Lecture 28: Product recovery from over-expressed cells

Introduction: In the lectures so far, we discussed the strategies to generate recombinant DNA and use them through over-expression in host system to produce biomolecule either in secreted form or inside the cell. The operation involved in isolation and purification of a product from a suitable organism to develop useful product is collectively known as **down-stream processing** (Figure 28.1). After the product is formed either in free (secreted) or associated with cell, the next process is to collect the product from cell. No specialized process is required to collect secreted product but several methods have been developed to released the product through cell disruption. Once the product is collected, additional extraction and purification steps are needed to develop final product for application.

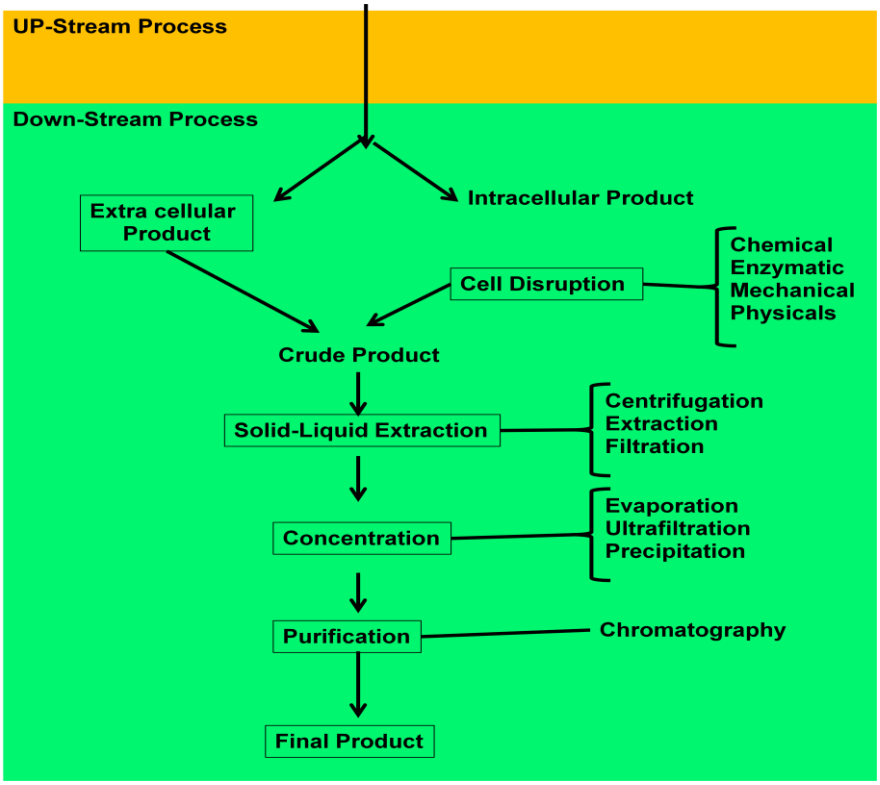

Figure 28.1: An over-view of different steps in down-stream processing.

Methods for Cell disruption : The methods of cell disruption used to release the product depends on the composition of plasma membrane, presence or absence of cell wall, choice of location and the kind of product (enzymatic/non-enzymatic or simply bioactive substance). The different methods utilizes a unique mechanism to disturb the integrity of plasma membrane. These methods can be classify into 3 major section:

1. Physical Methods- These methods play with the physical parameters to damage the cell to release the product.

A. Thermolysis-This method is easy, economical and require no additional specialized equipment. It can be used only if the product is thermostable.This method gives a heat shock to kill the organism and as a result it disturb the cellular integrity without affecting the product. The effect of heat shock depends on ionic strength, presence of chelating agents such as EDTA and presence of other proteolytic enzymes.

B. Osmotic Shock-Most of the mammalian cell have a plasma membrane with active transporter to maintain the osmotic balance. Maintaining an osmotic balance is an active process with expenditure of energy. Prolonged exposure to the cell with hypotonic liquid such as water causes osmotic imbalance and ultimately causes lysis of cell. In this process due to inflow of water, cell swell and ultimately burst to release the products. According to the Hoff's equation, osmotic pressure Π is directly propotional to the concentration of solute and temperature.

$$\Pi = RT(C_i - C_o) \text{……..Eq 28.1}$$

R= Gas constant, T=absolute temp (K), C_i-C_o=difference between total solute concentration inside and outside the cell (moles per litre)

Each mammalian cell has differential suspectibility towards osmotic shock. Red blood cells as shown in Figure 28.2, will be lysed with the addition of a tiny drop of water. Plant or bacterial cells are more resistant towards osmotic lysis due to presence of thick cell wall.

C. Sonication- A typical laboratory sonicator is given in Figure 28.3. A sonicator generates the ultrasound waves of frequency more than 20kHz to cause cell disruption by cavitation. The interaction of ultrasound with liquid causes compression and decompression very rapidly. The bubble formed in liquid, compresses several thousand atmospheres and gives shock waves to the cell wall or plasma membrane to cause cell lysis (Figure 28.3). Generation of ultrasonic waves in liquid causes rapid change in temperature and may cause thermal denaturation. Hence, ultrasonication medium needs to be cool and a long duration should be avoided.

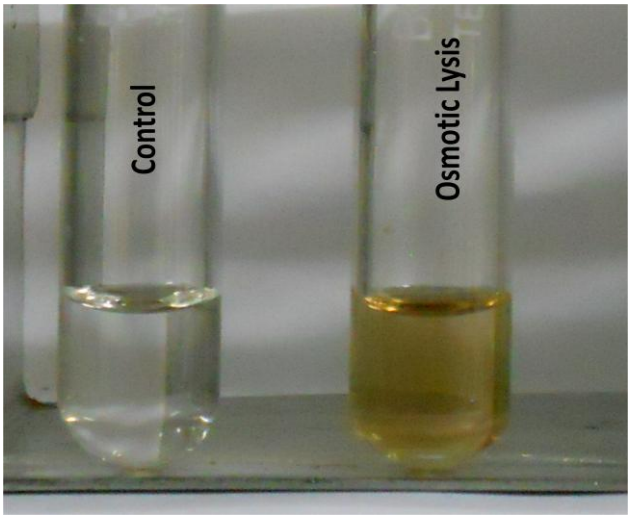

Figure 28.2: Osmotic Lysis of Red Blood Cells.

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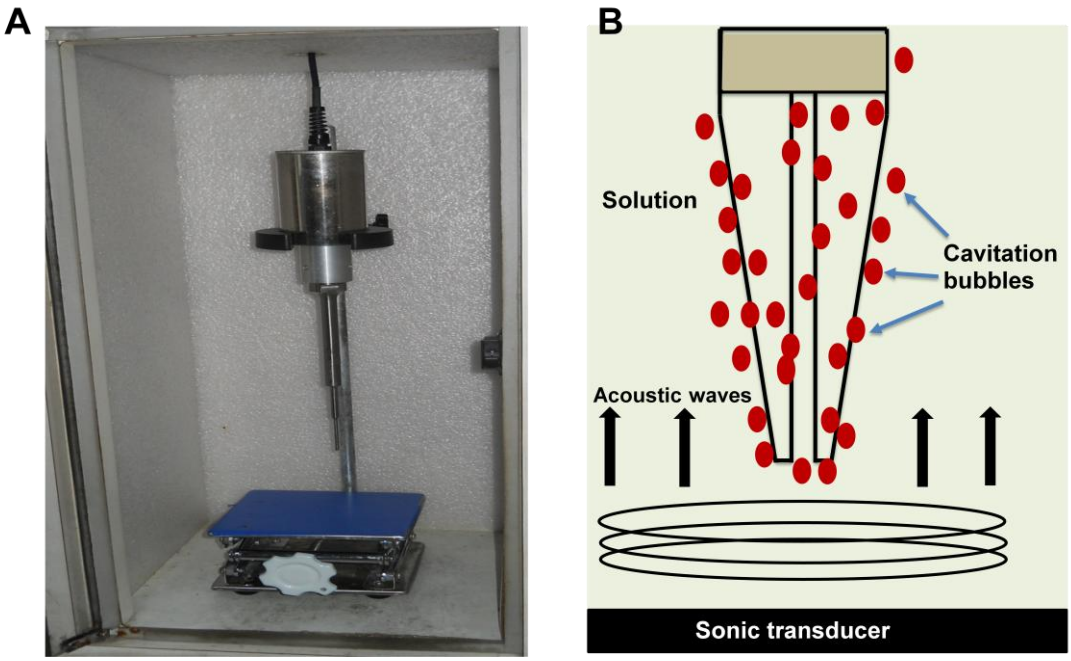


Figure 28.3: Ultrasonication. (A) Ultrasonic probe (B) Principle of sonic waves mediated cell disruption.

2. Chemical and Enzymatic Methods

A. Alkali Treatment-This is a harsh but effective chemical treatment to lyse the cells. Alkali treatment causes lipid saponification which disturbs the lipid packing and affects the cell wall integrity.

B. Detergent: Addition a detergent solution to the cell causes solubilization of lipid to form micelle. The effect of detergent on cell wall increases linearly with concentration. The detergent concentration which causes abrupt change in lipid solubility and forms micelle is known as critical micelle concentration (CMC). Example of detergents are SDS, CTAB, Triton X-100, saponin, digitonin.

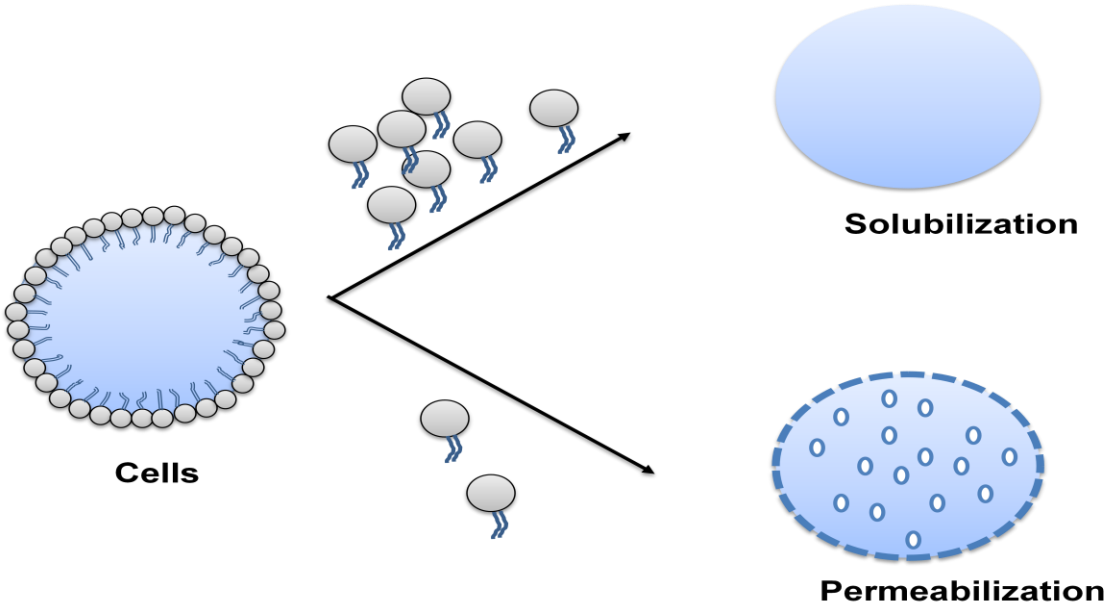


Figure 28.4: Effect of detergent on plasma membrane integrity. (A) Solubilization (B) Permeabilization

C. Permeabilization: A partial cell wall disruption or permeabilization is achieved by organic solvent such as toluene (Figure 28.4). The organic solvent is absorbed by the cell wall resulting in its swelling and ultimate rupture. But at low concentration, organic solvent permeablizes the cell wall without disturbing cellular integrity. This process allow to use cell as a reaction vessel to catalyze a reaction and get desired product.

D. Enzyme Digestion: Enzymatic methods are specific, gentle and most effective but costly. Lysozyme is commercially available to treat bacteria to release intracellular products. In addition to lysozyme, there are three other types of bacteriolytic enzymes, glycosidases, acetylmuramyl-L-alanineamidase and endopeptidase. Few protease are also found to be bacteriolytic. Yeast cell lysis requires a mixture of different enzymes such as glucanase, protease, mannanase or chitinase. Plant cells can be lysed by cellulose and pectinase. In most of the enzyme mediated cell lysis method, the rupture of cell wall depends on the osmotic pressure of the external medium. In few cases, enzymatic digestion is performed to remove external cell wall and then in second step protoplast is disrupted by gentle agitation.

3. Mechanical Methods: In the simpliest mechanical cell disruption method used in laboratory is waring blender and pestle-morter. Both are effective towards animal and plant tissue as well as filamentous organisms. In industrial scale, cell disruption is carried out by (i) bead mill or (ii) high pressure homogenizer.

1. Bead Mill Disruption-The bead mill (horizontal or vertical) consists of a grinding cylinder with a central shaft fitted with a number of impellers which can move in clockwise or anti-clockwise direction with the help of a motor (Figure 28.5). The grinding cylinder is filled with the beads made up of glass, alumina, titanium carbide, zirconium oxide or zirconium silicate. There is an inlet to supply the cell suspension and a outlet to collect the sample after the process. When bead mill runs cells experience a shear forces between produced between moving beads and cells. The rate and degree of cell disruption depends on cell type, thickness of cell wall, localization of product, type and agitation speed of impeller, bead size, its density and loading, residence time and temperature. Cell disruption in a bead mill and release of a produce is a first order kinetics and it may be given by first order equation

$$\ln\left(\frac{C\text{max}}{C\text{max} - C}\right) = -\text{kt}$$

Here, Cmax is the concentration of product that can be released from a given amount of cell suspension, C is the concentration of product released at a given time "t" and *k* is the first order constant. This relationship holds true only for batch mode of operation. The value of k depends on type of impeller, bead size and loading, speed of agitation and temperature.

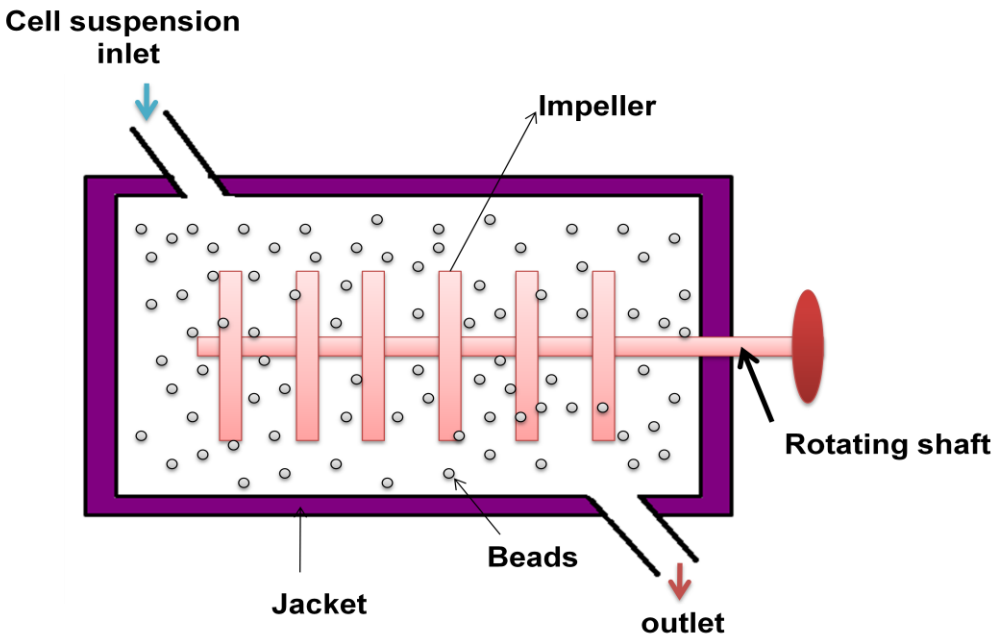


Figure 28.5: Schematic diagram of bead mill disruption

2. High Pressure Homogenizer-A high pressure homogenizer consists of a high pressure positive displacement pump connected to the an adjustable discharge valve with a restricted opening (Figure 28.6). The cell suspension is send into the homogenizer through a small homogenizing valve at a very high pressure (200-1000 atmospheric pressure). Cell passing through a small valve experience stress developed within the fluid and as result it get disrupted. The stress developed is expressed as dynamic pressre Ps and it is expressed as-

$$\mathbf{Ps} = \frac{\mathbf{1}}{\mathbf{2}}\mathbf{pv^2}$$

Ps is dynamic pressure, v is jet velocity and p is the density of fluid.

Cell disruption in high pressure homogenizer and release of a produce is a first order kinetics and it may be given by first order equation

$$\frac{Cmax}{Cmax - C}) = kN$$

Here, N is number of passes through the valve and and *k* is the first order constant. As high pressure homogenization passes the cell at a very high speed through a narrow valve, it disrupt the cells and simulatenously it lower down the temperature as well.

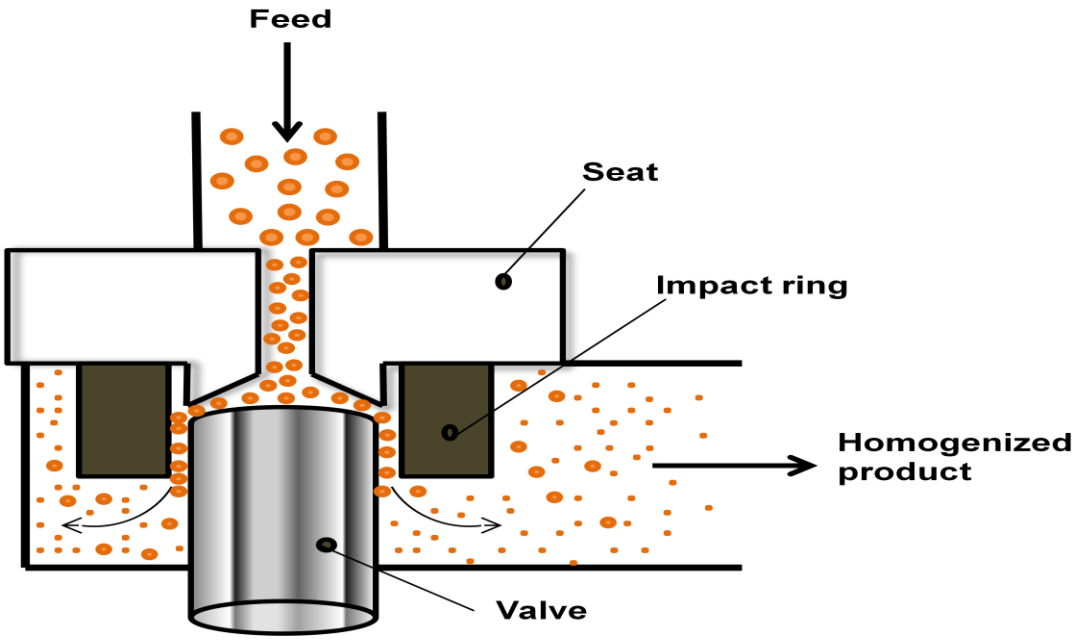


Figure 28.6: High Pressure homogenizer.

