بسم الله الرحمن الرحيم

BIOCHEMISTRY

Lecture 32 (and the last)

Protein Analysis

﴿ وَإِن تَتَوَلَّوْاْ يَسْتَبْدِلْ قَوْمًا غَيْرَكُمْ ثُمَّ لَا يَكُونُوٓاْ أَمْثَٰلَكُم ﴾

اللهم استعملنا لنصرة دينك

والحمد لله لها ختـــــــــام
ثم الصلاة بعد والسـلام

على النبي المصطفى وآله
وصحبه وتابعي منواله

Done by:

Muthanna Khalil

Relation between L31 and L32

- In the last lecture, we talked about the purification of protein samples.
- In this lecture, we will mainly focus on the techniques used for the analysis of these proteins.
- We have many techniques (as you will see in the lecture)
  1. SDS-PAGE
  2. Isoelectric Focusing
  3. 2D-PAGE
  4. Immunoblotting
  5. ELISA
  6. Sequencing
     - Edman Degradation
     - Chemical Digestion
     - Endopeptidase Cleavage
     - Exopeptidase Cleavage
  7. Crystallography
  8. NMR Spectroscopy

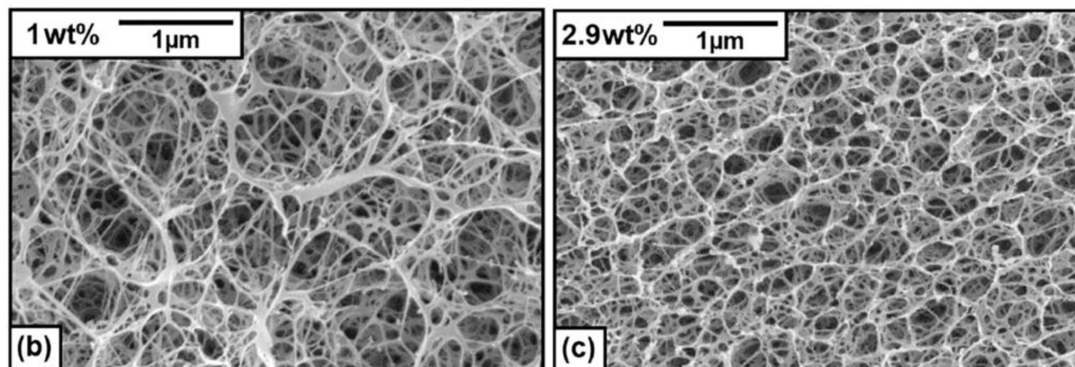
Gel electrophoresis

Electrical Movement

- A molecule 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.

غربال

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

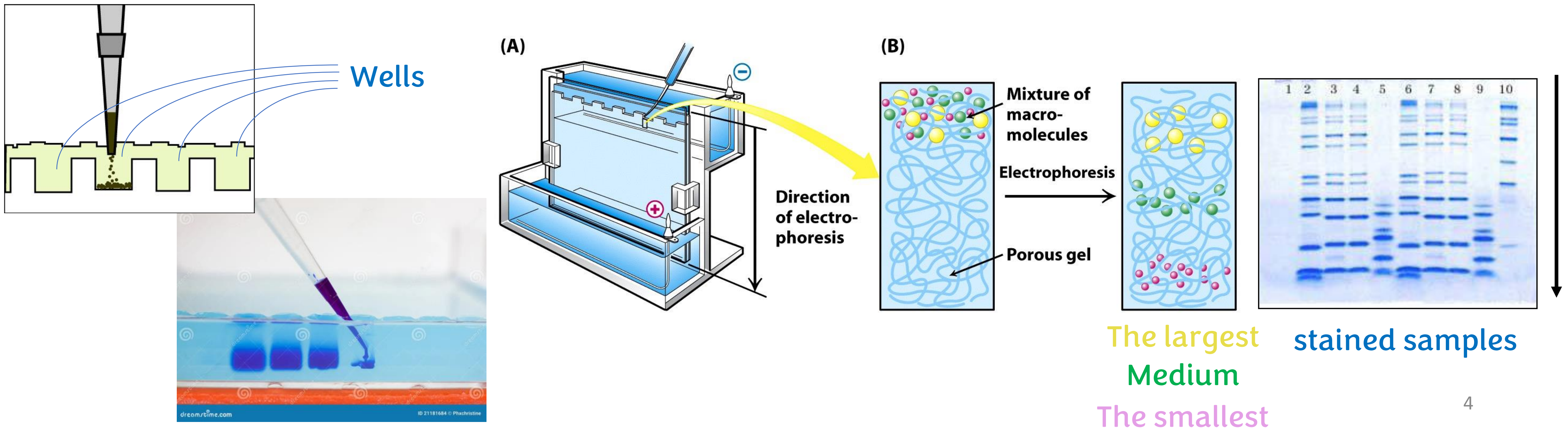


The gel is a network of fibers (see slide 6) that have molecular pores which permit the movement of proteins. The smaller the proteins is, the easier its movement through the gel is, and thus the faster it is.

The process

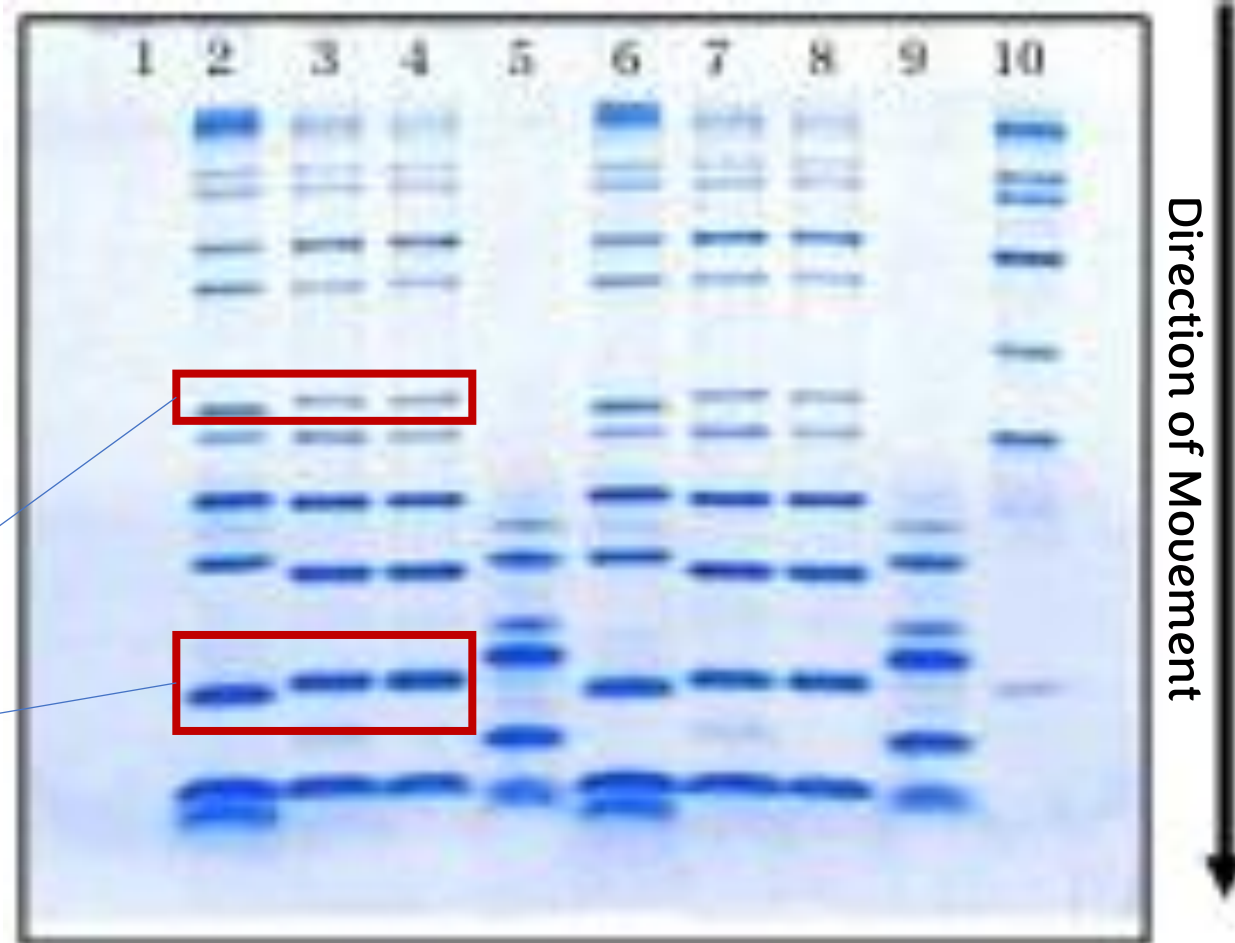
All proteins will move across the gel from top (cathode (-)) to bottom (anode (+))
Why?
Because they all have a total negative charge (see slides 7, 8, & 9)

- The most commonly used protein electrophoresis technique is termed **S**odium **D**odecyl **S**ulfate (SDS) **P**oly**A**crylamide **G**el **E**lectrophoresis **(SDS-PAGE)**.
- It is performed in a thin, vertical gel.
- The top of the gel consists of wells onto which samples are loaded.



Proteins in a specific band share similar molecular weights, so they do not have to be of the same type; they can be totally different proteins.

