Doctor 022

Biochemistry

Sheet no. 27

Protein Analysis II

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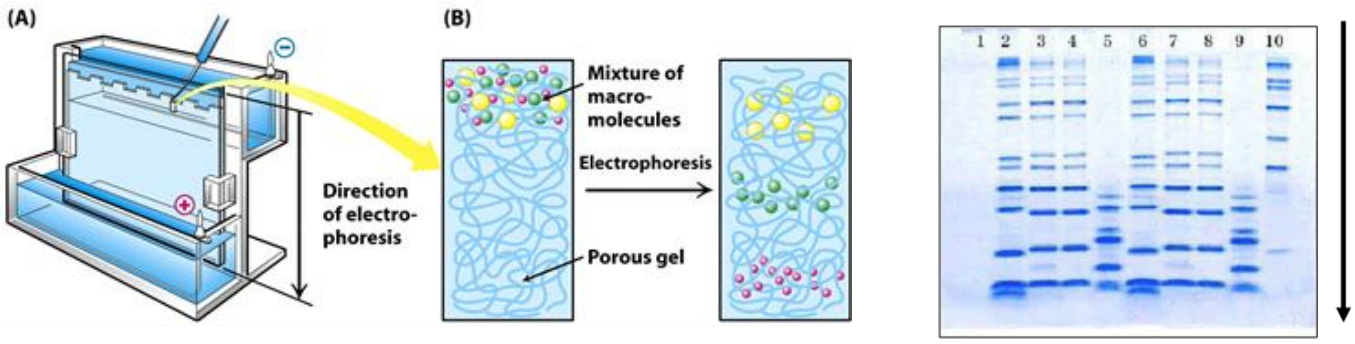
Protein Analysis II

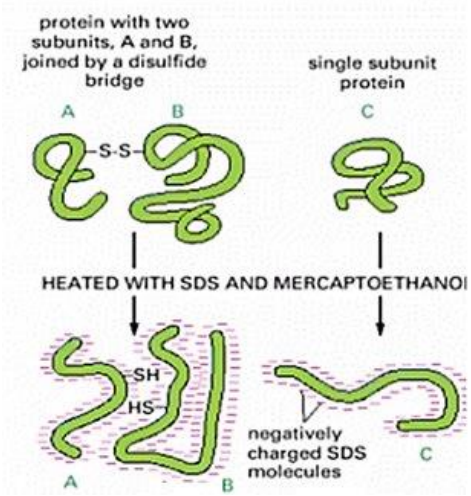
SDS-PAGE:

- **Sodium Dodecyl Sulfate SDS** (the detergent) **Polyacrylamide Gel Electrophoresis is a technique used to separate proteins <u>based on their sizes</u>. (not according to their charge because they all become negatively charged due to SDS)**

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



- **We often use reducing agents to break di-sulfied bonds → reducing SDS-PAGE.**
- **If a reducing agent isn't used → non-reducing SDS-PAGE.**

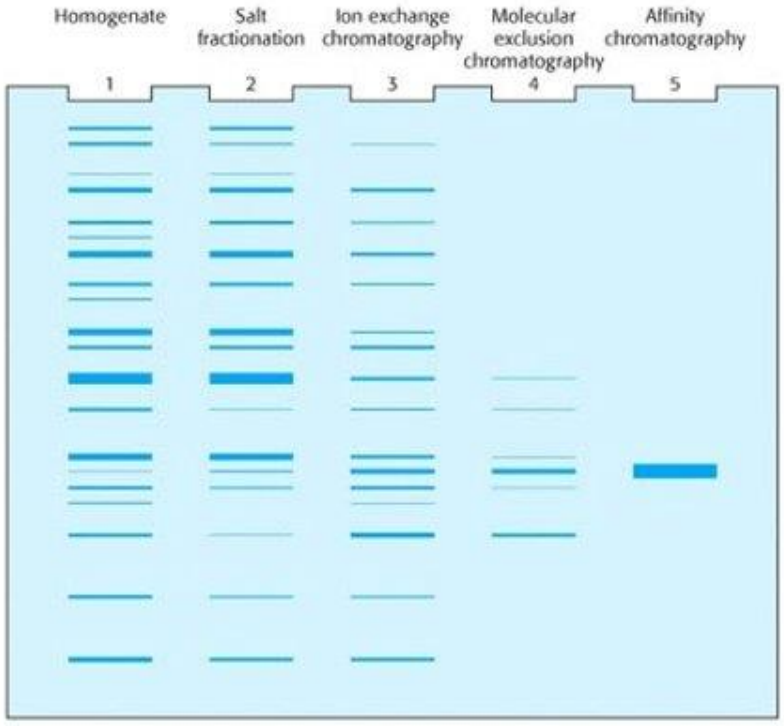


- **In non-reducing SDS-PAGE, the 2 unseparated polypeptide chains will migrate together across the gel reaching the anode.**

- **For example, If one has a weight of 40 kDa and the other has 50 kDa, they'll both have a weight of 90 kDa.**
- **Then we stain proteins and compare them to each other on the gel.**

***kDa = kilodalton (10^3 dalton)**

We can use SDS-PAGE to compare between different samples:



- **Homogenate: all proteins extracted from the cells are present in the sample.**

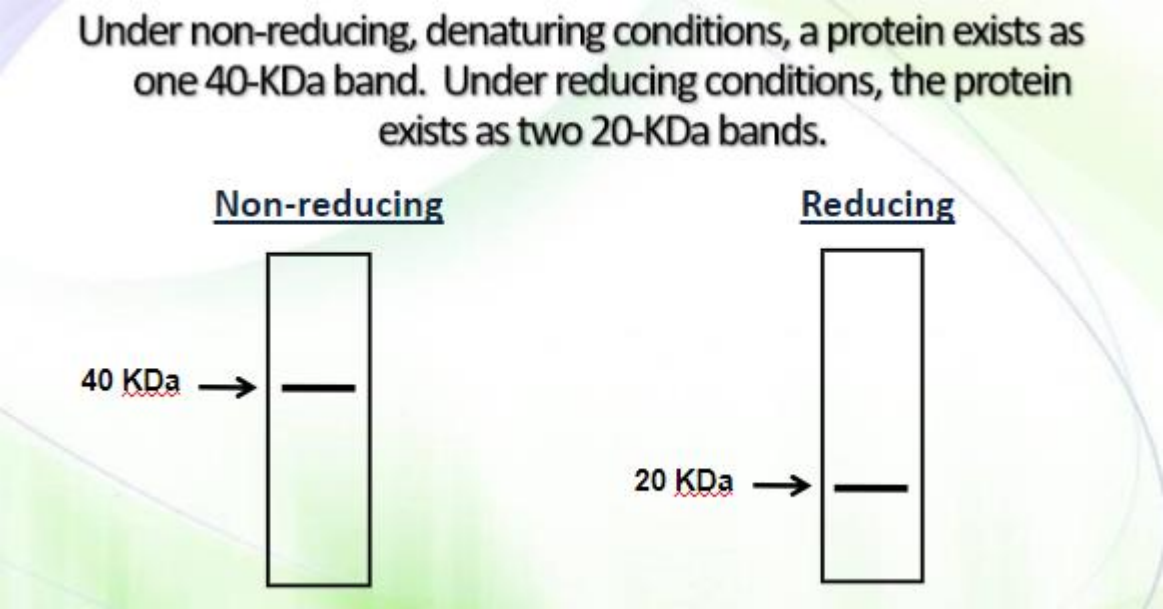
- **Salt fractionation (dirty way) and Ion exchange chromatography : poor purification.**