Lecture 29: Basics of Chromatography

Introduction: The molecules present in biological system or in synthetic chemistry are produced through a series of reactions involving intermediates. As discussed in previous lecture, at any moment of time biological organism has major fraction as desired product but has other compounds in minute quantities. The minor species present in a product is always referred as "impurities" and these compounds need to separate from desired product for biotechnology applications. **How two molecules can be separated from each other?** To answer this question we can take the example of three molecules given in Figure 29.1. These 3 molecules (benzene, phenol, aniline) are similar to each other but have distinct physical and chemical properties which can be used as a criteria to separate them. The physical and chemical properties which can be use to separate molecules are-

Physical Properties

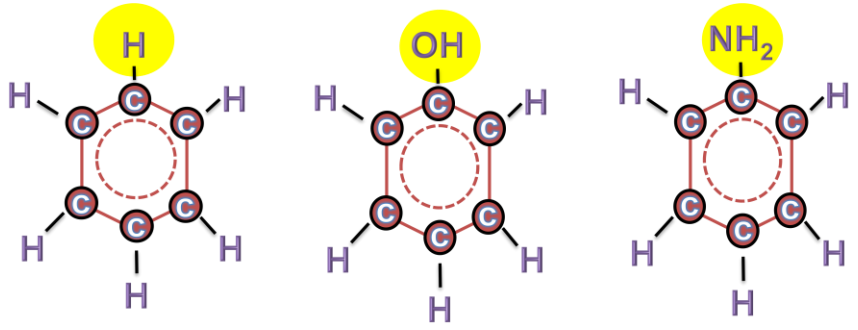
1. Molecular weight
2. Boiling point (in case both are liquid, as in this case)
3. Freezing point
4. Crystallization
5. Solubility
6. Density

Chemical Properties

1. Functional Group, for example, phenol has –OH where as aniline has NH2.

2. Reactivity towards other reagent to form complex

Now for example you have a mixture of compound 1 (benzene) and compound 3 (Aniline) and you would like to purify benzene rather than aniline. In this situation, you can take the physical and chemical properties of benzene into the account and isolate it from the mixture.



Name	Benzene	Phenol	Aniline
Molecular formula	C_6H_6	C_6H_6O	$C_6H_5NH_2$
Molar mass (g mol^{-1})	78.11	94.11	93.13
Density	0.8765 g cm^{-3}	1.07 g cm^{-3}	1.0217 g ml^{-1}
Melting point (°C)	5.5	40.5	-6.3
Boiling point (°C)	80.1	181.7	184.13

Figure 29.1: Chemical Structure and physical Properties of benzene, phenol and aniline.

Principle of Seperation: <u>How a physical or chemical property will allow to isolate a particular substance?</u> The mixture of compound 1 and 3 is shown in Figure 29.2 and assume if we are using boiling point as a criteria to isolate them. As we will heat the mixture there will two phase forms, one liquid phase and other is vapor phase. The molecules of compound 1 and 3 will distribute between these two phases and as the temp is near to boiling point of compound 1, more amount of 1 will be present in vapor phase than liquid phase. Where as more number of compound 3 will be in liquid phase. Eventually as this process will continue, at the end two molecules will get separated from each other. The distribution coefficient (Kd) to describe the distribution of compound 1 between two phase A and B is as follows:

$$Kd = \frac{\text{Concentration in Phase A}}{\text{Concentration in Phase B}}$$

Similarly one can also exploit other physical & chemical parameters as well. With each and every physical and chemical parameter the molecule present in the mixture will distribute as per their behavior in each parameter.

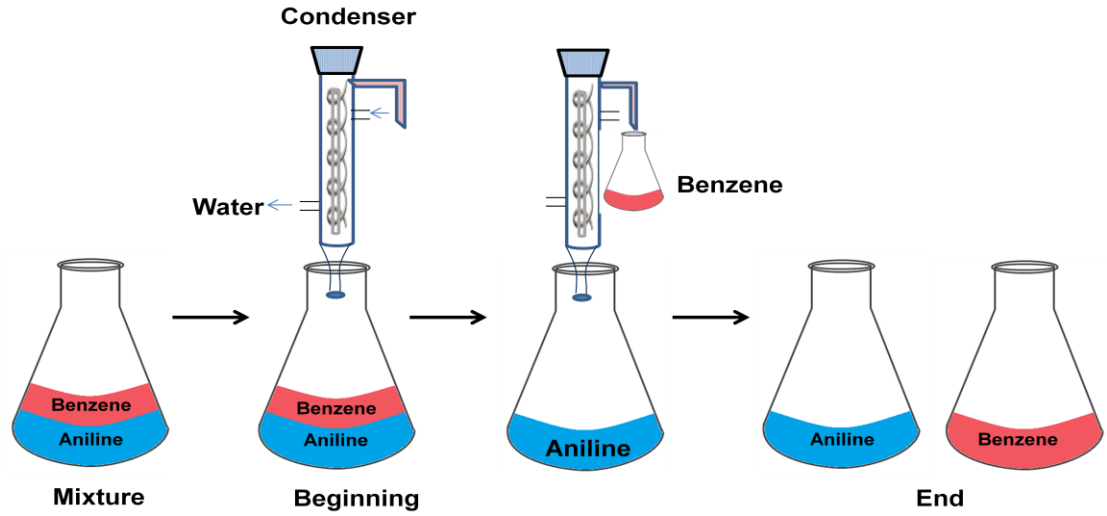


Figure 29.2: Distribution of molecules during distillation.

Chromatography: The purpose of chromatography is to separate a complex mixture into individual component exploiting the partition effect which distribute the molecules into the different phases. As discussed above, a distribution of a molecule between two phases A and B is given by a distribution coefficient, Kd. In most of the chromatography techniques, phase A is stationary phase or matrix and phase B is mobile phase or buffer.

Column Chromatography: In column chromatography, a stationary phase is filled into a cylindrical tube made up of glasss or steel. The mixture of analyte is loaded on the top and it runs from top to bottom. **How Kd is exploited in column chromatography ?** Assume two molecules, X and Y with a Kd value of 1 and 9 and they are traveling through a column with water as mobile phase as given in Figure 29.3. As they will travel, X and Y will partition between stationary phase and mobile phase. As there is a huge difference in Kd, Y will be associated with the matrix and remain on the top of the column where as X will move along the water. At the end of chromatography, X will come out first whereas Y will come out last.

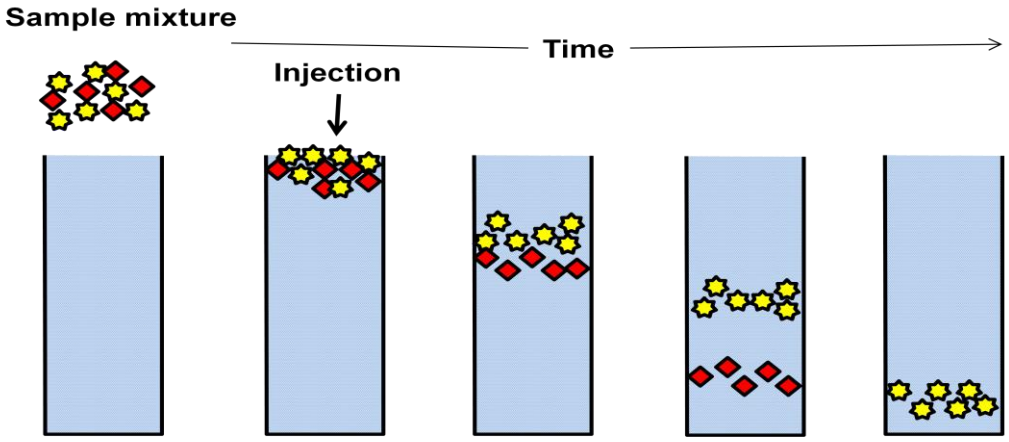


Figure 29.3: Seperation of two molecules on a column.

Chromatogram: The plot of elution volume along with the absorbance is known as chromatogram as given in Figure 29.4. The volume or time it takes for a analyte to come out from the column is known as retention volume or time. The chromatogram may have separate peaks (A and B) or peaks (C and D) with overlapping base, these peaks are called fused peaks.

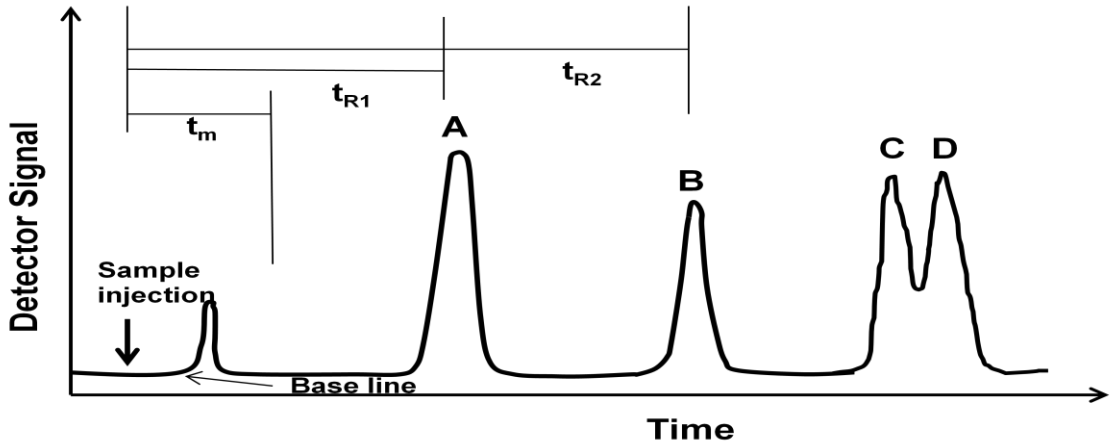


Figure 29.4: A typical chromatogram.

Resolution: The ability of a chromatography column to separate two analyte peak from another is known as resolution. It is defined as the ratio of difference in retention time between two peaks and average of base of peak width. It is given by

$$Rs = \frac{\Delta \mathrm{tR}}{\mathrm{Wav}}$$

When Rs=1, the separation of two peaks is 97.7% and a column with Rs more than 1.5 considered good. The number of distribution events govern the ability of a column to separate the two analytes. In another words, resolution is directly proportional to the number of distribution events. In column chromatography, each thin plain of column matrix participate in distribution of molecule. Assume height of a distribution plain is **H** and length of a column is **L**, hence number (**N**) of distribution plain in a column is given by,

$$N = \frac{\mathrm{L}}{\mathrm{H}}$$

$$\mathbf{N=16\ (t_R/W)^2}$$

$$\mathbf{N=5.54\ (t_R/W_{1/2})^2}$$

Hence, Number of distribution plain in a column is controlling two parameters:

(1) As number of distribution plain will go up, it will allow the analyte to travel for longer period of time, consequently it will increase the distance between two peaks.

(2) As number of distribution plain will go up, it will reduce the width of the base of peak, as a result the peaks will be more sharp. A representative example, how number of distribution plain affects the base of the peak is given in Figure 29.5. As the number is increasing, the peak width is decreasing. Hence, number of distribution is an indirect way to measure the column efficiency, higher N number is desirable for better separation.

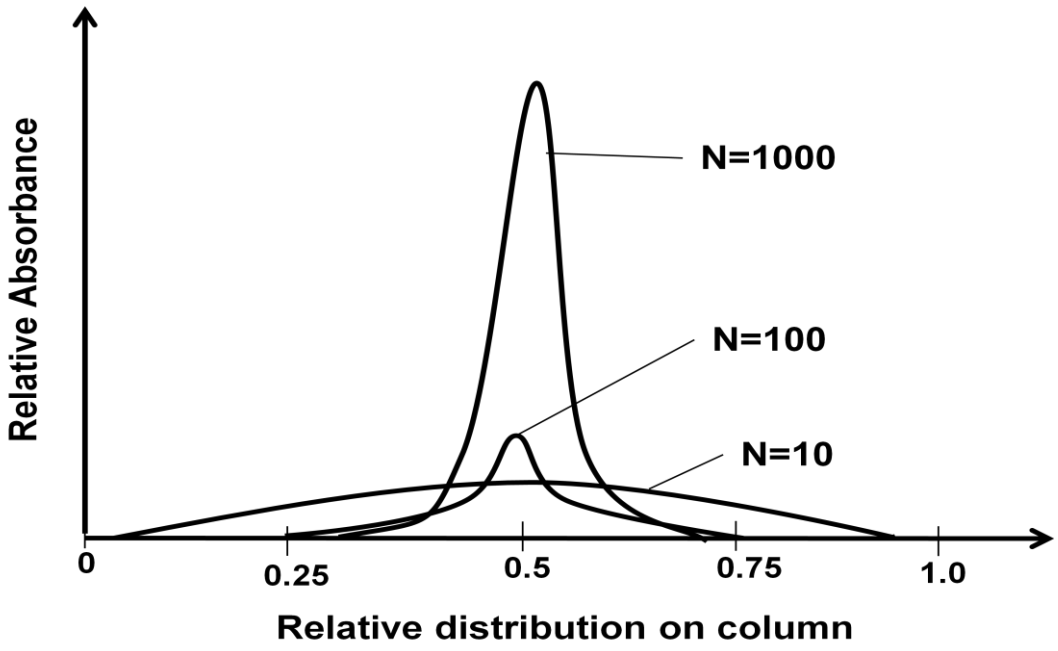


Figure 29.5: Relationship between number of distribution planes (N) and peak width.

Different components of chromatography system: The different components of a chromatography system is given in Figure 29.6. it has following components:

1. Reservoir : One or two reservoir for mobile phase (buffer).

2. Pump: One or two pump to flow the buffer from reservoir. Different types of pumps are used in chromatography system, mostly based on the pressure level required to perform chromatography.

3. Mixer: A mixer is required to mix the buffer received from both pumps to form a linear or step gradient.

4. Column: A column made up of glass or steel.

5. Detector: The elution coming out from column goes to the online monitoring system to test the presence of the analyte based on different properties. There are different types of detectors are known in chromatography such as UV-Visible detector etc.

6. Fraction Collection- The eluent can be collected in different fractions by a fraction collector.

7. Recorder: The profile of eluent with respect to the measured property in a detector can be plotted in the recorder.

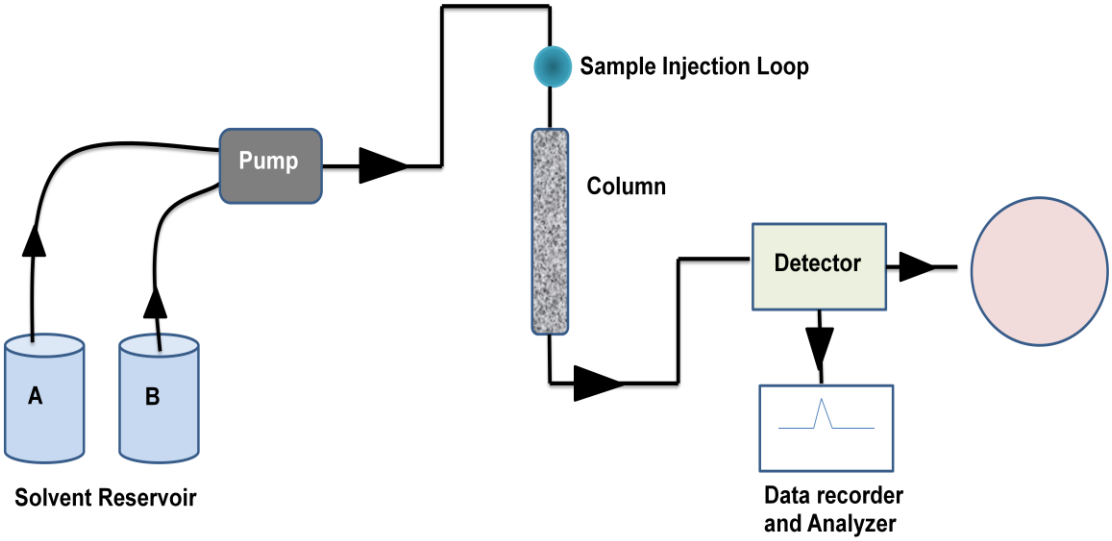


Figure 29.6: Different components of a chromatography system.

Different forms of chromatography:

Partition Chromatography: In this form of chromatography, an analyte distribute themselves into two phases, liquid stationary and mobile phase. The major advantage of this chromatography is that it is simple, low cost and has broad specificity. It is further divided into liquid-liquid chromatography and bonded-phase liquid chromatography. The example of this chromatography is cellulose, starch or silica matrix.

Adsorption Chromatography: In this form of chromatography, matrix molecule has ability to hold the analyte on their surface through a mutual interaction due to different types of forces such as hydrogen bonding, electrostatic interaction, vander waal etc. The example are ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography etc.

Lecture 30: Ion-Exchange Chromatography

Ion exchange chromatography: Ion-exchange chromatography is a versatile, high resolution chromatography techniques to purify the protein from a complex mixture. In addition, this chromatography has a high loading capacity to handle large sample volume and the chromatography operation is very simple.

Principle: This chromatography distributes the analyte molecule as per charge and their affinity towards the oppositively charged matrix. The analytes bound to the matrix are exchanged with a competitive counter ion to elute. The interaction between matrix and analyte is determined by net charge, ionic strength and pH of the buffer. For example, when a mixture of positively charged analyte (M, M^{+}, M^{-1}, M^{-2}) loaded onto a positively charged matrix, the neutral or positively charged analyte will not bind to the matrix where as negatively charged analyte will bind as per their relative charge and needed higher concentration of counter ion to elute from matrix (Figure 30.1).

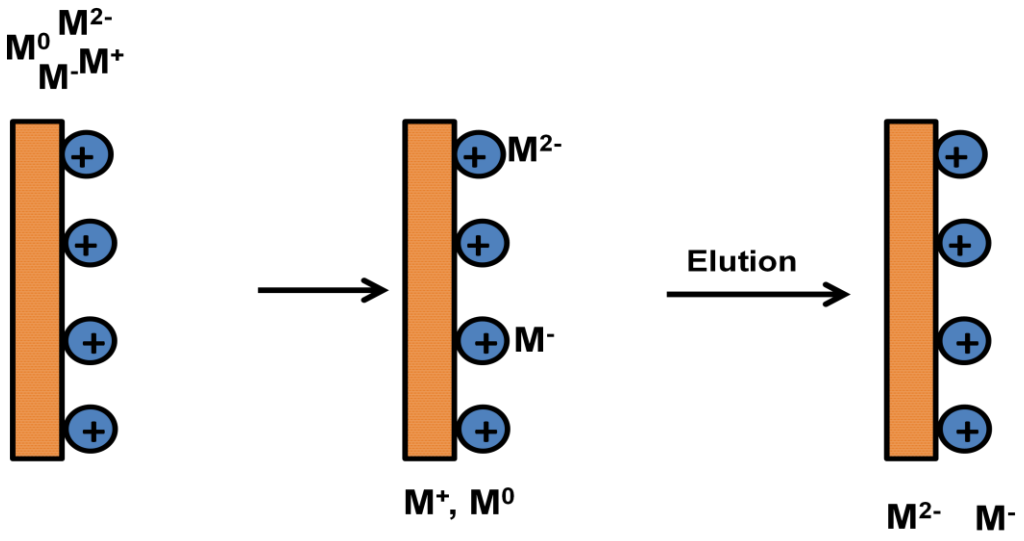


Figure 30.1: Affinity of analytes (M, M^{+}, M^{-1}, M^{-2}) towards positively charged matrix.

