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BIOCHEMISTRY

faculty of medicine - JU2017

Sheet Slides

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Gel Electrophoresis

This phenomenon, termed **electrophoresis**, offers powerful means of **separating charged proteins**. It works by adding the protein sample to a mold inside a **porous gel**, then have an **electrical current** pass through it causing the proteins to move through the gel *(molecules migrate towards the opposite charge)*, hence **separating** them.

Note: Charged molecules **move** in an electric field.

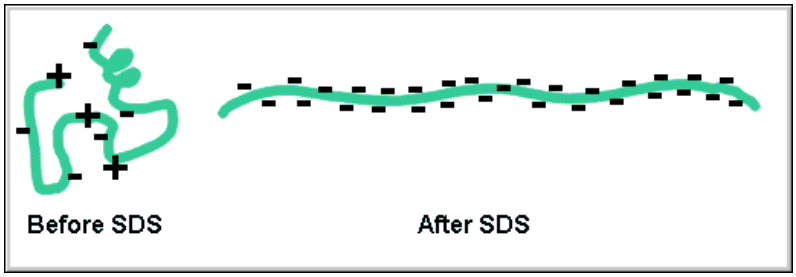
Types of electrophoresis:

1) Agarose electrophoresis → Tests **nucleic acids samples**
2) Polyacrylamide gel electrophoresis (PAGE) → Tests **proteins samples**

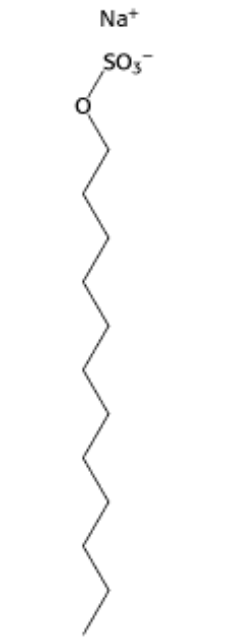
Types of polyacrylamide gel electrophoresis (PAGE):

1) **Native (Non-denaturing):** Protein sample is **unaltered** and placed in the gel, then with the electrical current running through it, proteins are separated based on their: **shape** *(Globular, linear, etc.)*, **size** *(molecular weight)* and **charge** *(+/ -)*.
   This method is **crude** *(flawed)* because **multiple** variables affect the results, thus nothing can be determined via the results. This method is only useful in **purifying** the desired protein.

2) **Denaturing (SDS-PAGE):** Protein sample is **treated with Sodium Dodecyl Sulphate (SDS).** SDS binds to the amino acids through its carbon skeleton, leaving its **negative** sodium end **exposed**, this **denatures** and **solubilize** proteins giving them a **negative** overall charge with a **linear** shape *(Same charge and size)*.



→ *They now migrate based on their molecular weight **only**.*



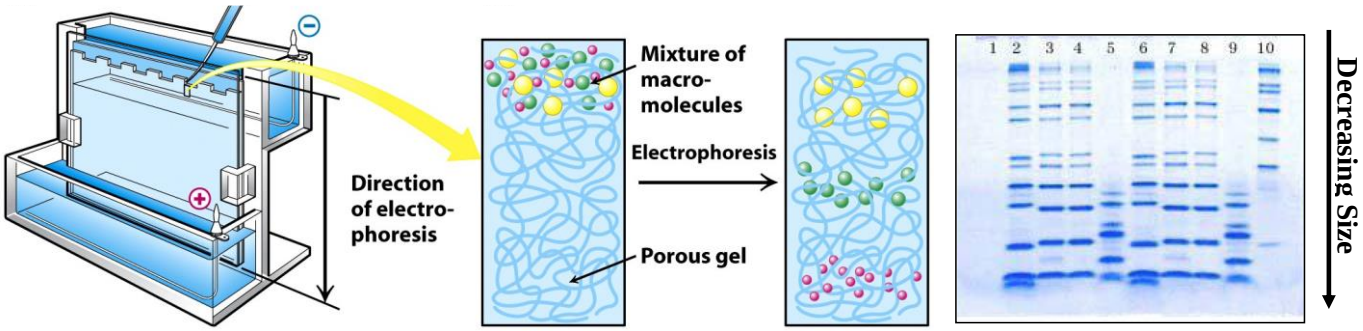
Sodium dodecyl sulfate (SDS)

This is the **most commonly used** protein electrophoresis technique.