- **In Molecular exclusion intermediate sized proteins were present as can be seen from the figure above.**

- **Affinity chromatography is the best way to isolate proteins because it is dependent on the specific interactions of proteins with molecules.**

- **Describe the protein's structure based on the following results of SDS-PAGE:** (describe the protein's structure: whether it's a dimer, trimer or tetramer / heterogeneous or homogeneous)

⚠️ **TRIGGER WARNER: DR. MAMOUN LOVES THIS TYPE OF QUESTIONS!!!!!** ⚠️

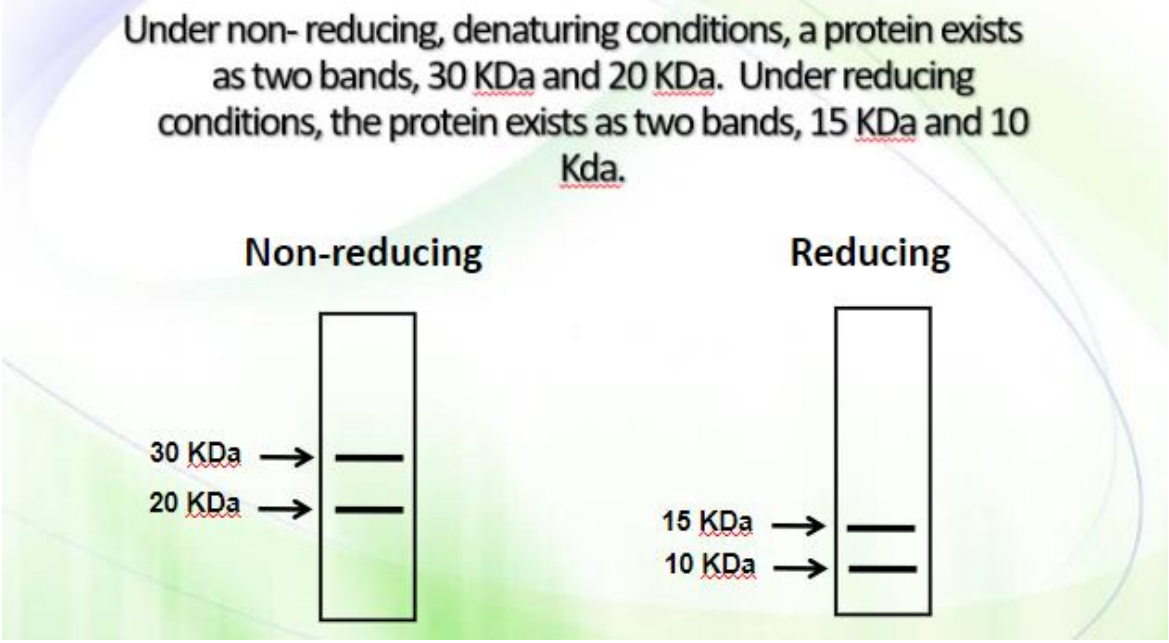


- **The word "denaturing" indicates that SDS was used. If SDS wasn't used, the protein would keep its shape, and when it migrates, the shape is going to affect the rate of migration.**

- **If the protein is elongated for example, it'll move fast and it'll move slowly if it's globular.**

- **ANS: The protein is a dimer. (when a reducing agent was added, the 2 polypeptide chains that had been covalently bonded -via disulfide bonds-, were separated) 2 polypeptide chains = 40 kDa (each is 20 kDa)**

- **We can't really decide if it's a homo or a hetero dimer! But we can assume that it is a homo dimer because both chains have equal molecular weight (20 kDa), so what are the odds that both of the chains are different? (Low possibility but we can't really tell)**



- **ANS: The protein is a tetramer (4 polypeptide chain). Disulfide bonds are present in both 30 kDa and 20 kDa bands. When a reducing agent is added to the sample, the 2 polypeptide chains that had been covalently bonded -via disulfide bonds-, will be separated.**