The matrix used in ion-exchange chromatography is present in the ionized form with reversibly bound ion to the matrix. The ion present on matrix participitate in the reversible exchange process with analyte. Hence, there are two types of ion-exchange chromatography:

1. Cation exchange chromatography- In cation exchange chromatography, matrix has a negatively charged functional group with a affinity towards positively charged molecules. The positively charged analyte replaces the reversible bound cation and binds to the matrix (Figure 30.2). In the presence of a strong cation (such as Na^+) in the mobile phase, the matrix bound positively charged analyte is replaced with the elution of analyte. The popular cation exchangers used are given in Table 30.1.

2. Anion Exchange chromatography- In anion exchange chromatography, matrix has a positively charged functional group with a affinity towards negatively charged molecules. The negatively charged analyte replaces the reversible bound anion and binds to the matrix (Figure 30.2, B). In the presence of a strong anion (such as Cl^-) in the mobile phase, the matrix bound negatively charged analyte is replaced with the elution of analyte. The popular anion exchangers used are given in Table 30.1.

Table 30.1: List of selected Ion-exchange matrix

S.No	Name	Functional Group	Type of Ion-exchanger
1	Carboxyl methyl (CM)	-OCH2COOH	Cation Exchanger
2	Sulphopropyl (SP)	-OCH2CH2CH2SO3H	Cation Exchanger
3	Sulphonate (S)	-OCH2SO3H	Cation Exchanger
4	Diethylaminoethyl (DEAE)	-OCH2CH2NH (C2H5)2	Anion Exchanger
5	Quaternary aminomethyl (Q)	-OCH2N(CH3)3	Anion Exchanger

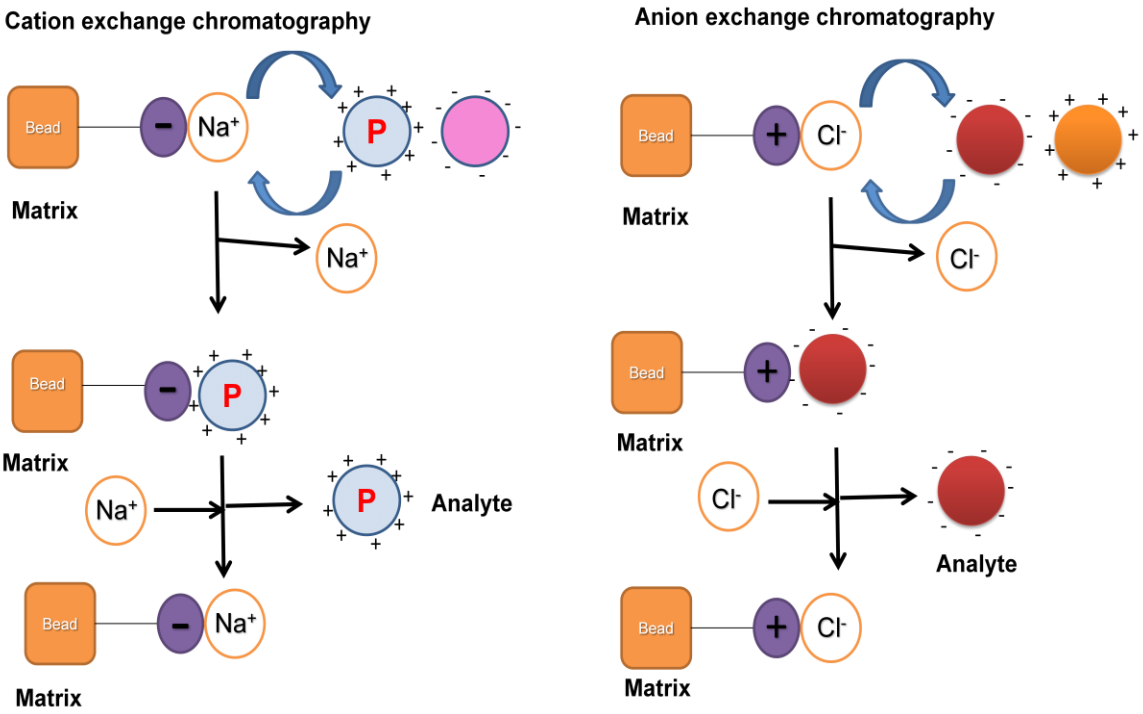


Figure 30.2: Cation and Anion exchange chromatography.

Isoelectric point and charge on a protein: Protein is a polymer made up of amino acids with ionizable side chain. At a particular pH, these amino acid side chain ionizes differentially to give a net charge (positive/negative) to the protein. The pH at which the net charge on a protein is zero is called as Isoelectric point (pI). The protein will have a net positive charge below the pI where as it has net negative charge above the pI value (Figure 30.3).

Choice of a Ion-exchange column matrix-Before starting the isolation and purification of a substance, a choice for a suitable ion-exchange chromatography is important. There are multiple parameter which can be consider for choosing the right column matrix.

1. pI value and Net charge- The information of a pI will be allow you to calculate the net charge at a particular pH on a protein. As discussed above, a cation exchange chromatography can be use below the pI where as an anion exchange chromatography can be use above the pI value.

2. Structural stability-3-D structure of a protein is maintained by electrostatic and vander waal interaction between charged amino acid, Π-Π interaction between hydrophobic side chain of amino acids. As a result, protein structure is stable in a narrow range around its pI and a large deviation from it may affect its 3-D structure.

3. Enzymatic activity-Similar to structural stability, enzymes are active in a narrow range of pH and this range should be consider for choosing an ion-exchange chromatography.

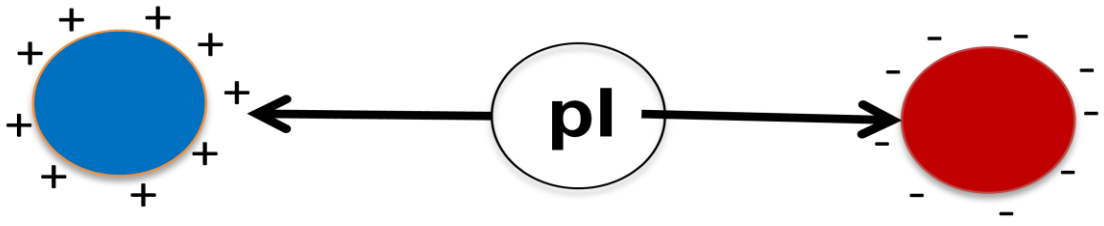


Figure 30.3: Change of charge with respect to the pI.

Operation of the technique-Several parameters needs to be consider to perform ion-exchange chromatography (Figure 30.4).

1. Column material and stationary phase-Column material should be chemically inert to avoid destruction of biological sample. It should allow free low of liquid with minimum clogging. It should be capable to withstand the back pressure and it should not compress or expand during the operation.

2. Mobile Phase-The ionic strength and pH are the crucial parameters to influence the property of the mobile phase.

3. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

4. Elution- There are many ways to elute a analyte from the ion-exchange column. (1) Isocratic elution (2) Step-wise gradient (3) Continuous gradient either by salt or pH (4) affinity elution (5) displacement chromatography

5. Column Regeneration- After the elution of analyte, ion-exchange chromatography column require a regeneration step to use next time. column is washed with a salt solution with a ionic strength of 2M to remove all non-specifically bound analytes and also to make all functional group in a iononized form to bind fresh analyte.

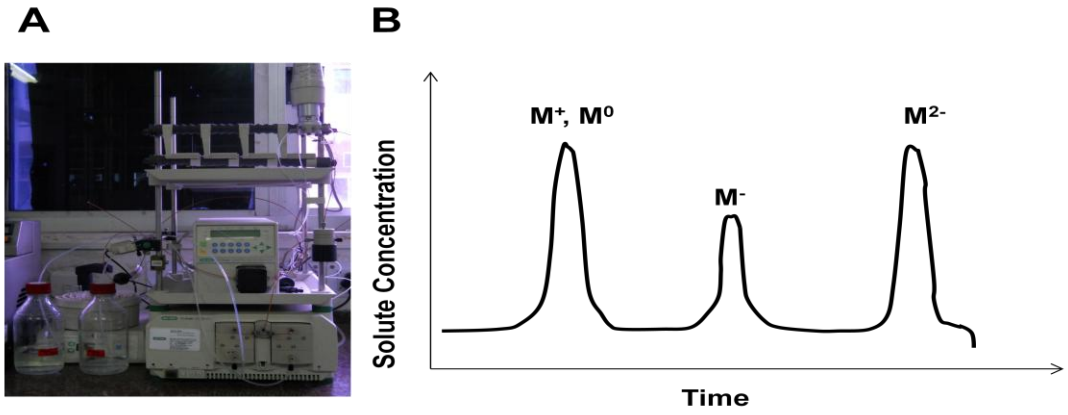


Figure 30.4 : Operation of the Ion-exchange Chromatography. (A) Chromatography system to perform gradient elution of analytes to give an (B) elution profile.

Lecture 31: Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography: Hydrophobic interaction chromatography exploits the ability of a strong interaction between hydrophobic group attached to the matrix and hydrophobic patches present on an analyte such as protein. Protein is made-up of amino acids with acidic, basic, polar and non-polar (aliphatic or aromatic) side chain. Protein is synthesized from ribosome as a linear chain and afterwards it gets folded into a 3-D conformation mostly guided by the environment of side chain and the outer medium. Local environment in a cell is aqeous and it favors the folding of protein to keep the polar or charged amino acids on the surface and non-polar side chain within the inner core (Figure 31.1). Most of the hydrophobic amino acids are shielded from the outer polar environment where as polar amino acid present on the surface has bound water molecule to form a hydration shell.

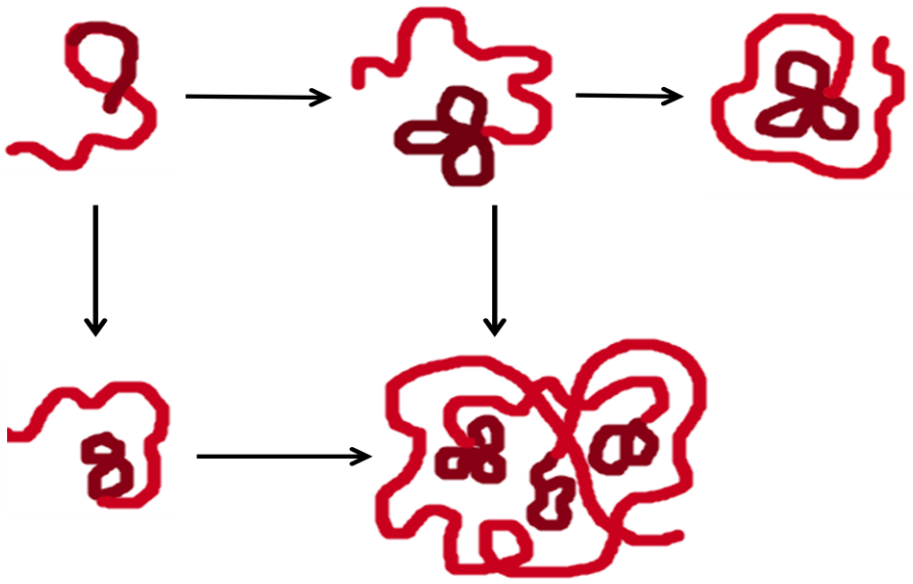


Figure 31.1: Folding of Protein in an aqueous environment. Following a series of folding stages, protein adopts a 3-D conformation with hydrophobic patches present in the core.

Addition of low amount of salt to the protein solution results in the displacement of bonded water molecule with an increase in protein solubility (Figure 31.2). This effect is called as **"salting-in"**. In the presence of more amount of salt, water molecule shielding protein side chains are displaced completely with an exposure of hydrophobic patches on protein surface to induce protein precipitation or decrease in protein solubility. This effect is called as **"salting-out"**. The phenomenon of salting out is modulated so that addition of salt induces exposure of hydrophobic patches on protein but does not cause precipitation or aggregation. The exposure of hydrophobic patches facilitates the binding of protein to the non-polar ligand attached to the matrix. When the concentration of salt is decreased, the exposed hydrophobic patches on protein reduces the affinity towards matrix and as a result it get eluted (Figure 31.3).

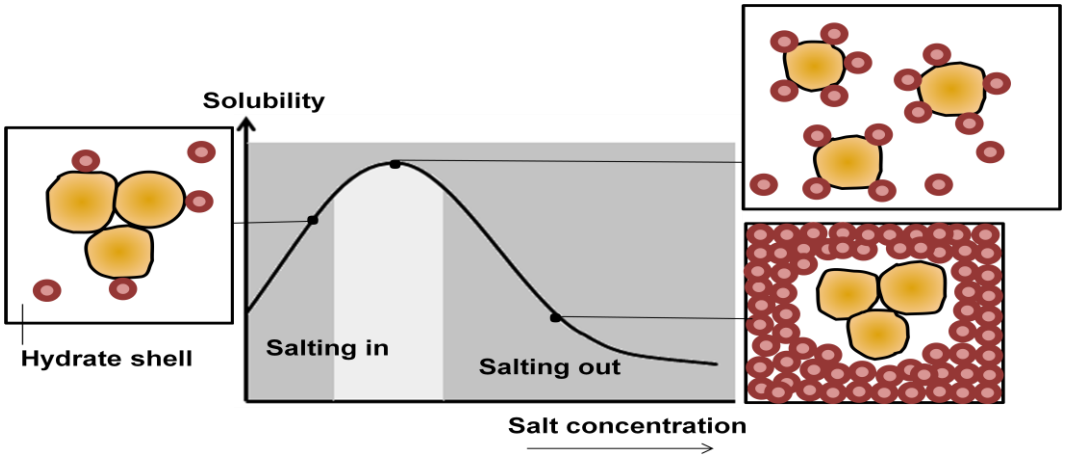


Figure 31.2: Effect of salt on protein, salting in and salting out effect.

The choice of HIC gel-The different commercially available HIC matrix are given in Table 31.1. Choosing a suitable HIC matrix is essential to achieve best result. The strength of the binding of analyte on a HIC column is governed by the length of the aliphatic linear ligand. Matrix with aromatic ring containing ligand makes additional Π-Π interaction and they will bind analyte more strongly than same number of carbon aliphatic ligand. In addition, presence of Π-Π interaction gives selectivity as well, such as ring containing aromatic ligand, phenylalanine. At last, ligand density plays a vital role in the strength of binding of an analyte to the matrix. Hence, these points should be consider to choose a suitable matrix for purification.

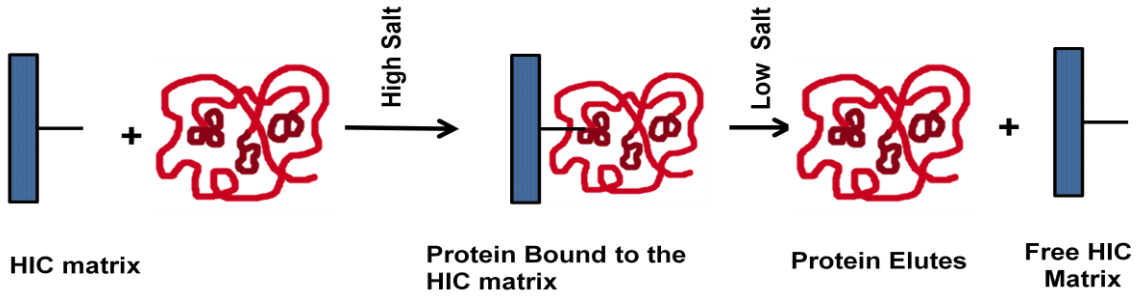


Figure 31.3: Principle of the hydrophobic interaction chromatography.

Table 31.1: Selected list of popular HIC column matrix.

S.NO	Column Material	Functional Group
1	Butyl-S-Sepharose	-Butyl
2	Phenyl Sepharose (Low Sub)	-Phenyl, low density
3	Phenyl Sepharose (High Sub)	-Phenyl, high density
4	Capto phenyl sepharose	-Phenyl
5	Octyl Sepharose	-Octyl

Operation of the technique-Several parameters needs to be consider to perform hydrophobic interaction chromatography.

1. Equlibration-HIC column material packed in a column and equilibrate with a buffer containing 0.5-1.5M ammonium sulphate (mobile phase). The salt must be below the concentration where it has salting-out effect.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- There are many ways to elute a analyte from the hydrophobic interaction column. (1) decreasing salt concentration, (2) changing the polarity of the mobile phase such as alchol, (3) By a detergent to displace the bound protein.

4. Column Regeneration- After the elution of analyte, HIC column require a regeneration step to use next time. column is washed with 6M urea or guanidine hydrochloride to remove all non-specifically bound protein. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

Lecture 32 Gel Filtration Chromatography

Introduction: This chromatography distributes the protein or analyte, based on their size by passing through a porous beads. The first report in 1955 described performing a chromatography column with swollen gel of maize starch to separate the protein based on their size. '**Porath and Floidin**'coined the term "gel filtration" for this chromatography technique separating the analytes based on molecular sizes. Since then the chromatography technique evolved in terms of developed of different sizes beads to separate protein of narrow range, as well as performing the technique in aqueous and non-aqueous mobile phase. The beads used in gel filtration chromatography is made up of cross linked material (such as dextran in sephadex) to form a 3-D mesh. These 3-D mesh swell in the mobile phase to develop pores of different sizes (Figure 32.1). The extent of cross linking controls the pores size within the gel beads.

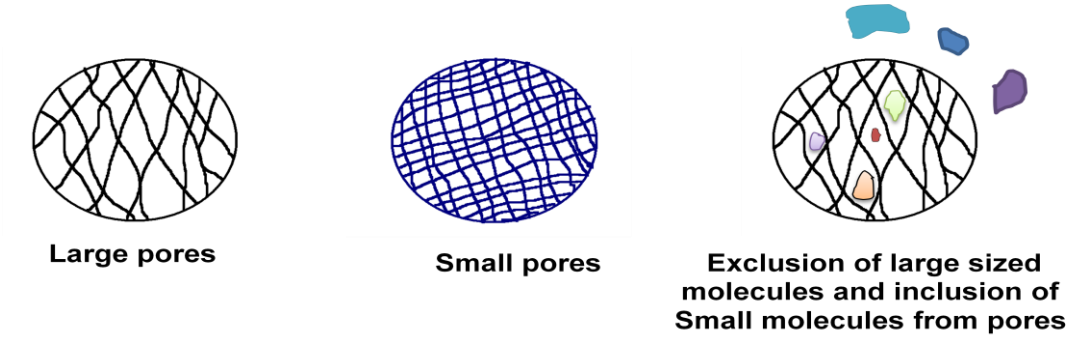


Figure 32.1: Gel Filtration Matrix has Beads with different pore sizes.