Cathode (-)



Anode (+)

Largest Proteins
Highest MW

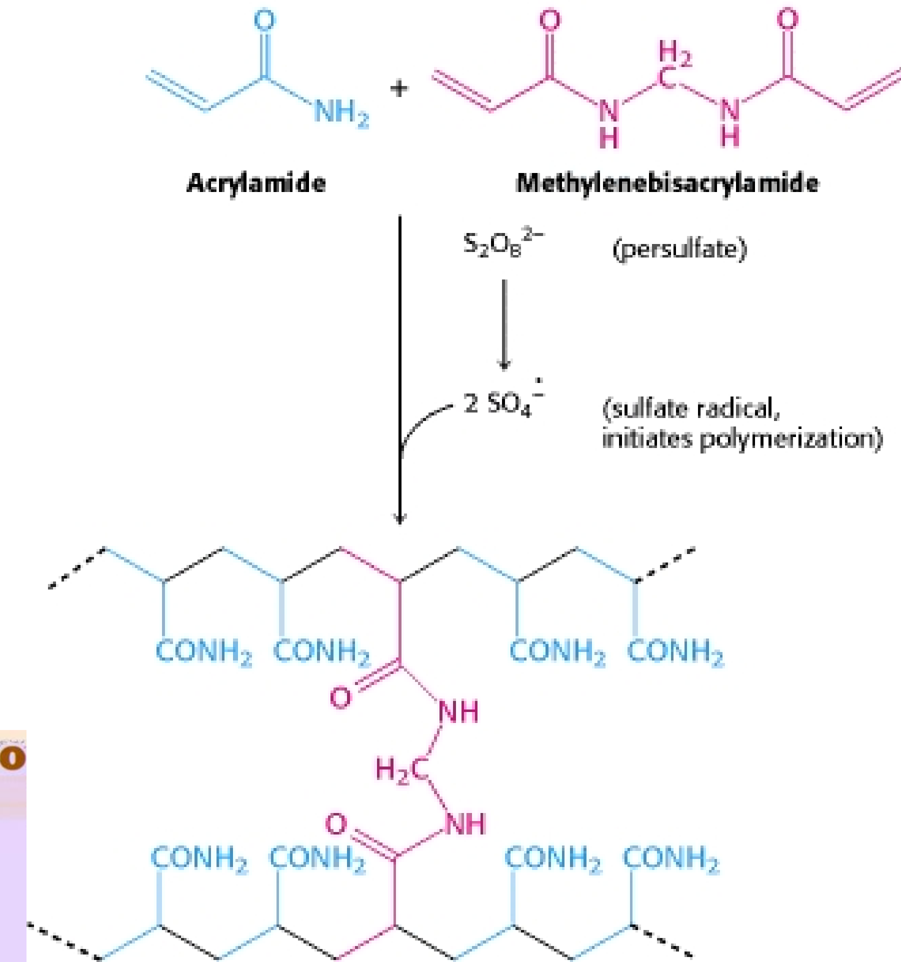
Smallest Proteins
Lowest MW

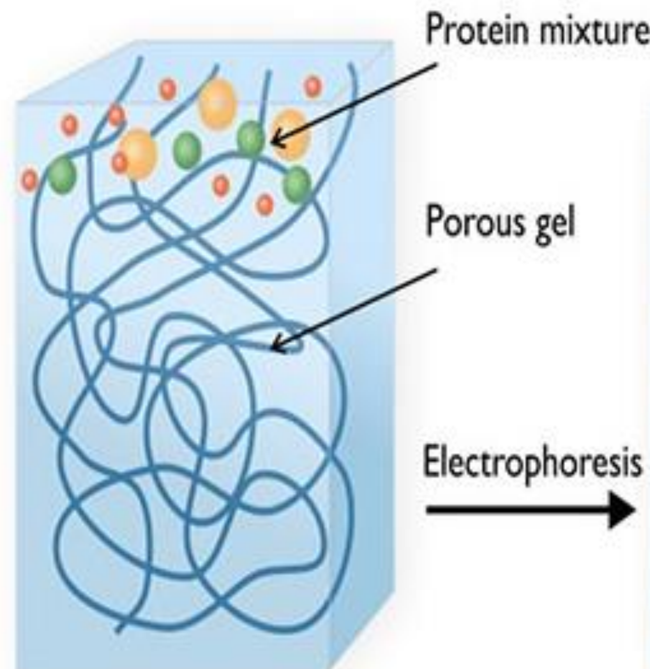
Proteins in these bands in well-2 are slightly smaller than their counterparts in well-3 and well-4

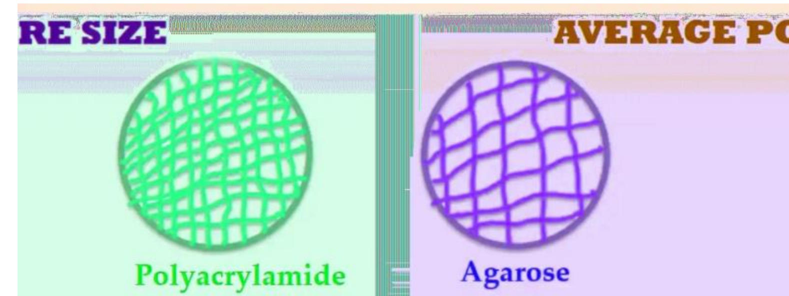
Know that the gel is a polymer of a molecule called acrylamide. Forget about the other names.

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.





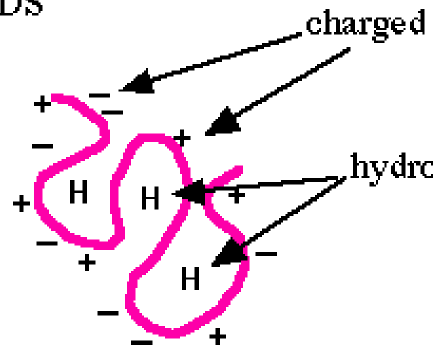


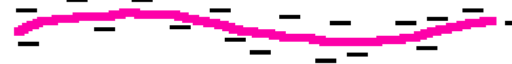
We mean this one

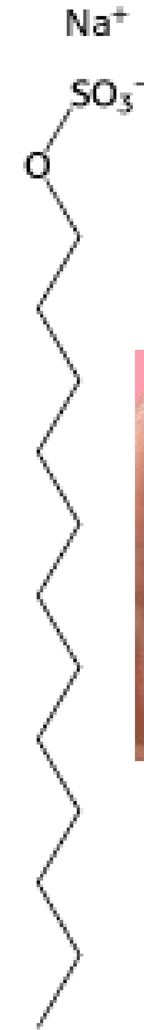
We will take this later

Purpose of SDS

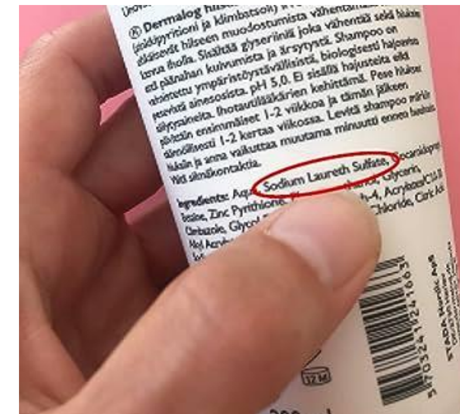
- This technique utilizes a negatively charged detergent **(sodium dodecyl sulfate)** to denature and solubilize proteins (denaturing condition). ignoring the effect of shape
  - ***Otherwise***, *non-denaturing condition or native condition where proteins maintain their original structure and shape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







Sodium dodecyl sulfate (SDS)



SDS is present in shampoo

Do not memorize the names of reducing agents

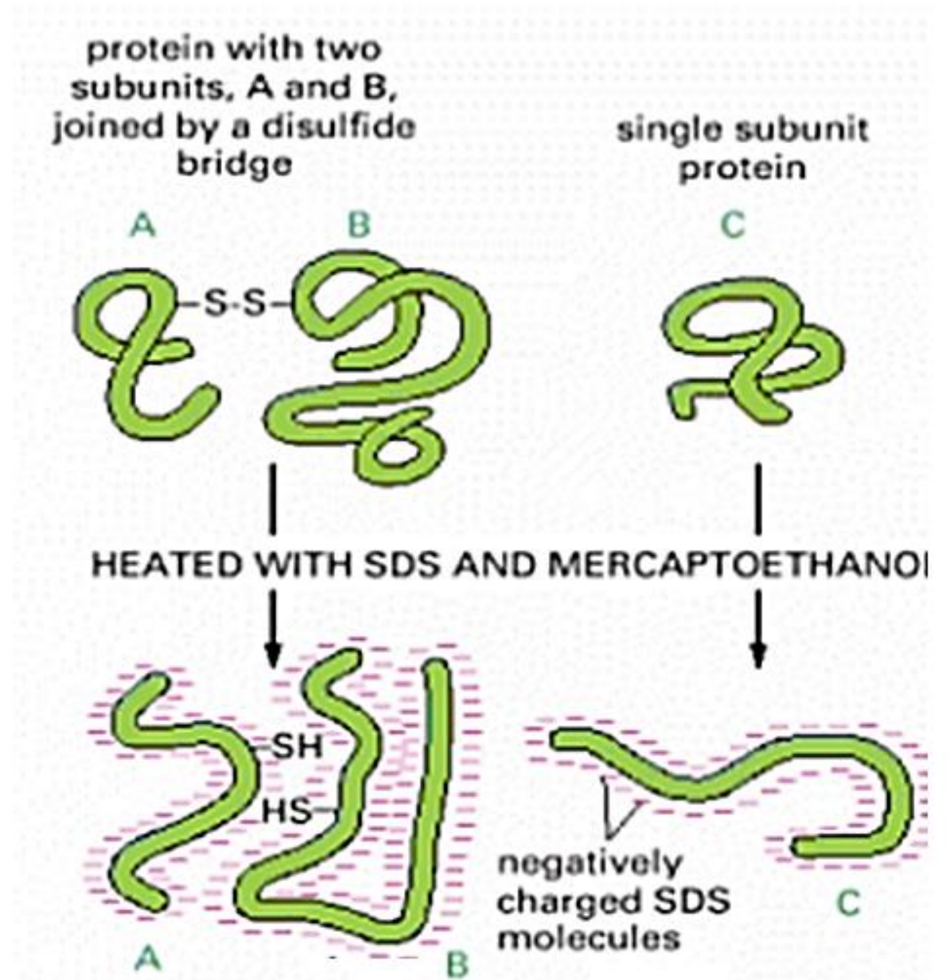
Purpose of reducing agents

- 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

SDS can break down non-covalent interactions only.

In case of the presence of disulfide bonds, we need a reducing agent to break them down.