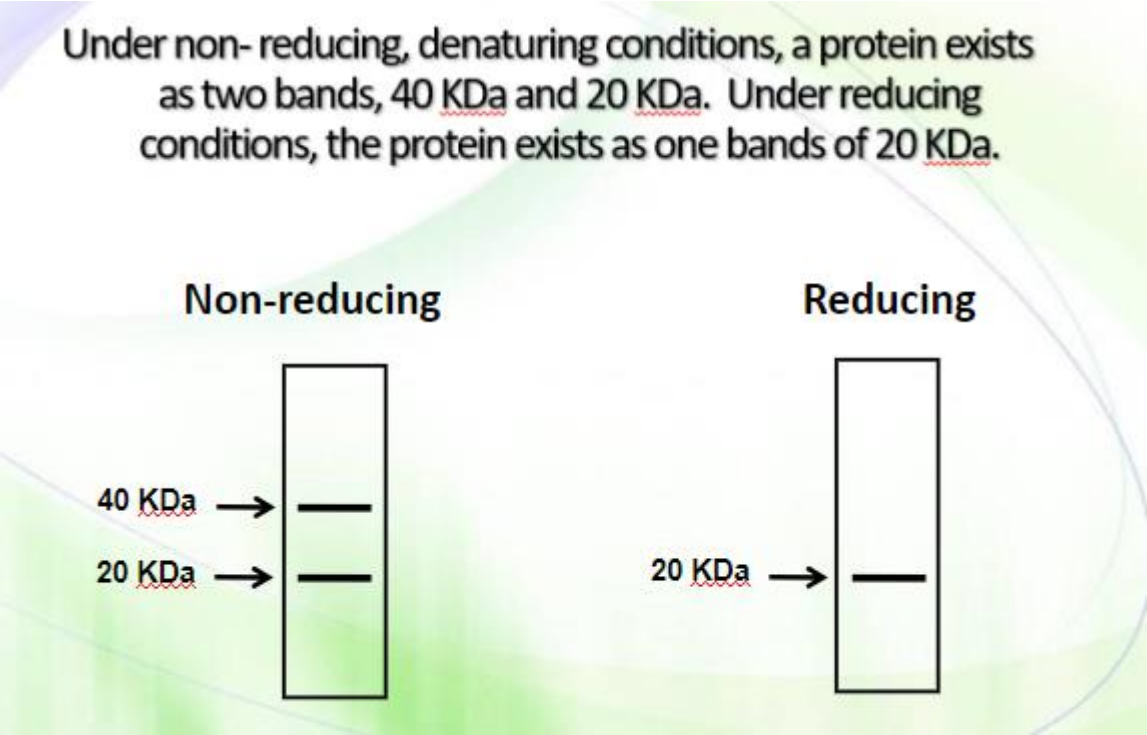
- **As if the proteins has two dimers separated from each other in the non-reducing sample, so they are connected to each other via non-covalent interactions.**

- **The protein is heterotetramer, because its chains don't have the same molecular weight; so it is highly probable that the chains are different.**

- **Q: how did we know that there were only 2 polypeptide chains?**

    - **<u>The 15 kDa band came from 30 kDa it didn't come from 20 kDa. Because if it did, we would see a 5 kDa band.</u> So, 30 kDa definitely had 2 polypeptide chains and each one has a molecular weight of 15 kDa.**
    - **<u>The 10 kDa band came from 20 kDa. It didn't come from 30 kDa because if it did, we wouldn't see a 15 kDa band.</u> So, 20 kDa definitely had 2 polypeptide chains and each one has a molecular weight of 10 kDa.**

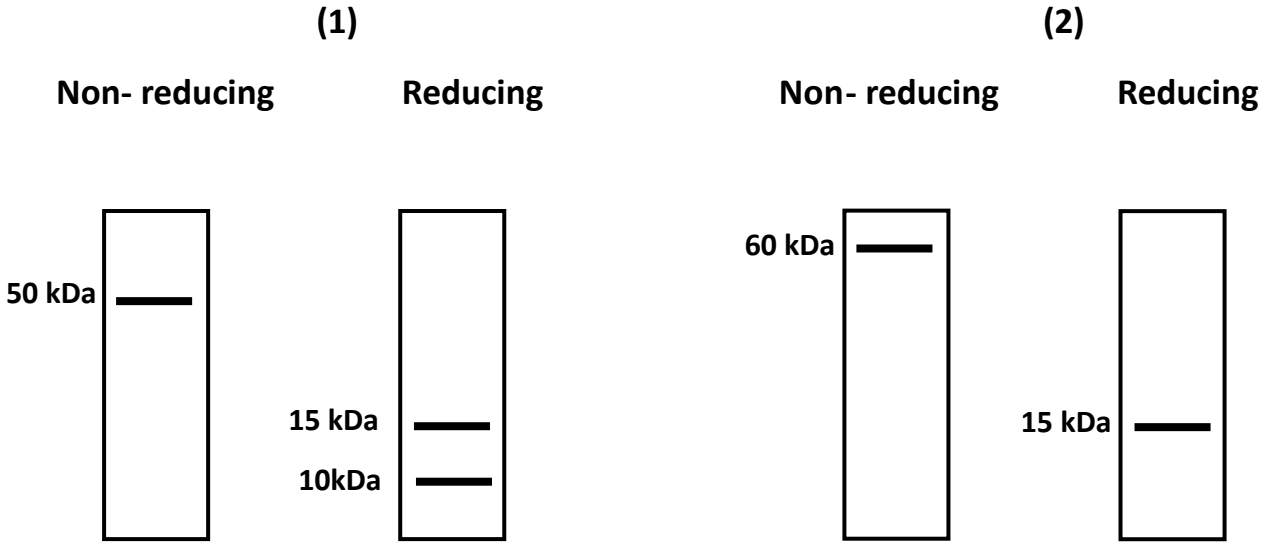
- **Note: breaking disulfide bonds within the polypeptide chain denatures it more, but it doesn't affect its molecular weight or cause it to split ***REMEMBER: when SDS denatures the protein, it breaks down the non-covalent interactions. 30 kDa and 20 kDa are bound to each other non-covalently.**



- **ANS: The protein is probable to be a trimer (3 polypeptide chains). We can assume it's a homotrimer because all 3 chains have equal molecular weight (20 kDa) but it's not necessarily homo.**

- **2 polypeptide chains are covalently bonded via di-sulfide (40 kDa). The third polypeptide chain (20 kDa) is bound to them non-covalently.**

- **EXTRA EXAMPLES: Describe these 2 proteins according to their SDS-PAGE results under denaturing conditions:**



(1): The protein is a tetramer with all its subunits connected to each other via disulfide bonds.

The subunits of the protein are: 2 (15 kDa) subunits & 2 (10 kDa) subunits.

(2): The protein is a tetramer with all its subunits connected to each other via disulfide bonds.

The subunits of the protein are: 4 (15 kDa) subunits.