Principle: The principle of the chromatography technique is illustrated in Figure 32.2. The column is packed with the beads containing pores to allow entry of molecules based on their sizes. Smallest size in the inner part of pore followed by gradual increasing size and largest molecule excluded from entering into the gel. The separation between molecules occur due to the time they travel to come out from the pores. When the mobile phase pass through the column, it takes protein along with it. The small molecules present in the inner part of the gel takes longer flow of liquid (or time) and travel longer path to come out where as larger molecules travel less distance to come out. As a result, the large molecule and small molecule get separated from each other. A schematic gel filtration chromatogram is given in Figure 32.3.

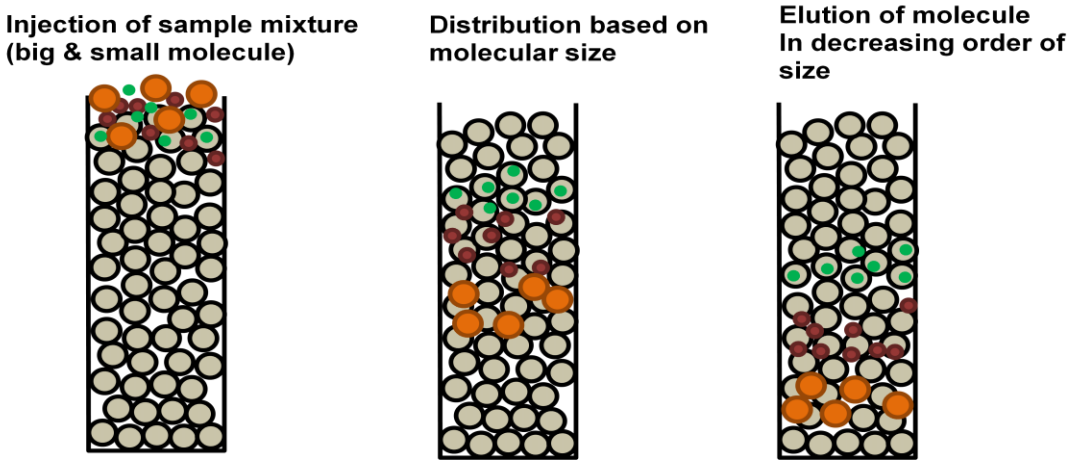


Figure 32.2: Principle of Gel Filtration Chromatography.

Suppose the total column volume of a gel is V_t and then it is given by-

$$V_t=V_g+V_i+V_o \quad \text{.....Eq (32.1)}$$

V_g is the volume of gel matrix, V_i is the pore volume and V_o is the void volume. The volume of mobile phase flow to elute a column from a column is known as elution volume (V_e). The elution volum is related to the void volume and the distribution coefficient Kd as given below

$$V_e=V_o+KdV_i \quad \text{.....Eq (32.2)}$$

$$Kd = \frac{Ve-Vo}{Vi} \quad \text{.....Eq (32.3)}$$

Kd is the ratio of inner volume available for an analyte and it is independent to the column geometry or length. As per relationship given in Eq 32.3, three different type of analytes are possible:

1. Analyte with **Kd=0**, or $V_e=V_o$, these analytes will be completely excluded from the column.

2. Analyte with **Kd=1** or $V_e=V_o+V_i$, these analytes will be completely in the pore of the column.

3. Analyte with **Kd>1**, in this situation analyte will adsorb to the column matrix.

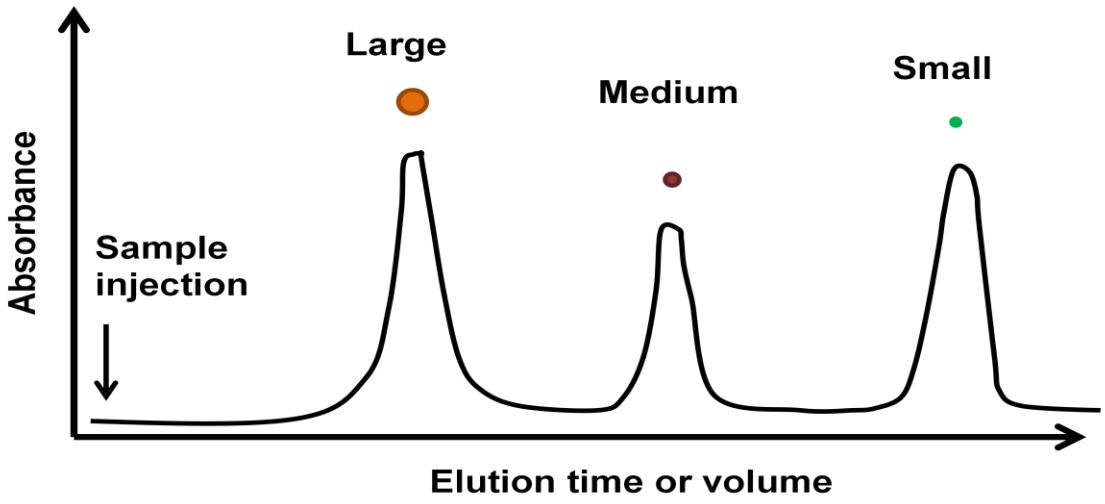


Figure 32.3: A typical Gel Filtration Chromatogram.

Choice of matrix for gel filtration chromatography-The choice of the column depends on the range of molecular weight and the pressure limit of the operating equipment. A list of popular gel filtration column matrix with the fractionation range are given in Table 32.1.

Operation of the chromatography-

1. Column packing-The column material is allowed to swell in the mobile phase. It is poured into the glass tube and allow the beads to settle without trapping air bubble within the column. Once the matrix is settled to give a column, it can be tested for presence of air channel and well packing by flowing a analyte with Kd=1, it is expected that the elution volume (Ve) in this case should be V_o+V_i.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- In gel filtration column, no gradient of salt is used to elute the sample from the column. The flow of mobile phase is used to elute the molecules from the column.

4. Column Regeneration- After the analysis of analyte, gel filtration column is washed with the salt containing mobile phase to remove all non-specifically adsorb protein to the matrix. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

Table 32.1: List of popular gel filtration matrix

S.No	Name of the matrix	Fractionation Range (Daltons)
1	Sephadex G10	Upto 700
2	Sephadex G25	1000-5000
3	Sephadex G50	1500-30,000
4	Sephadex G100	4000-150,000
5	Sephadex G200	5000-600,000
6	Sepharose 4B	60,000-20,000,000
7	Sepharose 6B	10,000-4,000,000

Determination of native molecular weight of a protein using gel filtration chromatography

The molecular weight and size of a protein is related to the shape of the molecule and the relationship between molecular weight (M) and radius of gyration (R_g)is as follows-

$$Rg \propto M^a \quad \text{……Eq 32.4}$$

here "a" is a constant and it depends on shape of the molecule, a=1 for Rod, a=0.5 for coils and a=0.33 for spherical molecules.

The set of known molecular weight standard protein can be run on a gel filitration column and elution volume can be calculated from the chromatogram (Figure 32.4). A separate run with the analyte will give elution volume for unknown sample. Using following formula, Kd value for all standard protein and the test analyte can be calculated.

$$\mathbf{Kd} = \frac{Ve - Vo}{Vi}$$

A plot of Kd versus log mol wt is given in Figure 32.4, B and it will allow us to calculate the molecular weight of the unknown analyte.

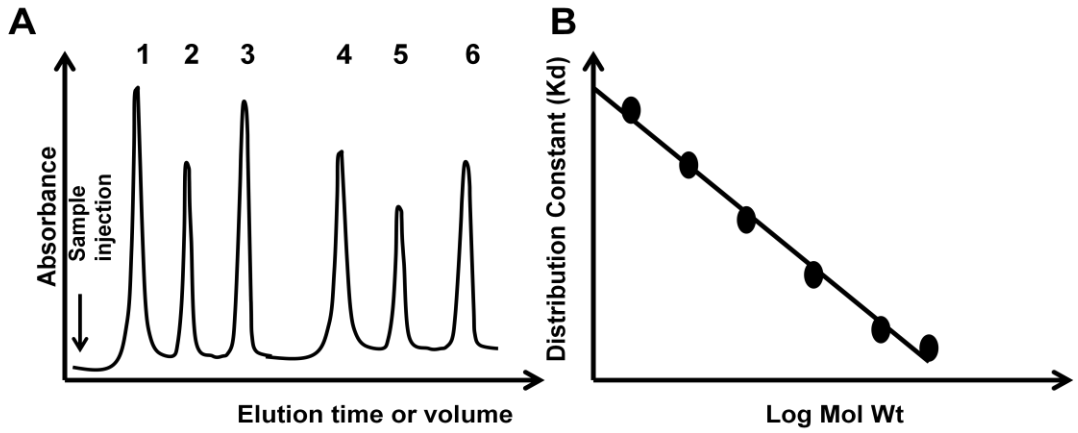


Figure 32.3: Determination of molecular weight by gel filtration chromatography. (A) Gel filtration chromatogram with the standard proteins (1-6), (B) Relationship between distribution constant (Kd) and Log Molecular weight.

Besides determining molecular weight of the protein as discussed above, gel filutration chromatography can be used to determine following properties of a protein:

1. Oligomeric status

2. Desalting

3. Protein-ligand interaction

4. Folding pathway.

Lecture 33 Affinity Chromatography

Introduction: The chromatography techniques we discussed so far were exploiting different types of interactions between the matrix and the group present on the analyte but these chromatography techniques are not specific towards a particular analyte per se. The generalized chromatography approaches needs higher sample volume to isolate the molecule of interest. In the current lecture we will discuss another chromatography technique where a chromatography matrix is specific for a particular molecule or group of protein.

Principle: The affinity chromatography works on the principle of mutual recognition forces between a ligand and receptor. The major determinants, responsible to provide specificity are shape complementarity, electrostatic, hydrogen bonding, vander waal interaction between the groups present on the ligand-receptor pair (Figure 33.1). A mutual interaction between a ligand (L) and receptor (R) forms ligand-receptor complex (RL) with a dissociation constant Kd, which is expressed as follows-

$$\mathbf{R + L \leftrightharpoons RL}$$

$$\mathbf{Kd = \frac{[R][L]}{[RL]} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 33.1}$$

Dissociation constant is specific to the receptor-ligand pair and number of interaction between them. when a crude mixture is passed through an affinity column, the receptor present on the matrix reacts with the ligand present on different molecules. The mutual collision between receptor on matrix and ligands from different molecule test the affinity between them and consequently the best choice bind to the receptor where as all other molecules do not bind and appear in flow through. A wash step removes remaining weakly bound molecules on matrix. Subsquently, a counter ligand is used to elute the bound molecule through a competition between the matrix bound molecule and counter ligand (Figure 33.2).

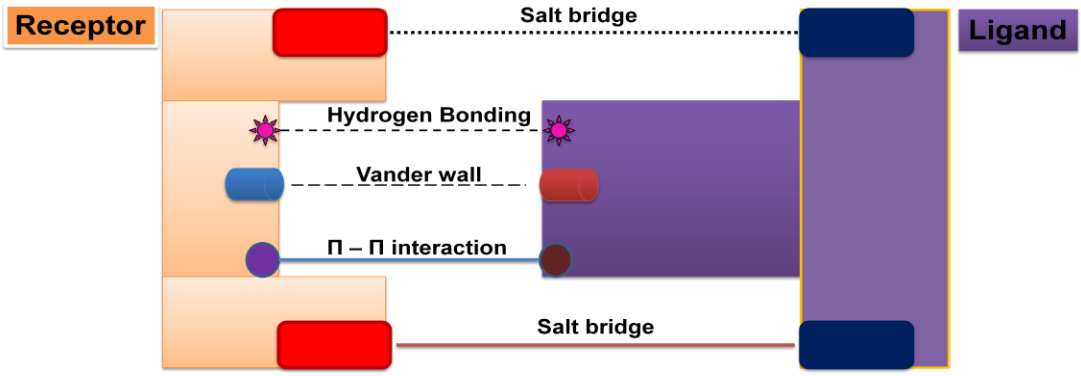


Figure 33.1: Interactions playing crucial role in providing specificity.

Advantages of Affinity chromatography-

1. Specificity: Affinity chromatography is specific to the analyte in comparison to other purification technique which are utilizing molecular size, charge, hydrophobic patches or isoelectric point etc.

2. Purification Yield: Compared to other purification method, affinity purification gives very high level of purification fold with high yield. In a typical affinity purification more than 90% recovery is possible.

3. Reproducible: Affinity purification is reproducible and gives consistent results from one purification to other as long as it is independent to the presence of contaminating species.

4. Easy to perform: Affinity purification is very robust and it depends on force governing ligand-receptor complex formation. Compared to other techniques, no column packing, no special purification system and sample preparation required for affinity purification.

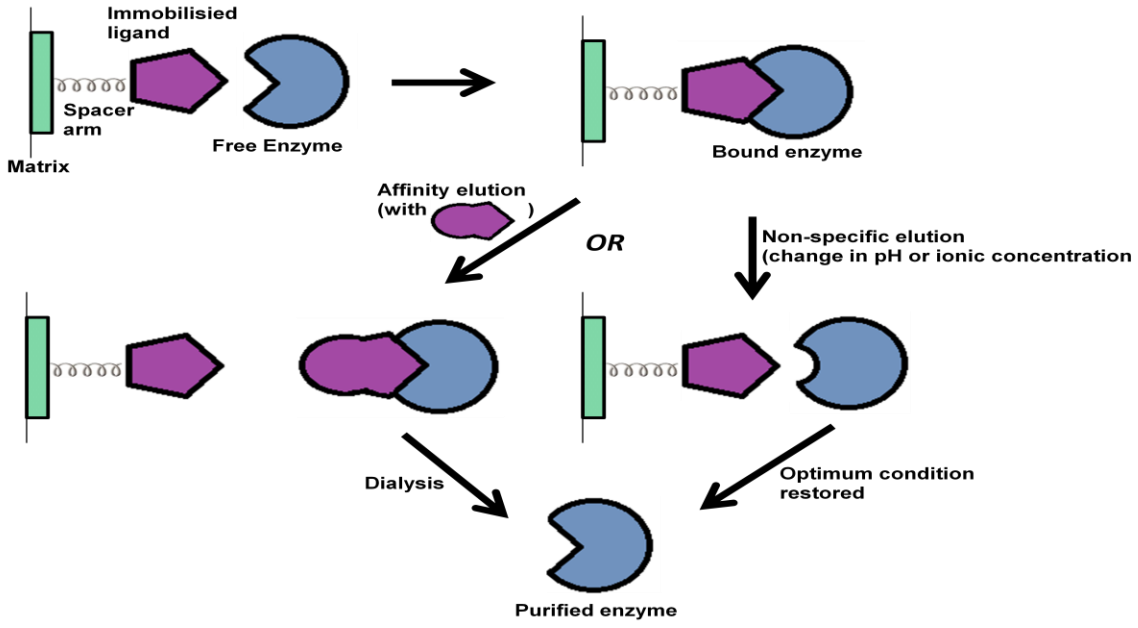


Figure 33.2: Principle of affinity chromatography.

Different types of affinity chromatography: Affinity chromatography is further divided into the different types based on the nature of receptor present on matrix to binds tag present on the analyte molecule. Different types of affinity chromatography are-

Bio-affinity chromatography- In this type of affinity chromatography, biomolecules are used as receptor present on matrix and it exploit the biological affinity phenomenon such as antibody-antigen. In addition, enzyme-substrate or enzyme-inhibitor is also belong to this class. Ex. GST-Glutathione.

Pseudo-affinity chromatography-In this affinity chromatography, a non-biological molecule is used as receptor on matrix to exploit the separation and purification of biomolecules. There are two specific example to this class-

A. Dye-affinity chromatography-In this method, matrix is coupled to the reactive dye and the matrix bound dye has specificity towards a particular enzyme. For ex. Cibacron Blue F3G-A dye coupled to the dextran matrix has strong affinity towards dehydrogenases.

B. Metal-affinity chromatography-In this method, transition metals such as Fe^{2+}, Ni^{2+} or Zn^{2+} is coupled to the matrix and the matrix bound metal form multidentate complex with protein containing poly-his tag (6x His). The affinity of protein for matrix bound metal is different and these differences are been exploited in metal affinity chromatography to purify the protein.

Covalent chromatography- This is a different type of chromatography technique where binding of analyte to the matrix is not reversible as it involves the formation a covalent bond between functional group present on matrix and analyte. Thiol group (-SH) present on neighbouring residues of protein forms disulphide bond after oxidation and under reducing environment, disulphide reversible broken back to free thiol group. The matrix in covalent chromatography has immobilized thio group which forms covalent linkage with the free thiol group containing protein present in the mixture (Figure 33.3). After a washing step to remove non-specifically bound protein, a mobile phase containing compound with reducing thio group is passed to elute the bound protein. The thio group containing compound present in mobile phase breaks the disulphide bond between protein and matrix thio group to release the protein in the mobile phase (Figure 33.3).

Choice of matrix for Affinity chromatography- Different popular affinity matrix used for protein purification is given in Table 33.1. The choice of matrix solely depends on the affinity tag present on the recombinant protein produced after genetic engineering.

Table 33.1: Matrix containing receptor for ligand present on protein.

S.No.	Receptor	Affinity towards protein ligand
1	5'AMP	NAD^+-dependent dehydrogenase
2	2'5'-ADP	$NADP^+$-dependent dehydrogenase
3	Avidin	Biotin-containing enzymes
4	Protein A and Protein G	Immunoglobulin
5	Concanavalin A	Glycoprotein
6	Poly-A	Poly U mRNA
7	Lysine	rRNA
8	Cibacron Blue F3GA	NAD+ Containing dehydrogenase
9	Lectin	Glycoprotein
10	Heparin	DNA binding site

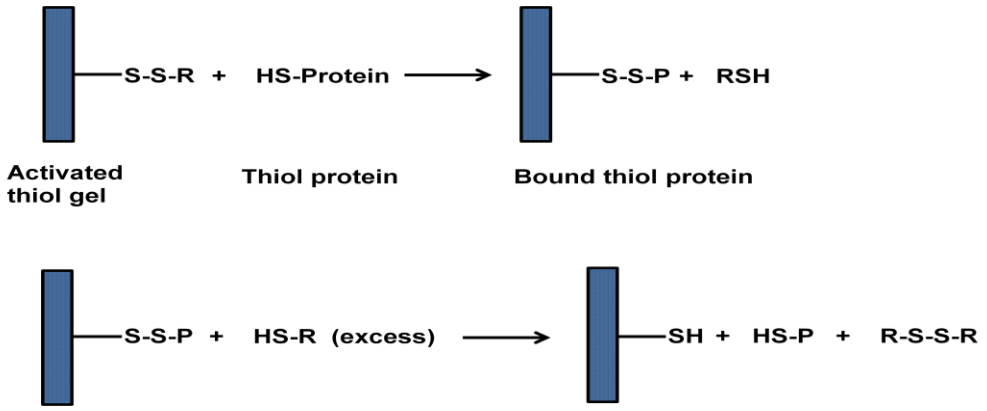


Figure 33.3: Principle of covalent chromatography.

