27

BIOCHEMISTRY

Faculty of medicine – JU2018

◉ Sheet ○ Slides

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GEL ELECTROPHORESIS (SDS-PAGE)

What does Electrophoresis mean?

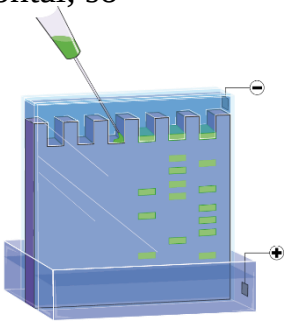
-It is a phenomenon indicates a molecule with a net charge that runs over a gel.

We used to separate DNA molecules according to its sizes using Gel Electrophoresis in the last semester and now we are also going to separate peptides according to their sizes using electrophoresis, but what is the difference between these two procedures

Well, we have some **differences**

1-The Gel Electrophoresis in the Peptides is Vertical rather than horizontal, so the wells are located above the Gel

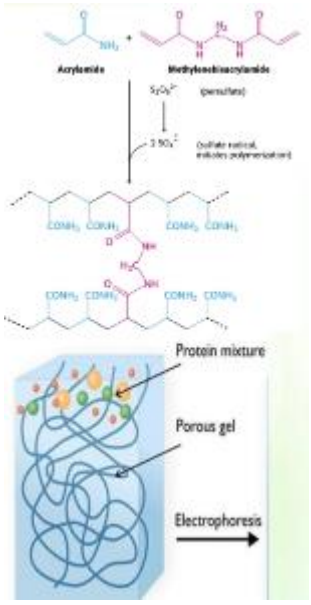
2-The Used Gel is **Polyacrylamide gel** rather than agarose



-Why don't we just use agarose?!

a-because **Polyacrylamide** is chemically **inactive**, so it doesn't interact with protein and doesn't disturb their structure

b-**Polyacrylamide gel** is formed by the polymerization of **Acrylamide** (forming the polyacrylamide) followed by the Cross linking by **Methylenebisacrylamide** this results in the formation of pores through the gel which allows the separation of the peptides with very high resolution so it can distinguish the peptides, even if the difference in length is one amino acid (Remember we separate in the basis of sizes) .



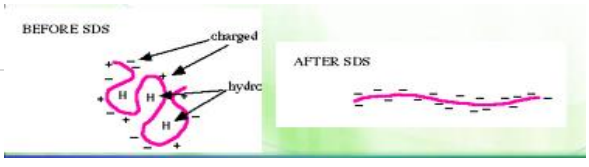
3- The third difference is about the charges, all the DNA molecules have a net negative charge allows it to move from the cathode (-) to the anode (+) (same direction different velocities), in the other hand different proteins have different net charges according to their ionizable side chains

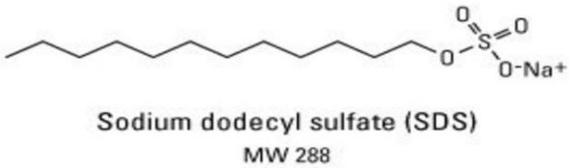
*To unify the charges on each protein, a detergent called **sodium dodecyl sulfate** (SDS) is used.

What does the SDS do?

a-Denature the protein by disturbing the hydrophobic interactions which hold the 3D structure of the protein

b-**SDS** attaches to the protein in a ratio 2:1 which means that every 2 adjacent amino acids are attached to one SDS molecule so that all the proteins will have a uniform negative net charge