SDS-PAGE process:

- It is performed in **thin**, **vertical** *(Top → Cathode, Bottom → Anode)* gels.
- The **top of the gel** consists of **wells** onto which samples are **loaded**.
- Since they **all are negative**; they can only migrate to the positive terminal *(Downwards)*, with their **velocities** related **inversely** with their **MW**; large proteins move slowly while small proteins move faster (*this means smaller proteins will be lower down than larger proteins*).

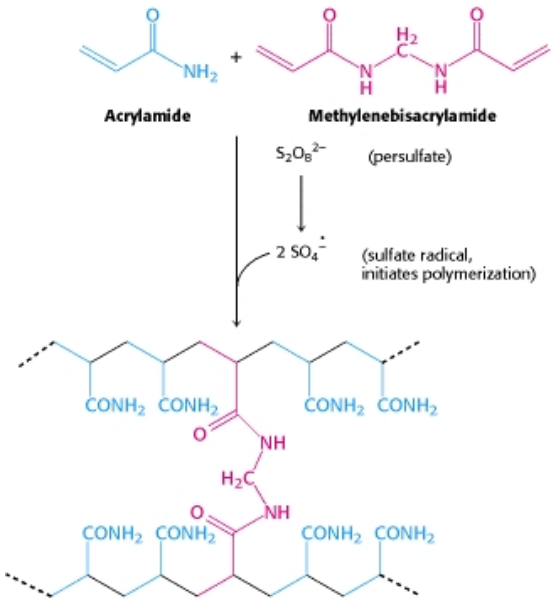
Note: Proteins will stick to their **vertical lanes** and will **not move** from left to right.



Gel Formation:

Acrylamide gets **polymerized** and **cross linked** by **methylenebisacrylamide**.

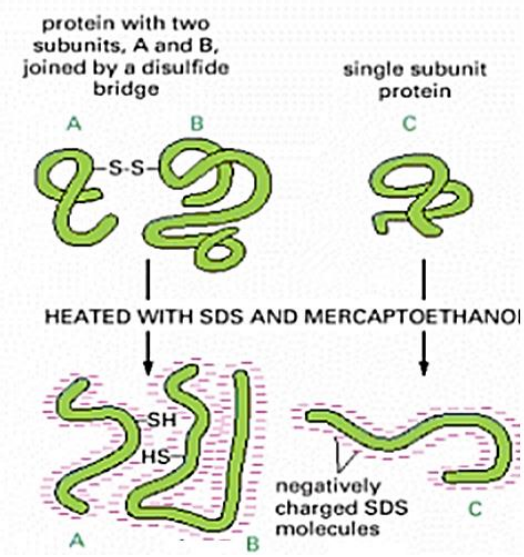
→ Polyacrylamide Gel



Note: Denaturing with **SDS** affects all noncovalent bonds **except disulfide bonds**.

Polyacrylamide gel electrophoresis (PAGE) can also be classified into:

1) **Reducing:** Insures the **complete distortion** of the protein using **reducing agents** like BME or DTT to reduce the **disulfide bonds** breaking down the protein into monomers.



2) **Non-reducing:** SDS was only used **without adding reducing agents**. Disulfide bonds are **present**.

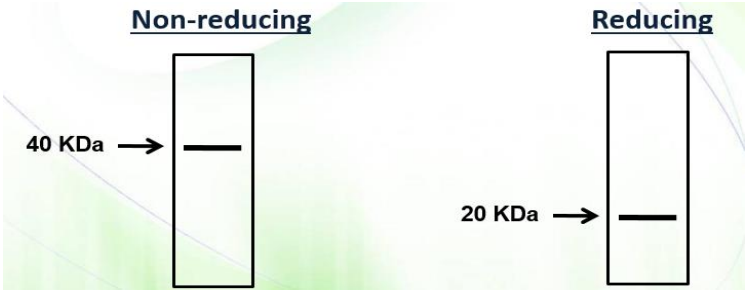
Questions:

Before solving the questions please know that:

Under non-reducing conditions**, means that the protein sample was treated by **SDS only**; only **non-covalent interactions are distorted.