Isoelectric focusing:

- **Isoelectric focusing is used to separate proteins based on their isoelectric point.**

- **Remember: Isoelectric point is the pH where the net charge for the molecule is 0 (neutral).**

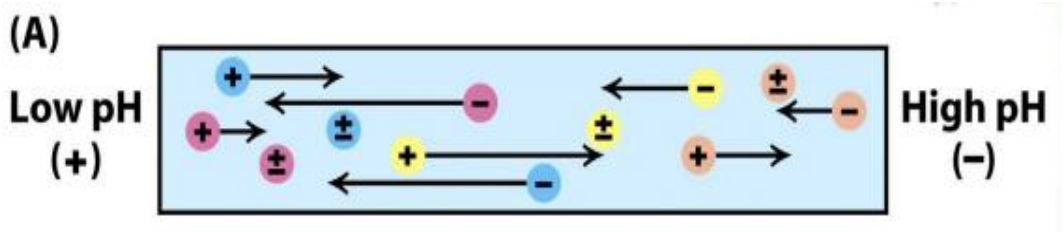
- **When the protein carries more negative charges it will migrate towards the anode.**

- **When the protein carries more positive charges it will migrate towards the cathode.**

- **So, when the protein is in its zwitterionic form (carries a neutral charge) it won't migrate through the gel.**

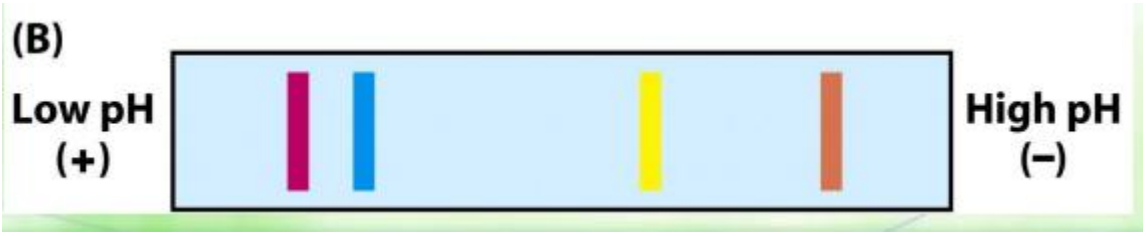
- **How it's done :**

  ⇨ **A gel is prepared with a pH gradient, this gradient is achieved by electrolytes, which are basically ions.**



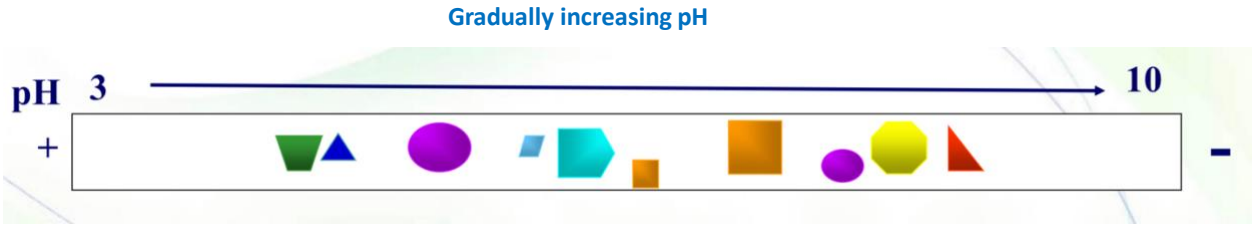
  ⇨ **As proteins migrate through the gel, they encounter regions of different pH, so the charge on the protein changes.**

  ⇨ **Eventually each protein reaches the point at which it has no net charge—its isoelectric point—and no longer migrates.**



  ⇨ **After that the bands are stained and the proteins are now arranged and separated based on their isoelectric point.**

⇨ **Each protein remains at the position on the gel corresponding to its pI, allowing for separation of proteins.**

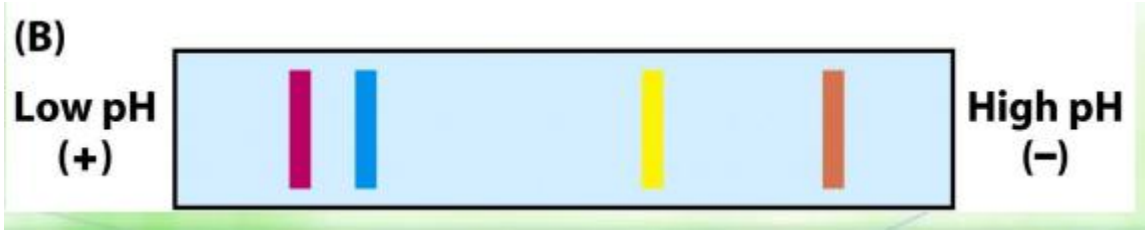


Two-dimensional gel electrophoresis (2D-PAGE):

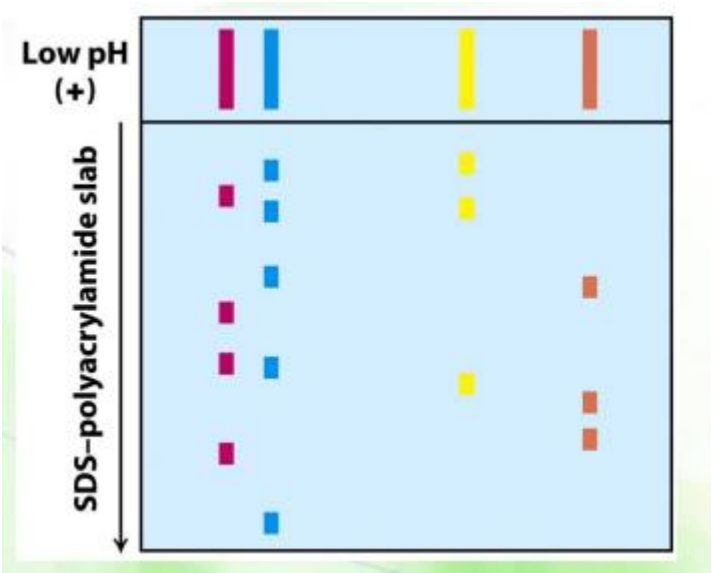
- **A better method to separate proteins more precisely is by combining the two methods together, <u>isoelectric focusing for charge</u> then <u>gel electrophoresis for size.</u>**

- **In 2D-PAGE, proteins are separated by, first, isoelectric focusing, then through an SDS-PAGE.**

- **Firstly, the sample is arranged in isoelectric focusing in the first dimension, horizontally for example (proteins are separated by charge).**