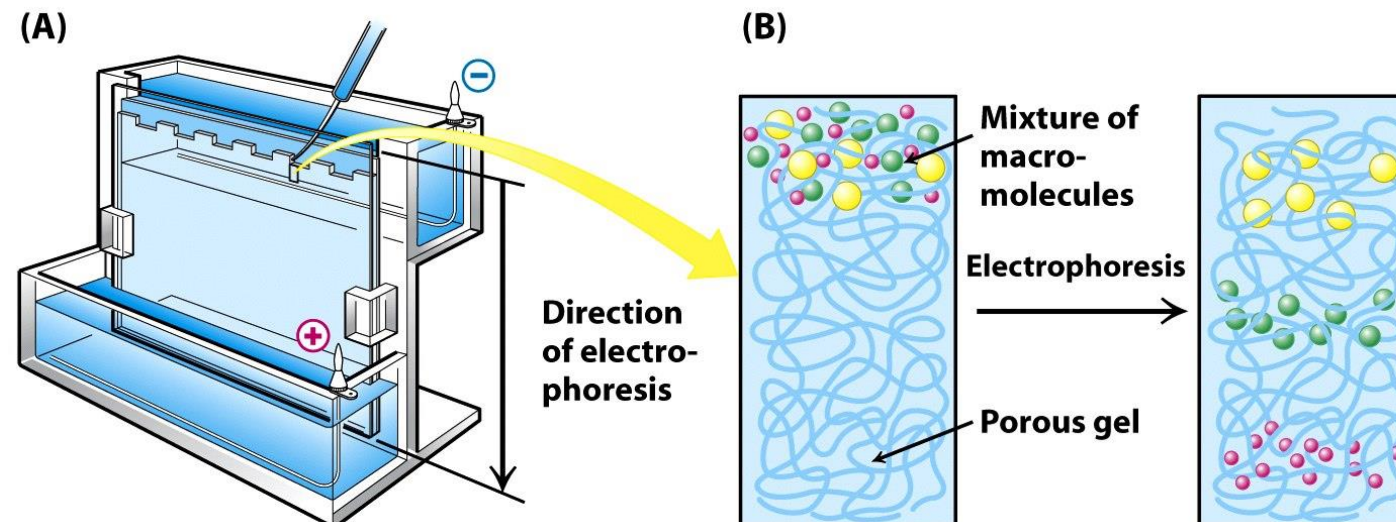
The combined effect is that proteins now lack any characterizing features apart from their MW (size).



Notice how SDS is distributed all over the proteins providing a net negative charge.

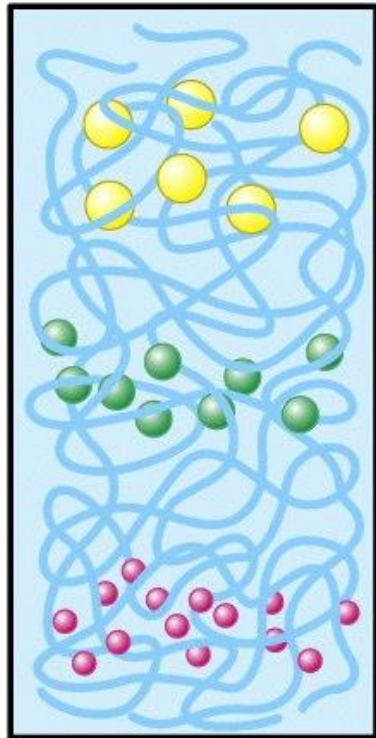
Migration of proteins

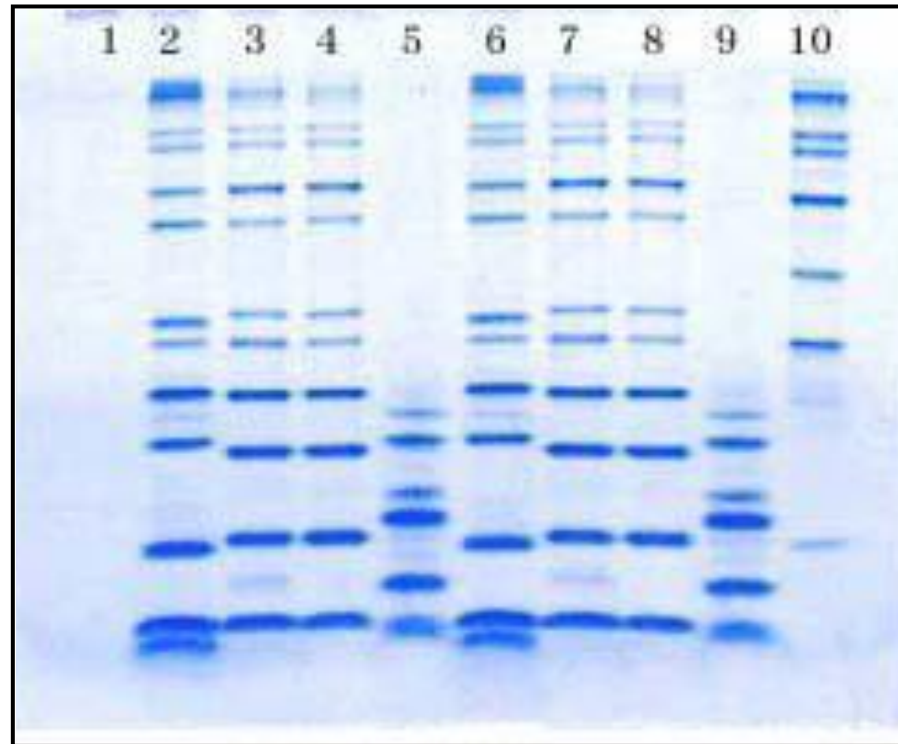
- When an electrical voltage is applied between the upper and lower ends of the gel, all proteins move in one direction towards the anode (positive) according to size only.
- The direction of movement is from top to bottom.
- Whereas smaller molecules move readily through the gel, larger molecules are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



Then…

- Once a gel has been "run", proteins are stained to reveal the positions of the proteins that appear as bands.





Questions

See next 3 slides for in-detail solutions

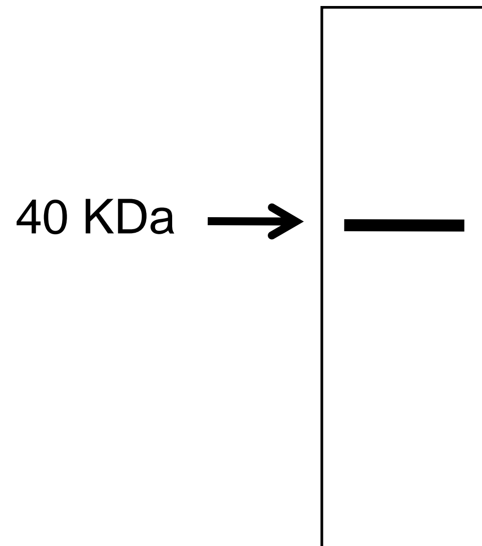
- Describe the protein's structure based on the following results of SDS-PAGE:
  - Under non-reducing condition, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands.
  - Under non- reducing condition, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein also exists as two bands, 15 KDa and 10 KDa.
  - 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.

Only SDS

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

Now both SDS and reducing agent

Non-reducing



Reducing

We can conclude that the protein was broken down into 2 chains, each 20-kDa, after the addition of a reducing agent. So, the bonds which have been holding the chains together must be (-S-S-) bonds.

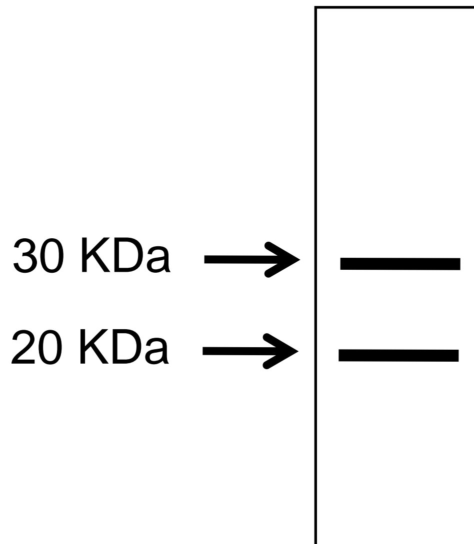
The original protein is most probably a homodimer.
It can be a heterodimer of 2 equal-size chains.
The 2 chains are connected by (-S-S-) bonds.

Only SDS

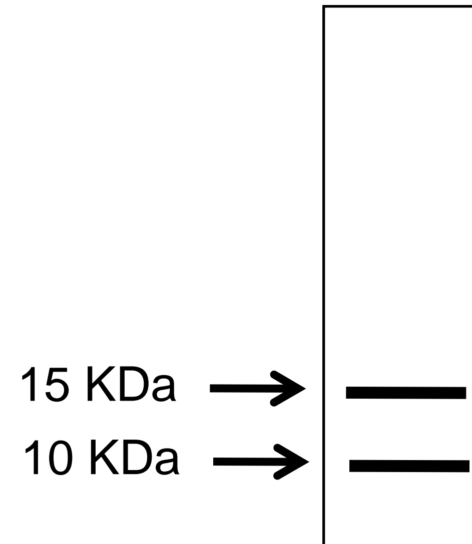
Under non- reducing, denaturing conditions, a protein exists as **two bands, 30 KDa and 20 KDa**. Under reducing conditions, the protein exists as **two bands, 15 KDa and 10 Kda.**

Now both SDS and reducing agent

Non-reducing



Reducing



The original protein is most probably a heterotetramer of 2 15-kDa chains and 2 10-kDa chains.

The two 15-kDa chains are connected by disulfide bonds.

The two 10-kDa chains are connected by disulfide bonds.

The two “dimers” are connected by **non-covalent** interactions

Only SDS

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

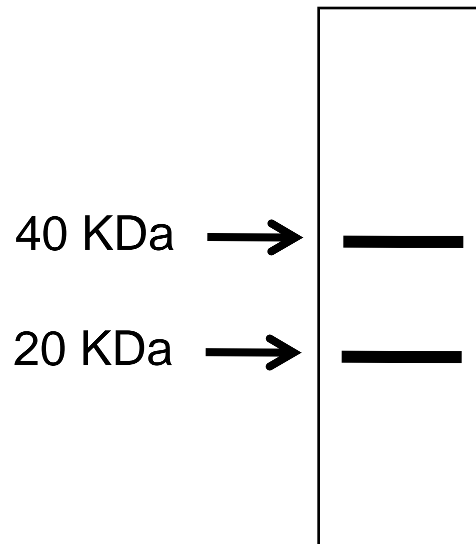
Now both SDS and reducing agent

The original protein is most likely a heterotrimer.