***Under reducing conditions,** means that the protein sample was treated by **SDS and a reducing agent**; **covalent and non-covalent interactions** are distorted.*

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

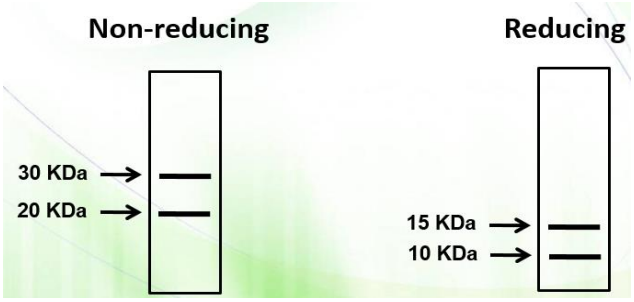


Non-reducing PAGE result: It shows **1 band**, therefore the protein originally can be a **monomer** or a **multimer**. MW= 40 KDa

Reducing PAGE result: It shows **1 band** with a MW= 20 KDa *(Half the original size)*, therefore the protein is made up of **2 identical monomers** joined by **covalent interactions** *(If they were joined by non-covalent interactions then under non-reducing conditions they would have been separated by the non-reducing PAGE and showed 2 bands).*

➔ **Homo-Dimer**

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.**

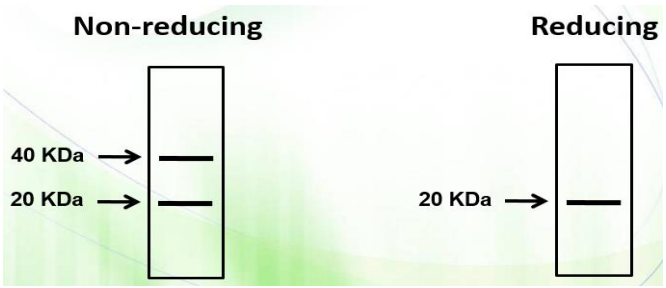


Non-reducing PAGE result: It shows **2 bands**, therefore the protein originally is **at least a dimer** joined by **non-covalent interactions** *(because they are separated in this condition)*, MW= 30 KDa and 20KDa.

Reducing PAGE result: It shows **2 bands** with a MW= 15 KDa and 10 KDa, therefore the protein is made up of **2 dimers** *(tetramer)* joined by **non-covalent interactions** *(explains the 2 separated bands under non-reducing conditions)*, consisting of **2 identical monomers** joined by **covalent interactions** *(each having half the original size)*.