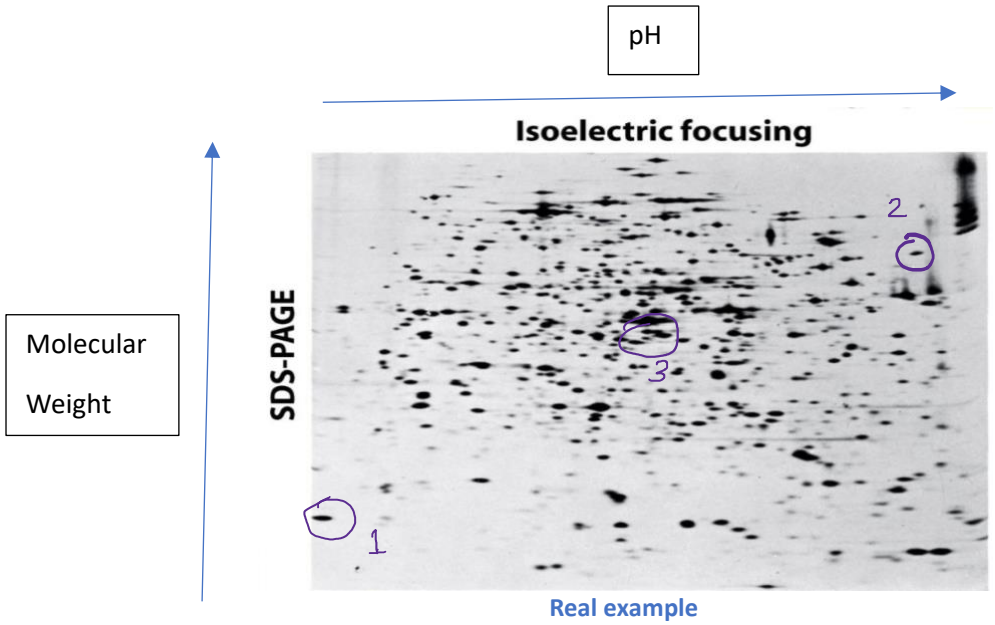
- **Then the proteins of the sample migrate through gel electrophoresis in the other dimension, vertically for example (proteins are separated by size)**



- **Thus, proteins are separated based on both charge and size.**

- **This way, we have more separation & resolution between proteins.**

These two bands could have been really close to each other in regular SDS-PAGE gel electrophoresis, but with 2-dimensional gel electrophoresis, they are also separated by charge.



Real example

(Every spot is several protein molecules not a single protein)

- **How can you describe the proteins in spot (1)?**

  - ⇨ **Acidic, because the proteins have a low isoelectric point.**
  - ⇨ **Low molecular weight, because of the relatively long distances of their migration through the gel.**

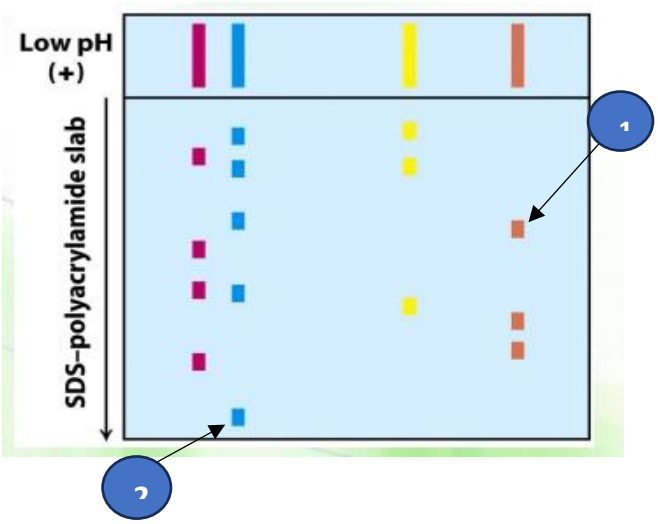
- **How can you describe the proteins in spot (2)?**

  - ⇨ **Basic, because the proteins have a high isoelectric point.**
  - ⇨ **High molecular weight, because of the relatively short distances of their migration through the gel.**

- **How can you describe the proteins in spot (3)?**

  - ⇨ **Neutral, because their isoelectric point is neither high nor low.**
  - ⇨ **Intermediate molecular weight, because they are present in the middle of the gel.**

- **We usually have a standard to compare the sample to, to collect quantitative data about the molecular weights of the sample proteins.**



- **Based on the above sample for example, we can conclude that fragment number 1 has high isoelectric point with intermediate molecular weight. And fragment number 2 has low isoelectric point with low molecular weight.**

Immunoblotting (Western blotting):

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

معنى blot لطخة (تدل على الصورة اللي بتظهر)

A) Proteins are separated (Based on size) in a gel using SDS-PAGE.

B) Gel is transferred (as is) to piece of paper or membrane & the distances between the proteins on the gel won't be affected.

C) Adding antibodies to the membrane (2 types of antibodies will be added), the 1st antibody will bind to proteins specifically, and the 2nd antibody (with enzyme on it) will bind to 1st. , then we add substrate to the enzyme and the enzyme will turn it to product with color , the color appears on the primary antibody (the 1st antibody).

D) We detect the color , it appears as blot (stain).

More detailed explanation:

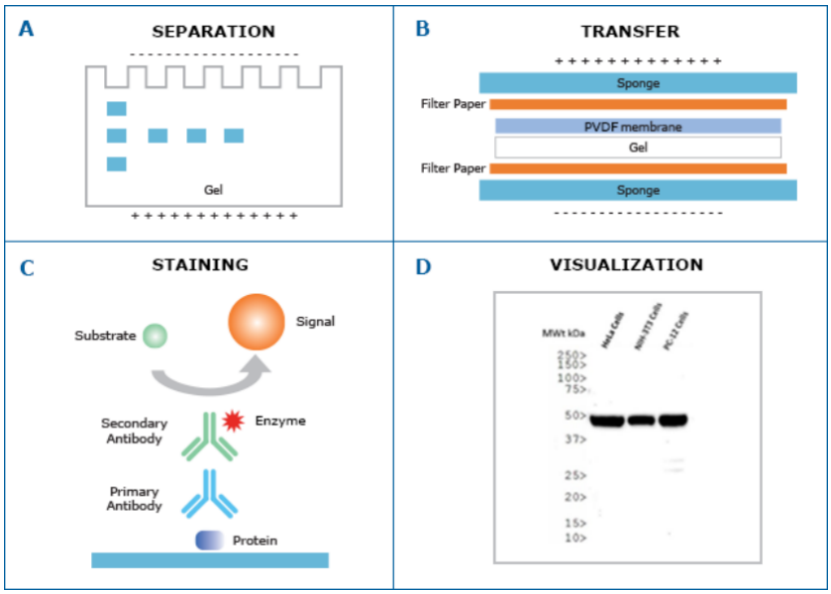
- **We use immunoblotting to detect proteins & to know if they are present in a certain sample or not.**
- **We get the primary antibody when we inject the protein (which we want to detect in the SDS-PAGE sample) in an animal (a rat for example), then their body makes antibodies that are specific for that protein, which are the primary antibodies.**
- **We get the secondary antibodies when we inject the primary antibody in another species so that their body makes antibodies that are specific for the primary antibody, & can bind by its Fc region to enzymes that catalyze a specific reaction which results in a blot or a stain on the membrane, those are the secondary antibodies.**
- **The stain or the blot on the membrane mean that the protein is present in the sample.**

