Mass Spectrometry Based Proteomics
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Mod 02 Lecture Number 06

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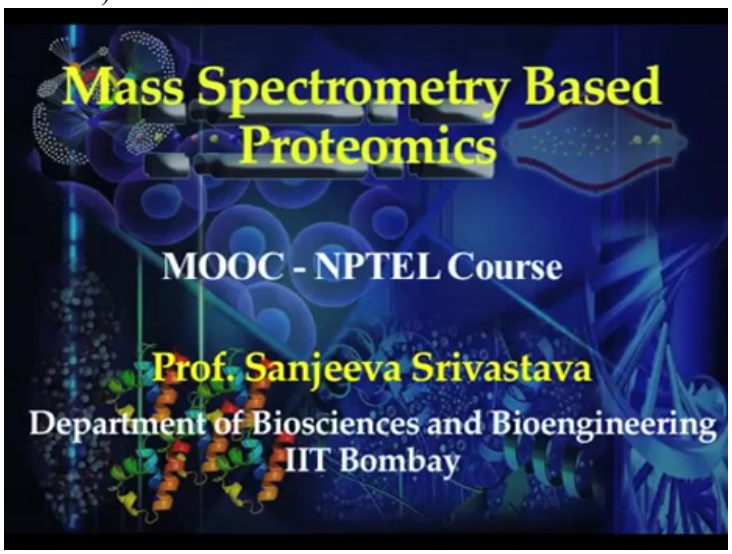

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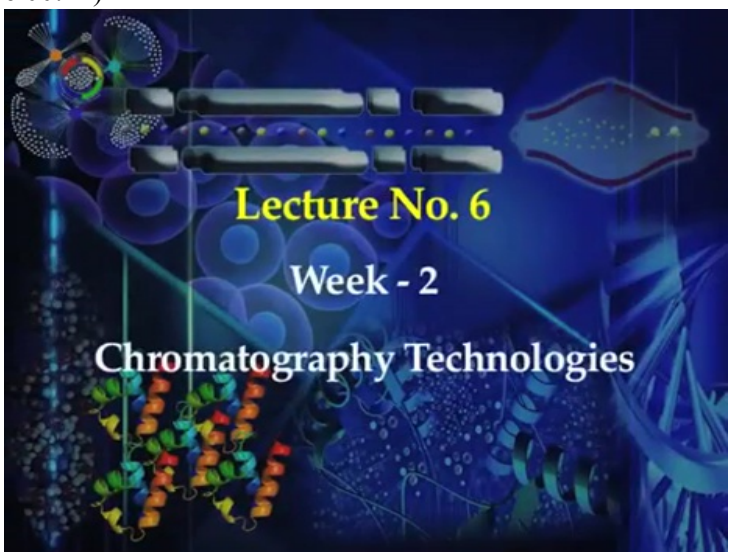

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Welcome to the Proteomics course. Today, we will talk about …

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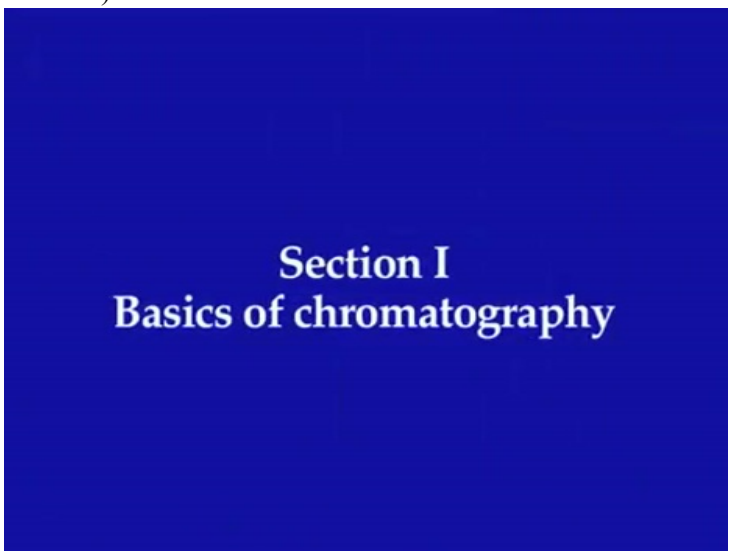

… protein purification and peptide isolation using chromatography methods

The chromatography techniques have always been considered prominent for the separation of enzymes and proteins. Protein purification by conventional chromatography is usually achieved by combining chromatographic methods such as gel filtration, ion exchange and affinity chromatography.

From complex proteome, it is challenging to purify a protein in a single chromatographic step. Therefore sequential pre-fractionation steps involving different modes and types of chromatographic methods are becoming necessary for proteome level analysis.

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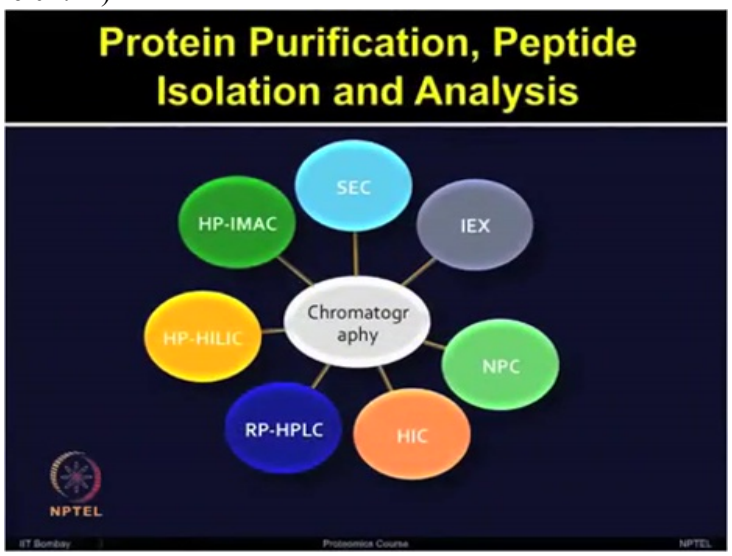

Protein purification and peptide isolation and analysis:

It can be performed by many methods but there are certain chromatography methods which are commonly used such as Size Exclusion Chromatography SEC, Ion EXchange chromatography IEX, Immobilized Metal Anion Chromatography IMAC, Normal Phase Chromatography NPC, Reverse phase-HPLC or RP-HPLC, Hydrophobic Interaction Chromatography HIC and HydrophILic Interaction Chromatography HILIC.

These are only few chromatographic methods but there are many more which are also used for different type of applications.

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So let's talk about what is chromatography.

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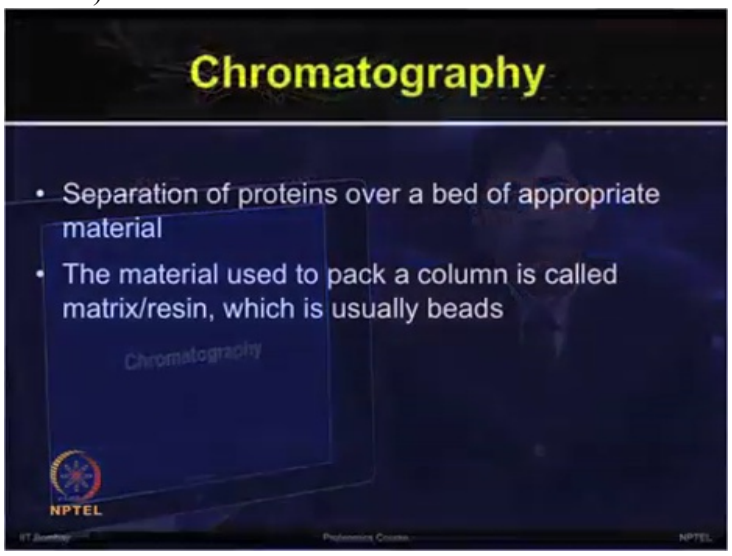

So, chromatographic methods involve separation of proteins over a bed of appropriate material. These materials are usually packed in a given column and known as matrix or resin. So these matrix and resins, they are usually beads with or without attached chemical groups.

Now the binding and interaction of proteins with the column matrix is an important feature of chromatography

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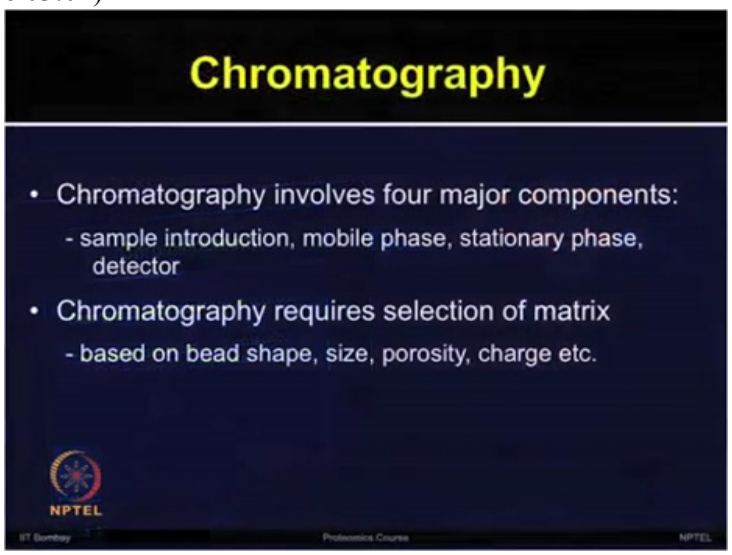

The chromatographic techniques involves four major components; an inlet for sample introduction, the mobile phase, stationary phase and a detector.

Each type of chromatography requires very educated and informed choice of matrix considering the bead shape, size and porosity.

In additional to the functional group type the charge, distribution and density as well as elusion conditions such as pH, the ionic strength and gradient shape.

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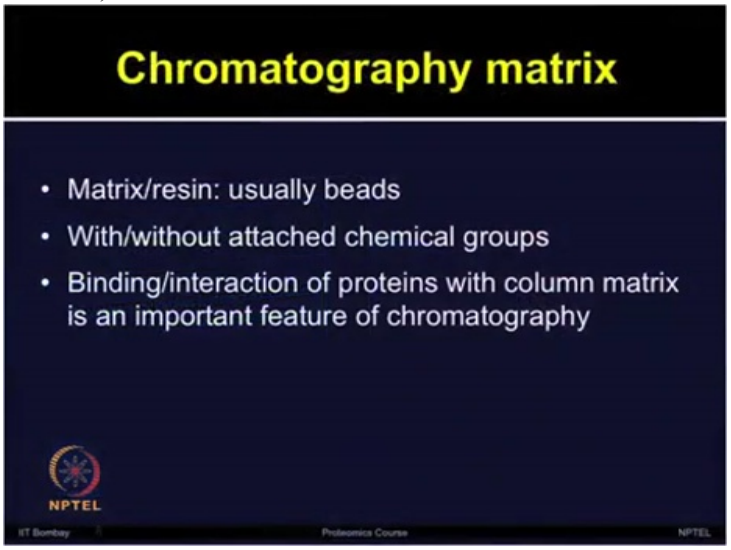

Since as we just talked, the chromatography involves the selection of right matrix. These matrix are very important for binding and interaction of proteins and they can determine the kind of achievement which we want to achieve from these types of chromatographic methods.