➔ **Hetero-Tetramer made up of 2 homo-dimers**

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.**



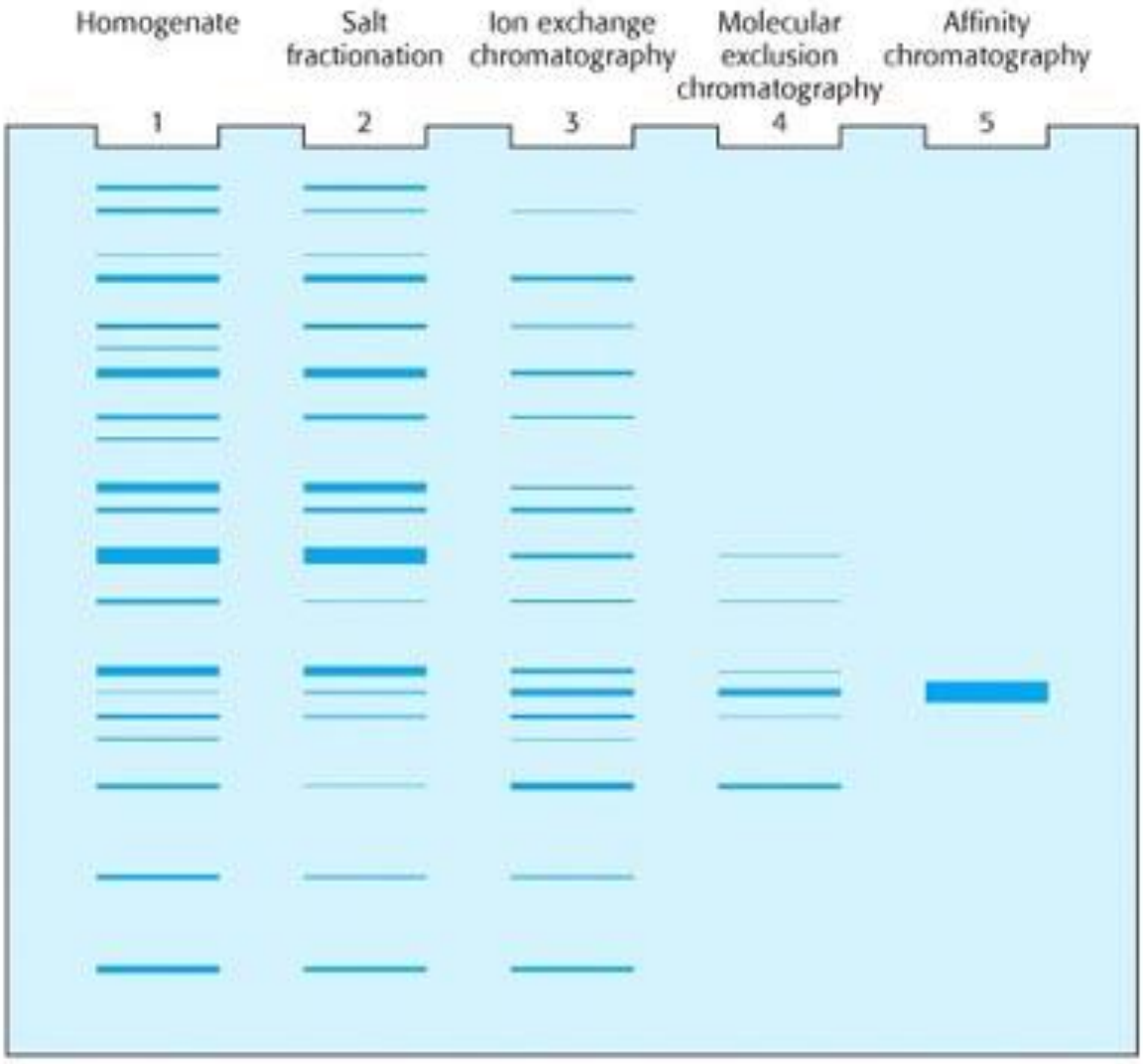
Non-reducing PAGE result: It shows **2 bands**, therefore the protein originally is **at least a dimer** joined by **non-covalent interactions**. MW= 40 KDa and 20 KDa.

Reducing PAGE result: It shows **1 band** with a MW= 20 KDa, therefore the protein is made up of **3 identical monomers** *(20+20+20);* a dimer *(Its subunits joined covalently because they weren't separated under non-reducing conditions)* and a monomer. Where the **monomer** is **non-covalently** joined with the **dimer** *(separated bands originally)*.

➔ **Hetero-Trimer**

"Please read and understand profoundly the previous questions before posting about it ;)"

Electrophoretic analysis of protein purification



Gel electrophoresis can be used to check the **purity** of protein samples; the **greater the variation** of the protein bands, the **less pure** the sample is.

From the electrophoresis results above, we can determine the purity of each of the protein purification mechanisms.

Affinity Chromatography, for example, has the **least protein** bands variations, therefore it is very pure. Its high purity makes it the best purification method.

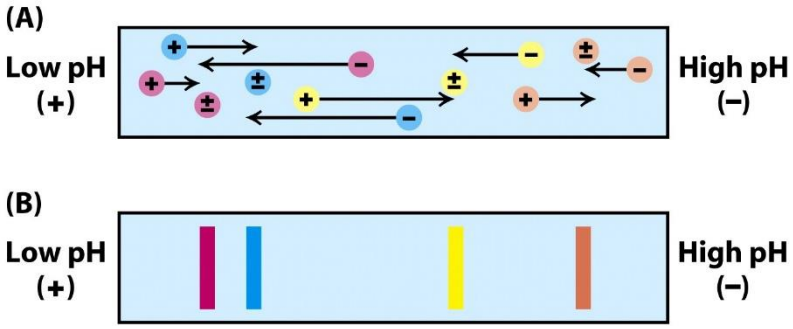
Isoelectric Focusing

It is a type of **electrophoresis**, usually performed on proteins in a prepared gel with a **pH gradient.**

As the proteins **migrate** through that gel under the influence of an applied electrical current, they **encounter different pH values.**