Operation of the Affinity chromatography-Different steps in affinity chromatography is given in Figure 33.4.

1. Equlibration-Affinity column material packed in a column and equilibrate with a buffer containing high salt (0.5M NaCl) to reduce the non-specific interaction of protein with the analyte.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- There are many ways to elute a analyte from the affinity column. (1) increasing concentration of counter ligand, (2) changing the pH polarity of the mobile phase, (3) By a detergent or chaotrophic salt to partially denature the receptor to reduce the affinity for bound ligand.

4. Column Regeneration- After the elution of analyte, affinity column requires a regeneration step to use next time. column is washed with 6M urea or guanidine hydrochloride to remove all non-specifically bound protein. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

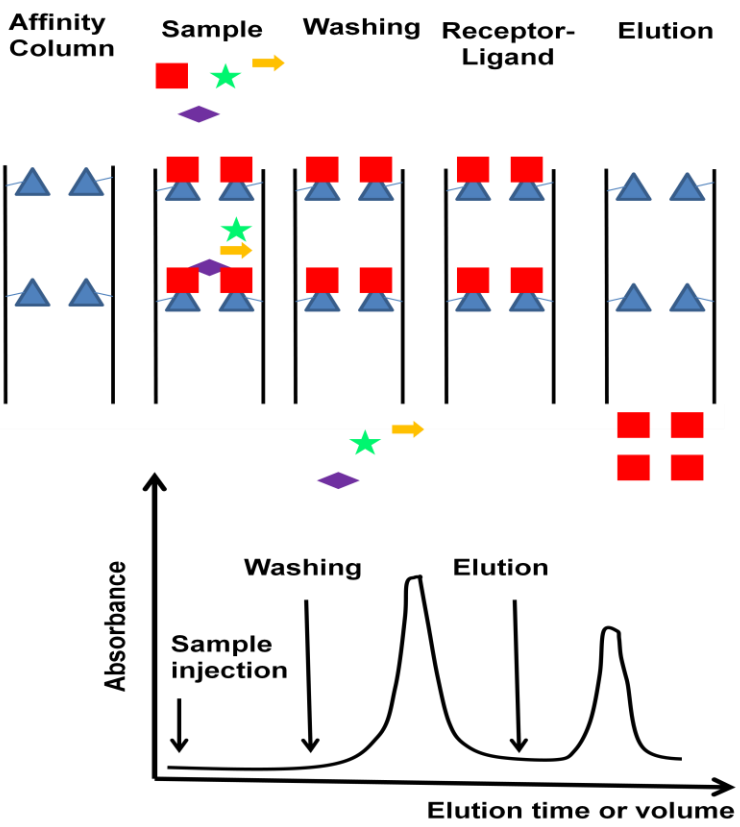


Figure 33.4: Performing Affinity chromatography

Applications of Affinity Chromatography

1. Purification of biomolecules present in traces
2. Recovery of biomolecules from crude extract
3. Scavenging of unwanted contaminants
4. Clinical diagnosis
5. Estimation of biomolecules
6. Immunoassay

Lecture 34 Thin Layer Chromatography

The thin layer chromatography technique is an analytical chromatography to separate and analyze complex biological or non-biological samples into their constituents. It is most popular for monitoring the progress of a chemical reaction or estimation of a substance in a mixture. It is also one of the popular technique for testing the purity of a sample. In this method, the silica or alumina as a stationery phase is coated on to a glass or aluminium foil as thin layer and then a sample is allowed to run in the presence of a mobile phase (solvent). In comparison to other chromatography techniques, the mobile phase runs from bottom to top by diffusion (in most of the chromatography techniques, mobile phase runs from top to bottom by gravity or pump). As sample runs along with the mobile phase, it get distributed into the solvent phase and stationery phase. The interaction of sample with the stationery phase retard the movement of the molecule where as mobile phase implies an effective force onto the sample. Supose the force caused by mobile phase is $\mathbf{F_m}$ and the retardation force by stationery phase is $\mathbf{F_s}$, then effective force on the molecule will be $\mathbf{(F_m\text{-}F_s)}$ through which it will move. The molecule immobilizes on the silica gel (where, $F_m=F_s$) and the position will be controlled by multiple factors.

1. Nature or functional group present on the molecule or analyte.

2. Nature or composition of the mobile phase

3. Thickness of the stationery phase.

4. Functional group present on stationery phase.

If the distance travelled by a molecule on TLC plate is $\mathbf{D_m}$ where as the distance travelled by the solvent is $\mathbf{D_s}$, then the retardation factor (R_f) of molecule is given by:

$$\mathbf{Rf} = \frac{\mathbf{Distance\ travelled\ by\ substance\ (Dm)}}{\mathbf{Distance\ travelled\ by\ solvent\ (Ds)}}$$

R_f value is characterstic to the molecule as long as the solvent system and TLC plate remains unchanged. It can be used to identify the substance in a crude mixture.

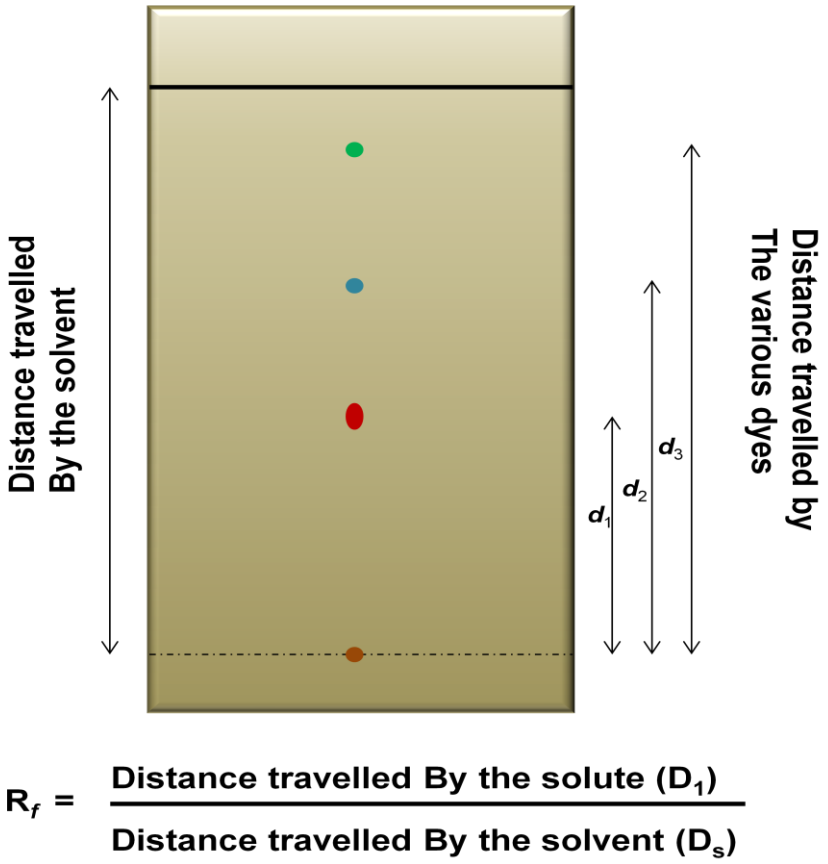


Figure 34.1: Principle of thin layer chromatography.

Operation of the technique-Several steps are required to perform a thin layer chromatography to analyze a complex samples. These preparatory and operational steps are as follows:

Thin Layer Chromatography Chamber- Thin layer chromatographjy chamber (rectangular or cylindrical) is made up of transparent non-reactive material, mostly glass (Figure 34.2). It is covered from top with a thick glass sheet and the joints are sealed with a high vacuum grease to avoid loss of solvent vapor. All three sides of the chamber is covered with a whatman filter paper to uniformly equilibrate the chamber. A solvent system is filled in the chamber and it is allowed to humidify the chamber with the solvent vapor. It is important for uniform running of solvent front during TLC.

Preparation of TLC plate- A silica slurry is prepared in water and spread on the glass or alumina sheet as a thin layer and allowed to dry. It is baked at 110^0C for 1hr in a hot air oven and then the plate is ready for TLC. The layer is thin (~ 0.1-0.25 mm) for analytical application and thick (0.4-2.1 mm) for preparative or bio-assay purposes.

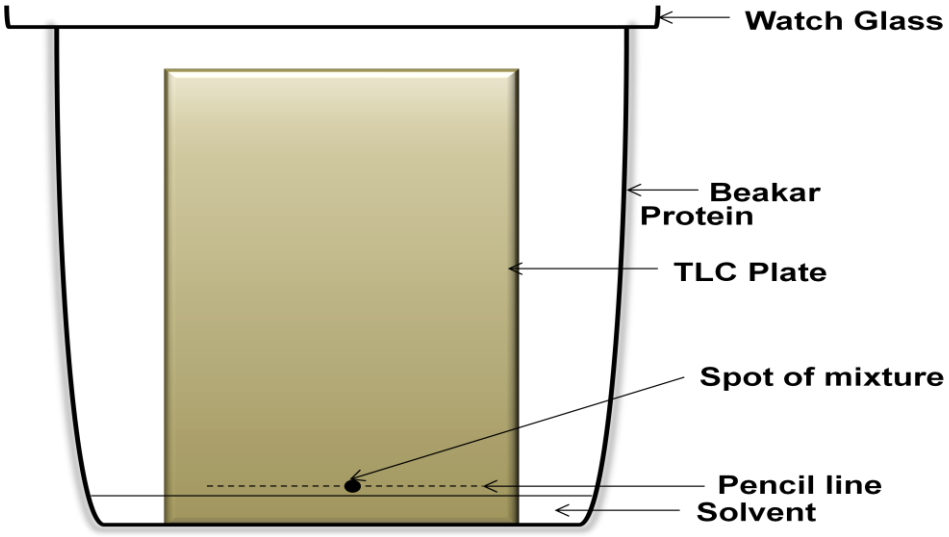


Figure 34.2: Thin Layer chromatography chamber

Spoting: The events involved in spotting is given in Figure 34.3. A line is draw with a pencil little away from the bottom. Sample is taken into the capillary tube or in a pipette. Capillary is touched onto the silica plate and sample is allowed to dispense. It is important that depending on the thickness of the layer, a suitable volume should be taken to apply. Spot is allowed to dry in air or a hair dryer can be used instead.

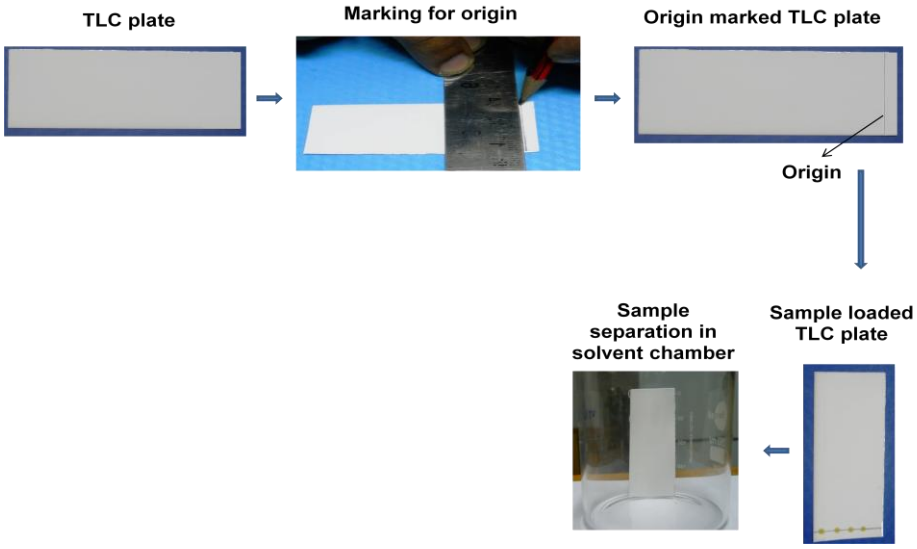


Figure 34.3: Events in spotting during thin layer chromatography.

Running of the TLC: Once the spot is dried, it is placed in the TLC chamber in such a way that spot should not be below the solvent level. Solvent front is allowed to move until the end of the plate.

Analysis of the chromatography plate- The plate is taken out from the chamber and air dried. If the compound is colored, it forms spot and for these substances there is no additional staining required. There are two methods of developing a chromatogram-

Staining procedure- In the staining procedure, TLC plate is sprayed with the staining reagent to stain the functional group present in the compound. Forx. Ninhydrin is used to stain amino acids.

Non-staining procedure- In non-staining procedure spot can be identify by following methods-

1. Autoradiography- A TLC plate can be placed along with the X-ray film for 48-72 hrs (exposure time depends on type and concentration of radioactivity) and then X-ray film is processed.

2. Fluorescence- Several heterocyclic compounds give fluorescence in UV due to presence of conjugate double bond system. TLC plate can be visualized in an UV-chamber (Figure 34.4) to identify the spots on TLC plate.

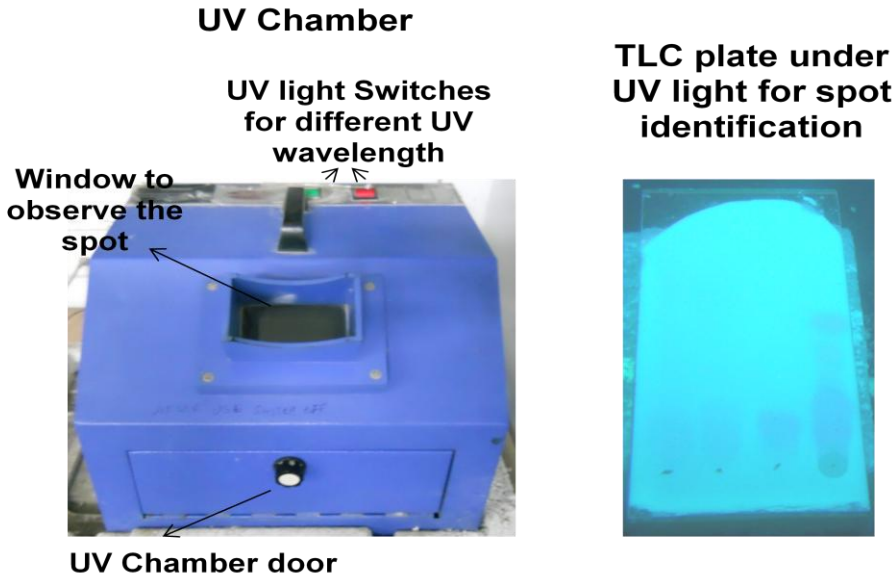


Figure 34.4: UV-Chamber and UV illuminated TLC plate.

Technical troubles with thin layer chromatography-

1. Tailing effect-In general sample forms round circular spot on the TLC plate. It is due to the uniform movement of the solvent front through out the plate. But in few cases instead of forming a spot, a compound forms a spot with long trail or rocket shape spot (Figure 34.5). it is due to few reasons as given below:

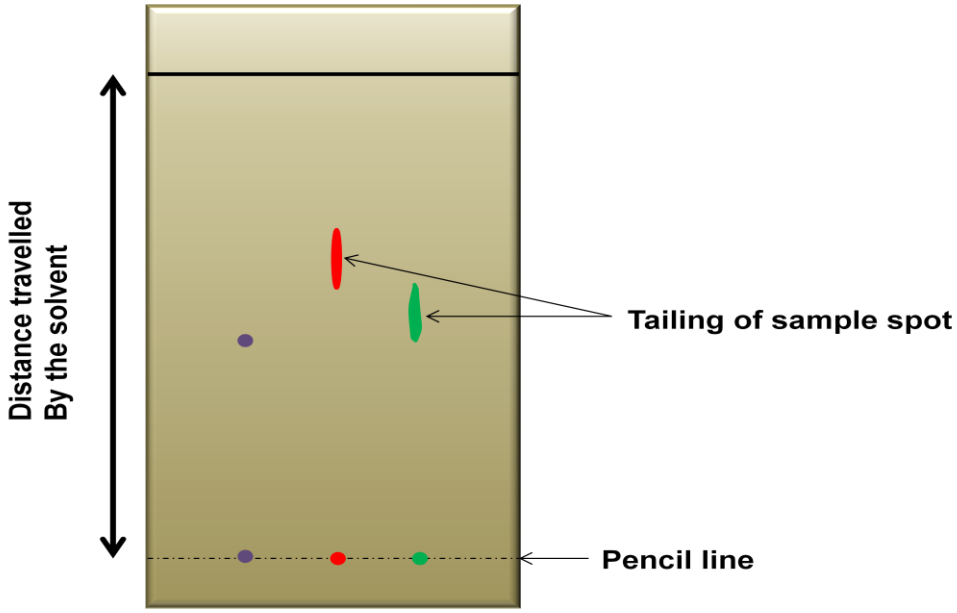


Figure 34.5: Tailing effect in thin layer chromatography.

A. Over-loading- if the sample is loaded much more than the loading capacity of the TLC plate, it appears as spot with trail or rocket shape spot. A diluted sample can be tested to avoid this.

B. Fluctuation in temp or opening of chamber- If there will be fluntuation in temperature or solvent saturation in the chamber (due to opening of the chamber during running), it disturb the flow of solvent front and consequently cause spot with trail. it can be avoided by maintaining a uniform temperature and the opening of the chamber should be minimized especially during running.

2. No movement of sample- In few cases, a sample doesn't move from the spot after the run is completed. These problems are common with high molecular weight substances such as protein or chemicals with large number of functional group. In this case, a change in polarity or pH of solvent system can be explored to bring the compound into the solvent front so that it run on silica plate to get resolved.

3. Movement is too fast-In few cases, the movement of a compound is too fast and does not give time to interact with the matrix to resolve into individual compounds. In this case, a change in polarity of solvent system can be explored to retard the running of the sample.

Applications of Thin layer Chromatography

1. Composition analysis of biomolecules/synthetic preparation
2. Quality testing of compound.
3. Identification of impurities in a sample
4. Progress of chemical reaction
5. Estimation of biomolecules
6. Bio-assay

Lecture 35 Electrophoresis (Part-I)

"Electrophoresis" literally means running in the electric field. The charged molecule moves to their counter charge electrodes but electric field is removed before it reaches the electrode. Movement of charged species in an electric field gives differential mobility to the sample based on the charge and consequently resolve them. Movement of the charged particle is retarded with the addition a polymeric gel so that a sufficient time is available for resolving the sample. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead it, forms pores of different size (depending on the concentration of polymer) and sample pass through these pore and as a result their electrophoretic mobility is reduced (Figure 35.1).