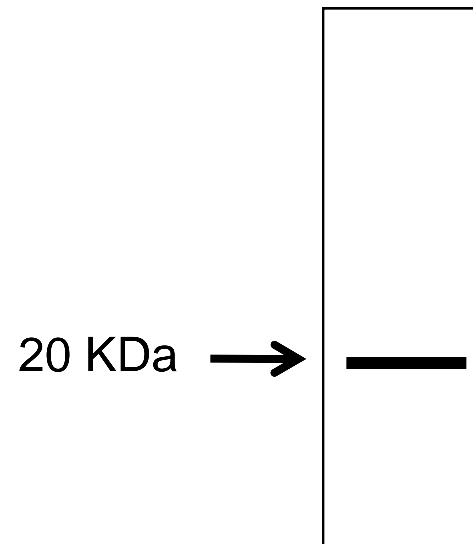
2 of its 3 chains are 20-kDa chains covalently attached by disulfide bonds.

The 3rd chain is also a 20-kDa chain, but it is non-covalently attached to the other 2 chains.

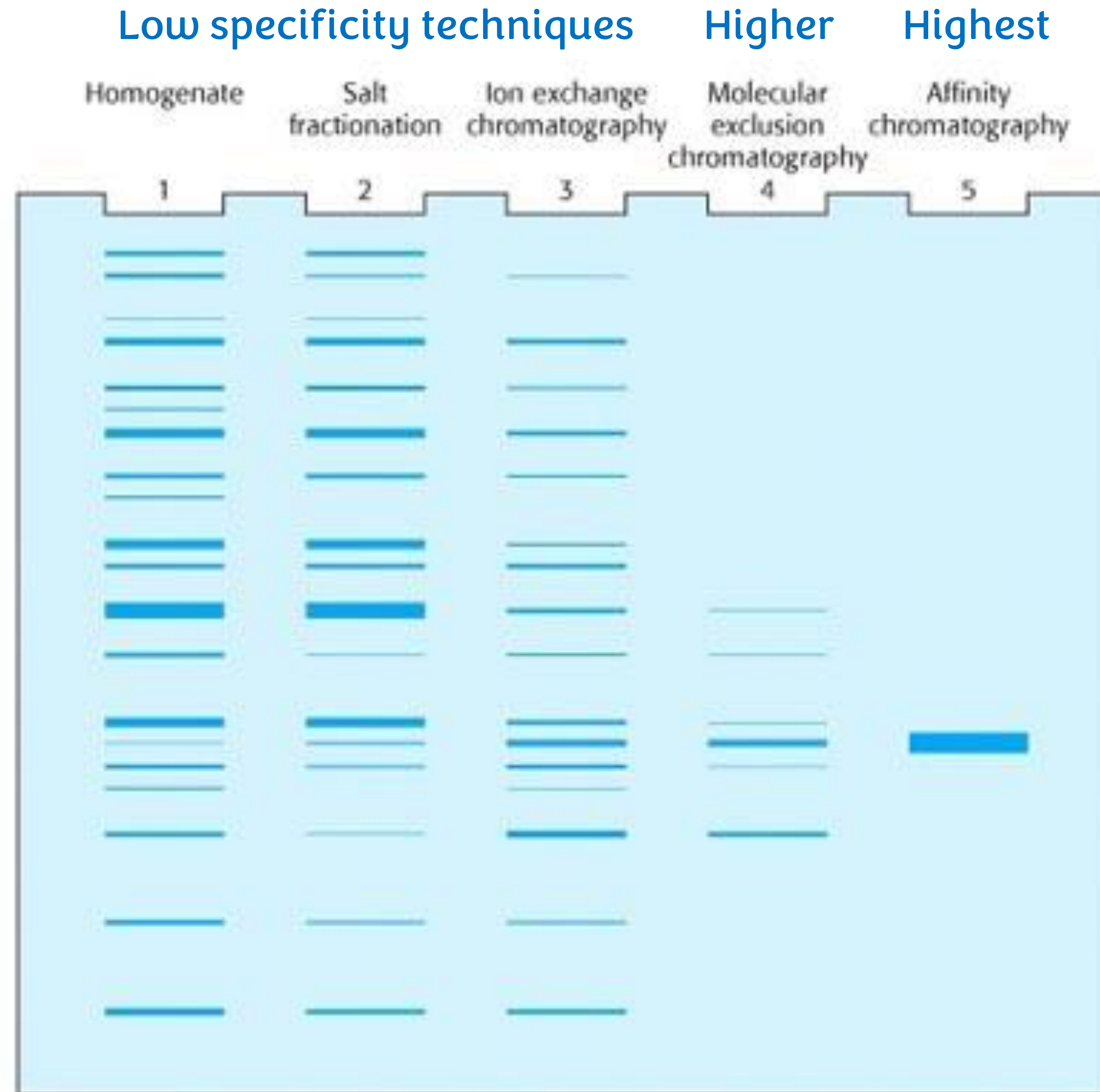
Non-reducing



Reducing



Electrophoretic analysis of protein purification



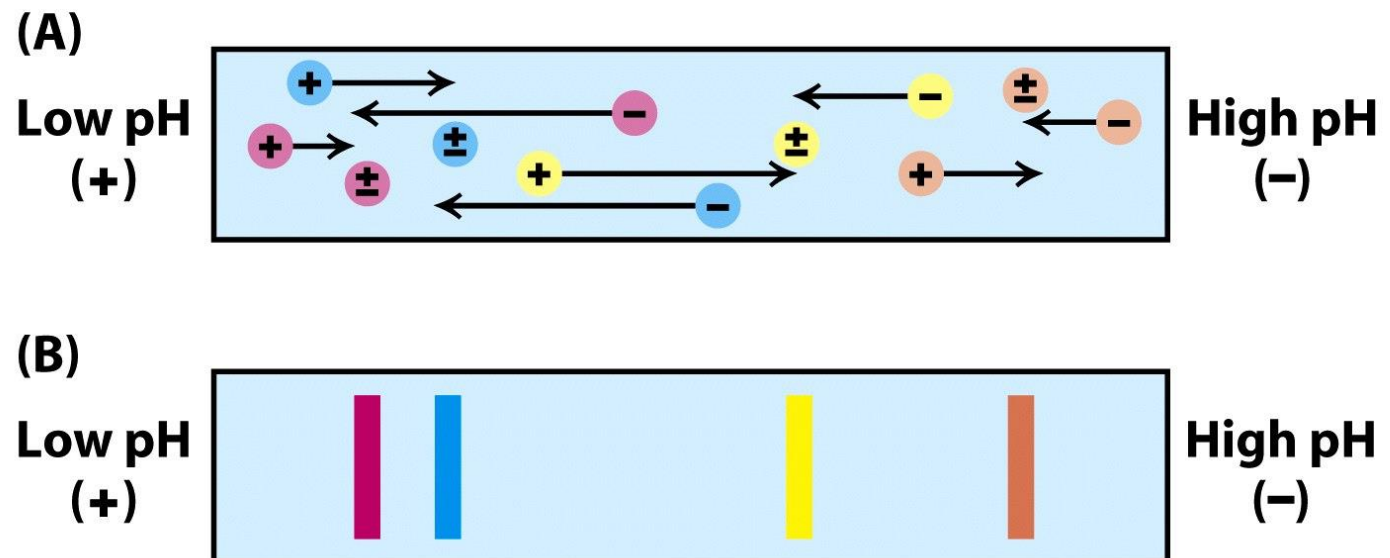
Isoelectric focusing

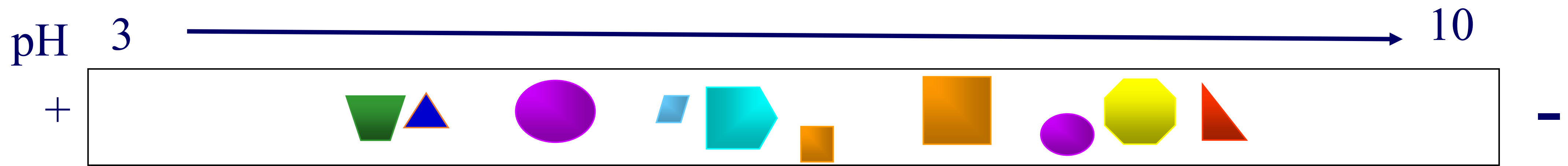
The driving force through the gel is the electric force.
F = q*$\mathbf{E}$; where **F** is the force, q the charge, **E** the field intensity.
You can notice that if the charge disappears, the force disappears as well → the object (protein) stops.
Recall that the net charge of a protein is zero when pH = pI.

- A gel is prepared with a **pH gradient**.
- As 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.

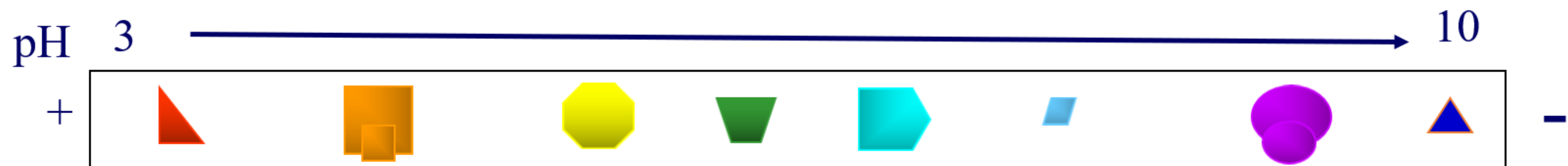
The nearer the protein is to the high pH zone (cathode), the higher its pI is → Basic.

The nearer the protein is to the low pH zone (anode), the lower its pI is → Acidic.