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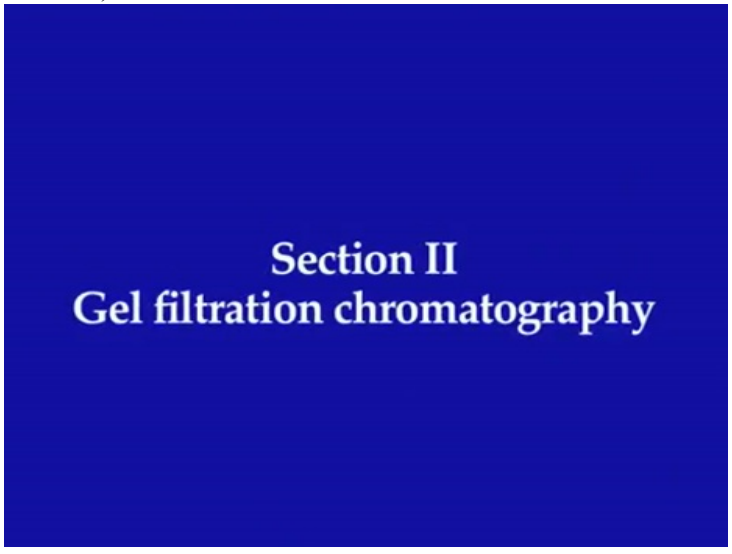

So let’s move on to more specific chromatographic techniques such as...

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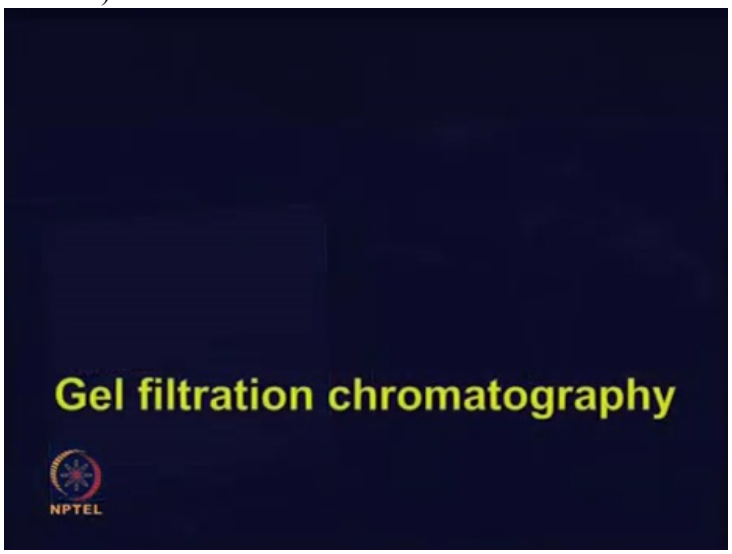

Gel Filtration Chromatography

The gel filtration chromatography, it separates proteins on the basis of difference in size. It is used to separate a protein of interest from a protein mixture that are of larger or smaller in size.

If the proteins are having similar sizes then the gel filtration or size exclusion chromatography is not an appropriate choice for doing the protein purification.

So this method is also used for various proteomic applications when there is need to remove even contaminants such as salts and low molecular size detergents

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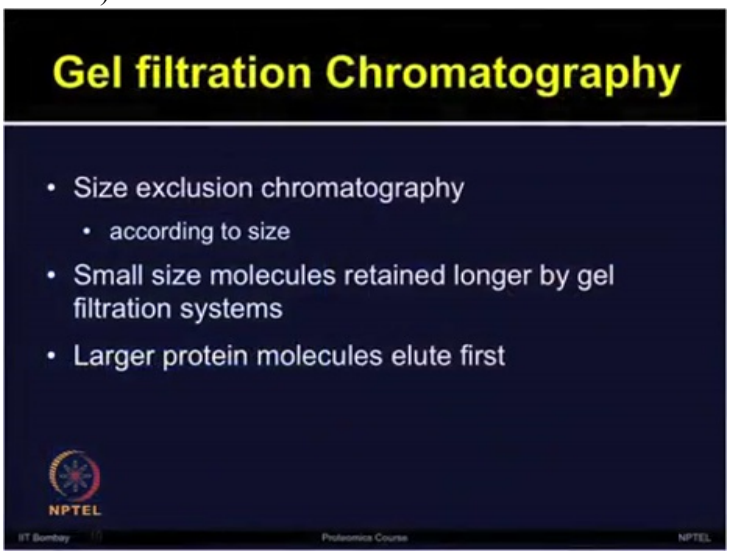

Gel filtration chromatography is also known as size exclusion chromatography. It means the molecules are separated according to the given size.

The small molecules or even if you are separating small molecules such as salt those will be retained longer by gel filtration systems and larger molecules such as proteins will elute first because they cannot enter inside these porous ah, these beads and they can be used to separate proteins based on the size.

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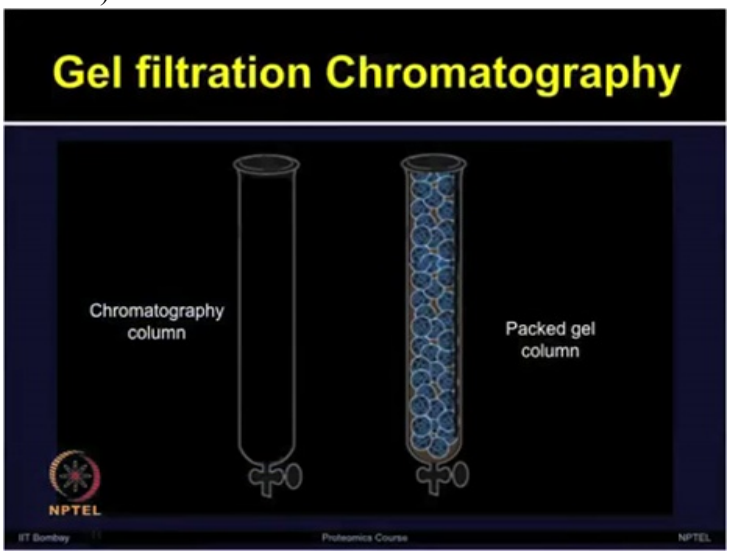

The gel filtration column is composed of porous beads which are made from polyacrylamide, dextran or agarose. Now these columns are packed with the hydrated porous gel matrix.

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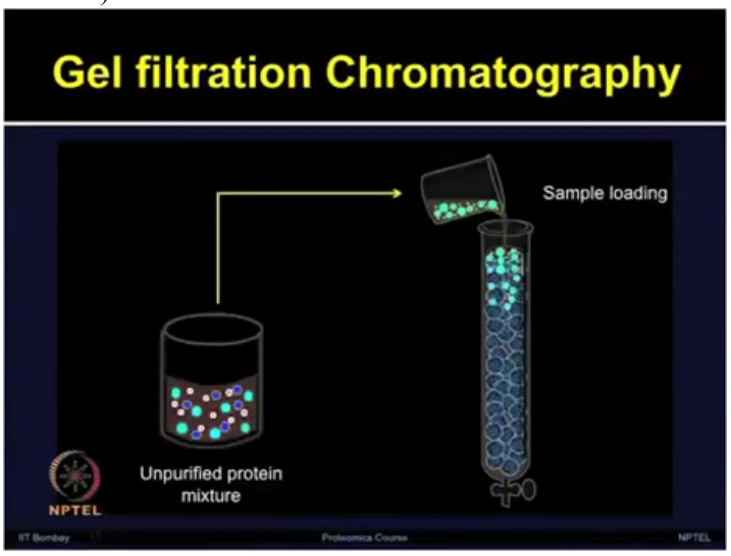

The protein sample which contains mixture of un-purified proteins of different size is then loaded on these columns.

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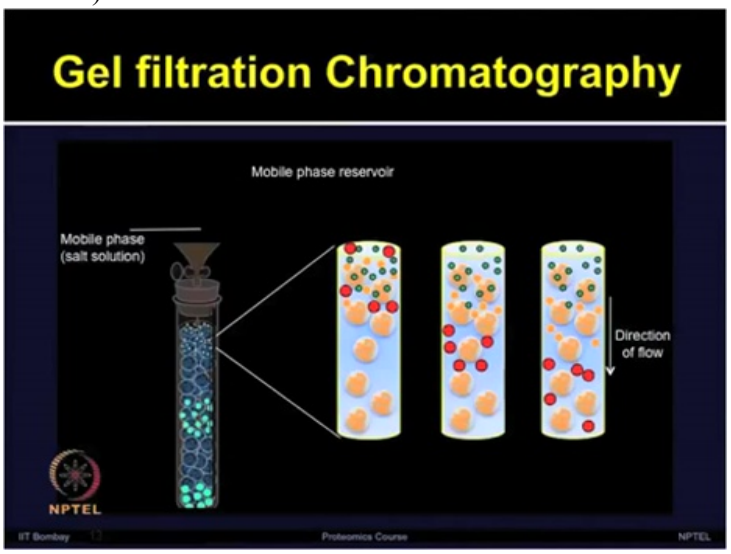

Now when protein sample is applied on to the column, the small proteins pass from the pores of the beads while the large proteins are excluded. Therefore this technique is also known as molecular exclusion chromatography.

The beads of different pore sizes or increasing retention time by adjusting the increasing column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

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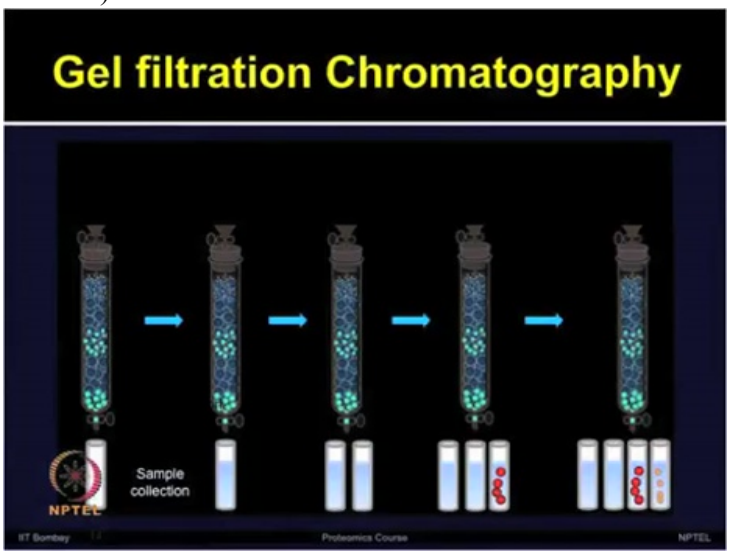

After doing these steps, the fractions are collected and analyzed for the protein content. You can expect that larger proteins will elute first and then the small molecules will be eluted later.

So gel filtration chromatography is useful for removal of contaminants, doing desalting steps as well s for buffer exchange. However its drawbacks are its low capacity, broad distribution of pore size and small sample volumes to be analyzed.

So let me describe gel filtration chromatography in following animation.