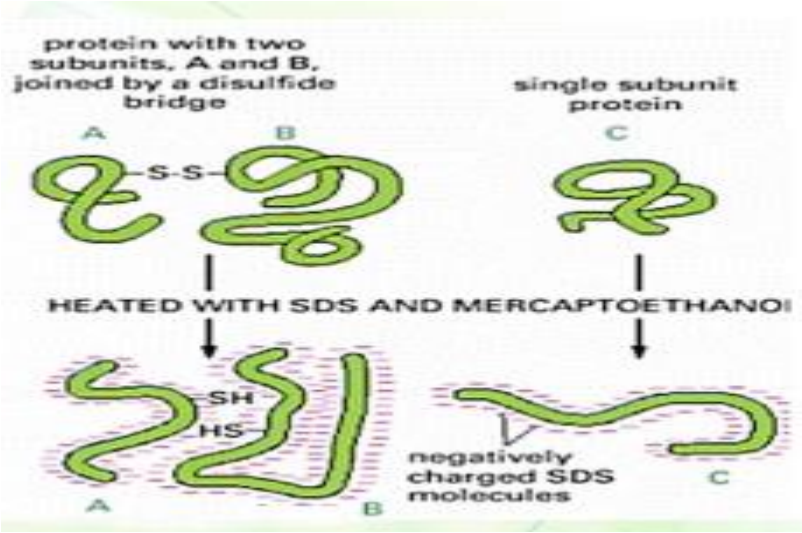


Sodium dodecyl sulfate (SDS)
MW 288

-A reducing agents could also be added such (**B-Mercaptoethanol**) and (**Dithiothreitol**) which is known as "DTT"

-**What does reducing agents do?**

-They break "reduce" disulfide bridges, which results in the loss of the tertiary and the quaternary structure of the protein (Separating the subunits from each other).



-**Describe the movement through the gel.**

-When the electrical current is applied, all the peptides start moving from the top to the bottom in one direction (toward the anode) according to size only

-Small molecules move readily through the gel whereas bigger molecules are slower

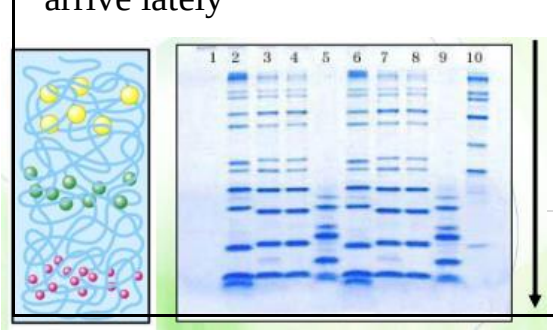
-After the run, proteins are stained to reveal the positions of the bands

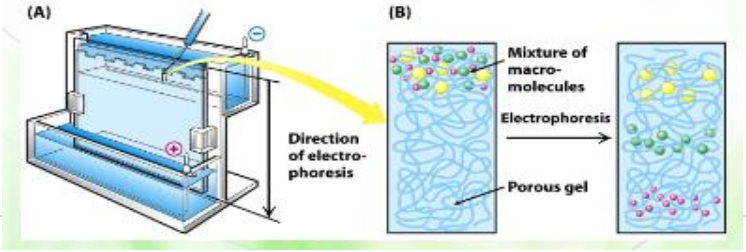
-They move as bands because they take the shape of the well

-The darker the band the more amount of proteins presents in this band

-**(Misunderstanding) As the bigger proteins contain higher number of amino acids, they will attach to higher number of SDS resulting in a higher negative charge, so why don't they reach the anode faster?**

-This is mainly because the bigger proteins experience a great frictional force while passing through pores, so the net force applied to them is small compared to small proteins so they arrive lately