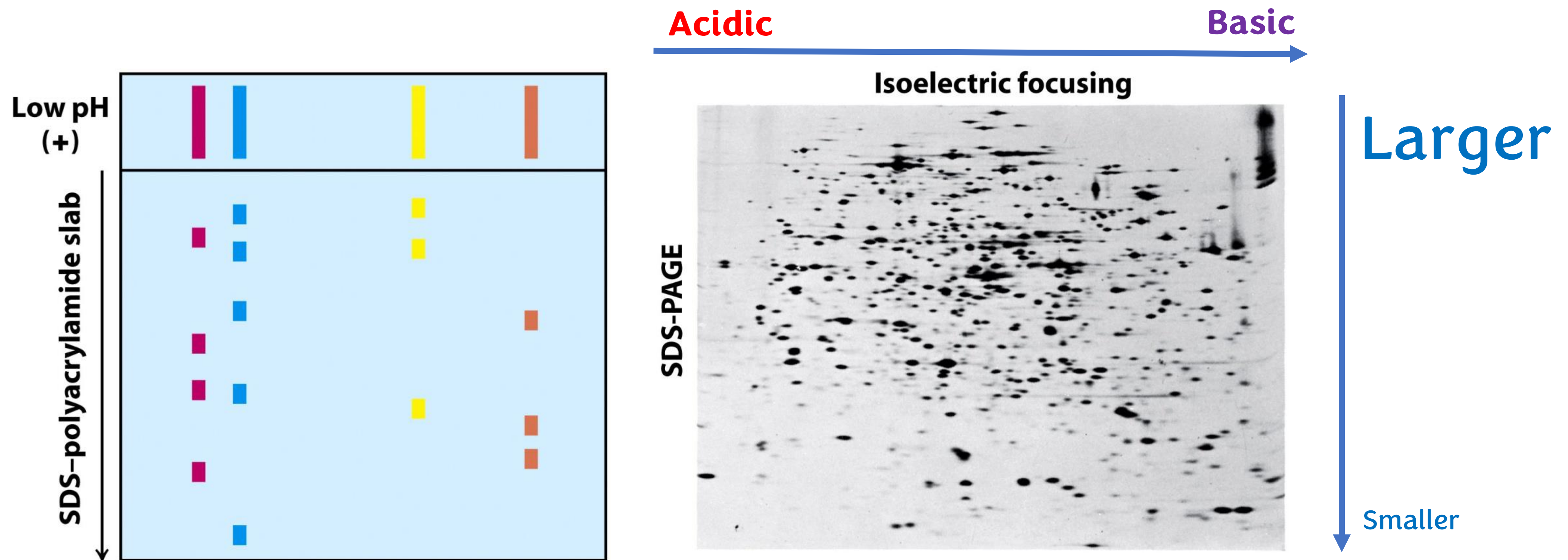


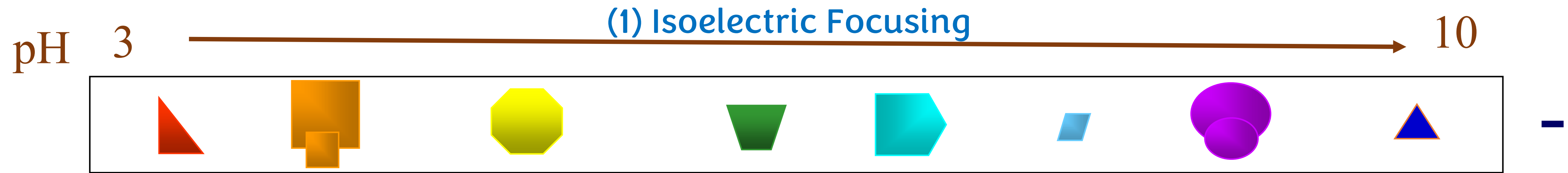
After the activation of the electric field



Two-dimensional gel electrophoresis (2D-PAGE)

- In 2D-PAGE, proteins are separated by, first, isoelectric focusing, then through an SDS-PAGE.
- Thus, proteins are separated based on **both charge and size**.





(2) SDS-Page

High MW

Low MW

+

Until now, none of the studied techniques can specifically identify a protein.
SDS-PAGE, isoelectric focusing, and 2D-PAGE can only classify proteins depending on their size and charge.

Immunoblotting (Western blotting)

- Specific proteins are detected by antibodies following SDS-PAGE.

A) Separation

Normal SDS-PAGE

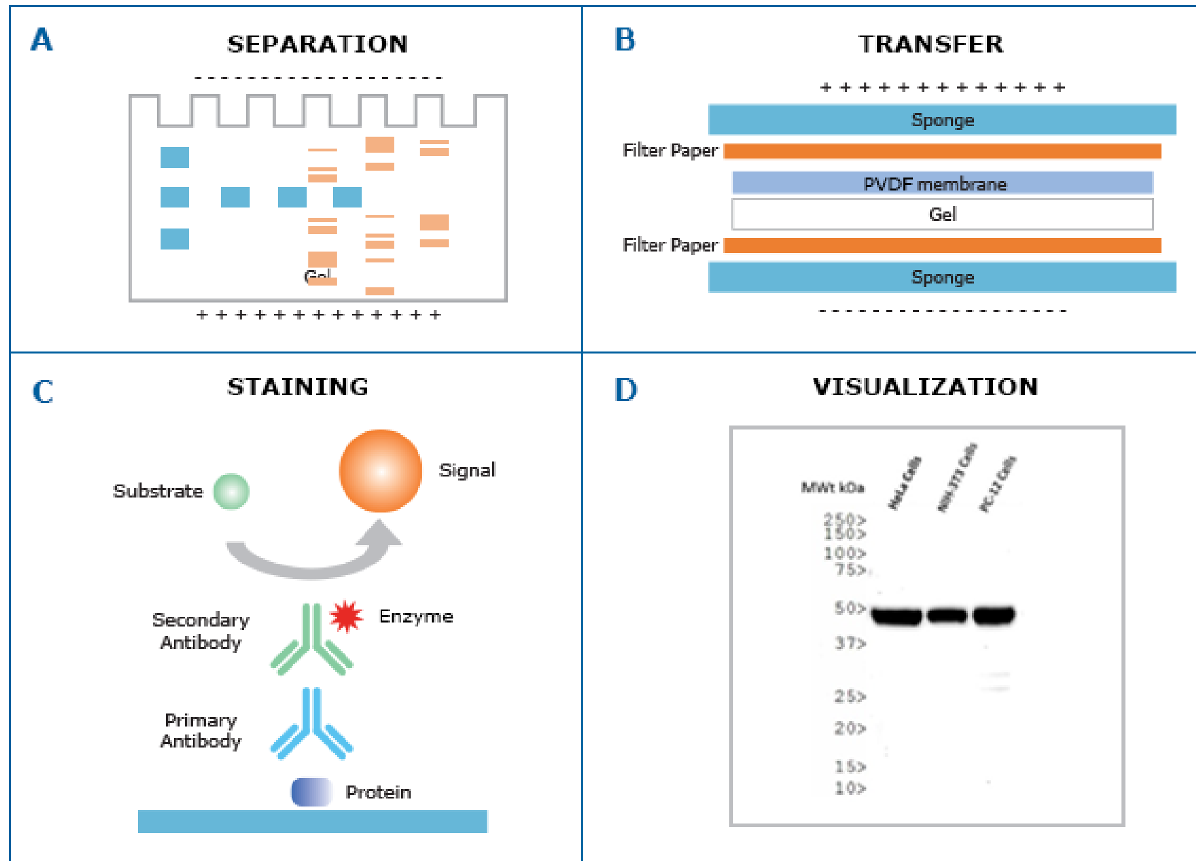
C) Staining

After the bands are oriented in the membrane, 2 types of antibodies are applied onto the membrane.

The first type is called a primary antibody, which binds directly to the specific protein.

Secondary antibodies are then added to cause a signal that is detected (see step D).

2* antibodies bind to 1* ones.



https://www.youtube.com/watch?v=Yh69yHJMWPc

B) Transfer

Protein bands on the gel are transferred into a membrane.
The bands in the membrane are in the same order as they were in the gel.
The driving force here is also electrical.

D) Visualizing

We can conclude 3 info.:

1) The presence of the specific protein in the studied sample (we seek for signal from 2*).
2) The amount of protein, by looking at the intensity of the signal observed.
3) The size of the protein (similar as regular SDS-PAGE)

ELISA

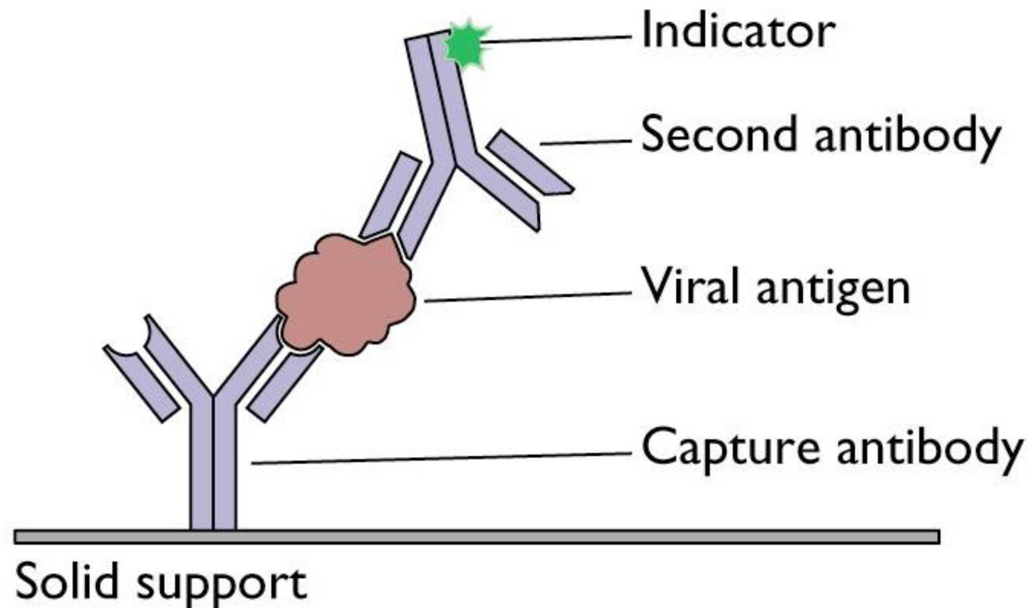
Experiment

- **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay
- Same concept as immunoblotting but rapid, convenient, and sensitive (less than nanograms (10^{-9} g) of a protein)
- http://www.genscript.com/gsfiles/flash/protein_a_elisa_protocol.swf

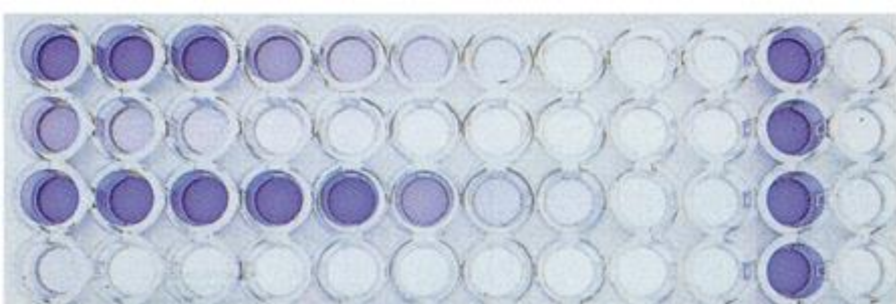
We first place capture antibodies on a solid surface (from their name you can conclude that they capture the protein).