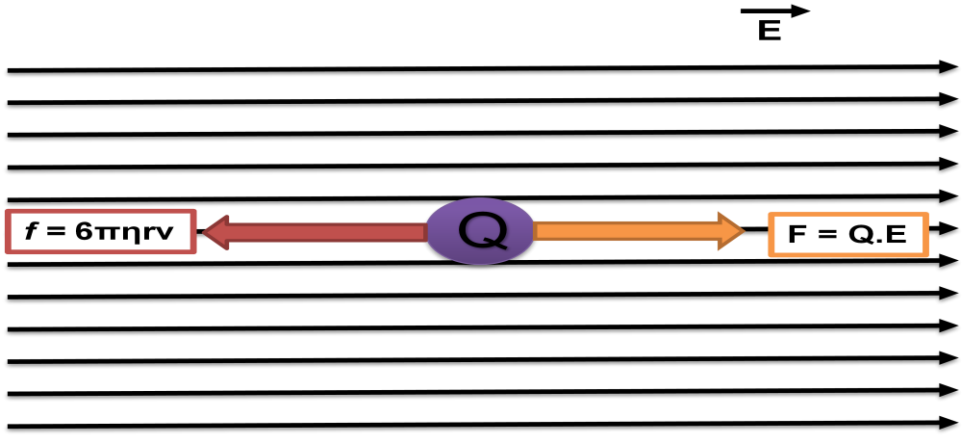


Figure 35.1: Movement of the charged particle in an external field.

Suppose a charged particle has net charge **Q** and the external electric field is **E**, then the force **F** responsible for giving electrophoretic mobility,

F= Q.E…….Eq (35.1)

The friction forces F which is opposing the movement of the charged particle is as follows

***F*= *f*. *v*……Eq (35.2)**, here *f* is the friction coefficient and the *v* is the velocity of the electrophoretic mobility. The movement of a spherical through a liquid medium (gel) of the viscosity η, the friction coefficient *f* is given by :

***f* = 6Πηr*v*……Eq (35.3)**

The place where, F=*F* or QE=**6Πηr*v***

The electrophoretic mobility *v* is given by: $$\boldsymbol{v = \frac{Q}{6\Pi\eta r}}$$

As Q=ze, where z is the valency and e is the electronic charge, the electrophoretic mobility can be expressed as:

$$\boldsymbol{v = \frac{ze}{6\Pi\eta r}}$$

Hence, electrophoretic mobility *v* is directly proportional to the charge and inversely proportional to the viscosity of the medium, size and shape of the molecule. In the case of relative mobility, it is directly related to the charge/radius of the molecule. For a globular protein, the radius (r) of the molecule is related to the molecular mass of the macromolecule. The relative mobility, *v'* is as follows

$$\boldsymbol{v' = \frac{Charge}{mass}}\textbf{……Eq (35.4)}$$

Electrophoretic techniques: Different types of electrophoresis techniques are designed depending upon whether it carried out in the presence or absence of a supporting media.

Moving boundary electrophoresis-In this method, the electrophoresis is carried in solution, without a supporting media. The sample is dissolved the buffer and molecules move to their respective counter charge electrodes. The resolution of the technique is very low due to the mixing of the sample as well as over-lapping of the sample components. The electrophoresis technique is not good to separate and analyze the complex biological sample instead it can be used to study the behavior of the molecule in an electric field.

Zone electrophoresis-In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The major advantage of presence of supporting media is that it minimizes mixing of the sample and immobilization of the molecule after electrophoresis. It makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. The gel electrophoresis is the best example of zone electrophoresis.

Gel electrophoresis:

Vertical Gel Electrophoresis: The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab. It has multiple modification in the running condition to answer multiple analytical questions.

1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Instrument- The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 35.2. It has two buffer chamber, upper chamber and a lower chamber. Both chamber are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

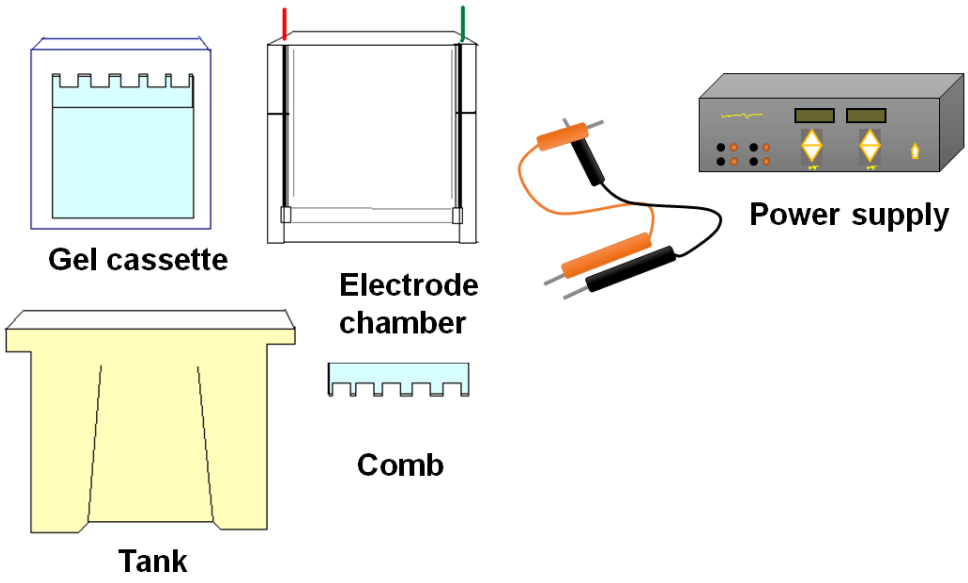


Figure 35.2: Different components of vertical gel electrophoresis apparatus.

Buffer and reagent for electrophoresis- The different buffer and reagents with their purpose for vertical gel electrophoresis is as follows-

1. N, N, N', N'-tetramethylethylenediamine (TEMED)-it catalyzes the acrylamide polymerization.

2. Ammonium persulfate (APS)-it is an initiator for the acrylamide polymerization.

3. Tris-HCl- it is the component of running and gel casting buffer.

4. Glycine- it is the component of running buffer.

5. Bromophenol blue- it is the tracking dye to monitor the progress of gel electrophoresis.

6. Coomassie brilliant blue R250-it is used to stain the polyacrylamide gel.

7. Sodium dodecyl sulphate-it is used to denature and provde negative charge to the protein.

8. Acrylamide- monomeric unit used to prepare the gel.

9. Bis-acrylamide- cross linker for polymerization of acrylamide monomer to form gel.

Casting of the gel: The acrylamide solution (a mixture of monomeric acrylamide and a bifunctional crosslinker bisacrylamide) is mixed with the TEMED and APS and poured in between the glass plate fitted into the gel caster. Ammoinum persupfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acryalide monomer to form a linear polymer (Figure 35.3). These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. IN a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organisc solvent (such as butanol or isoproponal) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After

polymerization of the resolving gel, a stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples (Figure 35.4).

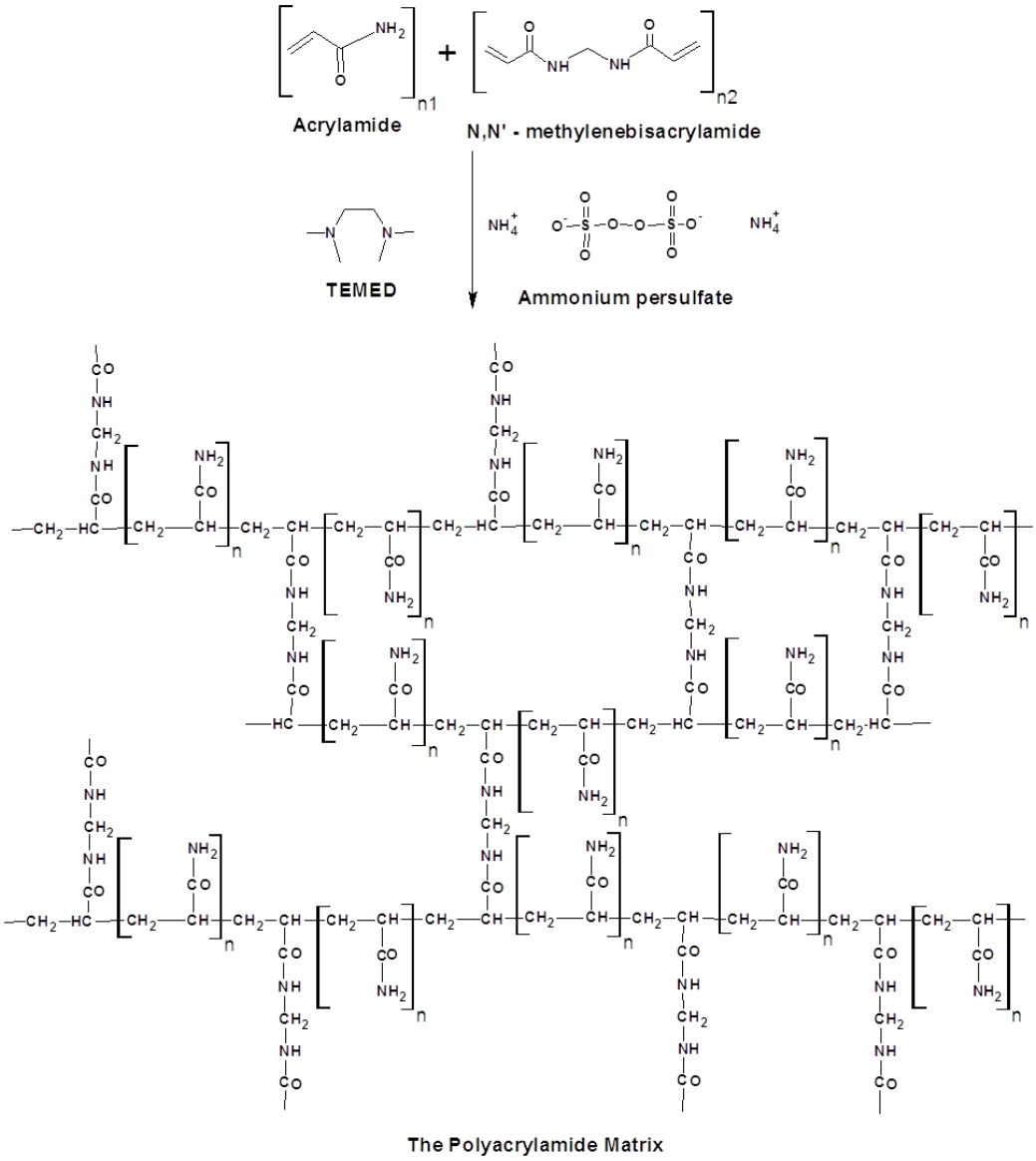


Figure 35.3: Mechanism of acylamide polymerization.

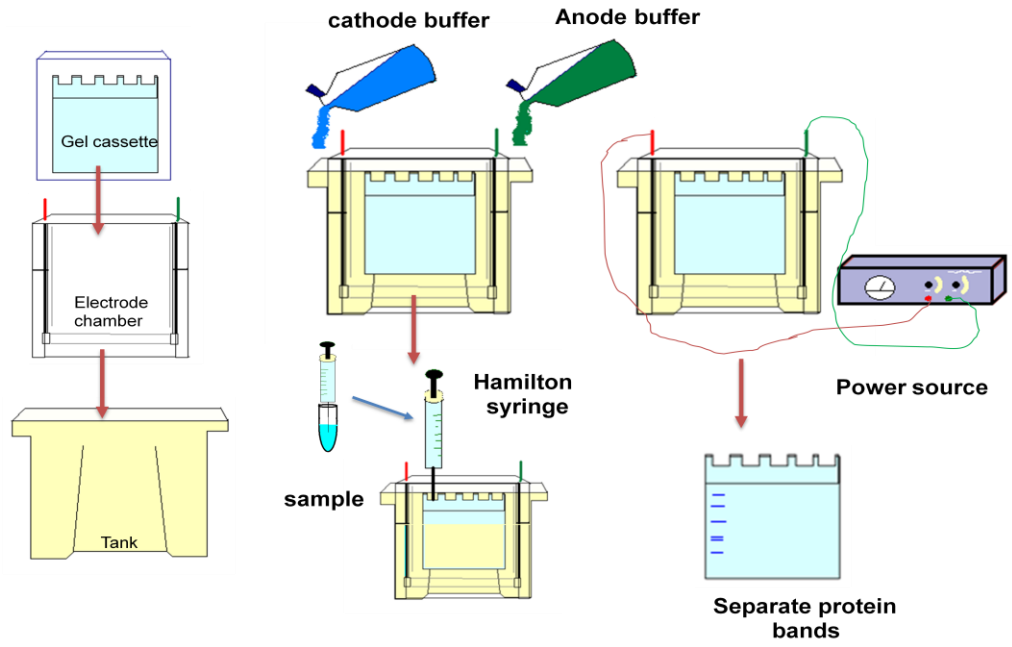


Figure 35.4: Different steps in performace of vertical gel electrophoresis to resolve sample.

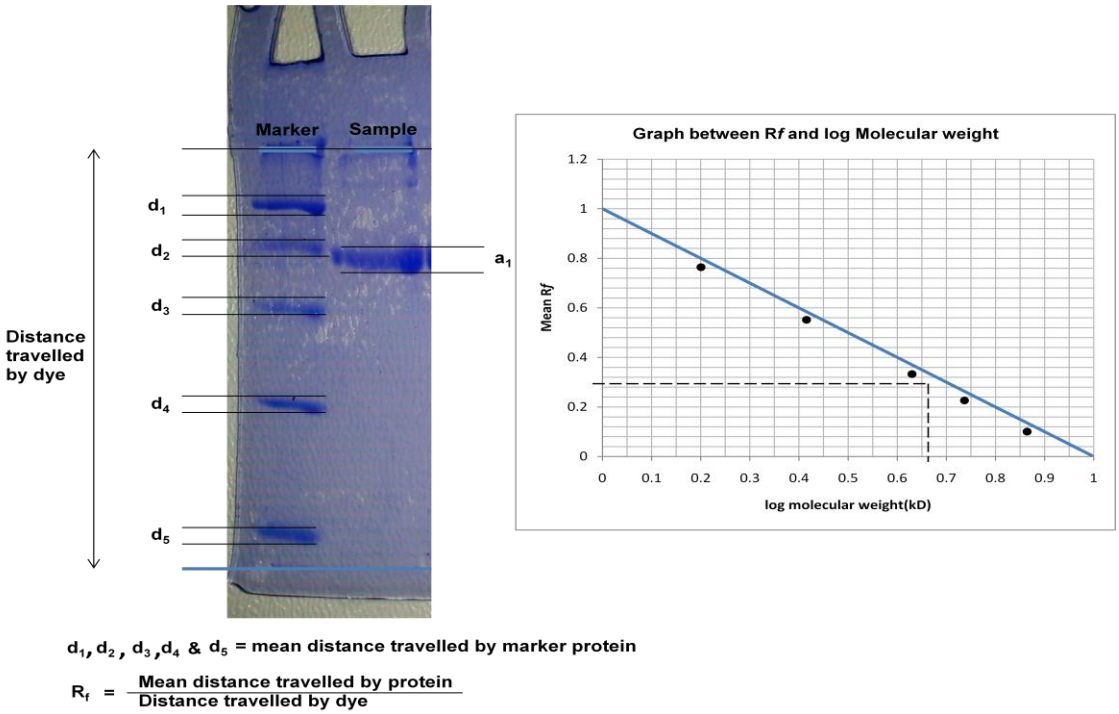
Running of the gel: The sample is prepared in the loading dye containing SDS, β-mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top Vs at the bottom in a lane. This problem is taken care once the sample run through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front where as Tris-HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula and it is stained with coomassie brilliant blue R250 dye. The dye stains protein present on the gel. A typical SDS-PAGE is given in the Figure 35.5.

In SDS-PAGE, the relative mobility and the log molecular weight as given by

$$v' = \mathrm{Vo}\,\frac{\mathrm{A}-\log \mathrm{M}}{\mathrm{A}} \quad \text{.....Eq (35.5)}$$

Molecular weight of a protein can be determined by plotting relative migration Rf with the log molecular weight of standard protein.

$$Rf = \frac{\text{migration of protein from the lane}}{\text{migration of tracking dye}} \quad \text{.....Eq (35.6)}$$



d_1, d_2, d_3, d_4 & d_5 = mean distance travelled by marker protein

$$R_f = \frac{\text{Mean distance travelled by protein}}{\text{Distance travelled by dye}}$$

Figure 35.5: Determination of molecular weight using SDS-PAGE. (A) SDS-PAGE (B) Determinaion of Rf.

Lecture 36 Electrophoresis (Part-II)

Summary of previous lecture: In the previous lecture, we discussed the principle of the electrophoresis. It is a mechanism of separating the charged species. In addition, we discussed the different type of electrophoresis techniques, details of vertical gel electrophoresis, instrument, reagent and performing the SDS-PAGE.

2. Native PAGE: SDS-PAGE discussed in the previous lecture is using anionic detergent sodium dodecyl sulfate and β-mercaptoethanol to give equal charge to all protein and breaks the disulphide linkage. As a result, the 3-D structure of the protein is destroyed and it migrate as per their subunit molecular weight. In the native PAGE, sample is prepared in the loading dye does not contains detergent or denaturating agent and as a result, sample runs on the basis of charge/mass. In native PAGE, the 3-D conformation as well as activity of the protein remains unaffected.

3. Urea PAGE: In this method, insoluable protein is dissolved in Urea and samples separate based on their charge/subunit mass. A gradient Ura PAGE is used to monitor the folding states of a protein.

2. Horizontal gel electrophoresis- The electrophoresis in this gel system is performed in a continuous fashion with both electrodes and gel cassette submersed within the buffer. The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 36.1. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc.

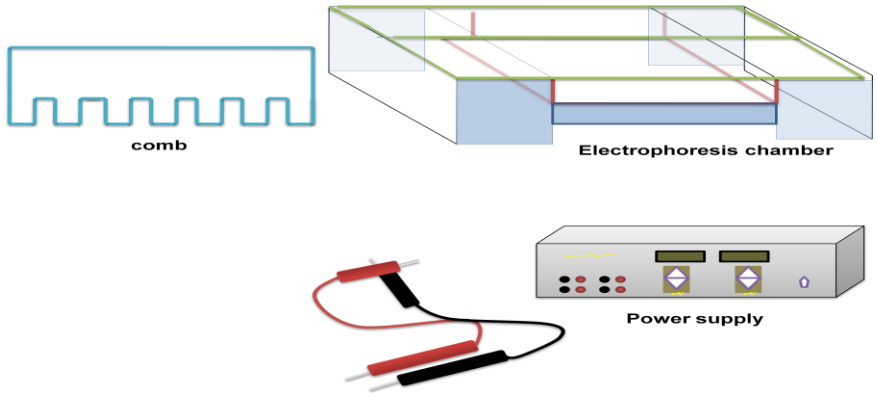


Figure 36.1: Different components of horizontal gel electrophoresis apparatus.

Buffer and reagent for electrophoresis- The purpose of each reagents used in horizontal gel electrophoresis are as follows-

1. Agarose-polymeric sugar used to prepare horizontal gel for DNA analysis.

2. Ethidium bromide- for staining of the agarose gel to visualize the DNA.

3. Sucrose-For preparation of loading dye for horizontal gel.

4. Tris-HCl- The component of the running buffer.

5. Bromophenol blue-Tracking dye to monitor the progress of the electrophoresis.

Casting of the agarose gel- Different steps to cast the agarose gel for horizontal gel electrophoresis are given in Figure 36.2. The agarose powder is dissolved in a buffer (TAE or TBE) and heated to melt the agarose. Hot agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot agarose to cast the well for loading the sample. In few cases, we can add ethidium bromide within the gel so that it stains the DNA while electrophoresis.