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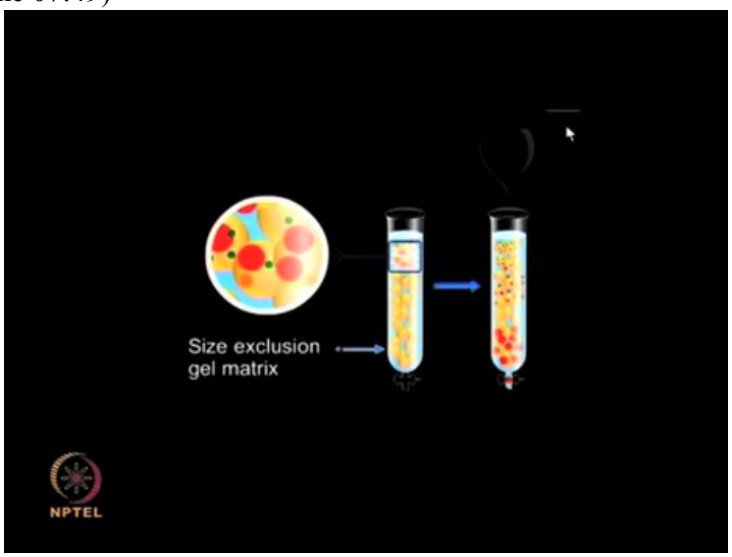

Let's first give the definition of few components

Size exclusion gel matrix: the matrix filling the gel filtration column consists of a highly hydrated polymeric material

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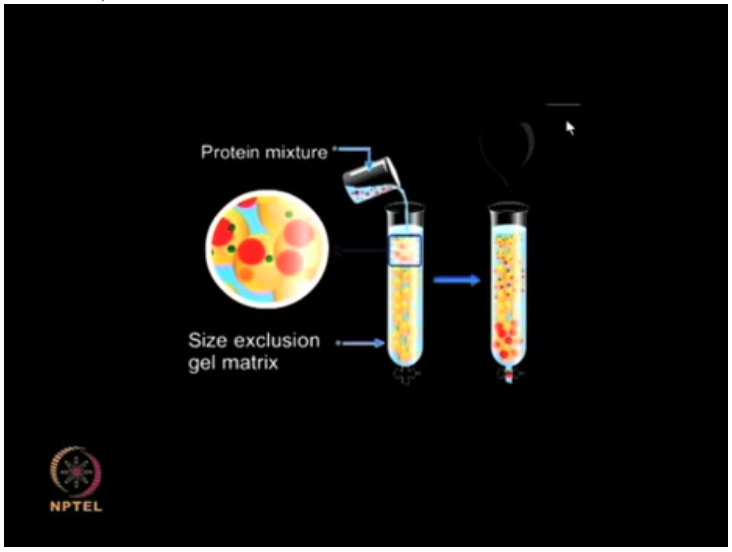

…commonly dextran, agarose and polyacrylamide

The protein mixture is a mixture of unpurified proteins of different sizes which is applied on top of the column.

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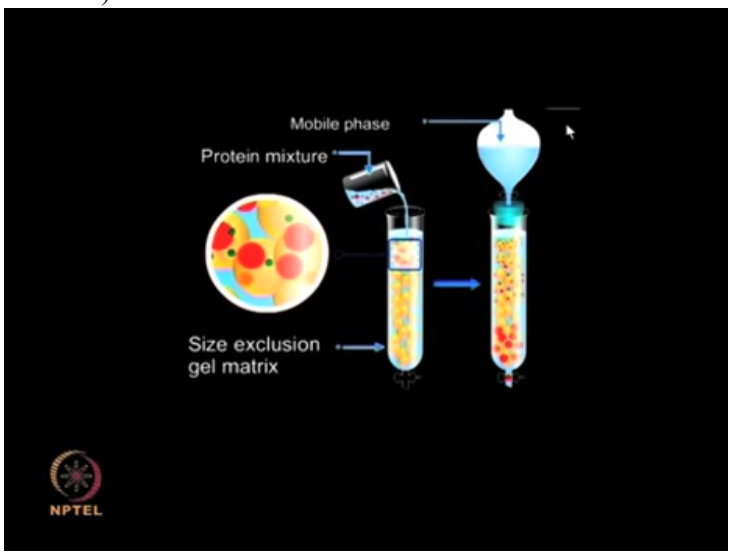

Mobile phase: the proteins are eluted out of the column by using a suitable mobile phase that carries the protein out for elution. For gel filtration a salt solution of appropriate strength is commonly used so that it will not have any effect on properties of proteins being purified.

Solvents or buffer systems are often used in other types of chromatography.

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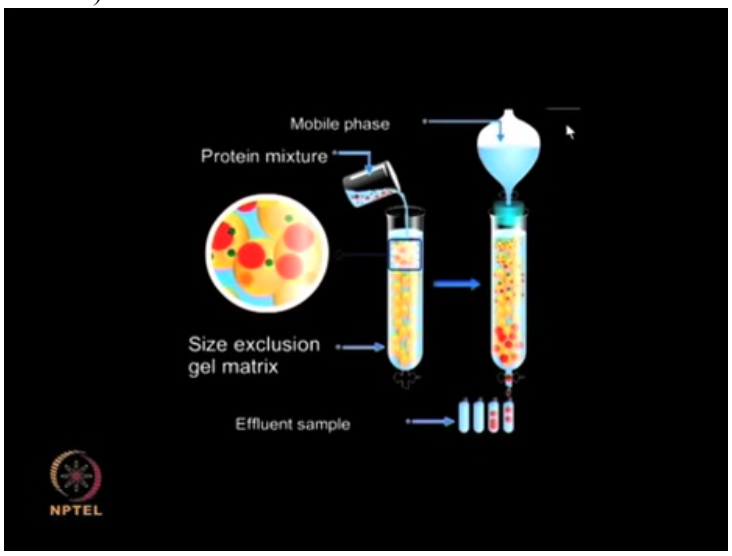

Effluent sample fractions: the solution leaving the column is collected in suitably sized fractions; initial fraction will contain only the mobile phase while later fractions will have purified proteins.

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Let me give an analogy of gel filtration with sieve. This process is very similar to separation of small particulate matter from food grains using a sieve.

The larger grains remain behind the sieve while the smaller sand or stone particles pass through them and are removed.

In keeping with this the gel filtration chromatography is also commonly referred as molecular sieve. Now let me show you how this process works.

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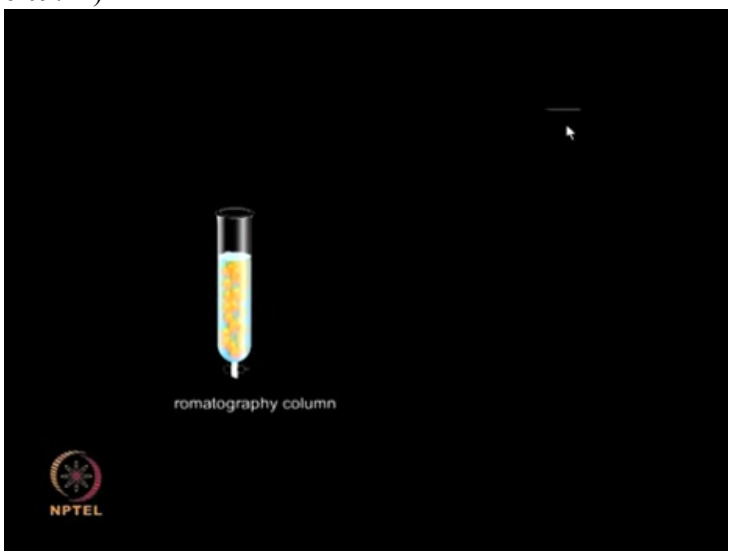

So first the matrix which is suitable for the required protein separation, this packed gel matrix is …

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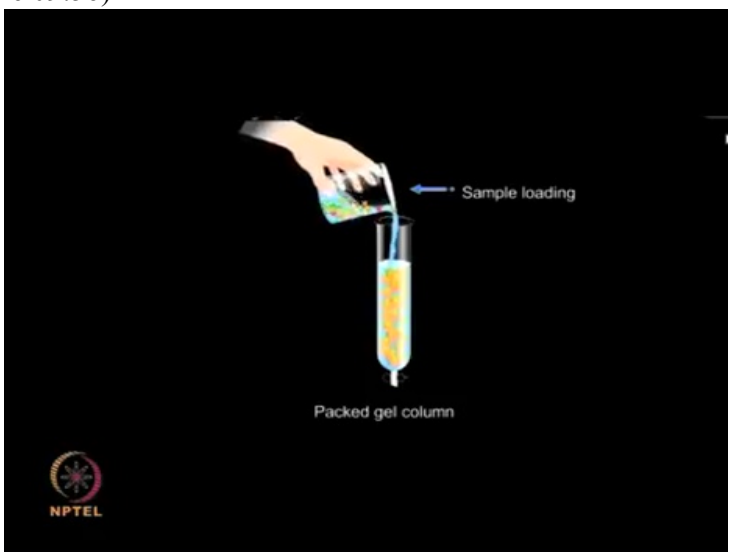

… then loaded with the protein sample contain a mixture of unpurified proteins of different sizes

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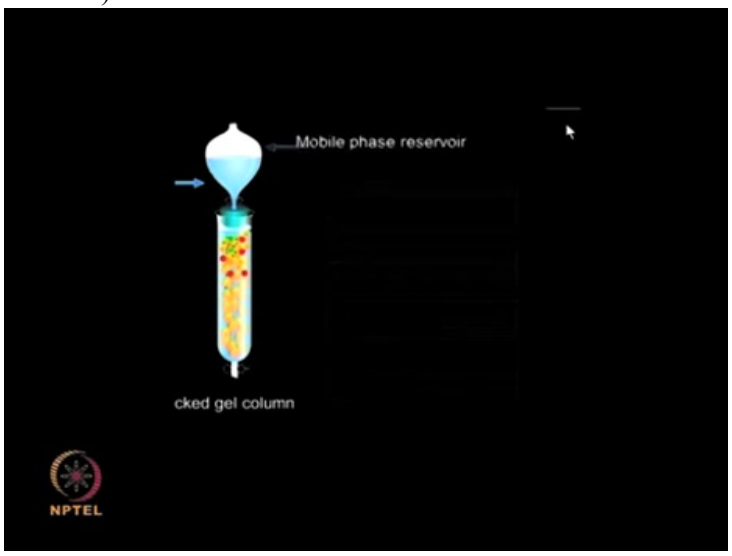

The column is then eluted with…

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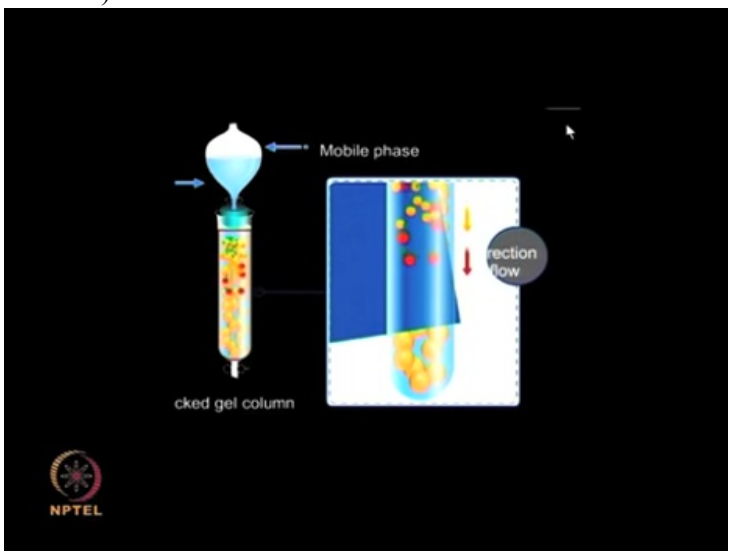

… a salt solution of appropriate concentration Large proteins that cannot enter into the pores of the gel move down through the interstitial spaces at a faster rate

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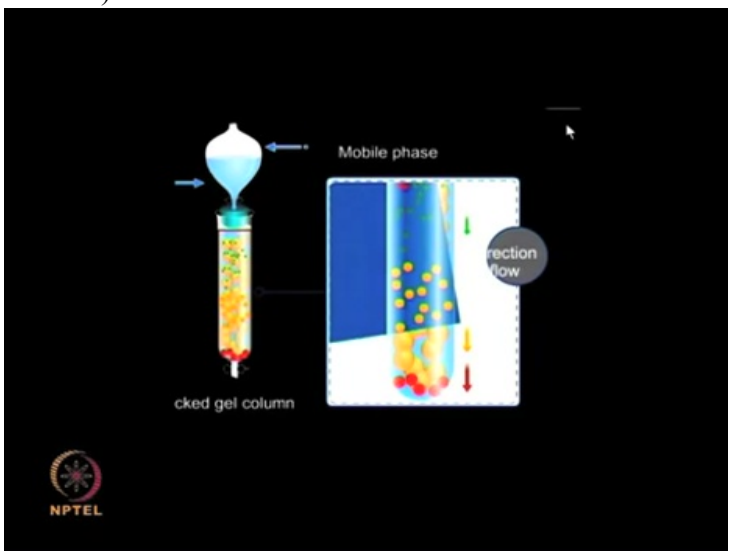

… and are eluted first

The smaller proteins move in and out thereby taking longer time to be removed from the column.