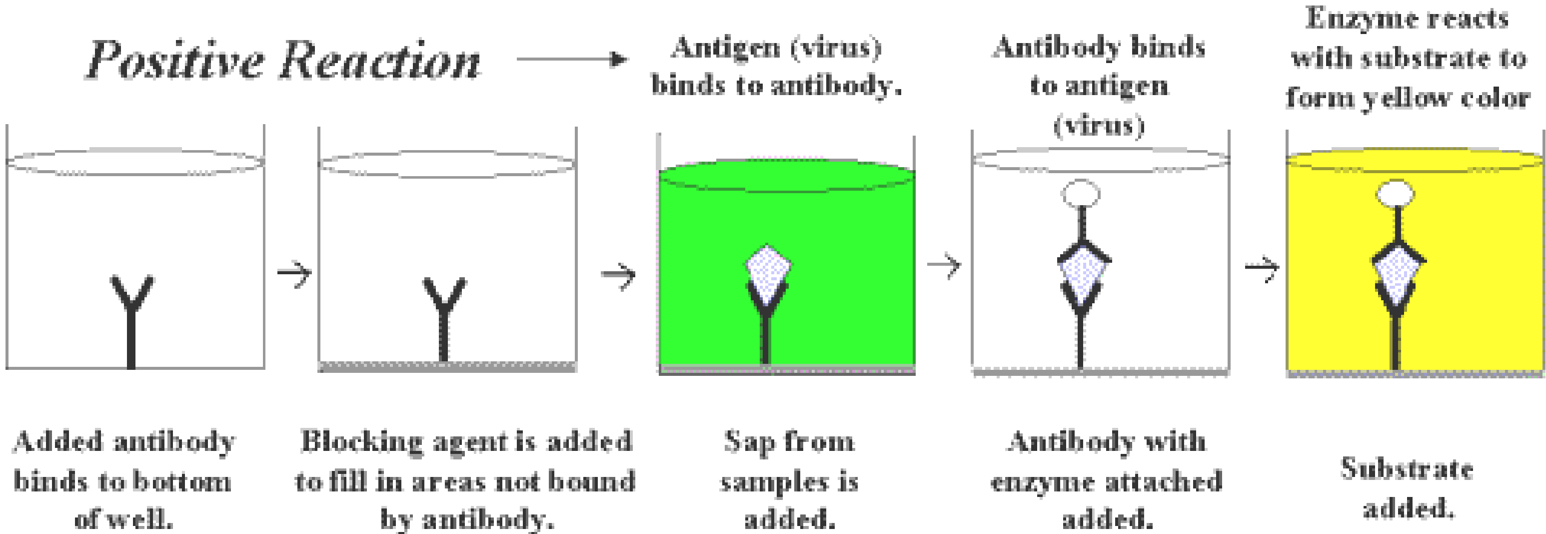
We then add another set of antibodies that bind to the antigen as well.

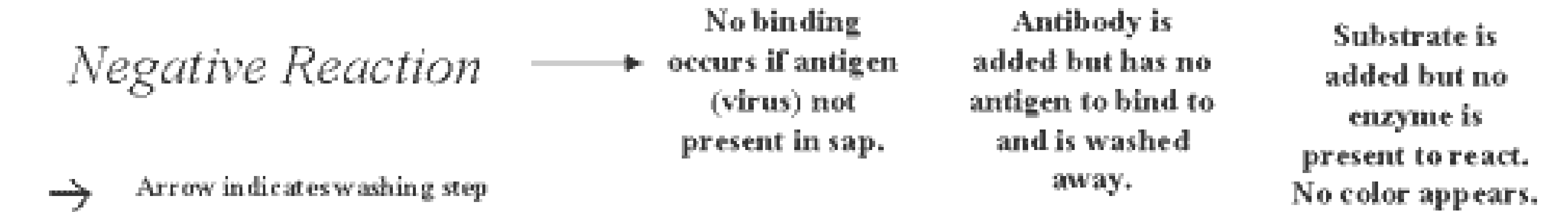
The second set is responsible for the signal visualization effect.



ELISA





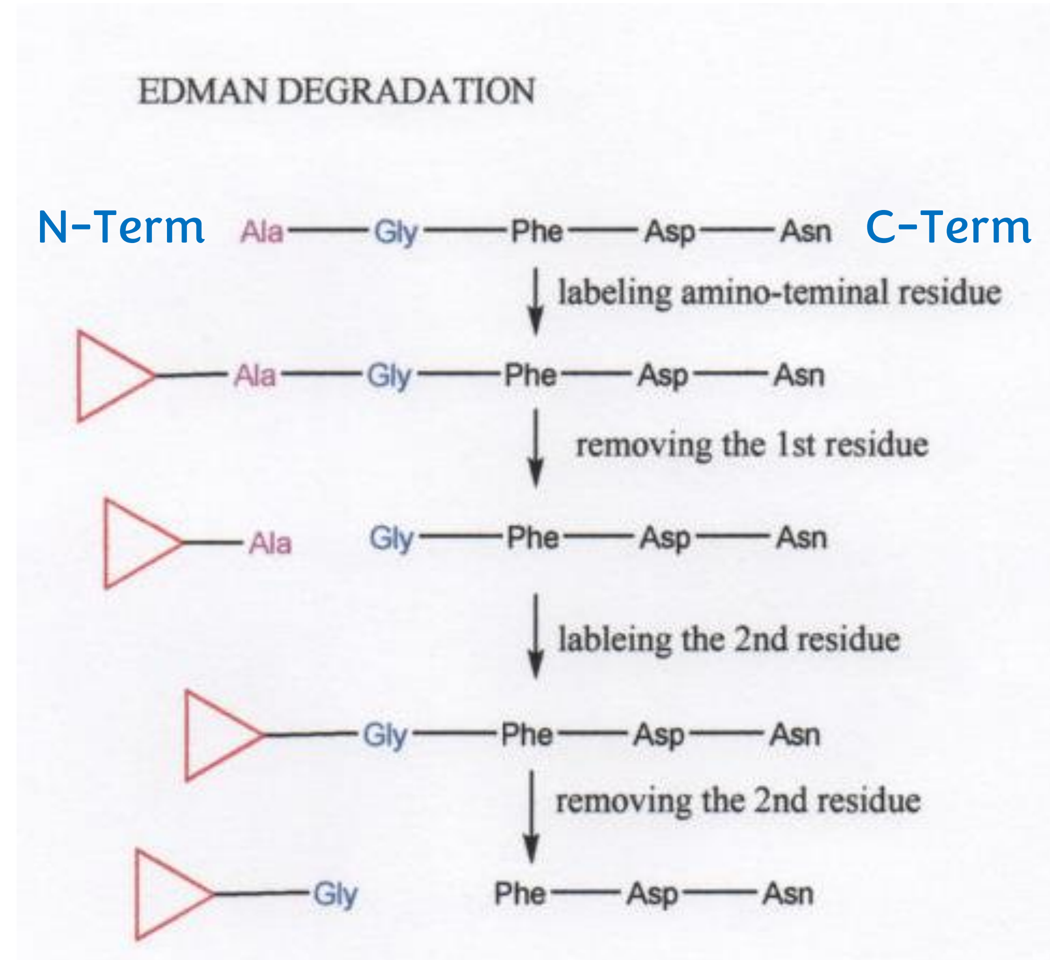


Protein sequencing

- 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

- This method utilizes phenylisothiocyanate (PITC) to react with the N-terminal residue. Don't memorize
- The resultant amino acid is hydrolyzed, liberated from the peptide, and identified by chromatographic procedures.



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

- 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

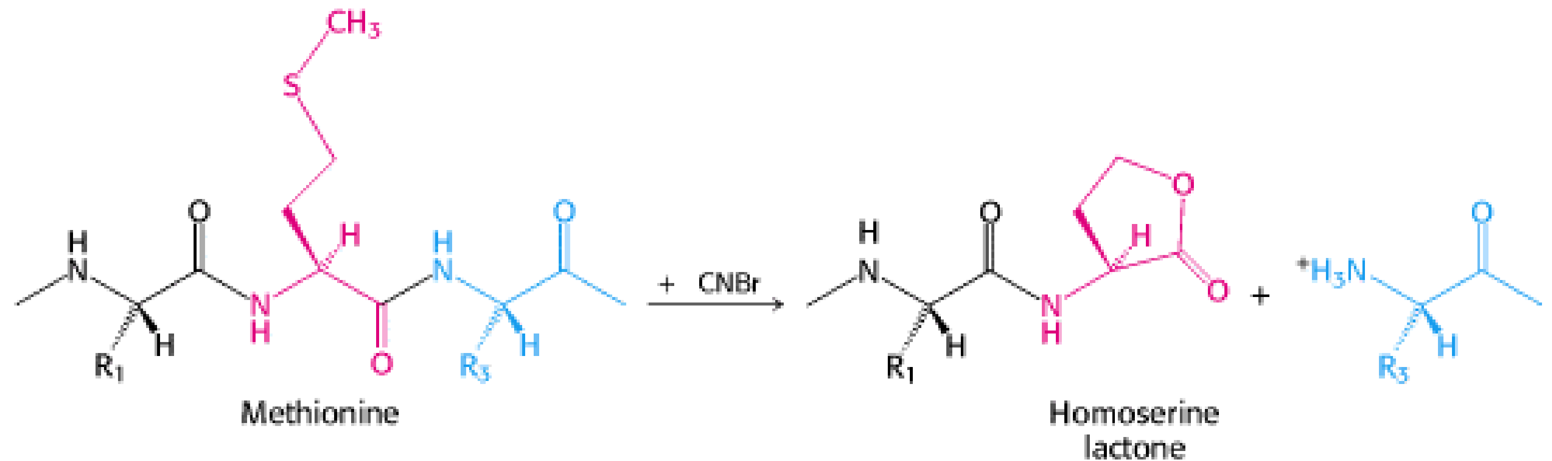
- The most commonly utilized chemical reagent that cleaves peptide bonds by recognition of specific amino acid residues is **cyanogen bromide (CNBr).**
- This reagent causes specific cleavage at the **C-terminal side of methionine** residues.
- A protein that has 10 methionine residues will usually yield 11 peptides on cleavage with CNBr.