- **The benefits of this method is : Knowing if a specific protein exists in this sample or not (when we detect signal this means the protein is there), knowing the size of the protein (low , high , or intermediate), and knowing the level of expression of the protein (the level : high or low in a cell).**

- **For example: when we are comparing between a sample of a patient & a sample of a healthy person, the Western blot technique can help me to know what protein is missing in the patient's sample & how it's level of expression has been affected.**

- **Remember: we know the size of the protein relative to other proteins in the sample from the distances they migrate in the SDS-PAGE gel.**

- **Note : We add the enzyme to the 2^{nd} antibody because the numbers of primary antibodies are large and it is hard to put the enzyme on it.**



ELISA:

- **Enzyme-linked immunosorbent assay.**
  When you see "immuno" word , guess that there is using of antibodies.

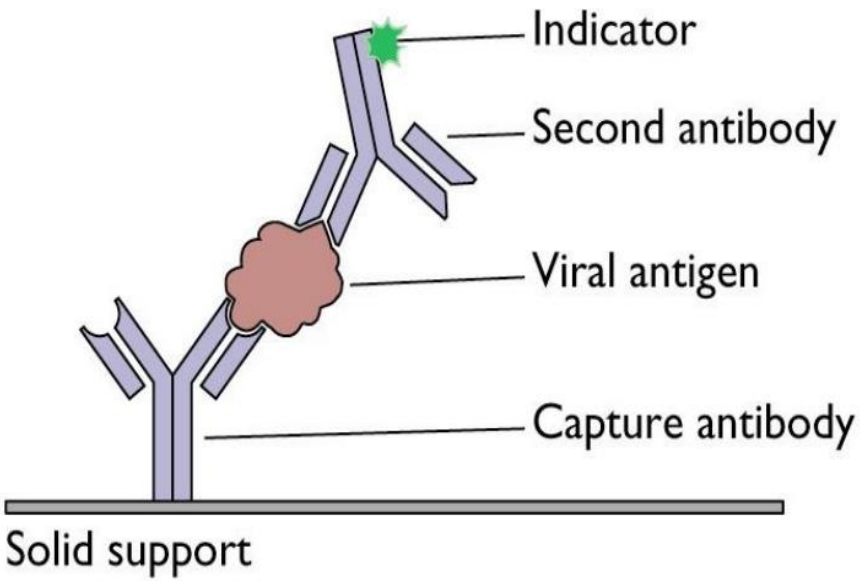
- **Same concept as immunoblotting but rapid, convenient, and sensitive (less than nanogram (10-9 g) of a protein)**

- **The concept : They take primary antibody against specific protein , then they put it on a surface , then they add patient sample to it , if the protein exist in the sample it'll bind to the antibody, if not, nothing will bind, then we remove the patient's sample , we only keep the antibody bound to the surface , then we add other antibody (with enzyme on it) that will bind to the same antigen on the 1st antibody (see the figure), & an enzyme will bind to the 2nd antibody.**

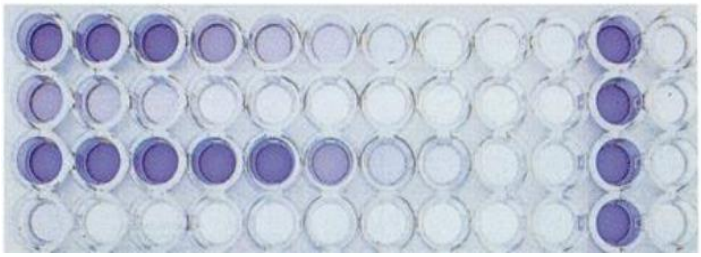
- **The enzyme (on the 2nd antibody) function is : bind to substrate & turn it to product and gives a color (signal/ indicator) , this happens after 2nd antibody bind to the antigen.**

- **Depending on the color amount we can guess the protein amount.**

- **So the benefits of this method os to know of the protein exist or not, and to know the amount of the protein.**

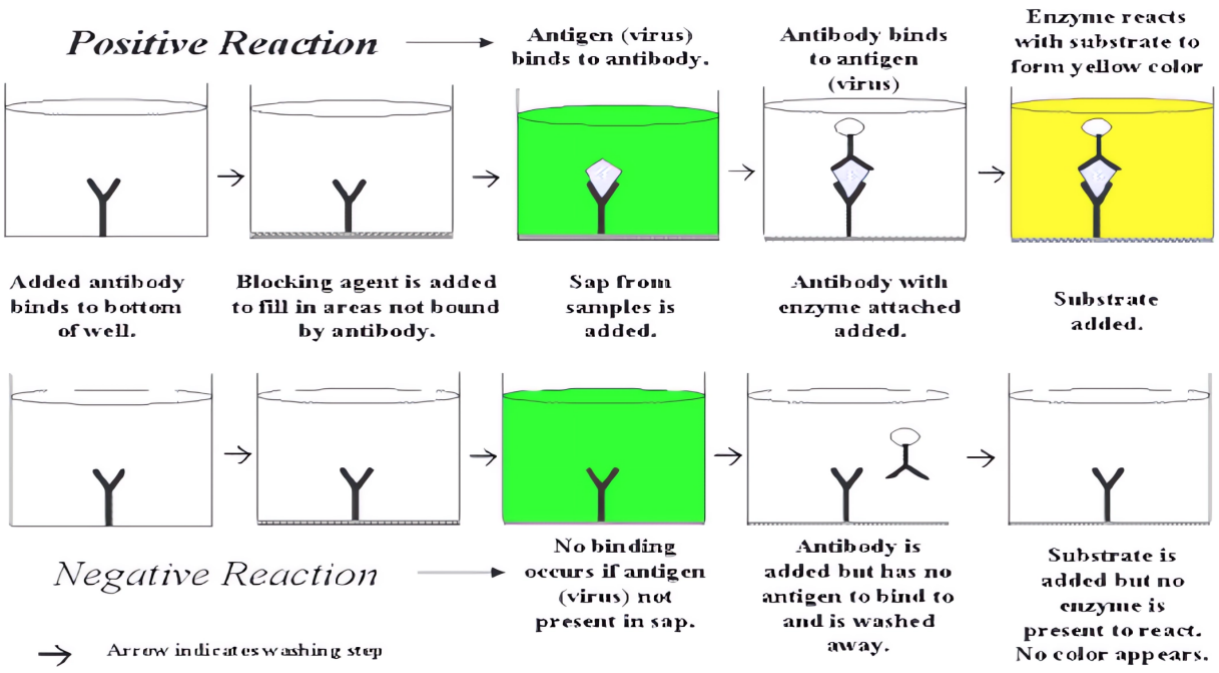


- **ELISA is the standard assay used in clinical laboratories.**
- **Low cost , fast , and automated.**
- **In ELISA the solid surface has wells as shown in the picture below:**