Eventually each protein reaches a point at which it has **no net charge** *(Isoelectric point)* near a **certain pH**, so they **no longer migrate**. *(Each protein stops at the position corresponding to its pI on the gel, because it has no net charge).*

➔ Proteins are **separated** according to their **pI** and not their **MW** like in **SDS-PAGE**

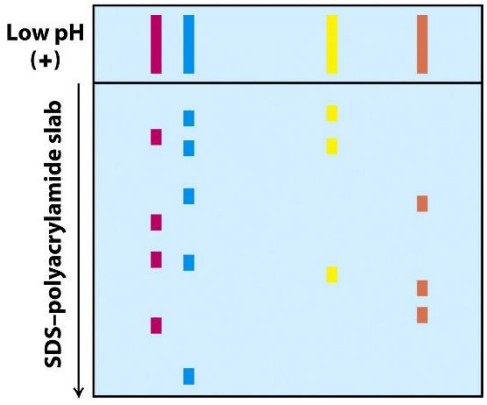


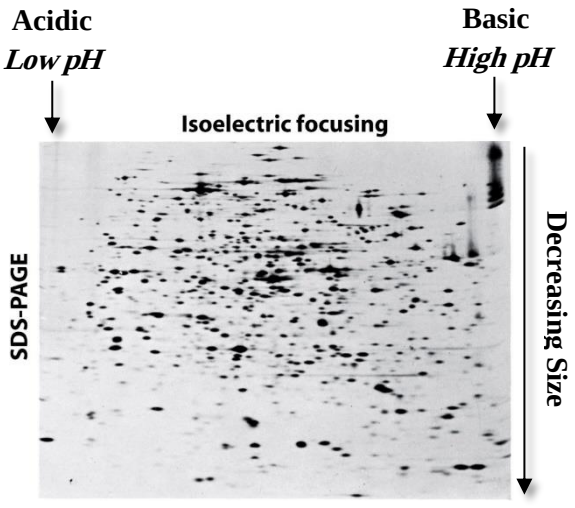
Note: The idea behind isoelectric focusing is **determining the pI** of the amino acids in the proteins and **separating** them according to it.

After separating the proteins according to their pI values, they can be separated according to their sizes by an SDS-PAGE.

This process is called **2D-PAGE**, where -again- proteins are separated according to their:

1) **Isoelectric Points.**
2) **Size (MW).**





Enzyme Linked Immunosorbent Assay (ELISA)

An assay, is an analysis done in **laboratories** that measures the **presence/concentration** of a **certain** substance. One example is **ELISA**, it is a test that uses **antibodies** and **antigens** to **identify** a substance.

Note: Before ELISA, **immunoblotting** was used which has the **same** concept of ELISA. But since ELISA is more **rapid**, **convenient** and **sensitive** *(identifies Nano-gram substances*); it is used instead of immunoblotting.

ELISA is done by the following steps *(understand it):*

1) A wells' bottom is **coated** with an **antibody**.

2) Blocking agent is added to **block** any **non-specific** binding sites to the **desired** antigen.

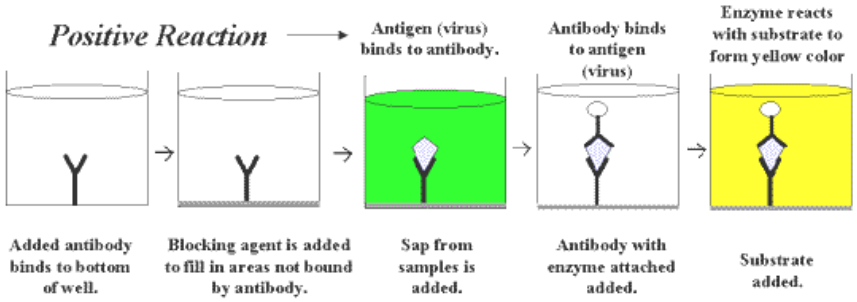
3) Added antigens **attach** to the antibodies.

4) The plate is washed to **remove unbound antigen**.

5) A **specific secondary antibody** *(attached with an enzyme)* is added, and binds to the **antigen** *(hence the antigen is stuck between two antibodies).*

6) A substrate is added.

If the reaction is positive, the enzyme on the antigen will **react** with the substrate and begin forming a fluorescent dye.