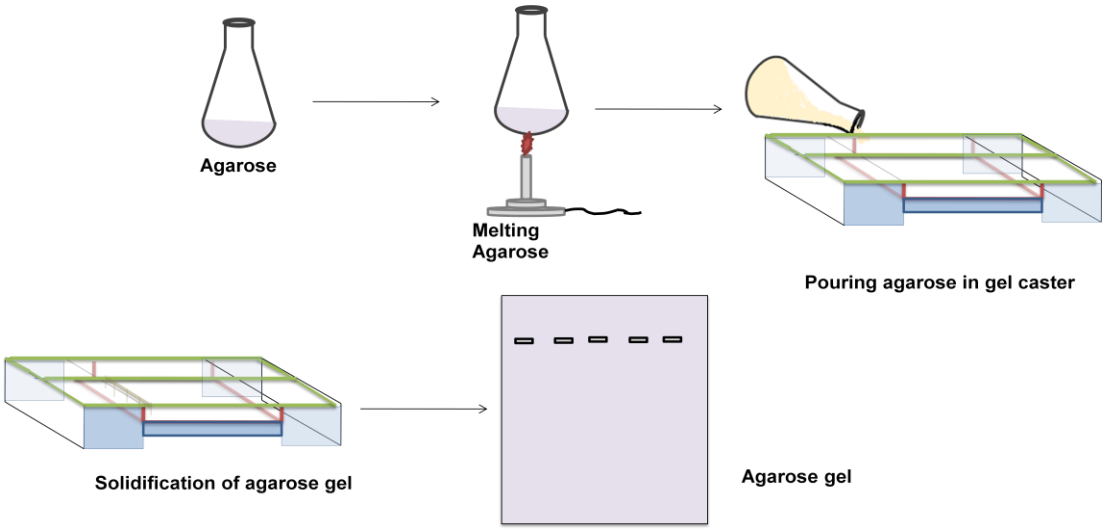


Figure 36.2: Different steps in casting of the agarose gel for horizontal gel electrophoresis apparatus.

Running and staining-The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of pipetman and run with a constant voltage. DNA runs from negative to positive end and ethidium bromide (EtBr) present in the gel stain the DNA. Observing the agarose gel in a UV-chamber shows the DNA stained with EtBr as organe colored fluorescence (Figure 36.3).

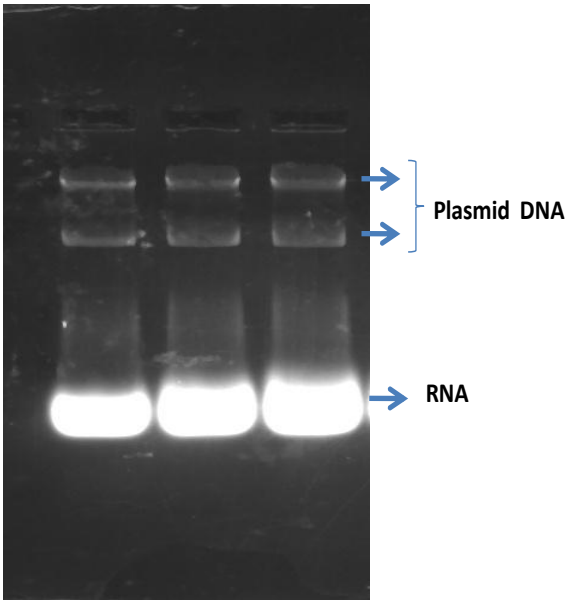
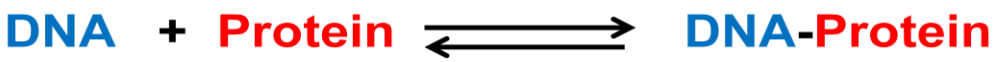


Figure 36.3: Observation of DNA stained with EtBr in a UV chamber.

Applications of Horizontal Agarose Gel Electrophoresis: Horizontal gel electrophoresis is used to answer several biological, molecular biology and cell biology question. Here, in today's lecture we will discuss few selected example of application of horizontal gel electrophoresis-

1. Determination of size of DNA-The size of a DNA can be determined by comparing the size of the known DNA molecules. The DNA of known sizes are resolved on 0.8% agarose along with the unknown sample. The value of the relative migration (Rf) of each DNA band is calculated from the agarose gel. The values of relative migration (Rf) and size of the DNA is used to draw the calibration curve to calculate the size of the unknown DNA samples.

2. DNA-Protein Interaction-DNA is a negatively charged molecule and it interact with positively charged protein to form DNA-protein complex. The size and the hydrodynamic volume changes when DNA is interacting with protein to form DNA-protein complex.



To study the DNA-protein interaction, a fix amount of DNA is incubated with the increasing concentration of protein (Figure 36.4). Due to the formation of DNA-protein complex, the hydrodynamic volume of the complex increases and a shift in band is observed. The DNA has a extended structure and it provides docking site for several protein molecules such as single stranded binding protein (SSB). As a result, a gradual shift in DNA band will be observed until the DNA binding site is not saturated with the protein molecules. Hence, at the end of the experiment, we can be able to understand several aspects of DNA-protein interaction:

1. Whether protein-X has a affinity for DNA and the interaction is specific or non-specific in nature.

2. What will be affinity parameters of the interaction of DNA to protein in making DNA-protein complex?

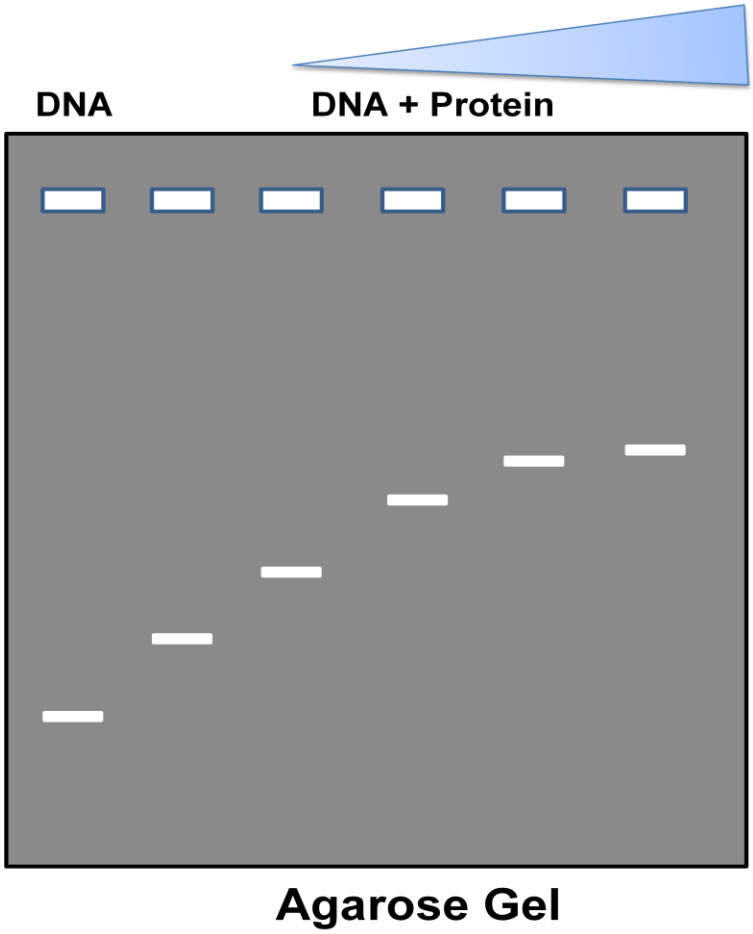


Figure 36.4: DNA-Protein interaction analysis by agarose gel electrophoresis.

3. Electroelution-As discussed in previous lecture, protein band present within the polyacrylamide gel block is removed by electroelution for further usage (Figure 36.5). In the electroelution, a gel band is cut from the SDS-PAGE and placed in a dialysis bag and sealed from both ends. The dialysis bag is choosen so that the molecular weight cut off of dialysis membrane should be lower than the protein of interest. The dialysis bag is placed in the horizontal gel apparatus with buffer and electrophoresis is performed with a constant voltage. During electrophoresis the protein band migrate and ultimately comes out from the gel block. Due to dialysis bag, salt and other small molecule contaminant moves out of the dialysis bag but protein remain trapped within the dialysis bag. Protein can be recovered from the dialysis bag for further use in downstream processing.

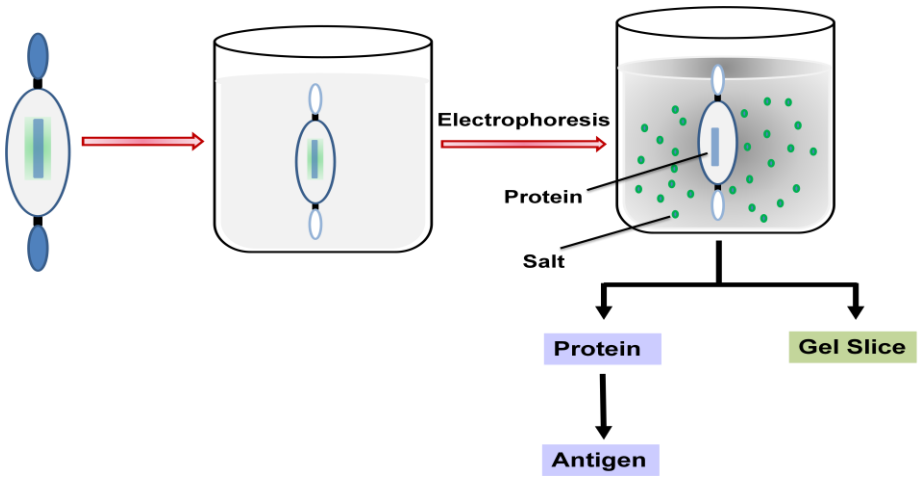


Figure 36.5: Electroelution using horizontal gel electrophoresis apparatus.

4. Southern blotting- In southern blotting, the genomic DNA is digested with the EcoRI or BamHI and the DNA fragments are resolved on the agarose gel. The gel is incubated in an alkaline solution to denature the double stranded DNA to single stranded form. DNA is transferred on the nitrocellulose membrane by capillary action by applying a uniform pressure either by suction pressure or by placing wet paper towels. The membrane is incubated with a non-specific DNA such as sonicated calf thymus genomic DNA to block the binding sites on the membrane. A single stranded radioactive probe is added to the membrane and allowed to bind. Membrane is washed and the blot is developed by autoradiography. The DNA fragment complementary to the probe sequence binds the radioactive probe and give positive signal (Figure 36.6).

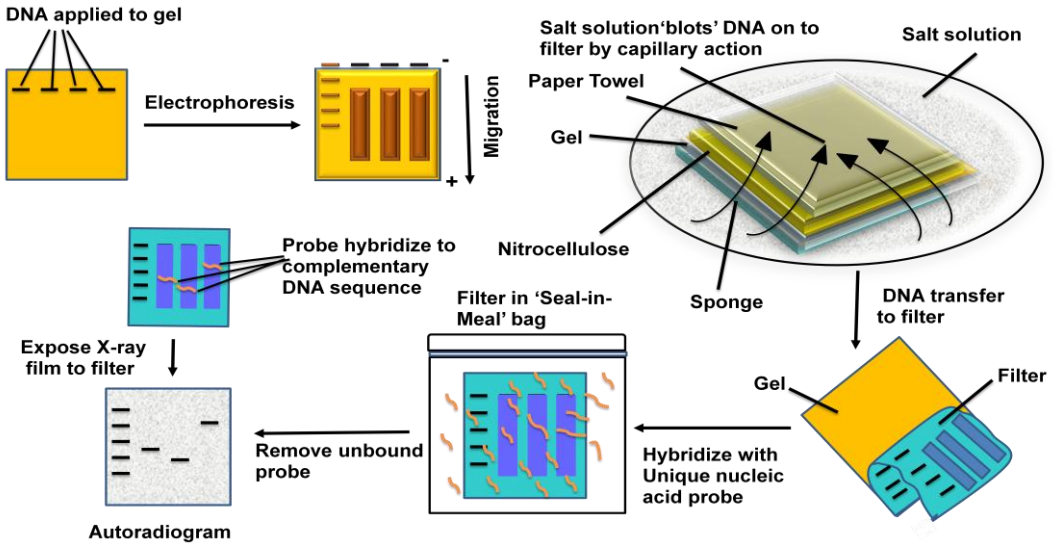


Figure 36.6: Southern Blotting using horizontal gel electrophoresis apparatus.

Lecture 37 Sequencing of Biomolecules (Part-I)

Introduction: Protein and DNA are polymeric molecules made up of the monomeric constituent. DNA is made up of 4 different types of nucleotide, A, T, G and C where as protein is made up 20 amino acids. Information present on the DNA is in the form of combination of these 4 nucleotides and responsible for phenotypic changes in an organism. After generation of recombinant DNA, it is also important to confirm its nucleotide sequence before further manipulation in the down-stream processing.

DNA sequencing-Historically there are two methods of DNA sequencing with a similar principle of breaking the DNA (chemical or enzymatic method) into the small fragment followed by separation and analyze them on a high resolution electrophoresis gel.

Di-Deoxy Chain termination or Sanger Methods: This method is originally developed by Frederick Sanger in 1977. In this method, a single stranded DNA is used as a template to synthesize complementary copy with the help of polymerase and in the presence of nucleotides (Figure 37.1). The polymerization reaction contains a primer and nucleotides, 3 normal nucleotides and 2'3'-dideoxynucleotide triphosphate (ddNTPs). When DNA polymerase utilizes ddNTPs as nucleotide, it gets incorporated into the growing chain but chain elongation stops at ddNTPs due to absence of 3'-hydroxyl group. In the typical sequencing reactions, 4 different ddNTPs are taken into the 4 separate reactions and analyzed on high resolution polyacrylamide gel electrophoresis. The ratio of NTPs/ddNTPS is adjusted so that chain termination occurs at each position of the base in the template.

Protocol for Di-deoxy sequencing- There are two protocols people adopt to sequence DNA following di-deoxy chain termination method (Figure 37.2).

Original sanger protocol uses klenow fragment as polymerase for DNA synthesis where as termination protocol uses a T7 polymerase or sequenase. **The DNA sequencing by original sanger protocol has following steps:**

Step 1: A primer is added and annealed to the 3' of the DNA template.

Step 2: The radiolabeled ^{35}S ATP to label the primer.

Step 3: The polymerease reaction is divided into 4 reactions.

Step 4: DNA synthesis continues until terminated by the incorporation of the specific ddNTPs (either A, T, G or C).

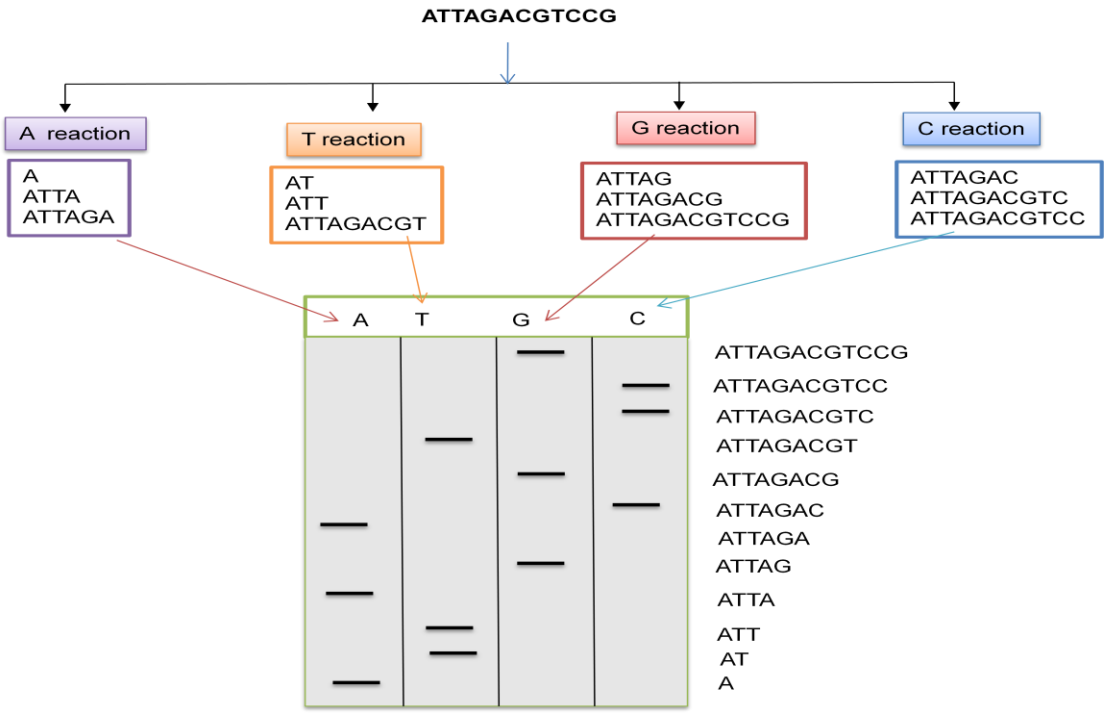


Figure 37.1: Principle of Di-Deoxy Method.

Step 5: A chase of polymerization reaction is performed in the presence of high concentration of NTPs to extend all non-terminated sequences into high molecular weight DNA. These high molecular sequences will not enter into the sequencing gel.

The different steps in labeling/termination protocol differ from sanger protocol after step1 and it has following steps:

Step 2: A limited amount of NTPs are added along with the one of the radiolabeled nucleotide to label the DNA through the length.

Step 3: The polymerease reaction is divided into 4 reactions.

Step 4: The polymerase reaction continues with 4 nucleotide and one ddNTPs. Synthesis is terminated at the specific ddNTPs (either A, G. C. T) to give DNA fragment of different length.

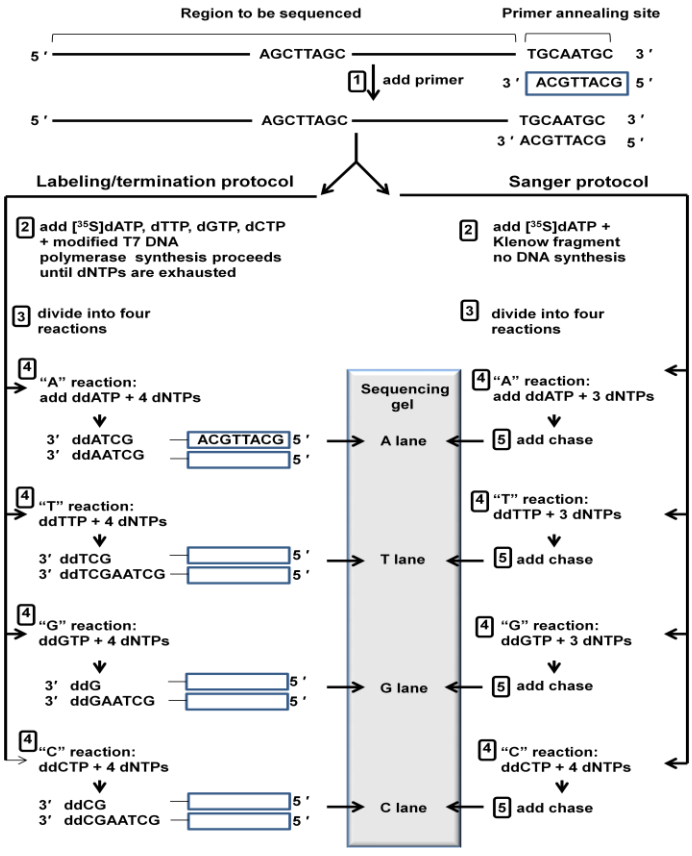


Figure 37.2: Different Steps in Di-deoxy Method

The polymerization reaction is analyzed on a high resolution polyacrylamide gel. The use of sequenase allow to perform sequencing of long DNA stretches where as original sanger method is more appropriate for short length DNA.