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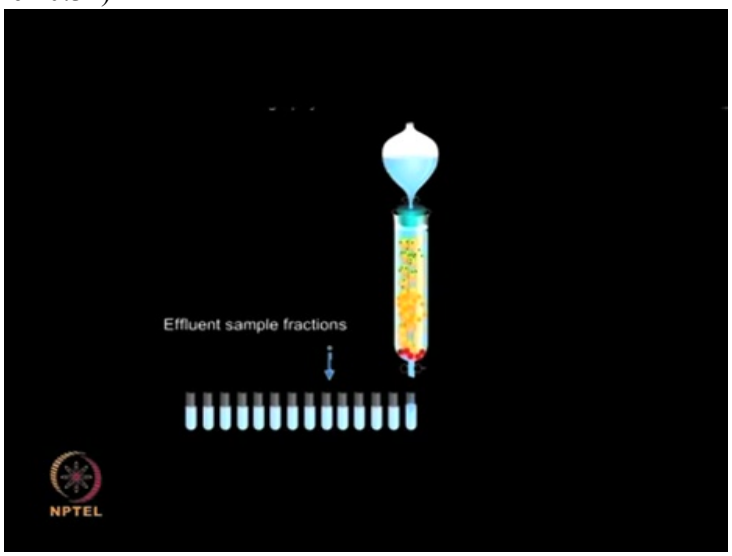

The fractions of appropriate size should be collected and analyzed for their protein content

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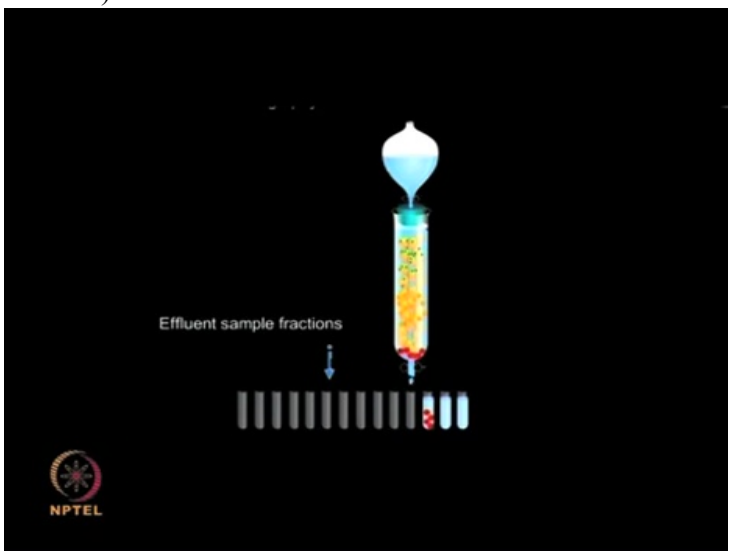

. The largest proteins eluting out first will be present in the initial fractions

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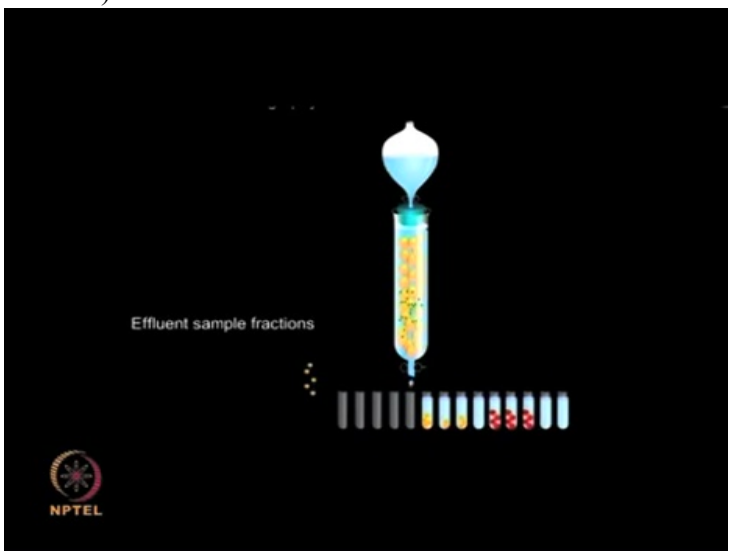

…while the smaller proteins which elute out later will be present in later fractions

Once all these fractions are collected, then it can be analyzed for their protein content

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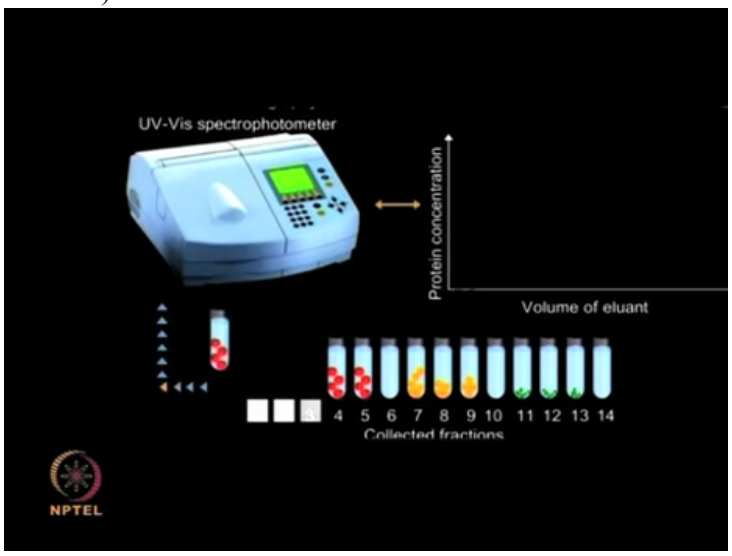

… by using a UV-visible spectrophotometer Now analyze the protein content by using UV-visible spectrophotometer at 280 nanometers. Once all the absorbance values are recorded

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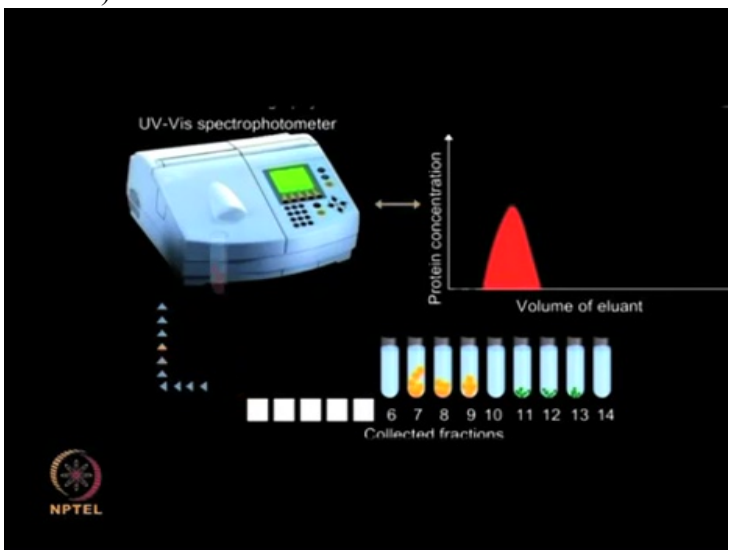

a graph of eluant volume versus protein concentration can be plotted.

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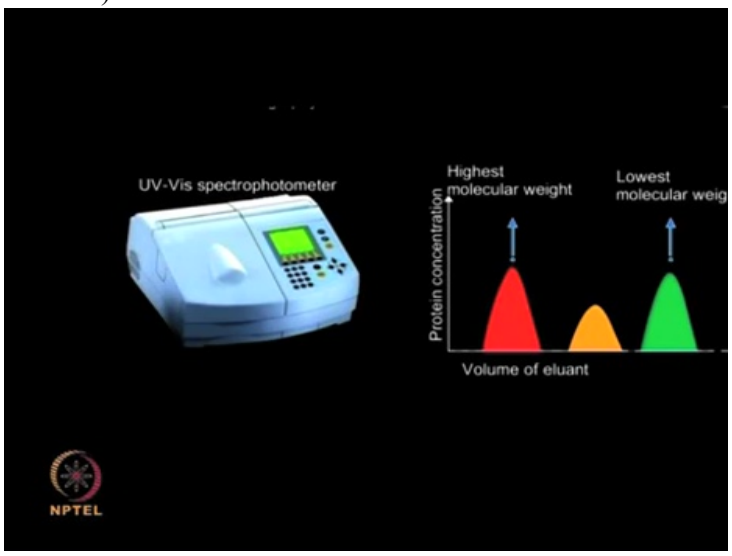

The highest molecular weight is in the beginning and lowest molecular weight at the end

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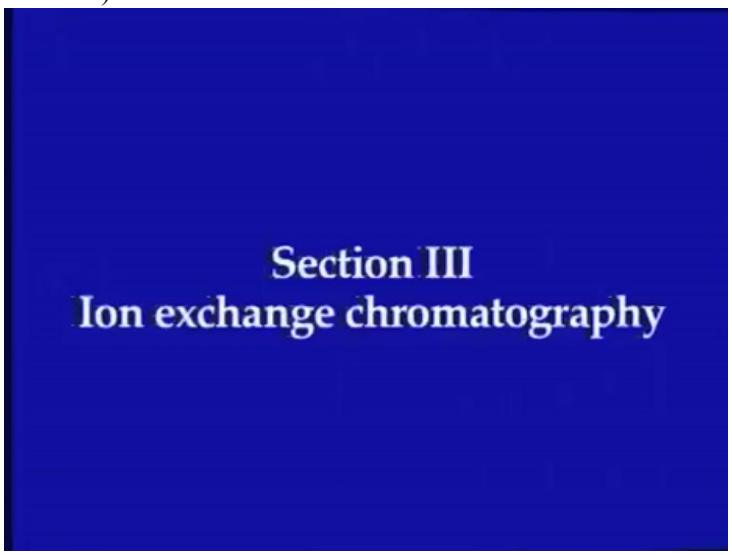

Let's now move on to next chromatography method which is …

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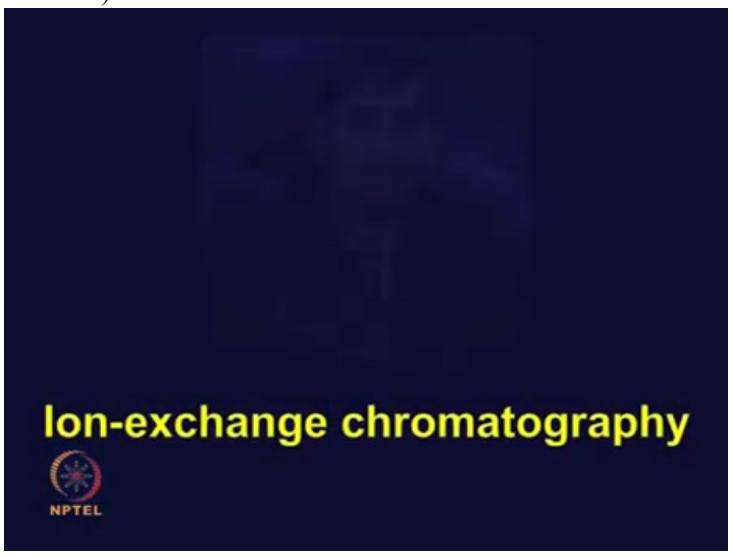

… Ion exchange chromatography

This is one of the most versatile chromatographic separation methods, which relies on differences between number of charges and distribution of charge groups in defined pH and solvent conditions.