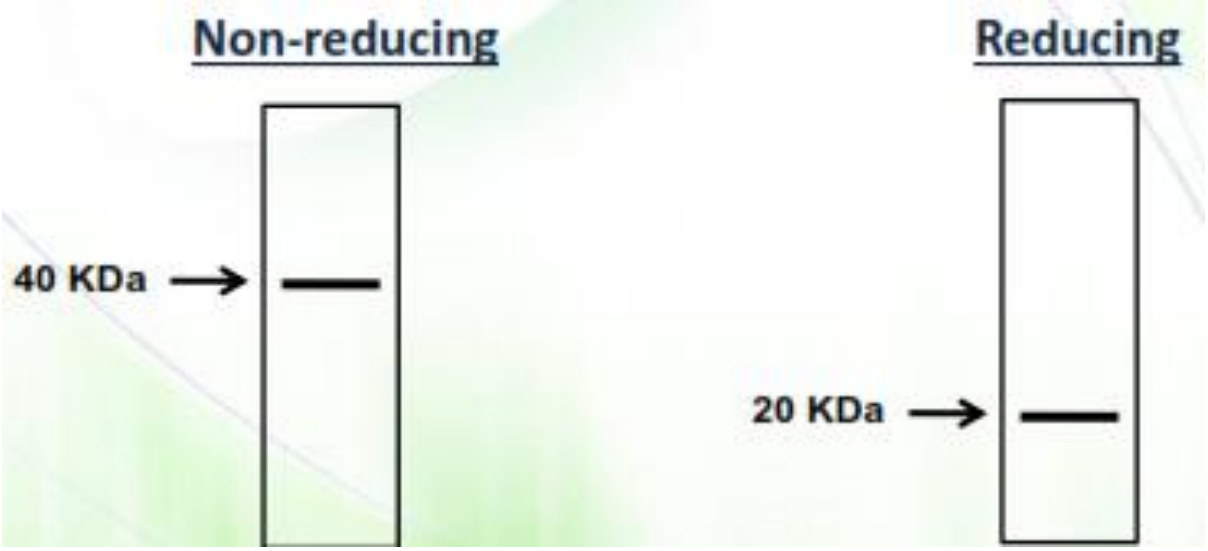




Describe the protein structure based on the following SDS-PAGE results

1- Under non-reducing condition, a protein exists as one 40KDaband. Under-reducing conditions, the protein exists as two20-KDa

-It is a dimer (Homodimer) since the result of SDS-PAGE is still one band after breaking the disulfide bonds
-It also could be a Heterodimer (2 different monomers with the exact molecular weight)

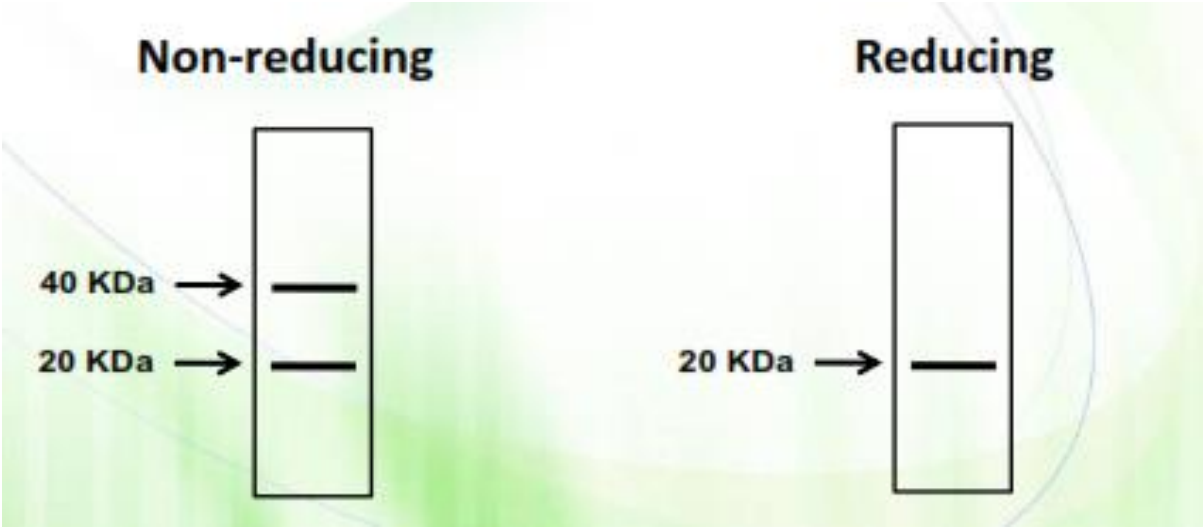


2- Under non- reducing condition, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein also exists as two bands, 15 KDa and 10 KDa

-This is tetramer consists of 2 homodimers attached to each other by noncovalent interactions (This is why it appears 2 bands before the addition of reducing agents, remember the effect of SDS)
-Now each dimer consists of 2 monomers attached to each other by disulfide bridges (This is why it requires reducing agents to separate it).
-Notice that even after reducing we still have two bands, which implies that the two dimers have different Mw, but they are homodimers (2 monomers have the same Mw)