Maxam-Gilbert method: DNA cloning and polymerization reactions made the sanger method less popular than maxam-gilbert DNA sequencing method. This method was discovered by Allan Maxam and Walter Gilbert in 1977 which is based on chemical modification and subsequent cleavage. In this method, a 3' or 5' radiolabeled DNA is treated with a base specific chemicals which randomly cleaves the DNA at their specific target nucleotide. These fragments are analyzed on a high resolution polyacrylamide gel and a autoradiogram is developed (Figure 37.3). The fragment with terminal radiolabel appears as band in the gel. The chemical reactions are performed in two steps;

Base Specific Reaction: Different base specific reagents are used to modify the target nucleotide.

Reaction 1: Dimethylsulfate (DMS) modifies the **N7** of guanine and then opens the ring between **C8** and **N9**.

Reaction 2: Formic acid acts on purine nucleotide **(G+A)** by attacking on glycosidic bond.

Reaction 3: Hydrazine breaks the ring of pyridine **(T+C)**.

Reaction 4: Where as in the presence of salt (NaCl), it breaks the ring of cytosine.

Cleavage reaction : After the base specific reactions, piperidine is added which will replace the modified base and catalyzes the cleavage of phsophodiester bond next to the modified nucleotide.

Intepretation of the band in autoradiogram: The fragment in G lane is read as "G" where as fragment present in G+A but absent in G is read as "A". Similarly fragment in C is read as "C" where as fragment present in T+C but absent in C is read as "T". To get the DNA sequence, the band with the lowest molecular weight is read followed by next band in the four lane. For example in the given figure 37.3, in G lane the band is of lowest molecular weight followed by band in A lane etc.

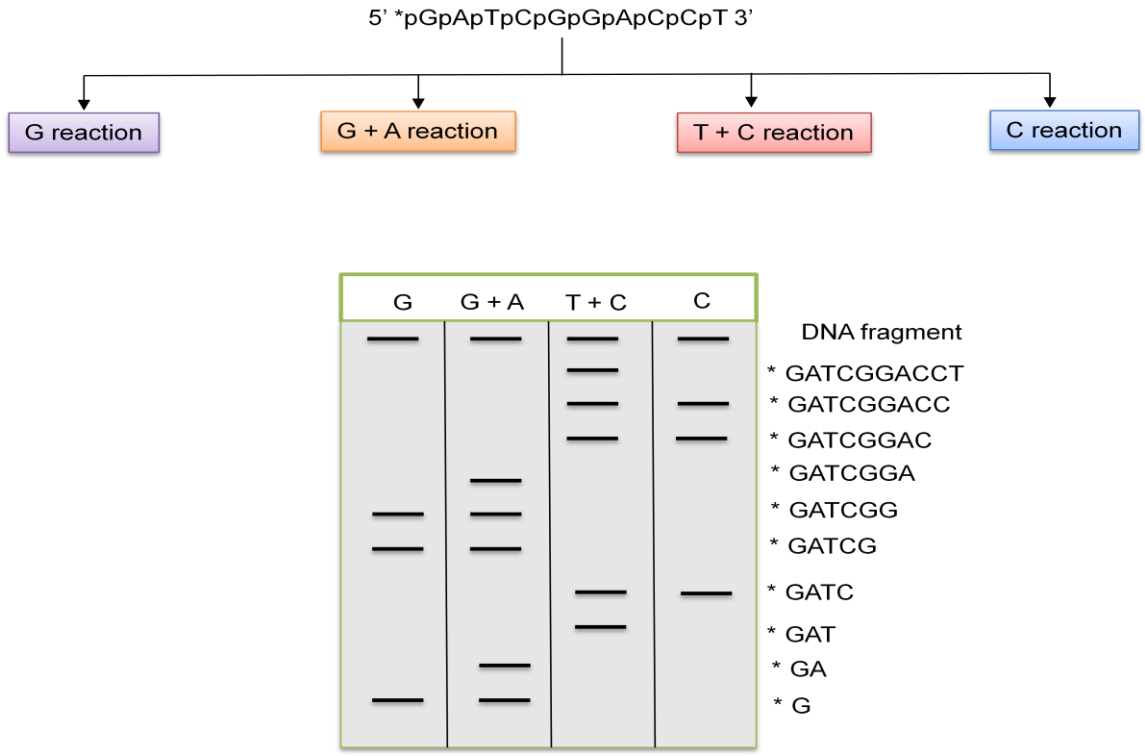


Figure 37.3: Different Steps in Maxam-Gilbert Method

Next generation sequencing methods: With the advancement in cloning and requirement for cheap sequencing methodology, several next generation sequencing methods are developed. These have lower cost of sequencing as well increased the output by processing multiple sample at the same time. The next generation DNA sequencing technologies developed as follows-

- Massively parallel signature sequencing (MPSS)
- Polony sequencing
- 454 pyrosequencing
- Illumina (Solexa) sequencing
- SOLiD sequencing
- Ion semiconductor sequencing
- DNA nanoball sequencing
- Heliscope single molecule sequencing
- Single molecule real time (SMRT) sequencing

Student are advised to follow these publications to know more about different next generation sequencing technologies and their comparison, (1) Quail, Michael; Smith, Miriam E; Coupland, Paul et al. "A tale of three next generation sequencing platforms: comparison of Ion torrent, pacific biosciences and illumina MiSeq sequencers". *BMC Genomics* 13 (1): 341 PMID 22827831 (2) Liu, Lin; Li, Yinhu; Li, Siliang et al. Comparison of Next-Generation Sequencing Systems. *Journal of Biomedicine and Biotechnology* 2012: 1–11.

Lecture 38 Sequencing of Biomolecules (Part-II)

Protein Sequencing: Protein contains 20 different types of amino acids connected by a peptide bond. Each protein has unique amino acid sequence which dictates the folding, 3-D conformation and functional outcome. Understanding the amino acid sequence and over-all bonding pattern help us to characterize the protein.

The sequencing of a long protein has multiple stages: A protein needs to go through following stages for elucidation of its sequence as well as bonding pattern. These stages are schematically given in Figure 38.1. Over-all, the complex protein first needs to break into the subunits, and sequential release of amino acids from N-terminus of each fragment following edman-degradation method. At the end, the sequence of each fragment can be put together to deduce the complete amino acid sequence of protein. The details of each stage is as follows-

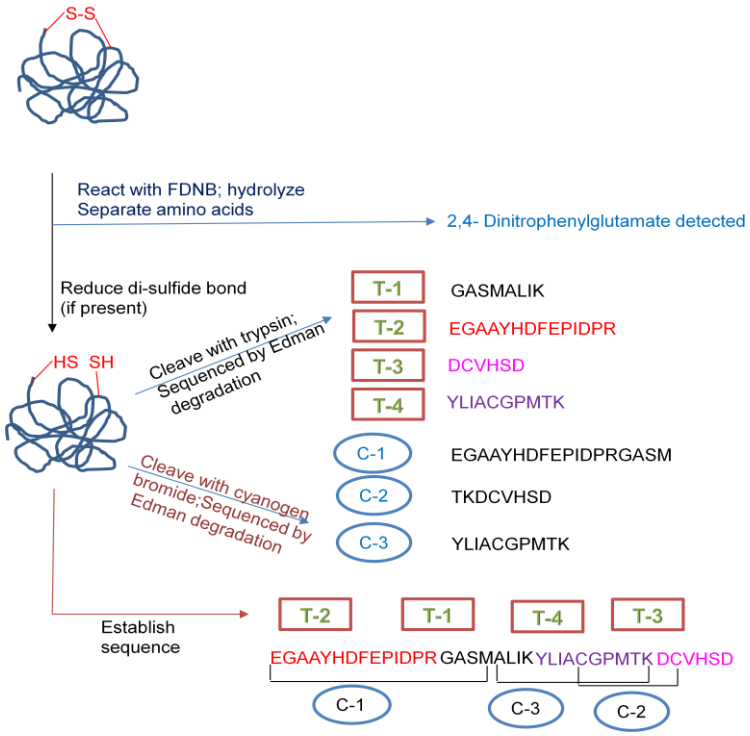


Figure 38.1: Over-View of the different stages in sequencing of a protein.

Stage 1. Breaking Disulphide Bonds: In protein two cysteine amino acids are linked by a disulphide linkage. The disulphide linkage interfere with the complete sequencing procedure as it doesn't allow the release of cleaved amino acid from the peptide chain. There are two approaches two disrupt the disulphide linkage in a protein sequence (Figure 38.2). In first approach, protein is oxidized with a performic acid to produce two cysteic acid residues. In another approach, protein is reduced by dithiothreitol (DTT) or β-mercaptoethanol (β-me) to form two cysteine followed by treatment with iodoacetate to form carboxymethyl-cysteine. Formation of carboxymethyl-cysteine stops the re-formation of disulphide bond.

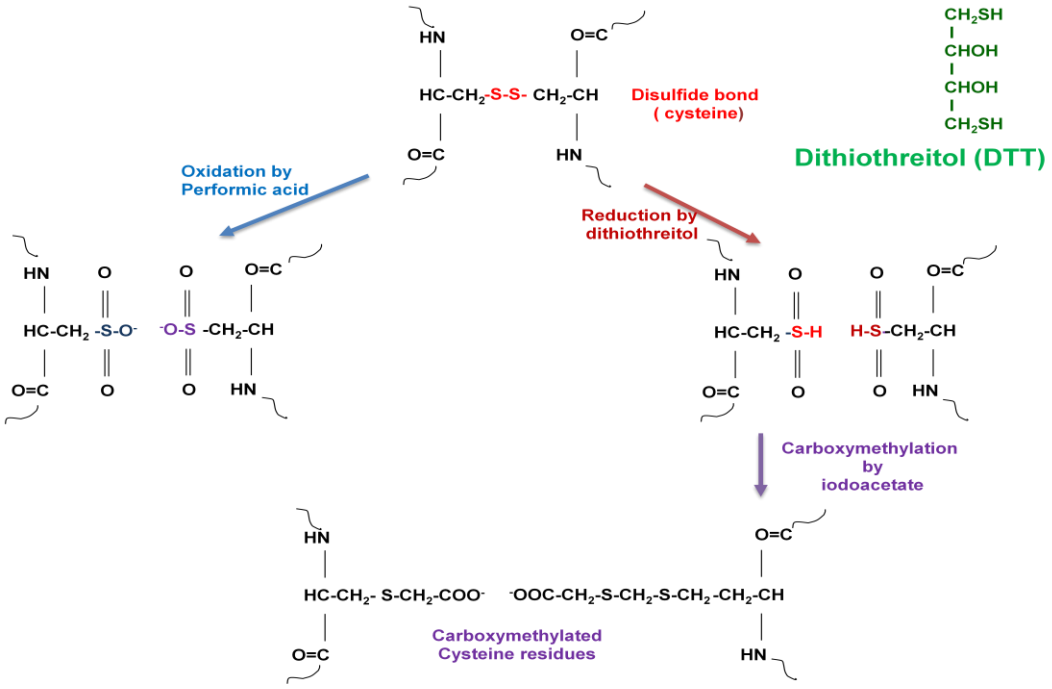


Figure 38.2: Disruption of disulphide bond by different approaches.

Stage 2. Cleavage of the polypeptide chain: Proteases and the chemical agents targeting proteins have a specific recognition sequence and they cleave after a particular amino acid. A list of protease and chemicals commonly used to digest the polypeptides into the small peptide fragment is given in Table 38.1.

Table 38.1: Some Common reagents for fragmenting polypeptide chain.

S.No	Reagent	Cleavage Point
1	Trypsin	After Lys, Arg
2	Chymotrypsin	After Phe, Trp, Tyr
3	Pepsin	After Leu, Phe, Trp, Tyr
4	Cynogen Bromide	After Met

Stage 3. Sequencing the peptides-Once the peptide fragments are generated, we can start the sequencing of each polypeptide chain. It has following steps:

A. Identifying the N-terminal residue: The N-terminal amino acid analysis is a 3 steps process.

1. Derivatization of terminal amino acid-The chemical reaction is performed to labeled terminal amino group with compounds such as sanger reagent 1-fluoro-2,4-dinitrobenzene (DFNB) and dansyl chloride. In most of the case these reagents also label free amino group present on basic amino acids such as lysine and arginine. In a reaction mechanism given in Figure 38.3, dinitrofluorobenzene reacts with the free amine group to form **dinitrophenyl-amino acid** complex.

2. hydrolyse the protein-Acid hydrolysis of dinitrophenyl-amino acid complex leads to the breaking of peptide bond to release dinitrophenyl-amino acid complex in solution.

3. Separation and analysis of derivatized amino acids-A HPLC or TLC separation of complex and comparing with the standard amino acids.

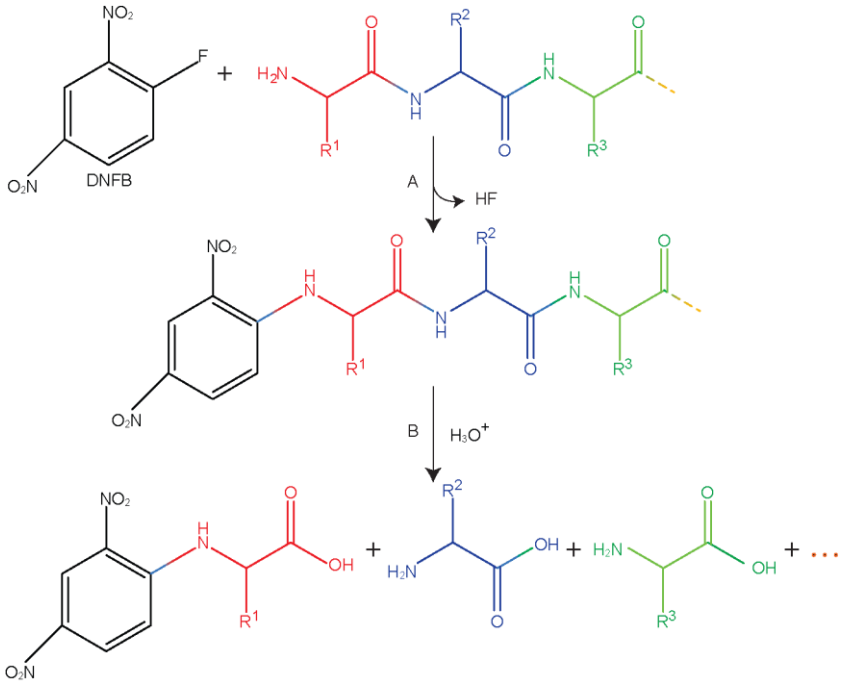


Figure 38.3: Derivatization of N-terminal amino acid with sanger reagent.

B. Edman Degradation sequencing: Edman Degradation sequencing methods is given in Figure 38.4 and it has following steps:

1. Similar to sanger reagent, phenylisothiocynate reacts with the terminal amino group to form a cyclic phenylthiocarbamoyl derivative.

2. Under acidic condition, the terminal amino acid is cleaved from the main chain as thiazolinone derivative.

3. Thizolinone derivative is extracted into the organic solvent and it forms phenylthiohydantoin-amino acid (PTH-amino acid) derivative in the presence of acid.

4. PTH-amino acid acid complex can be identify by HPLC or TLC and comparison with the standard amino acids.

5. Step 1-4 can be repeated again with the next amino acid residue in the peptide chain.

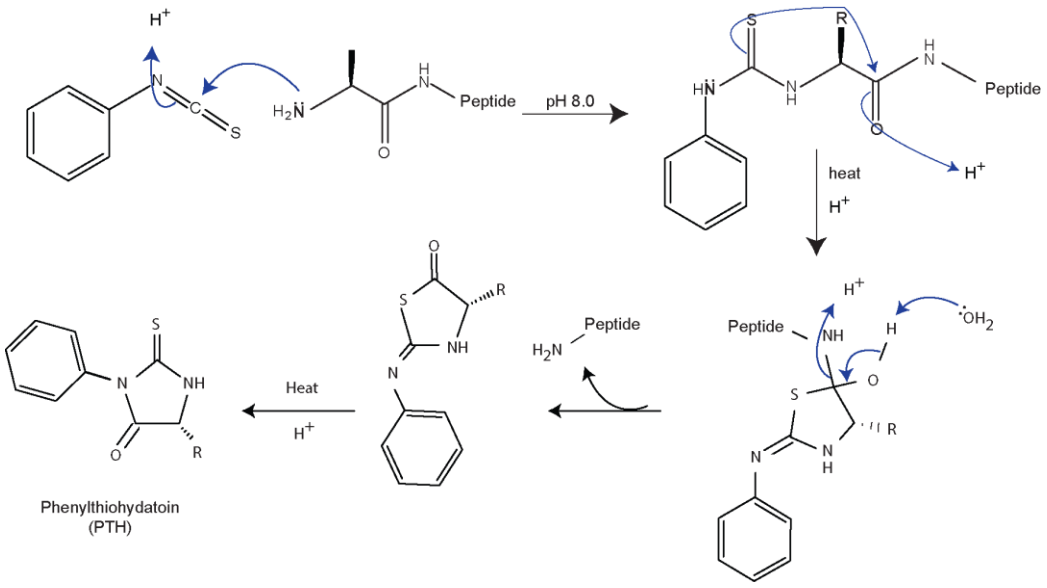


Figure 38.4: Steps in Edman degradation method of protein sequencing.

C. C-terminal residues: Not many methods are developed for c-terminal amino acid analysis. The most common method is to treat the protein with a carboxypeptidase to release the c-terminal amino acid and test the solution in a time dependent manner.

Stage 4. Ordering the peptide fragments: The usage of different protein cleavage reagent produces over-lapping amino acid stretches and these stretches can be used to put the whole sequence.

Stage 5. Locating disulfide bonds: The protein cleavage by typsin is performed with or without breaking di-sulphide linkage. Amino acid sequence analysis of the fragments will provide the site of disulphide bond. The presence of one disulphide will reduce two peptide fragment and will appear as one large peptide fragment.

Mass Spectrometry Method: In recent pass, mass spectroscopy in conjugation with proteomics information is also been popular tool to chacracterize each peptide fragment to deduce its amino acid sequence. The minor detail of this approach can be explored by following the article

[Collisions or Electrons? Protein Sequence Analysis in the 21st Century". *Anal. Chem.* 81 (9): 3208–3215.]