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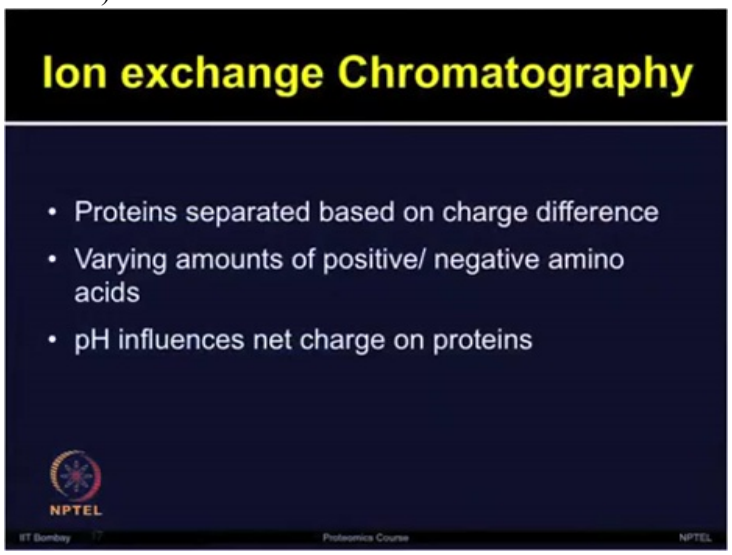

In ion exchange chromatography, the proteins are separated based on charge difference. The proteins with overall negative charge will interact with positive charges or vice versa. So, by varying the amount of positive and negative amino acids and, even pH can influence the net charge on proteins.

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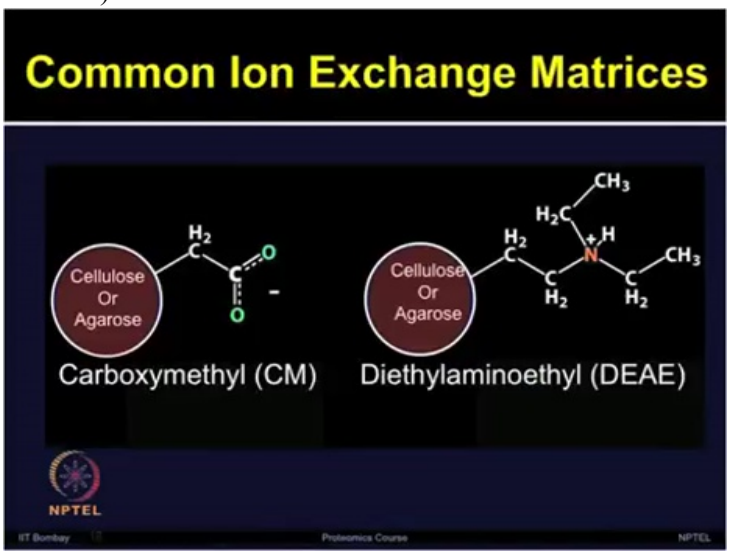

So in this slide some of the common ion exchange matrices are shown like CarboxyMethyl, CM and DiEthylAminoEthyl, DEAE.

So when a desired protein is positively charged, the cation exchange chromatography should be used. When a desired protein is negatively charged the anion exchange chromatography method should be used.

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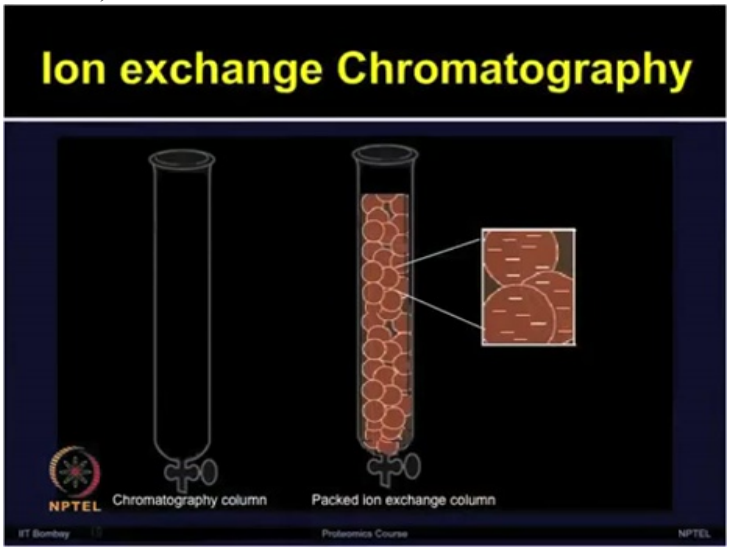

So in ion exchange chromatography, the column is packed with a resin, whether it is cation or anion exchanger depending upon the charge of the protein that need to be bound to the column and purified.

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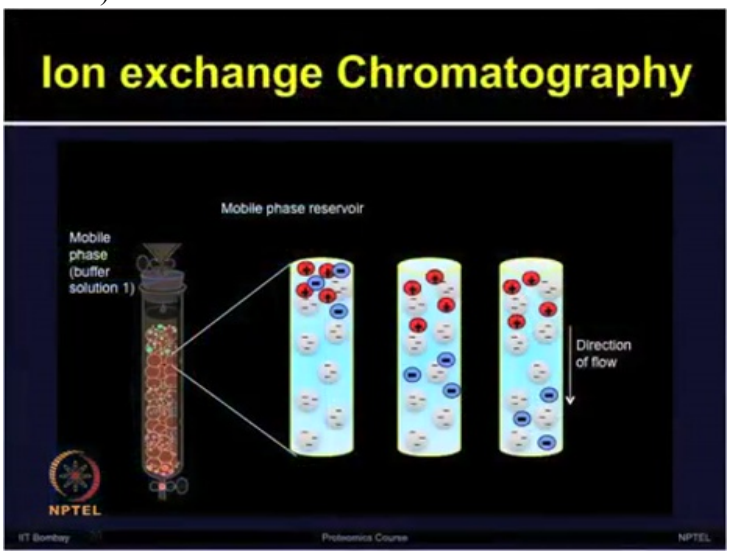

So proteins are adsorbed to the ion exchange column and then it can be desorbed by increasing the salt or altering the pH of the buffer which can change the charge on protein.

So various anionic buffers such as acetate and phosphate are used for cation exchange and cationic buffers such as Tris-chloride or ethanolamine are used for the anion exchange.

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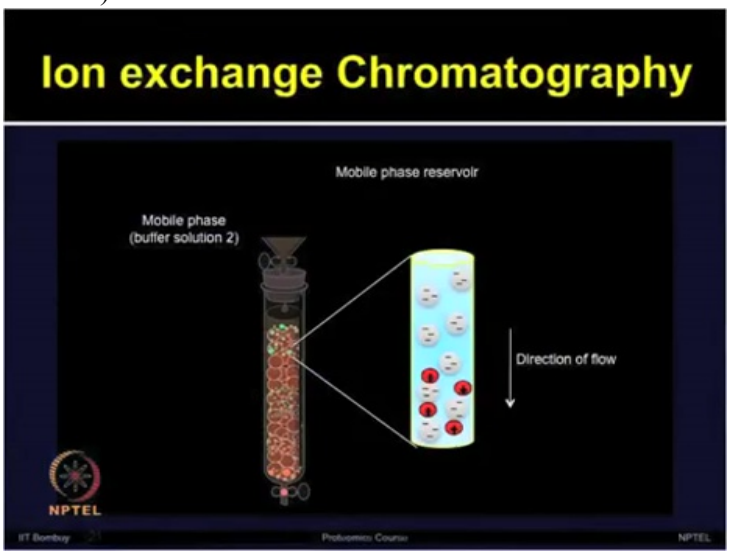

Now the buffer solution is changed so that the net pH of the protein of interest can be modified and it no longer binds to the ion exchange resin, therefore the bound protein can eluted out as shown in this slide.

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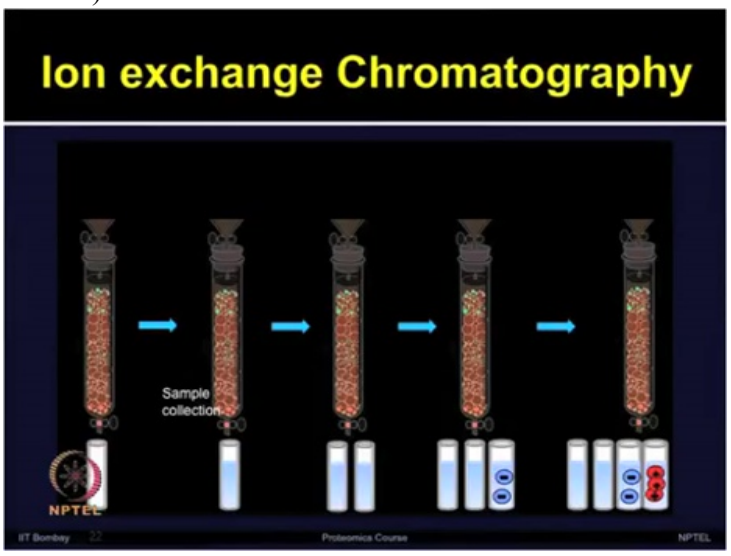

So if you have negatively charged protein which gets eluted firs will be present in the initial fractions while the positively charged protein that bound to the column will be eluted in the later fractions or it will vice versa.

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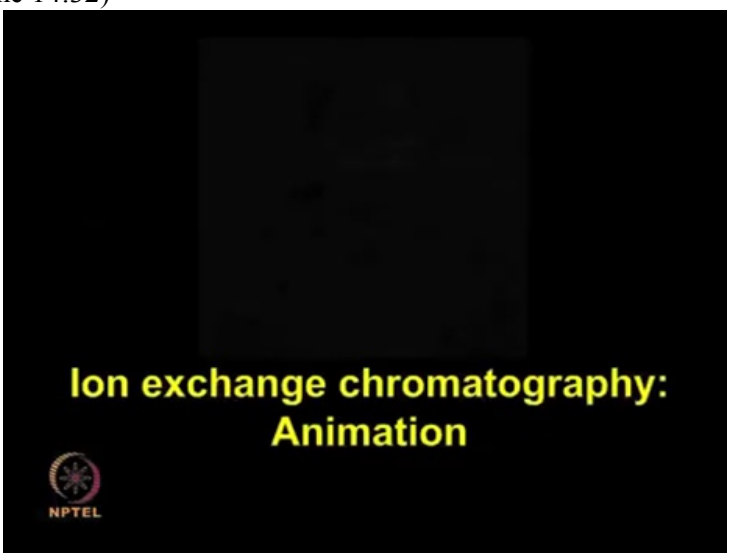

So let me describe how Ion exchange chromatography works, step by step in following animation.