- **Notice the difference in color intensity between the samples, which reflect the difference in antigen concentration.**

- **This figure shows the ELISA steps that we wrote up.**



- **ELISA is of significant use in researches, as it can be used as a bio-marker.**

- **After adding the sample to the wells, you need to wash the unbound proteins away from the well, so that the proteins bound to the antibody are the only ones left.**

- **Then, after adding the second antibodies which are bound to the enzymes, you need to wash the wells before adding the substrates so that only 2nd antibodies bound to the antigen remain & the color appears only when the antigen (protein) is present in the sample.**

- **To quantitavely estimate the amount of the protein in a sample, then we compare the intensity of the color of the unknown sample to a standard sample.**

Protein sequencing:

- **Protein sequencing is basically the process of knowing the amino sequence (primary structure) of a protein or a peptide.**

- **Knowing the primary structure of a protein is important, because if we know the primary structure, then we can know the secondary & tertiary structure of a protein & a lot of other features.**

- **One technique is known as Edman Degradation.**

- **The technique of Edman Degradation:**

- **A certain chemical is added to the peptide.**
  The chemical then binds to the first amino acid in the peptide (on the N-terminus).

- **The chemical then cleaves the first N-terminus amino acid, as it breaks the peptide bond between the first & the second amino acid.**
  We take the released amino acid, and identify it by chromatographic procedures.

- **Then we repeat the steps again so we can identify the amino acids & their sequence along the protein.**

- **But there is an issue here. The peptide can be very long, it can be as long as thousands of amino acids, and it is unpractical to use this way of protein sequencing.**

- **There are other methods which are more practical, such as cleavage methods, which work by cleaving the proteins by cleaving them into smaller peptides.**

- **There are several cleavage methods which facilitate protein sequencing:**
  1) **Chemical digestion**
  2) **Endopeptidases**
  3) **Exopeptidases**

- **Quick terminology:**
  - ⇨ **Peptidases: enzymes which cleave peptides**
  - ⇨ **Endo-: within the molecule**
  - ⇨ **Exo-: at the ends of the molecule**

- **There are (carboxy-) & (amino-) exopeptidases.**
  - ⇨ **Amino-exopeptidases cleave the first amino acid in the peptide**
  - ⇨ **Carboxy-exopeptidases cleave the last amino acid in the peptide**

Chemical Digestion:

- **We utilize a chemical called cyanogen bromide (CNBr).**

- **CNBr cleaves the peptide bond between every methionine residue & the amino acid which comes afterwards.**

- **So, a protein that has 10 methionine residues will usually yield 11 peptides on cleavage with CNBr, except if the protein has terminal methionine residues or consecutive methionine residues.**

  - ⇨ **If a peptide turns into smaller peptides upon the addition of cyanogen bromide, what does it mean?**
    It means that this peptide contains methionine residues.

  - ⇨ **If the number of those smaller peptides are 11 for example, what does this mean?**
    This means that the peptide contains 10 internal nonconsequtive methionine residues.

⇨ **If we already know that a peptide contains 10 methionine residues, but upon the addition of CNBr, the peptide yielded 10 smaller peptides, what does that mean?**
It means that one of the methionine residues is at the end of the peptide.

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.**

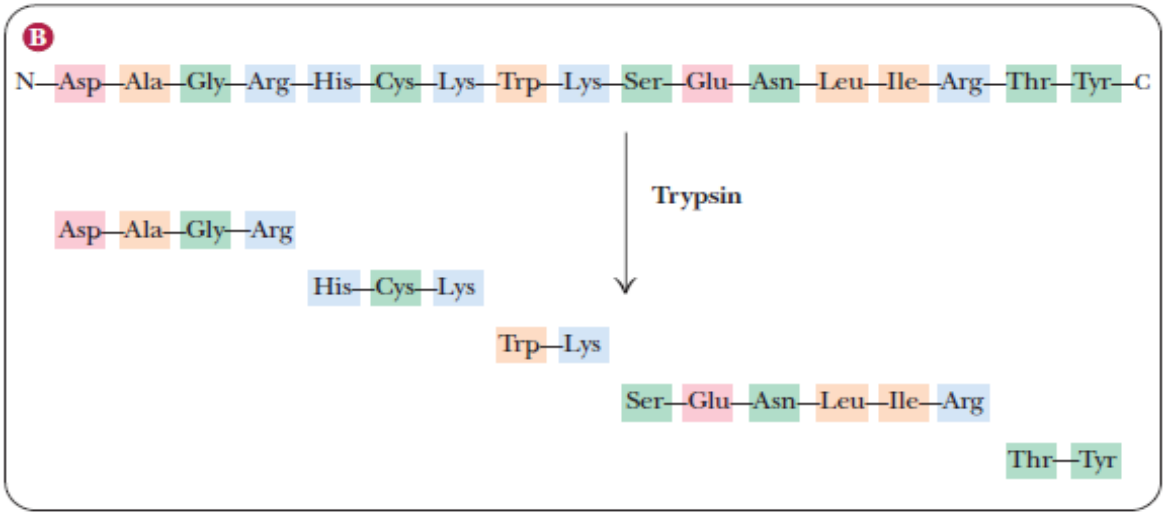
- **so, I can take these peptides, put them with Edman degradation because they become smaller and I can use it 50 times.**

- **For example: We have trypsin (we've said before that trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues). Therefore, A protein that contains 9 lysine and 7 arginine residues (16 residues) will usually yield 17 peptides on digestion with trypsin.**

- **If there is lysine followed by arginine what will happen??**

  There will be cleavage after lysine & after arginine also, so arginine will go out as a single amino acid that will result in 3 products, which are: 2 peptides chain and a single amino acid.