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

-It is a trimer consists of a monomer (20 KDA) and a homodimer (40 KDA).
-the monomer is attached to the dimer by non-covalent interactions (This why it appears as 2 bands under the effect of **SDS**)
-the dimer consists of two monomers each one is 20 KDA attached to each other by disulfide bonds.
- After reducing agents are added there are 3 monomers each one is 20 KDA (This is why it appears as one band in the gel)



ISOELECTRIC FOCUSING

-Each protein consists of a unique combination of ionizable amino acids that results in a different net charge around the neutral PH

-This also means that every protein has its own isoelectric point

-This characteristic can be used to separate proteins

-This Procedure includes a gel prepared with a PH gradient, in which the protein sample is added

-As proteins migrate through the gel "horizontally" after applying an electrical current, 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, it stops when it's neutral.

-Each protein remains at the position on the gel corresponding to its PI, allowing for separation of proteins

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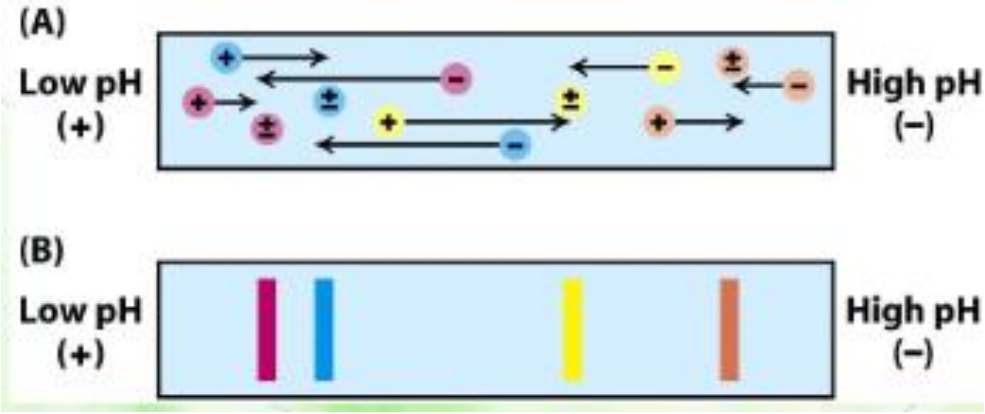


-A Cell consists around 6000 proteins, so that, "isoelectric focusing" is an insufficient method to separate all these proteins, so we combine it with "SDS-PAGE" method, which results in a method called **(2D-PAGE)**

Two-dimensional gel Electrophoresis

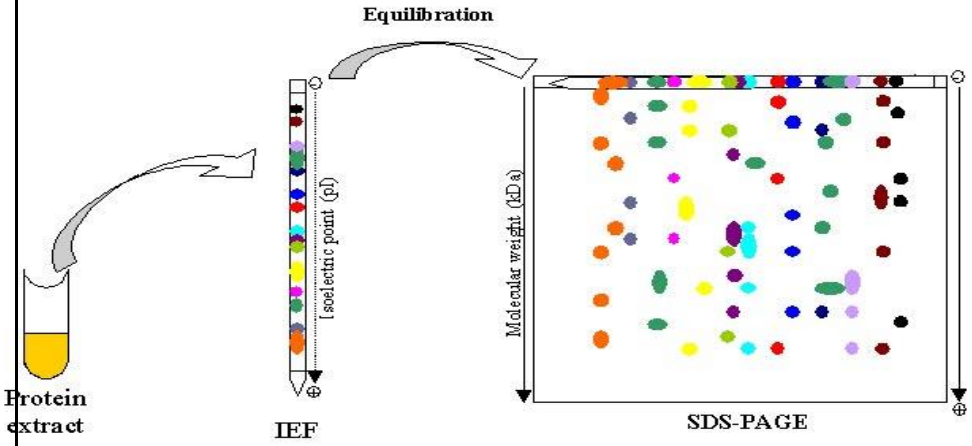
-In 2D-PAGE, proteins are separated first, by isoelectric focusing, then, through SDS-PAGE.