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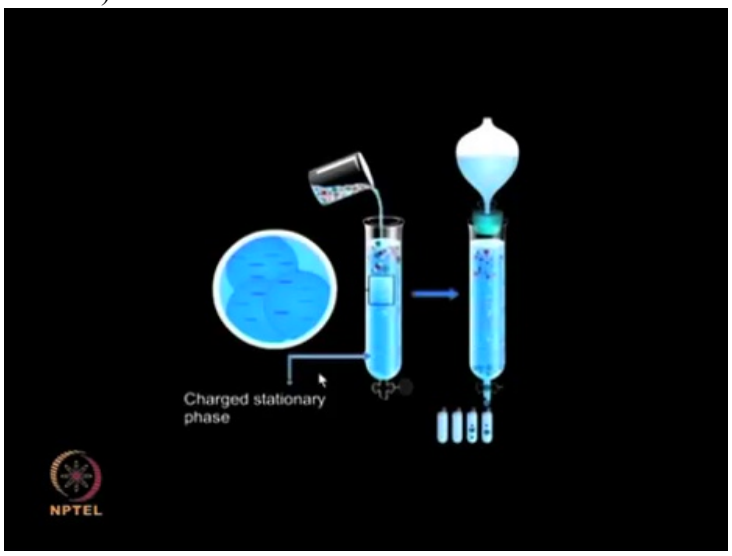

The Charged stationary phase

The column stationary phase consists of a positively or negatively charged polymeric matrix which will bind the molecules of opposite charge.

Commonly used ion exchangers are included negatively charged carboxy-methyl cellulose or CM cellulose which is a cationic exchanger...

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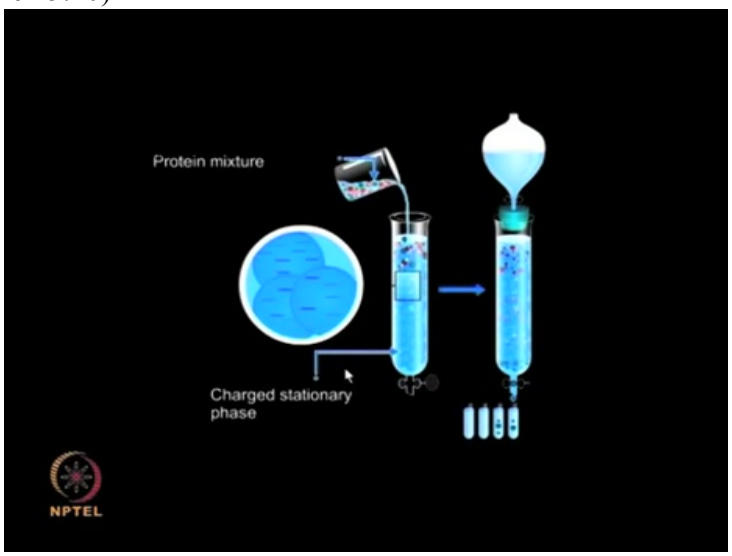

… and positively charged DEAE cellulose which is an anion exchanger

The protein mixture- unpurified mixture of proteins which consist of proteins of different net charges is loaded on to the column.

The proteins having charges opposite to that of stationary matrix will bind to it while remaining proteins will be eluted.

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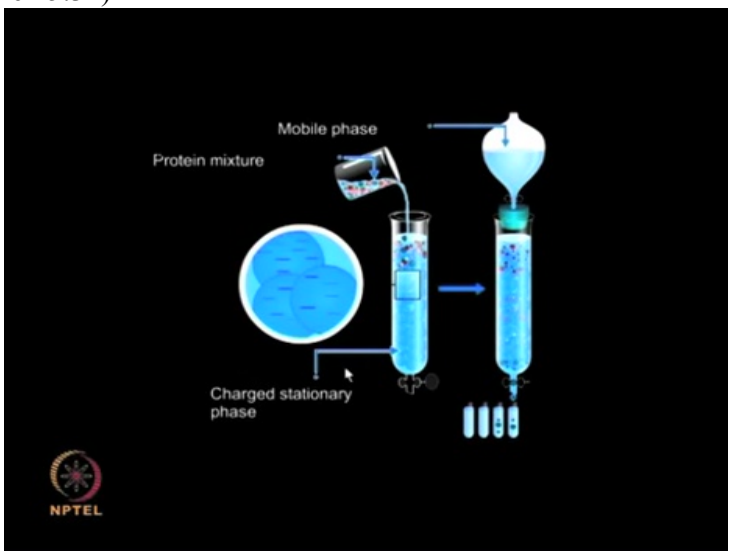

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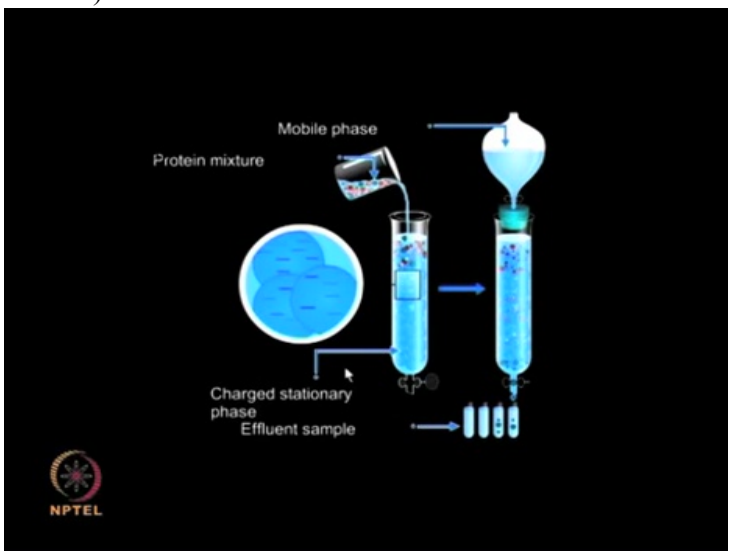

Mobile phase: The proteins are eluted of the column by using suitable mobile phase and then samples are collected by using different sample fractions.

The solution leaving the column can be collected in suitably sized fractions for further analysis.

After giving you the brief description of the components, let me show you

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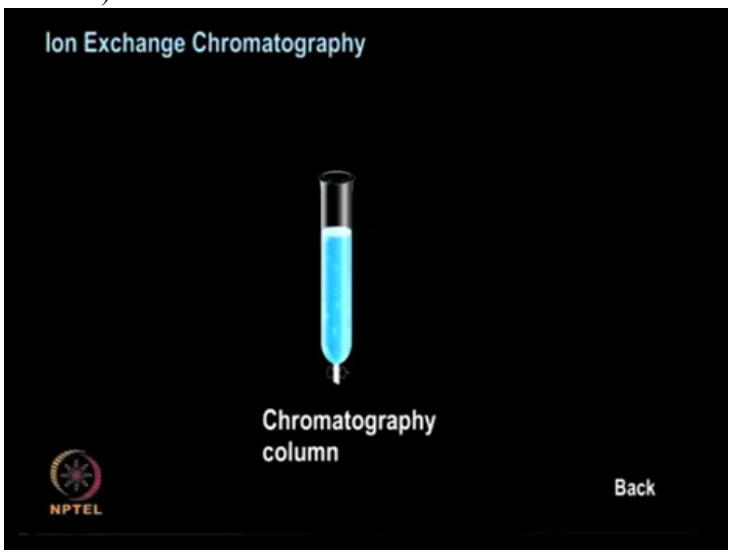

… the process in animation The column is packed with a suitable cation or anion exchange resin

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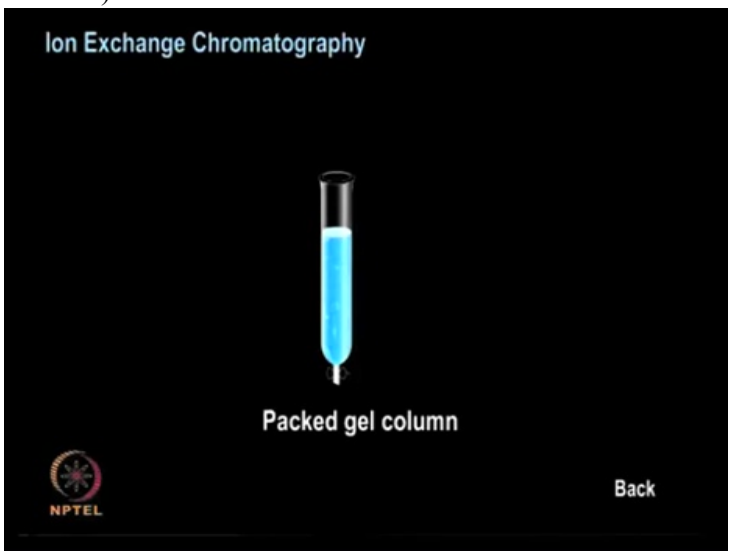

…depending upon the charge of the protein that needs to be …

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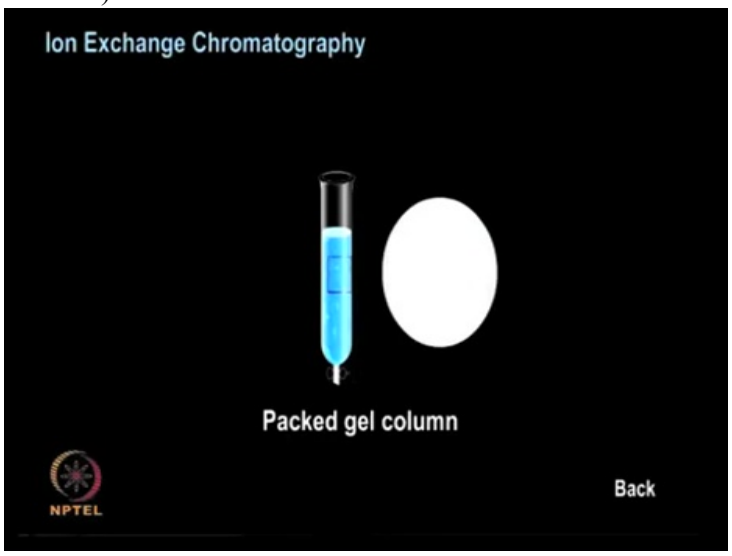

… bound to the column and purified The anion exchange column is then …

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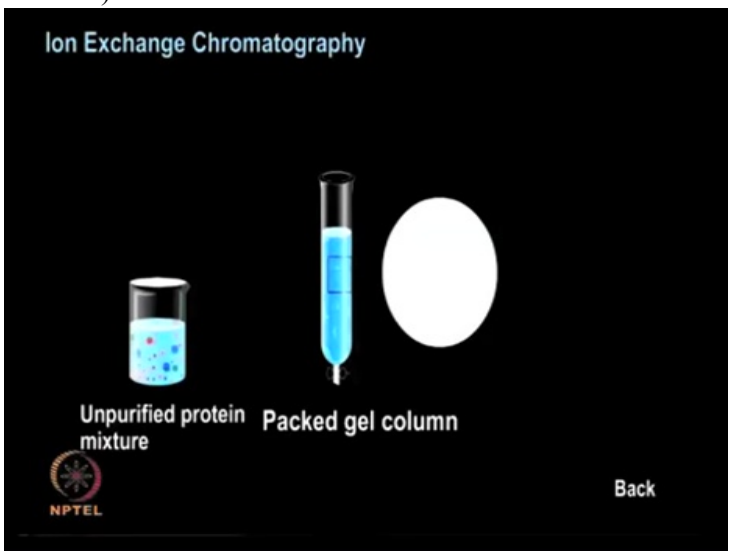