- **If we are talking about CNBr that cleaves after Met and it is the first amino acid residue, it will go out as a single amino acid.**



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 next to Pro

- **Pepsin is different from others it cut before Leu, Phe, Trp not after them and if the Pro before any of them it can't cut peptide bond.**

Q: 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 thesequence 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

To solve this question, you look to the similar sequence of amino acids & compare them between different aliquots & connect them, and so on (like solving Lego or puzzles)

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—Gly—Phe

Q) 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.

You should know the amino acids (positive, negative) and where exactly did the chymotrypsin cleave to solve these problems.

Answer:

Met—Val—Ser—Thr—Lys—Leu—Phe—Asn—Glu—Ser—Arg—Val—Ile—Trp—Thr—Leu—Met—Ile

Do this exercise 

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^-

Also do questions 45 and 46 Campbell (9th edition)

Exopeptidase:

These are enzymes that cleave amino acids starting at the end of the peptide.

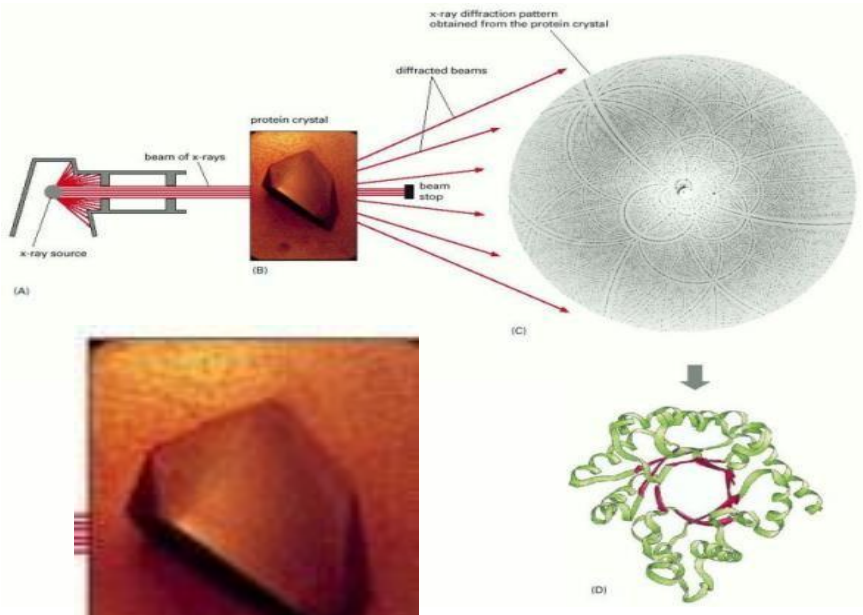
There are two types:

1) **Aminopeptidases that cleave at the N-terminus**
2) **Carboxypeptidases that cleave at the C-terminus**

Crystallography:

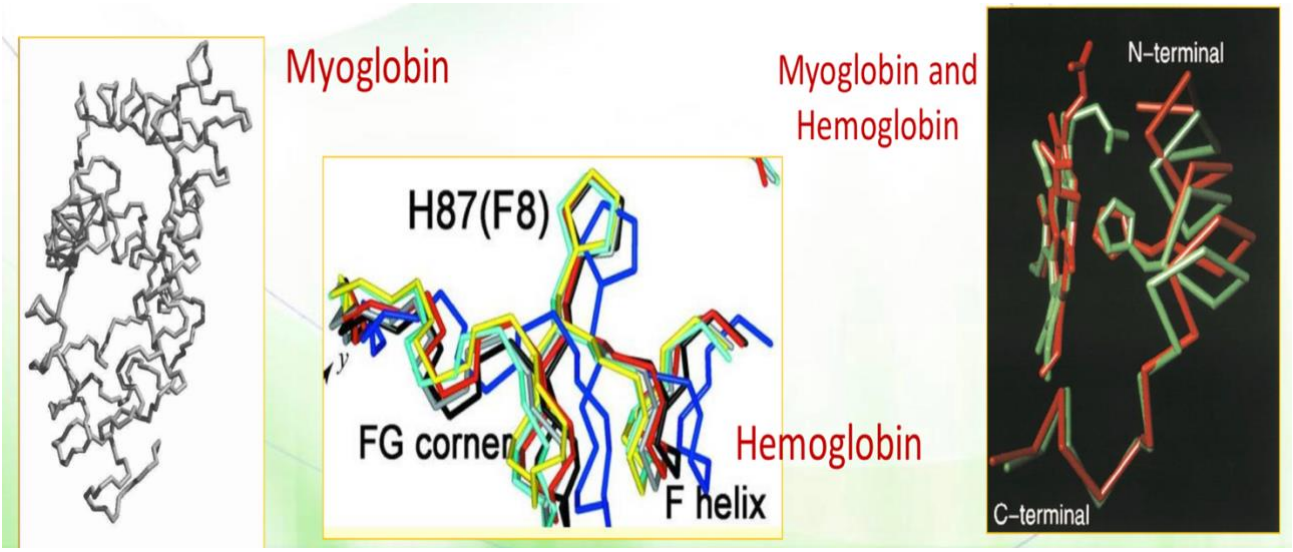
- **Which is a really old technique.**
- **Basically, the protein is taken pure (by chromatography), then we take a single protein molecule, and we turn it into a crystal by removing water from it, and then, we expose the crystallized protein to x-rays, & the atoms of the protein will scatter the x-rays.**
- **Depending on the pattern of the detected scattered x-rays that appear on the plate, we can determine the structure of the protein.**
- **It is a lengthy process.**
- **X-ray crystallography is used to determine the three-dimensional structure of proteins.**
- **The protein structure is really important to know the function, make drugs against specific proteins.**

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



Nuclear magnetic resonance (NMR) spectroscopy

- **The advantage of this technique is that the protein is present in liquid solution (right environment & the same one as in the body).**
- **If we take a protein and dry it up (take water from it) as in crystallography, some structural features might change because it's not in the right environment (different from the environment in our body).**
- **We use the magnet to know the structure.**
- **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.**
- **For example: we can use crystallography or NMR with hemoglobin when it is bound & unbound to oxygen, and then compare the structure of both states (T-state or R-state) to know how is the structure of hemoglobin affected by the binding of oxygen.**



These are the techniques which we use to know the structure of the protein, but nowadays we use AI.