Specific cases do not abide

Such as:

If Met is at C-Term, no cleavage occurs since no amino acid is to the C side of Met.

Understand the concept



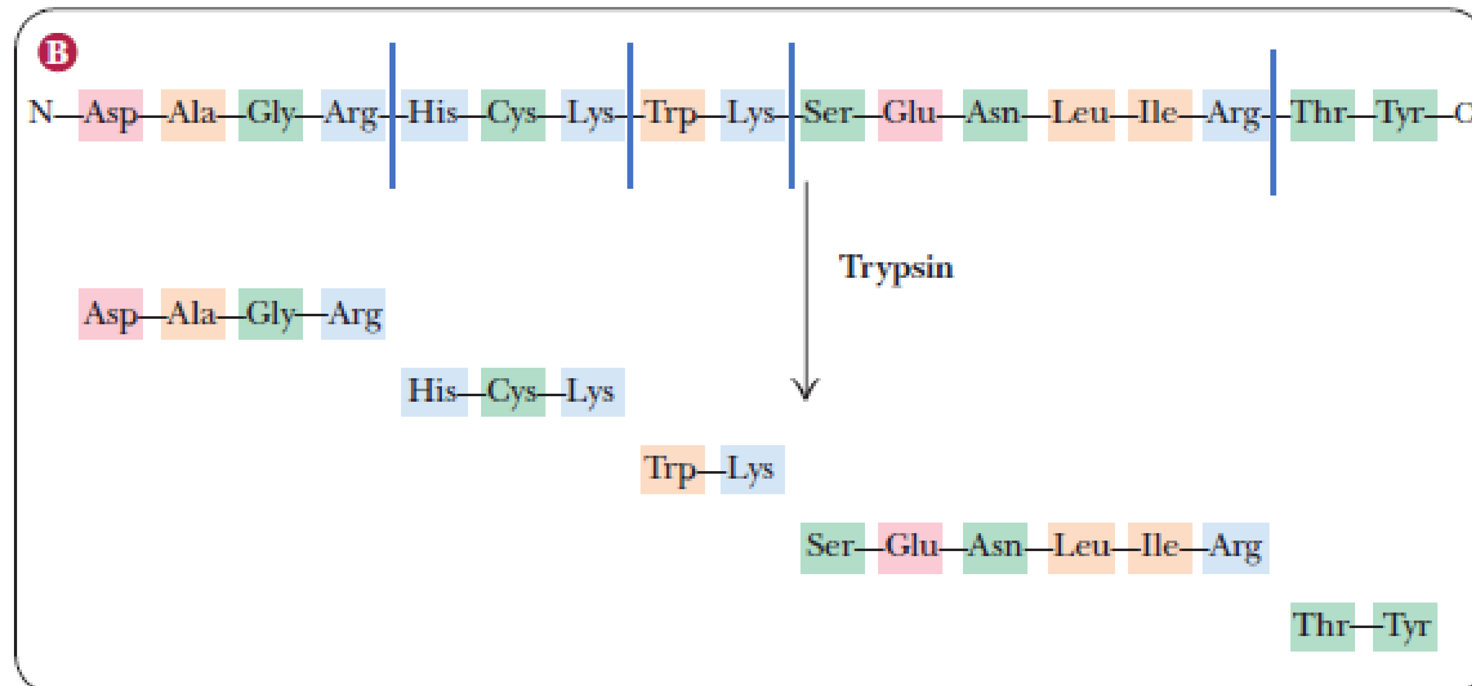
Endopeptidases

- 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.

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 <u>usually</u> yield 17 peptides on digestion with trypsin.

Another example



Other examples

	Enzyme	Specificity
Memorize	Trypsin	peptide bond C-terminal to Arg or Lys, but not if next to Pro
Memorize	Chymotrypsin	peptide bond C-terminal to Phe, Tyr, or Trp, but not if next to Pro
Memorize	Elastase	peptide bond C-terminal to Ala, Gly, Ser, or Val, but not if next to Pro
Don't memorize	Pepsin	peptide bond N-terminal to Leu, Phe, Trp, or Tyr, but not if next to Pro

Exopeptidases

- 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

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^-

- Do questions 45 and 46 (9th edition)

Check the practice questions that will be posted on TEAMS

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

Solve it as a puzzle

Try to locate similar portions of consecutive amino acids

Hint:
In this case Phe is on the C-Term because it is the only way it can exist as a C-term residue (no cleavage by trypsin) to it must be already at C-Term.

Solution:

Val—Leu—Gly—Met—Ser—Arg —Asn—Thr—Trp—Met—Ile—Lys—Gly—Tyr—Met—Gln—Phe

Use the same way as the previous slide

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

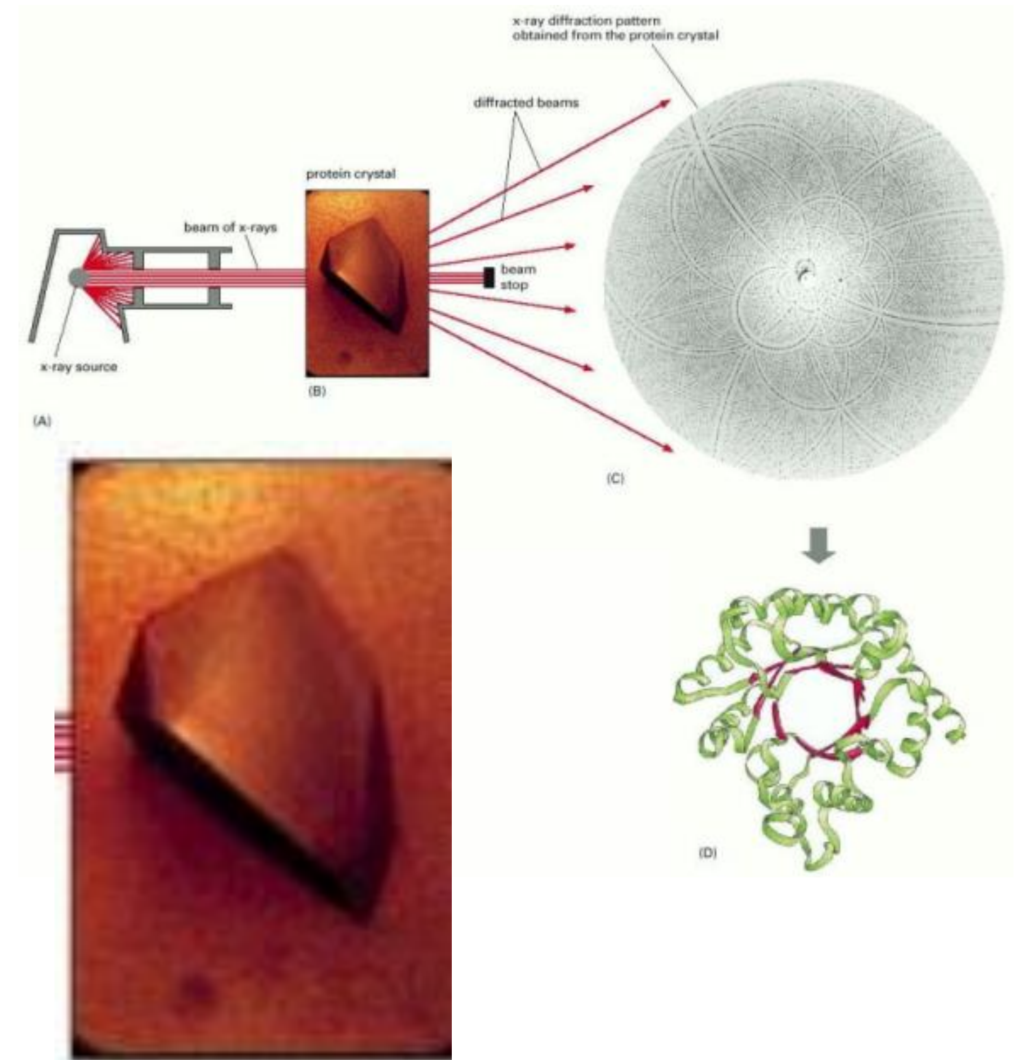
Deduce the sequence of the original peptide.

Crystallography

The sample must be pure protein.

- X-ray crystallography is used to determine the three-dimensional structure of proteins.
- A protein must first be turned into a crystal before being exposed to x-rays, which are scattered by the electrons of the molecule.