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

-**Horizontal Dimension** – Isoelectric point

-**Vertical Dimension** – (SDS-PAGE)

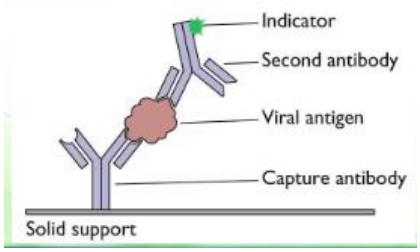
First Step Second Step



When 2 Proteins are in
1-The same **column** they have the same **PI**
2-The same **raw** they have the same **size**
3-The same spot they have the same **PI** and the same **size**

*I highly recommend to watch the animation for deep understanding

ELISA



-Stands for (**Enzyme-Linked immunosorbent assay**)
-The same as **immunoblotting**
-The procedure is

1- An antibody will be tightly bound to the bottom of well, now this antibody has a variable region which is highly specific and recognize a certain protein.

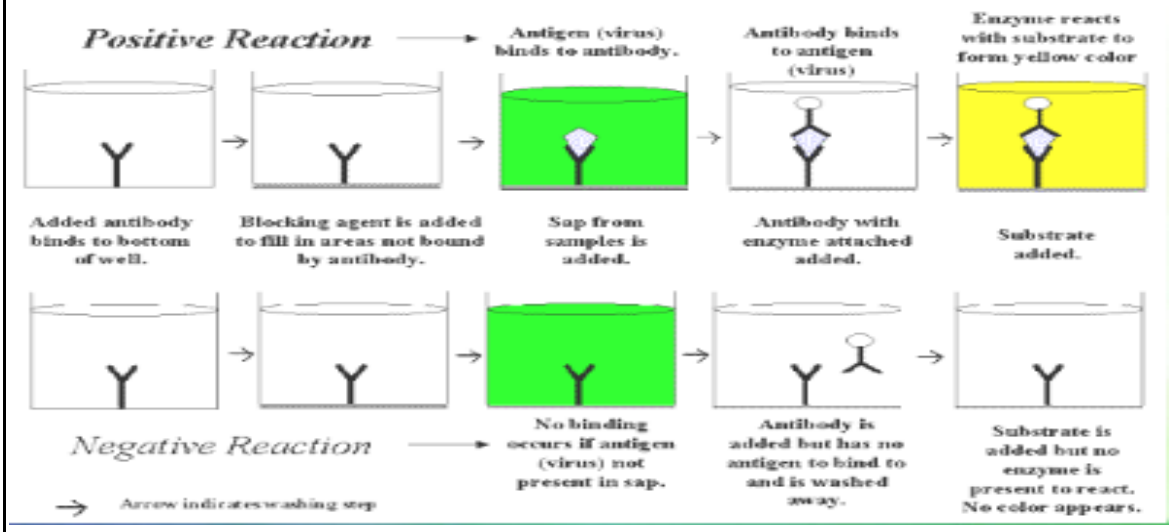
2- The sample is then added to the well, if it contains the protein that we are trying to detect, it will tightly bind to it.

3- After a certain period the sample is washed, and the only remaining are the antibody and the protein (if present).

4- Second type of antibodies is added to the sample, this type contains a different variable region that recognizes another Epitope on the same protein, in addition, it contains an indicator that is capable to change the sample color and indicates the presence of the protein.

5-The final step is to wash the sample again, now if the protein is present, the second antibody with the indicator would bind to it, and change the color of the well
(The darker the color the higher the amount of protein is present)

6-If not, no change on the color of the sample could be detected, so the protein of interest isn't there.



Extra Info: **not required from us, for further understanding and were not mentioned in the lecture**

You may be wondering what type of indicators is present in this procedure, well it is usually an enzyme that catalyzes a certain type of reactions that results in changing the substrate's color, so what happens is that after the second wash this substrate is added, now if the protein was present in the sample, the second antibody with its "indicator enzyme" bound to it (the enzyme is present and it interacts with the added substrate resulting in color changing) .