… loaded with the impure proteins mixture consisting of …

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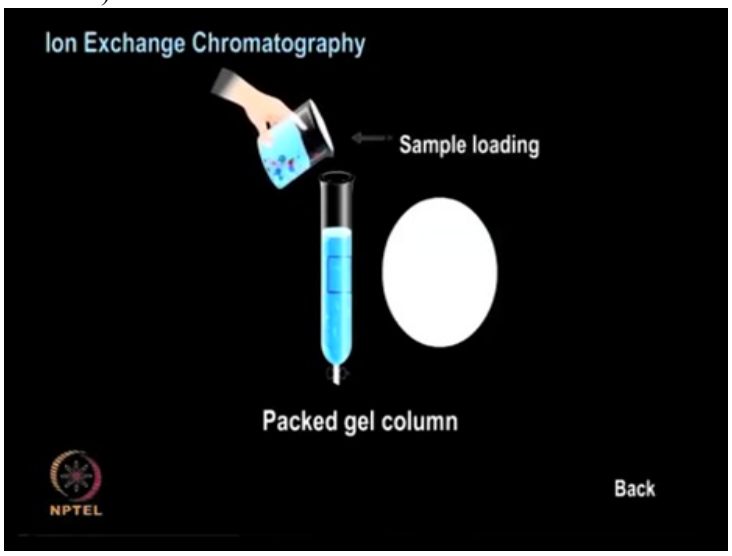

… various positively and negatively charged proteins The column is eluted with a buffer solution of suitable pH such that the negatively charged molecules are removed from the column

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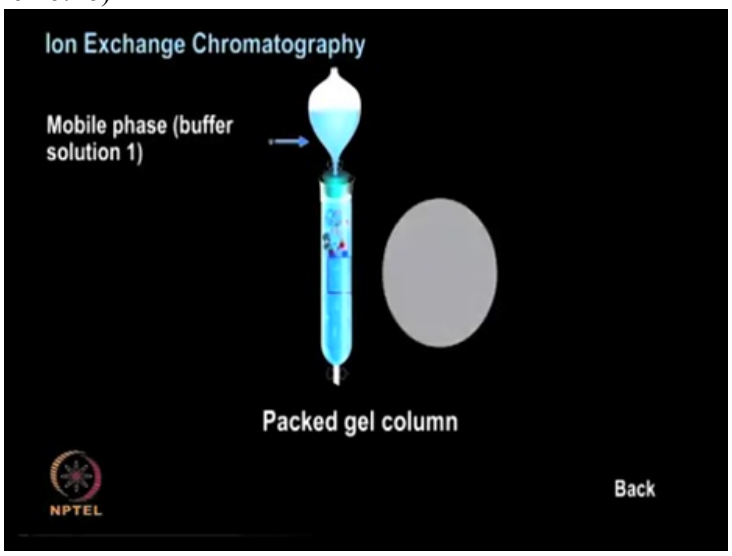

…while …

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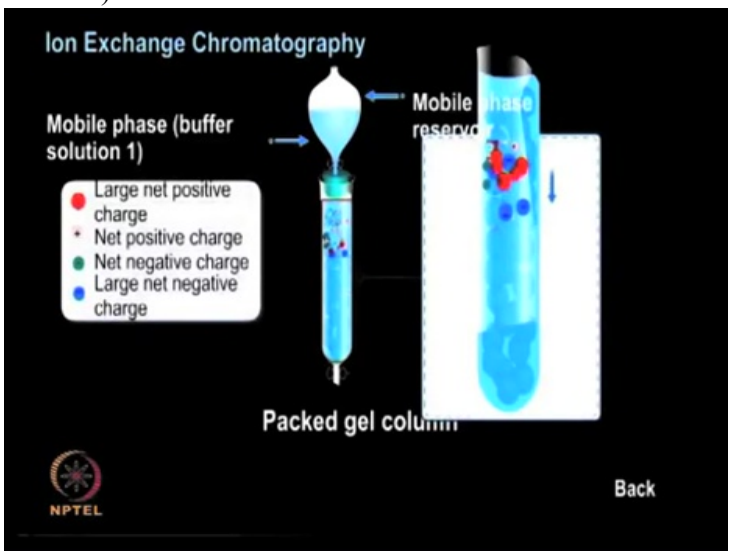

… the positively charged …

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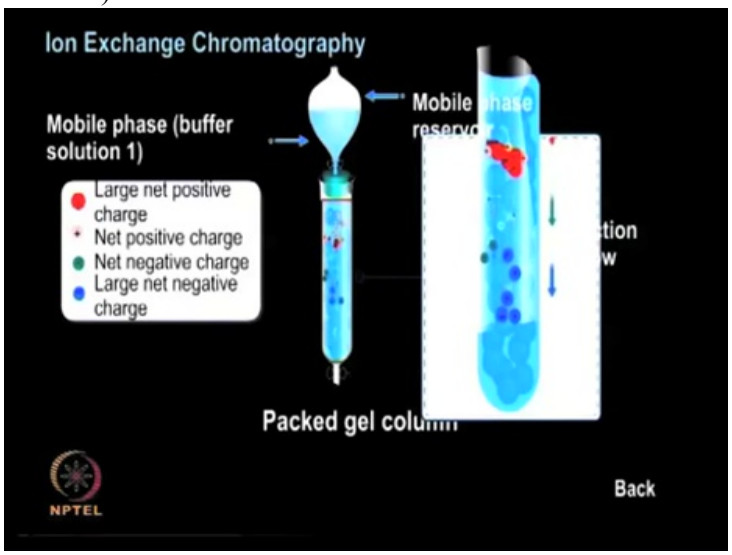

… molecules remain bound to the anion exchange resin.

The buffer solution is then changed such that the net pH of the protein of interest is modified and no longer binds the ion exchange resin.

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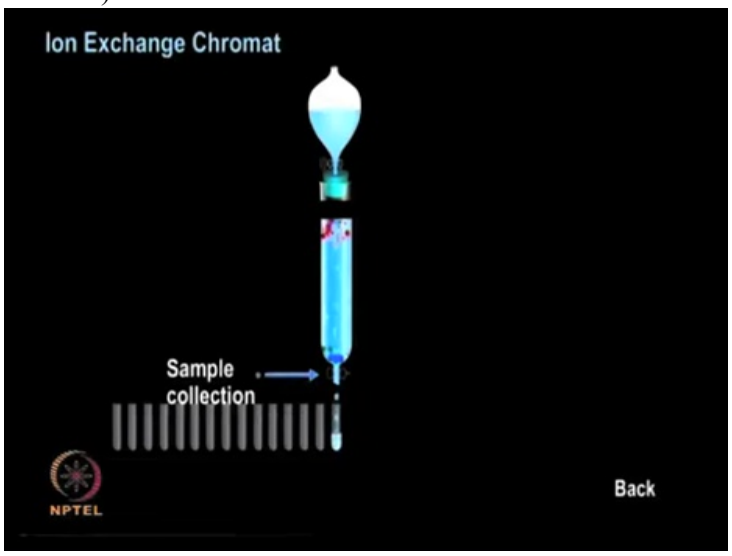

Therefore the bound protein also gets eluted out of the column in this manner.

The fractions of appropriate size must be collected and analyzed for their protein content. The negatively charged proteins which get eluted first …

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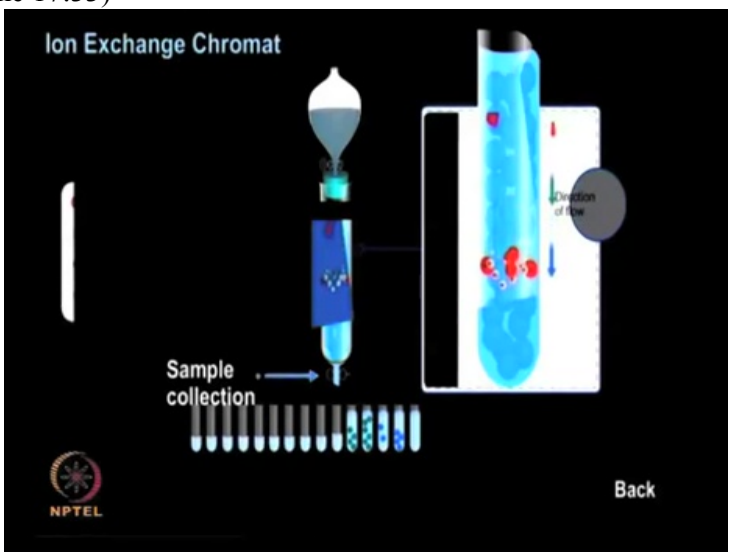

… will be present in the initial fractions while positively charged proteins that bound to the column are eluted in later fractions Once all the fractions are collected then the protein content can be analyzed by using spectrophotometer.

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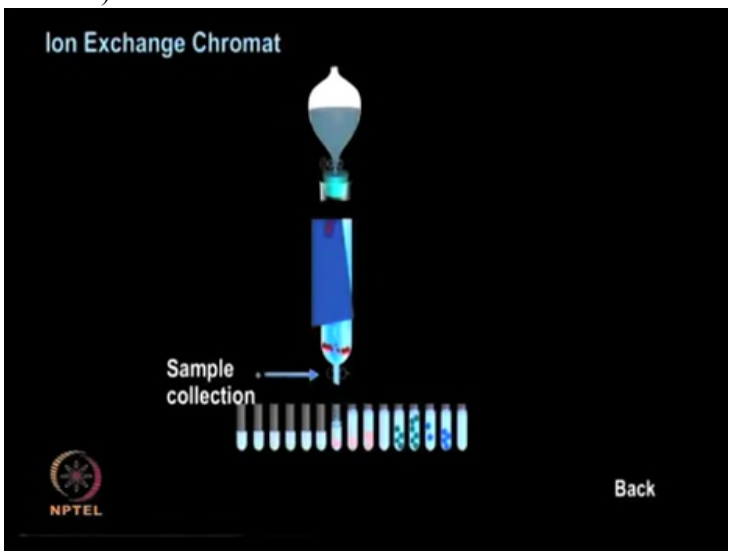

So analyze the contents these fractions for their protein content by using a UV-visible spectrophotometer at 280 nanometers.

A graph of eluant volume versus protein concentration can be then plotted.

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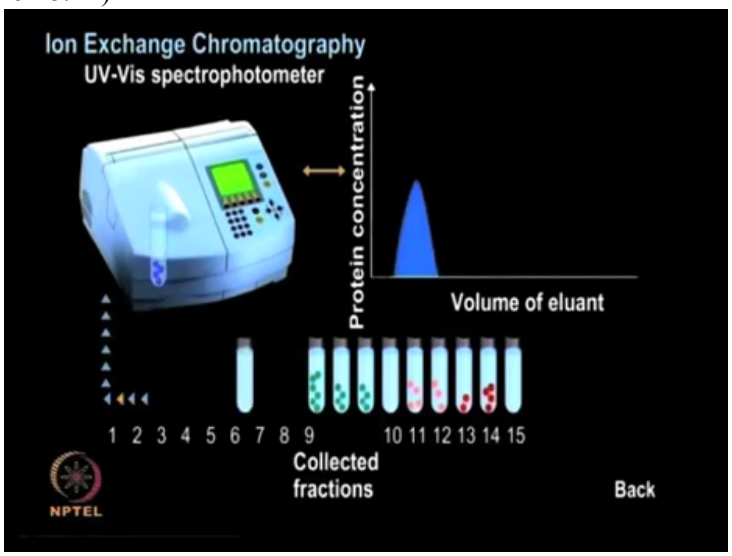

In this particular example the negative charged large molecules coming first and then positively charged fractions are coming later.

