize exact numbers)

Here are all of them

Arg: 12.5
Lys: 11
Tyr: 10
His: 6
Cys: 8
Asp: 4
Glu: 4

Homework

Chymotrypsin	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe
Cyanogen bromide	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met
Chymotrypsin	His—Met—Thr—Met—Ala—Trp
Cyanogen bromide	Thr—Met
Cyanogen bromide	Ala—Trp—Val—Lys—COO^-
Chymotrypsin	Val—Lys—COO^-
Overall sequence	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met—Thr—Met—Ala—Trp—Val—Lys—COO^-

A sample of an unknown peptide was divided into two aliquots. One aliquot was treated with trypsin; the other was treated with cyanogen bromide. Given the following sequences (N-terminal to C-terminal) of the resulting fragments, deduce the sequence of the original peptide.

Trypsin treatment

Asn—Thr—Trp—Met—Ile—Lys

Gly—Tyr—Met—Gln—Phe

Val—Leu—Gly—Met—Ser—Arg

Cyanogen bromide treatment

Gln—Phe

Val—Leu—Gly—Met

Ile—Lys—Gly—Tyr—Met

Ser—Arg—Asn—Thr—Trp—Met

Answer :Val–Leu–Gly–Met–Ser–Arg–Asn–Thr–Trp–Met–Ile–Lys–Gly–Tyr–Met–Gln–Phe
(Detailed explanation on how to solve these questions in the next slide)

How to solve the previous question:
1. List and Analyze Your Fragments
First, write out your fragments and remember the rules for each cleavage method:
Trypsin cuts after Arginine (Arg) and Lysine (Lys).
Cyanogen Bromide (CNBr) cuts after Methionine (Met).
Trypsin fragments:
1. Asn–Thr–Trp–Met–Ile–Lys (Ends in Lys - expected)
2. Gly–Tyr–Met–Gln–Phe (Ends in Phe - unexpected)
3. Val–Leu–Gly–Met–Ser–Arg (Ends in Arg - expected)
Cyanogen Bromide fragments:
1. Gln–Phe (Ends in Phe - unexpected)
2. Val–Leu–Gly–Met (Ends in Met - expected)
3. Ile–Lys–Gly–Tyr–Met (Ends in Met - expected)
4. Ser–Arg–Asn–Thr–Trp–Met (Ends in Met - expected)

2. Identify the C-Terminus (The End)
Look for fragments that do not end in the expected amino acid for their specific enzyme/chemical.
Trypsin fragment 2 ends in Phe. Trypsin does not cut after Phenylalanine.
CNBr fragment 1 ends in Phe. CNBr does not cut after Phenylalanine.
Because neither agent cuts after Phe, ...Gln–Phe must be the absolute tail end (C-terminus) of the entire original peptide.

3. Identify the N-Terminus (The Start)
Look for fragments that share a sequence but differ in where they were cut.
Notice Trypsin fragment 3 (Val–Leu–Gly–Met–Ser–Arg) and CNBr fragment 2 (Val–Leu–Gly–Met).
Because the CNBr fragment perfectly matches the beginning of the Trypsin fragment, we can confidently place Val–Leu... at the very beginning (N-terminus) of the peptide.

4. Build the Sequence via Overlaps
Now, piece it together like a puzzle, starting from the N-terminus and using the overlapping ends.

A sample of a peptide of unknown sequence was treated with trypsin; another sample of the same peptide was treated with chymotrypsin. The sequences (N-terminal to C-terminal) of the smaller peptides produced by trypsin digestion were as follows:

Met—Val—Ser—Thr—Lys

Val—Ile—Trp—Thr—Leu—Met—Ile

Leu—Phe—Asn—Glu—Ser—Arg

The sequences of the smaller peptides produced by chymotrypsin digestion were as follows:

Asn—Glu—Ser—Arg—Val—Ile—Trp

Thr—Leu—Met—Ile

Met—Val—Ser—Thr—Lys—Leu—Phe

Deduce the sequence of the original peptide.

Answer :Met–Val–Ser–Thr–Lys–Leu–Phe–Asn–Glu–Ser–Arg–Val–Ile–Trp–Thr–Leu–Met–Ile

Quiz!

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			

Additional Resources:

Reference Used:
Prof. Mamoun Ahram recorded lecture

Extra References for the Reader to Use:
Marks’ Basic Medical Biochemistry (6th edition)

رسالة من الفريق العلمي:

الحمدلله الذي بنعمته تتم الصالحات،
الحمدلله حمداً كثيراً طيباً مباركاً،،
بفضل الله وتوفيقه، أنهينا كتابة وتدقيق جميع ملفات
المحاضرات للفصل الصيفي للسنة الأولى، سائلين المولى
عز وجل أن يوفقنا وإياكم في طاعاته وعباداته.
اللهم يسر علينا وسهل أمرنا وانفعنا بما علمتنا وعلمنا ما
ينفعنا في الدنيا والآخرة. 💛💛