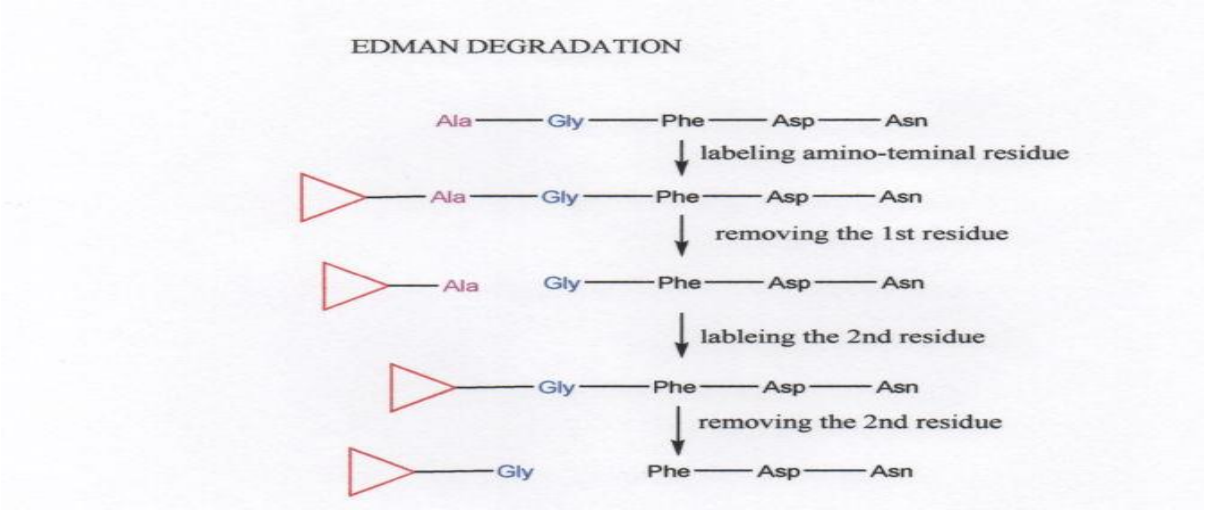
PROTEIN SEQUENCING

- Protein sequencing is basically the process of knowing the amino acids sequence of a protein or a peptide, basically it's knowing its primary structure.

-**Why do we want to know this sequence?**
- This helps in further studying of the protein, and it also helps in designing drugs against proteins that cause diseases.

-One type of it is called **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
- The N-terminal residue is first labeled by **phenylisothiocayante** (**PITC**)
- After labeling the peptide bond, the whole peptide is exposed under special conditions that result in cleavage of the labeled peptide bond only (Usually hydrolyzed)
- Now the N-terminal residue "which is the 1st amino acid" is free from peptide, and can be identified by **chromatographic procedures**
- This process is repeated over and over to identify the following amino acids in the sequence
- The Edman degradation technique does not allow peptides more than **50** residues to be sequenced



So how would identify the amino acid sequence for longer peptides?

-Simply we use some cleavage methods to cut the long peptide into smaller pieces and do Edman degradation for each one alone

Cleavage methods

- Chemical digestion
- Endopeptidases
- Exopeptidases

The process

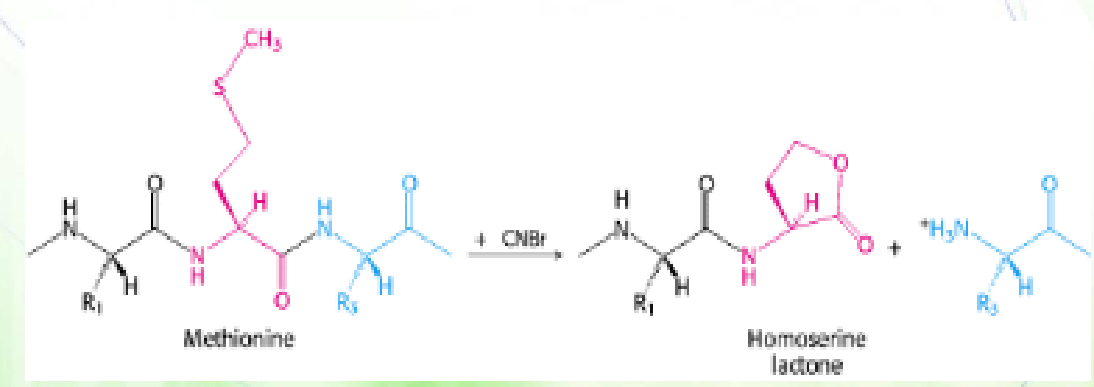
Cleavage Method
- Chemical digestion
- Endonuclease
- Exonulclease