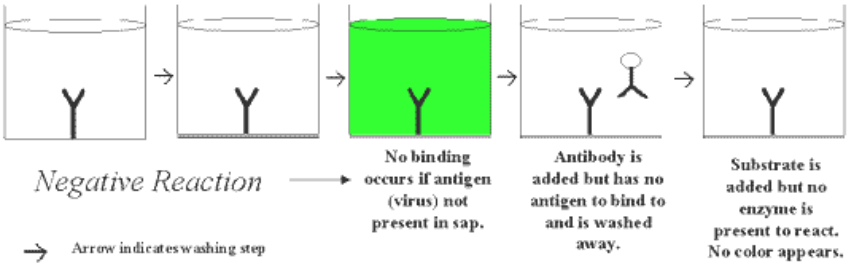


If the reaction is negative, there will be **no binding** of the antigen to the first antibody;

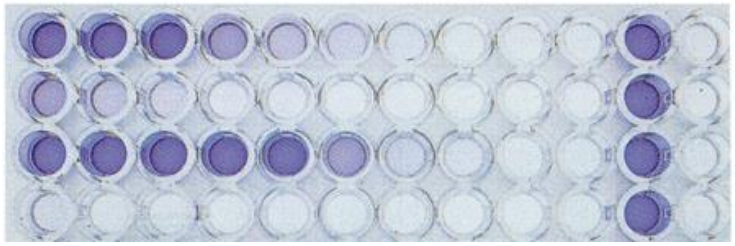
→ The secondary antibody finds **no bound antigen** to bind to since it **got washed away**.

→ Reaction **doesn't occur** after adding the substrate.

→ No color.



Note: Colors' **appearance** indicates the **presence** of a certain substance (antigen).
. Colors' **intensity** indicates the **concentration** of the substance.



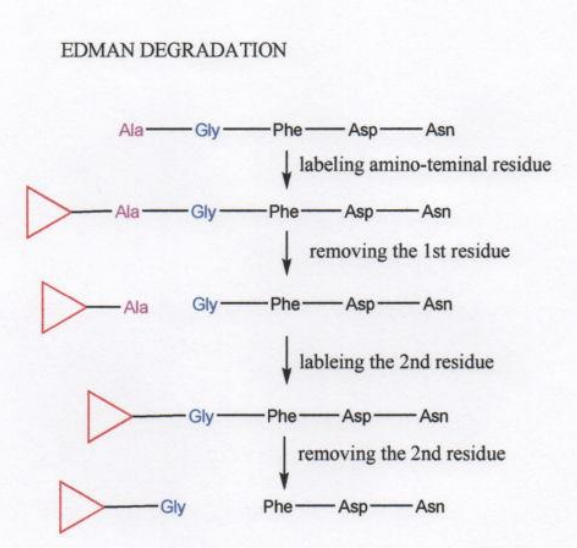
ELISA is used in screening for various hormones, pregnancy tests (detection of HCG), food allergies and HIV.

Protein Sequencing

*It is basically the process of determining the **amino acids sequence** (primary structure) of a protein or a peptide. These techniques are:*

1) Edman Degradation

This procedure involves a step by step **cleavage** of the **N-terminal residue** of a peptide, allowing the **identification** of each cleaved residue.



Phenylisothiocyanate (PITC) binds to the **N-terminal amino acid**, and the resultant amino acid is **hydrolyzed** and **liberated** from the peptide, then it is identified through **chromatography**. *(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).*

This technique is **very accurate** but it is **not practical**; it takes a lot of time and money. So it can only be used for peptides with **less than 50** amino acid.

2) Cleavage Methods

It is possible to sequence a whole protein with **50+ amino acids** by cleaving the protein into smaller peptides. This is facilitated by the following methods:

a- Chemical digestion **b- Endopeptidases** **c- Exopeptidase**

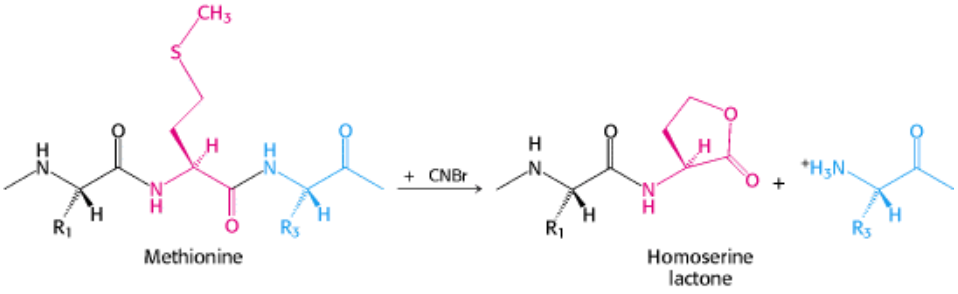
Now let us discuss each cleavage method:

1) Chemical digestion

An example for the use of chemicals in cleavage is the use of **Cyanogen Bromide** *(CNBr)*, which is the **most commonly utilized chemical reagent** that cleaves peptide bonds by recognition of specific amino acid residues. This reagent causes cleavage at the **C-terminal side** of **methionine residues only.**

Imagine the following situations:

- A protein has **10 methionine residues** and it would yield **11 peptides** when cleaved with CNBr. It may have **11 methionine residues** in the protein, in the case that the **last** amino acid is actually methionine.
- If **two methionine** were found consequently, then cleavage by CNBr would yield a **peptide** and an **amino acid** *(methionine).*



2) Endopeptidases

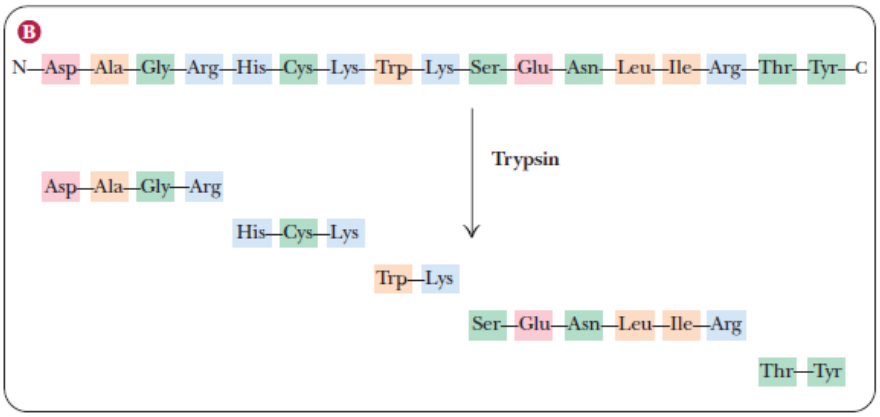
These are enzymes that cleave at specific sites within the **primary sequence of proteins**, and the resultant smaller peptides can be **chromatographically** separated and subjected to **Edman degradation** sequencing reactions.

Examples on Endopeptidases:

1) **Trypsin:** It cleaves polypeptide chains on the **carboxyl side** of **arginine** and **lysine** residues but not if they are next to a proline residue.

   Example 1: A protein that contains **9 lysine and 7 arginine** residues will usually yield **17 peptides on** digestion with **trypsin**.

Example 2: Observe the following polypeptide and how it got cleaved using Trypsin. We can see that at every **carboxyl side** of **arginine** or **lysine** cleavage occurred.



Which peptide do you think has a total net charge of +2 (At physiological pH)?

His-Cys-Lys

2) **Chymotrypsin:** It cleaves polypeptides on the **carboxyl side** of the aromatic amino acids **phenylalanine**, **tyrosine**, and **tryptophan**, *but not if they are next to proline.*

3) **Elastase:** It cleaves polypeptide chains on the **carboxyl side** of alanine, **glycine**, **serine**, and **valine**, *but not if they are next to proline.*

4) **Pepsin**: It cleaves polypeptide chains on the **nitrogen side** of **aromatic amino acids** and **lysine**, *but not when next to proline*

Note: These enzymes **can't cleave** an amino acid **next to proline**; as proline is **bulky** so it **won't fit** to the active site.