Each protein has its own pattern

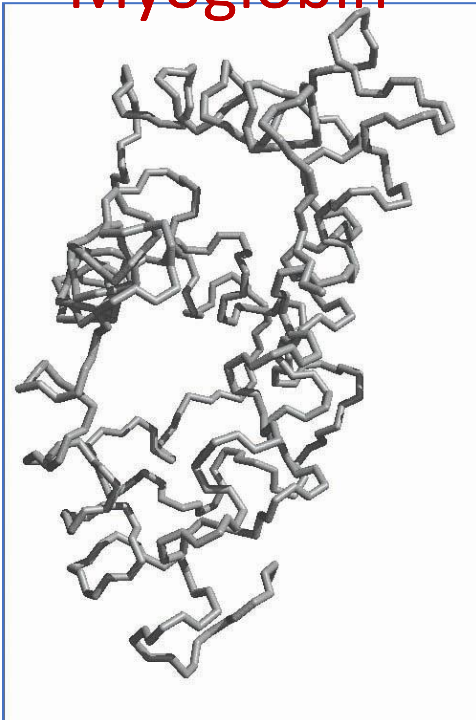


http://www.dnatube.com/video/279/Protein-Structure-Revealed-xray-crystallography

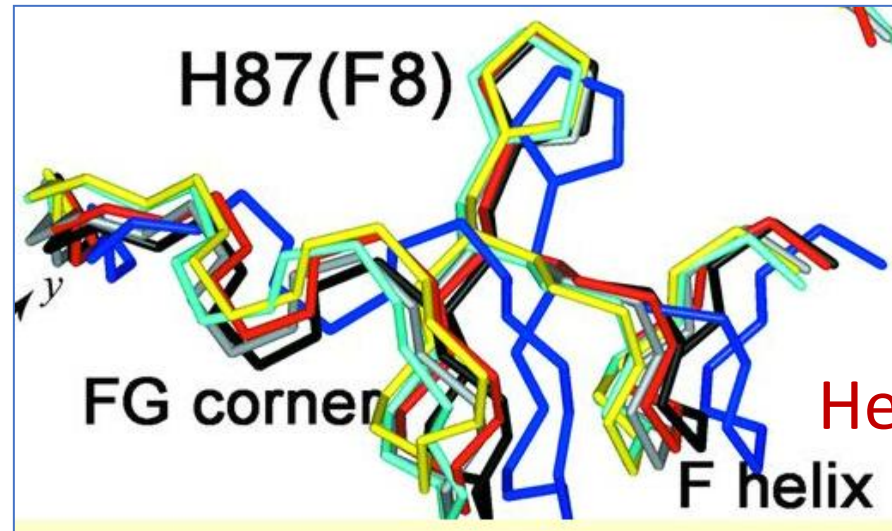
Nuclear magnetic resonance (NMR) spectroscopy

- Nuclear magnetic resonance (NMR) spectroscopy reveals the structure and dynamics of proteins in solution (not crystals).
- This is important with protein binding to other molecules like enzymes to their substrates, receptors to their ligands, etc.

Myoglobin

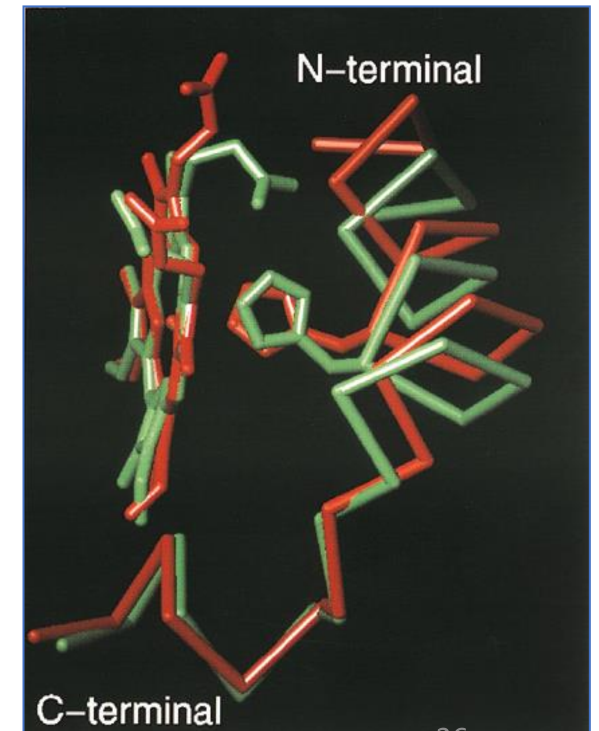


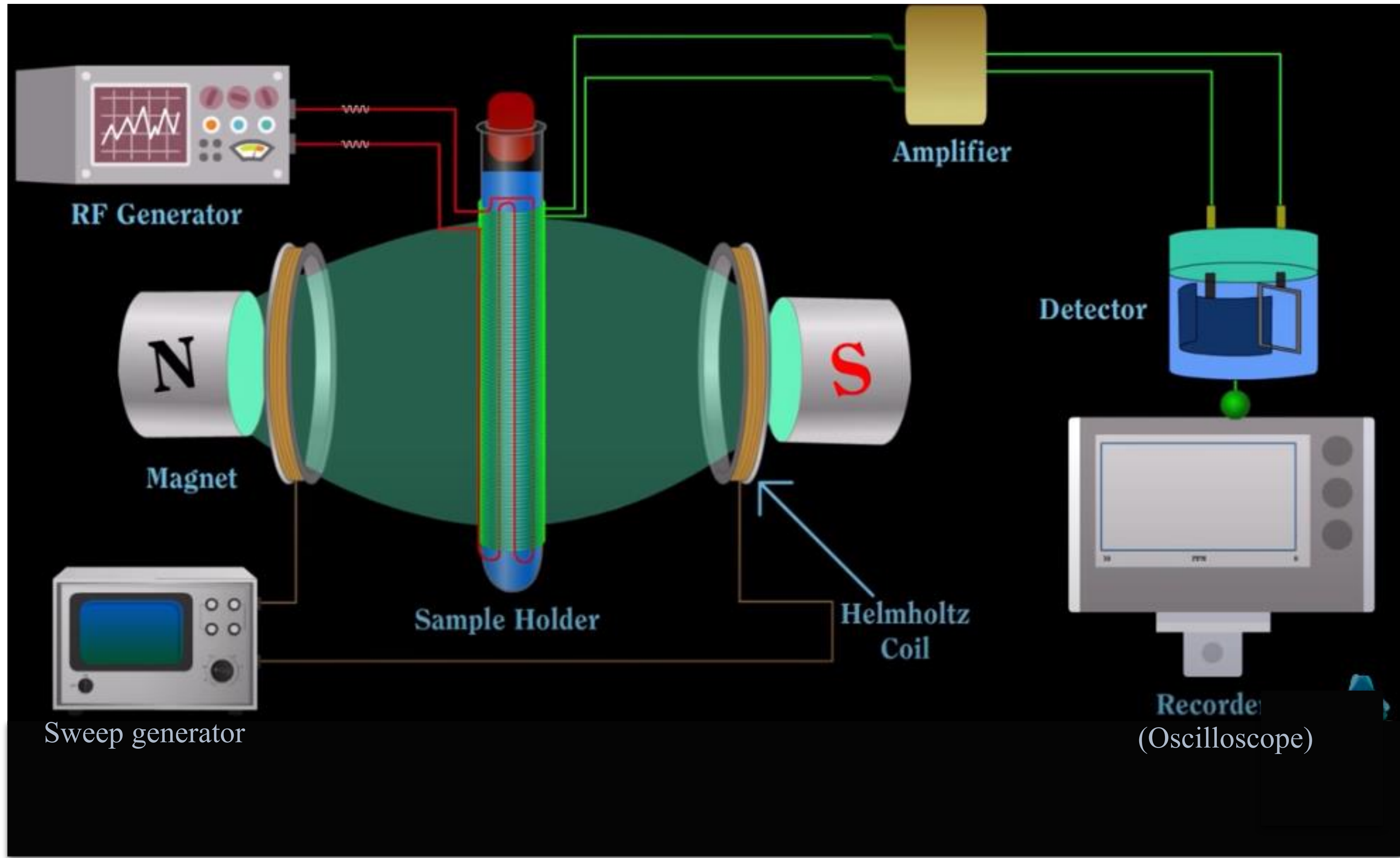
It can differentiate between T and R forms!



Hemoglobin

Myoglobin and Hemoglobin




RF Generator
Amplifier
Detector
N
S
Magnet
Sample Holder
Helmholtz
Coil
Recorde
Sweep generator
(Oscilloscope)

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

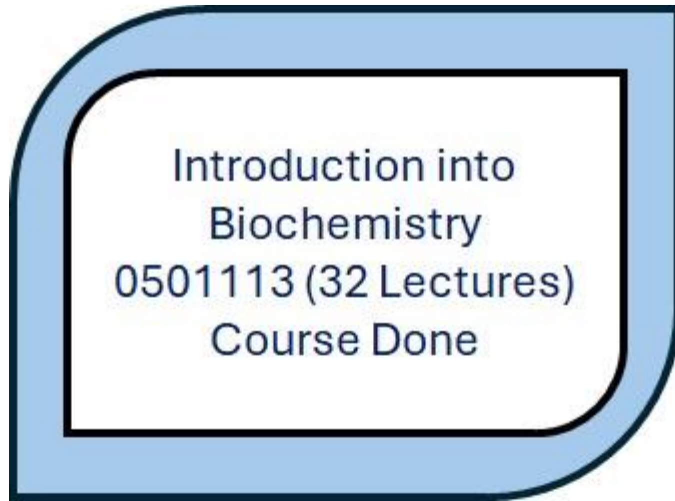
Corrections from previous versions:

Versions	Slide # and Place of Error	Before Correction	After Correction
V1 → V2			
V2 → V3			

رسالة من الفريق العلمي:

الحمد لله الذي منّ علينا بتمام هذا العمل المبارك
من يهد الله فهو السديد الرشيد
ومن يضلله فهو الطريد البعيد
اللهم لا حول لنا ولا قوة إلا بك
ارحم شيوخا ونساء وأطفالا
اللهم عليك بالظالمين فإنهم لا يعجزونك
الله الطف بغزة وأهلها يا كريم
اللهم اقهر عدونا وأخرجه من أرضنا ويسر لنا النصر التمكين
لا إله إلا أنت سبحانك، إنا كنا من الظالمين

سبحانك اللهم وبحمدك
نشهد أن لا إله إلا أنت
نستغفرك ونتوب إليك



تم بحمد الله وفضله
شكر خاص خالص لأعضاء الفريق فردا فردا
بوركتم وبورك مسعاكم

دكاترة دوبامين، لا تنسونا من صالح الدعاء