Purification
- Gel Electrophoresis
- Gel Filtration
- used mainly to separate the cleave peptides from each other

Edman Degredation
- Identify The amino acid sequence for each cleaved peptide
- The overlabbing between cleaved peptides is used to identify the sequence of the original polypeptide we are working on

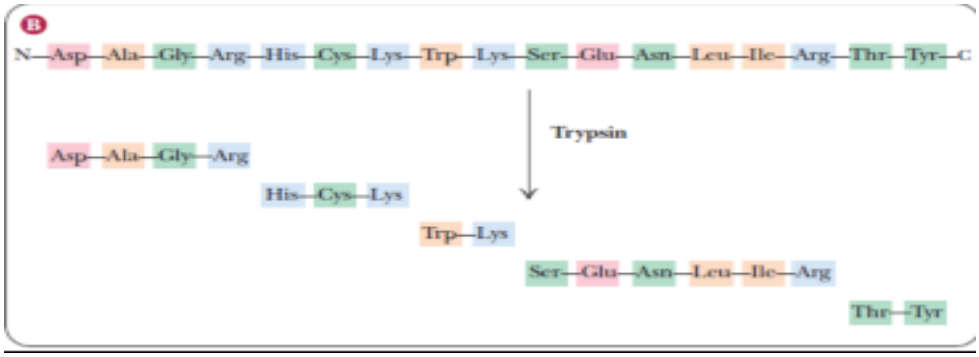
Chemical Digestion

- 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
- If you have 10 Methionine residues and the cleavage results is 10 peptides this indicates than on of these residues represent the C-terminus of the whole peptide (The last residue in the peptide is Met)



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.
- an example of it is the digestive enzyme **Trypsin**.
- Trypsin cleaves polypeptide chains on the carboxyl side "After" of arginine and lysine residues.
- A protein that contains 9 lysine and 7 arginine residues, (9+7=16) so 16 cutting sites, so it will yield 17 pieces (9+7+1) or "peptides" when digested with trypsin



❖ **Another Examples of Endopeptidases**

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

Note that the following information has not been explained in Dr.mamon videos nor must be included in this sheet, but we decided to present a whole complete idea so we included them

-notice that in the previous schedule none of the digestive enzymes can cut or cleave an amino acid if it was next to a *"Proline residue".*
- note that these enzymes do not recognize the peptide bonds, because peptide bonds are the same for all A.Acids, instead they recognize their side chains and they cut depending on it.

EXOPEPTIDASES

- They are enzymes that cleave the amino acids starting at the ends *"both edges"* of the peptide.
- Types:
  1- Aminopeptidases that cleave the N-terminus
  2- Carboxypeptidase that cleave the C-terminus

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^-

- When we are sequencing, we must use at least two types of enzymes, that cut in different sites, *why?*

  -because we can't identify which piece come first. For example, if we only use chemical digestions "which only cuts after Met" you wouldn't be able to know their order of arrangement.
  that's why we need another enzyme that will serve as a ruler and used for "overlapping sequences"

- In the picture above, the overlapping sequences are allied above and below each other to create the original primary sequence "without repetition "of the peptide.

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

Val-Leu-Gly-Met
Val-Leu-Gly-Met-Ser-Arg
Ser-Arg-Asn-Thr-Trp-Met
Asn-Thr-Trp-Met-Ils-Lys
Ils-Lys-Gly-Tyr-Met
Gly-Tyr-Met-Gln-Phe

The sequence is:
Val-Leu-Gly-Met-Ser-Arg-Asn-Thr-Trp-Met-Ils-Lys-Gly-Tyr-Met-Gln-Phe
*notice that the amino acids with the same color are the overlapping parts.

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.

The sequence is

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

💎✨**Never Quit Whatever It Takes**✨💎