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^-

3) Exopeptidases

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

There are two types:

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

Questions:

1) 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 *(Cleaves at Arg/Lys):*

Asn—Thr—Trp—Met—Ile—Lys
Gly—Tyr—Met—Gln—Phe → *We can tell that this is the last residue since it doesn't end with Arg/Lys*
Val—Leu—Gly—Met—Ser—Arg

Cyanogen bromide treatment *(Cleaves at Met):*

Gln—Phe → *We can tell that this is the last residue since it doesn't end with Met*
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

2) 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

A tip on how to solve such questions: Always point out which is the last residue, then move backwards along the amino acids sequences given in the question

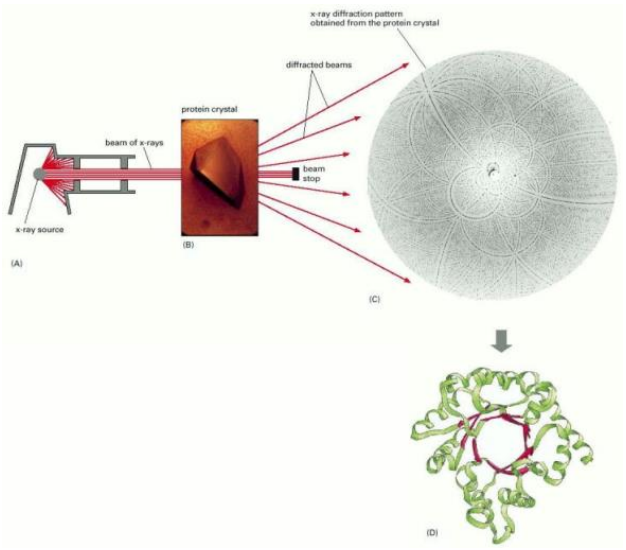
.

Crystallography

To determine the **tertiary structure of a protein**, many methods can be used, but the most common one is through **x-ray crystallography** or **nuclear magnetic resonance (NMR).**

1) X-ray Crystallography

A protein firstly is turned into a **crystal** *(can be produced by dissolving the protein in water and evaporating it under special conditions).* Then, it is **exposed to x-rays** which are scattered by the electrons of the molecules giving a series of **diffraction patterns** and information on the molecular coordinates.



Side Information: *These coordinates are extracted by an analysis called a Fourier series.*

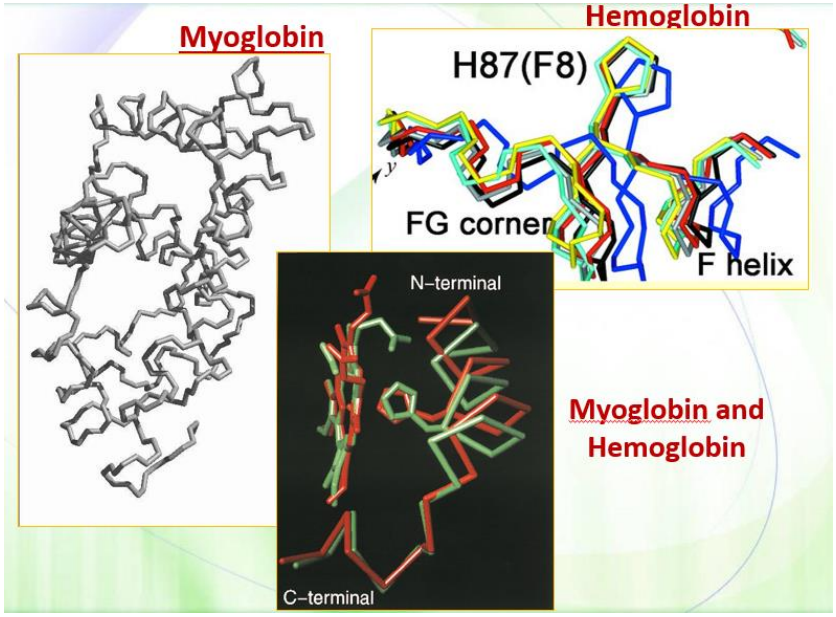
The main **disadvantage** of this technique is that when proteins get **crystallized** they become **rigid,** when they are **dynamic** in nature. This is solved by using **NMR**.

2) Nuclear Magnetic Resonance

In this method, proteins can be **dissolved in solutions**, thus **preserving** its natural **dynamic activity.**

This is important when analyzing proteins **binding to other molecules** (*like enzymes to their substrates, or like receptors to their ligands).*

Side information: *NMR depends on the change in the spin of electrons in a protein when a magnetic field is applied, where different vibrations can be detected from different atoms depending on its environment (the surrounding atoms).*



Do not study because you need to. Study because you want to know more. Study because it enhances you. Study because knowledge is power.

Best wishes and Good luck ❤