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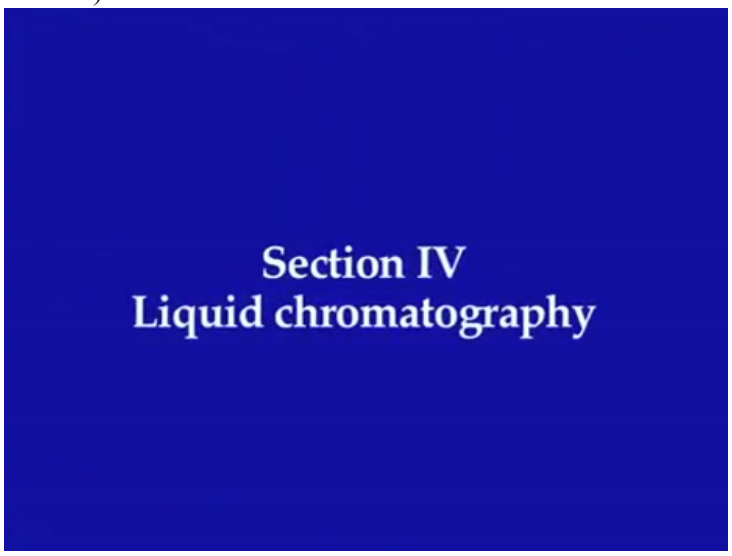

Let's now talk about …

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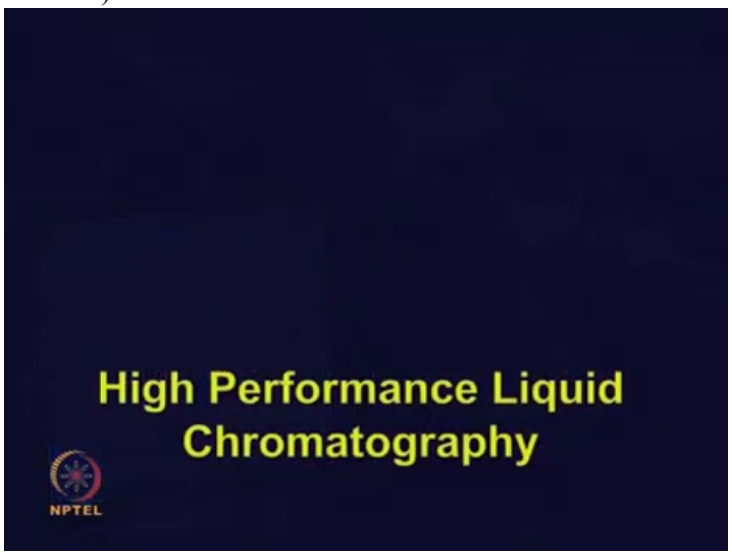

High Performance Liquid Chromatography HPLC, HPLC is a separation technique that separates molecules based on their differential adsorption and desorption between stationary phase matrix in column and mobile phase. Better separation and resolution of the components can be achieved by HPLC.

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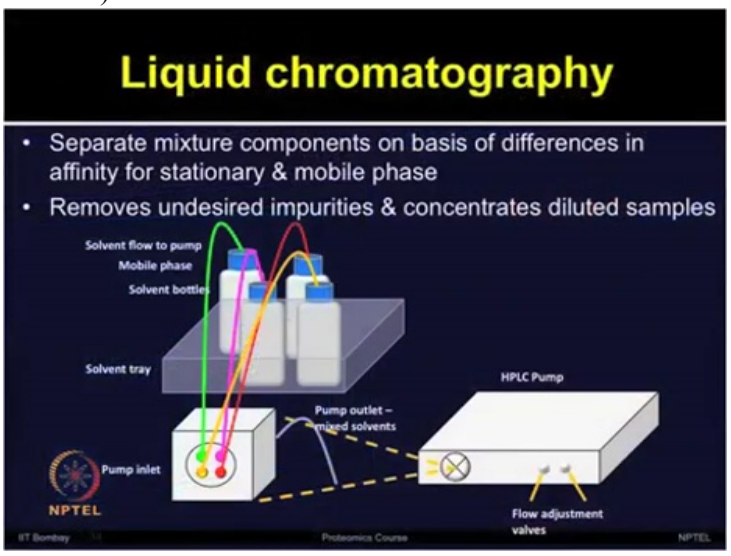

So what is Liquid chromatography?

Liquid chromatography separates mixture components on basis of differences in the affinity for stationary and mobile phase.

It can remove the undesired impurities therefore for various type of mass spectroscopy based applications; it increases the reproducibility and robustness for analysis of peptides and proteins.

It also concentrates the diluted samples and therefore it increases the sensitivity and detection of low level proteins by using... when you are applying the different type of proteomic technologies. It is mostly used for separating the peptide mixtures.

One of the diagrams is shown here; which is a typical liquid chromatography setup which consists of solvent bottles, de-gasifiers, dual or quaternary pump, the sample injector, column and detectors.

Different solvents can be placed in solvent bottles depending upon the purification requirements.

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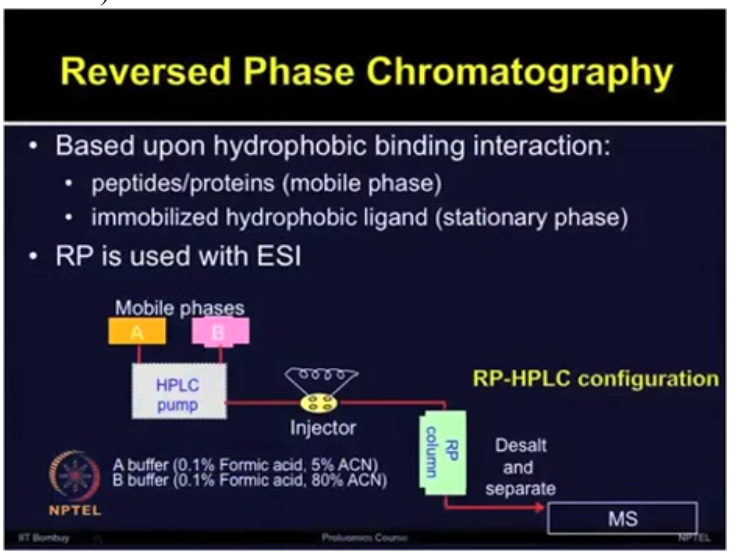

Lets first talk about Reversed Phase RP chromatography-

The Reversed Phase chromatography is based upon the hydrophobic binding interaction between the peptides or the proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase.

In Reversed Phase, the stationary phase consists of long aliphatic carbon chains which is highly hydrophobic in nature.

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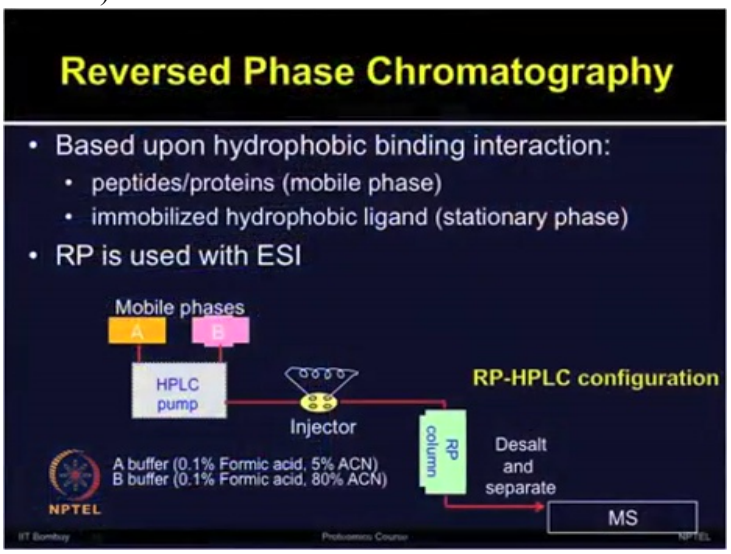

Molecules are bound on the column by means of hydrophobic interactions and are eluted out when the solvent polarity is modified.

The Reversed phase chromatography is mostly used with Electro-Spray Ionization in mass spec due to its compatibility of its acidic aqueous and polar mobile with ESI. It is also used for desalting the peptides before injecting for electro-spray ionization.

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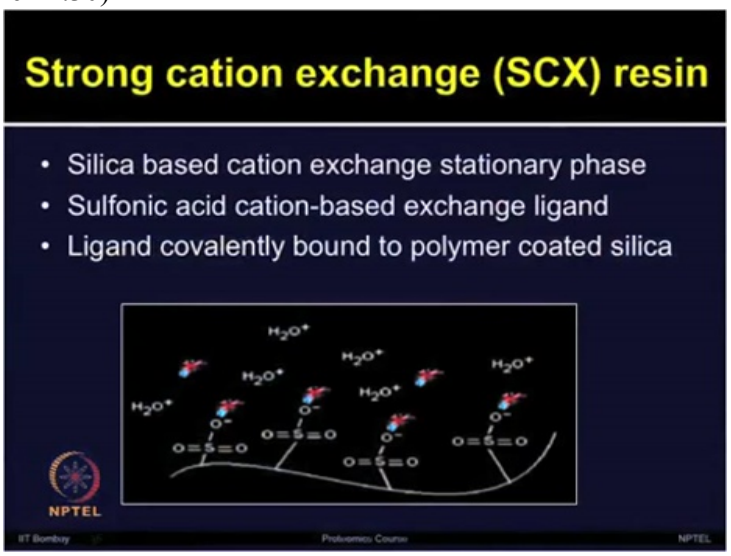

Let's now talk about Strong cation exchange or SCX chromatography

SCX consist of stationary phase matrix which is made up of negatively charged sulfonic acid groups which bind the oppositely charged peptide molecules.

These molecules can be eluted out using a positively charged mobile phase which binds the analyte molecules more firmly.

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So what is MudPIT or Multi-dimensional Protein Identification Technology?

So MudPIT method combines cation exchange and reversed-phase HPLC chromatographic separation of tryptic peptides for the proteome based analysis.

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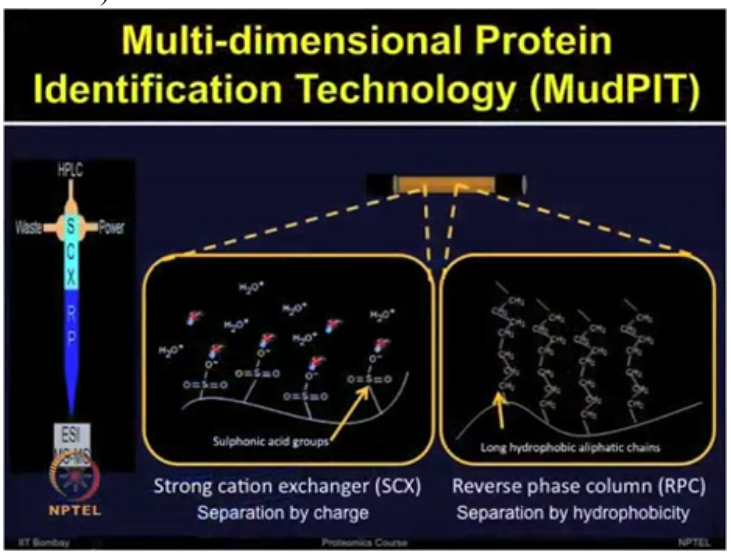

So we have already discussed two separate methods, SCX and RP.

Strong cation exchange is based on the electrostatic attraction between the negatively sulfonic acid and positive peptides and the elution can be caused by addition of positively charged mobile phase.

The Reversed Phase shown on the right side is based on the hydrophobic interactions between the analyte and stationary phase.

The elution can be brought about by modifying the mobile phase polarity. The Strong cation exchange can be used offline and then each fraction can be analyzed by Reversed Phase HPLC followed by mass spectrometry.

Alternatively, both RP and Strong cation exchange resins can be packed into single column and by introducing buffers in the series, the multidimensional separation can be achieved.

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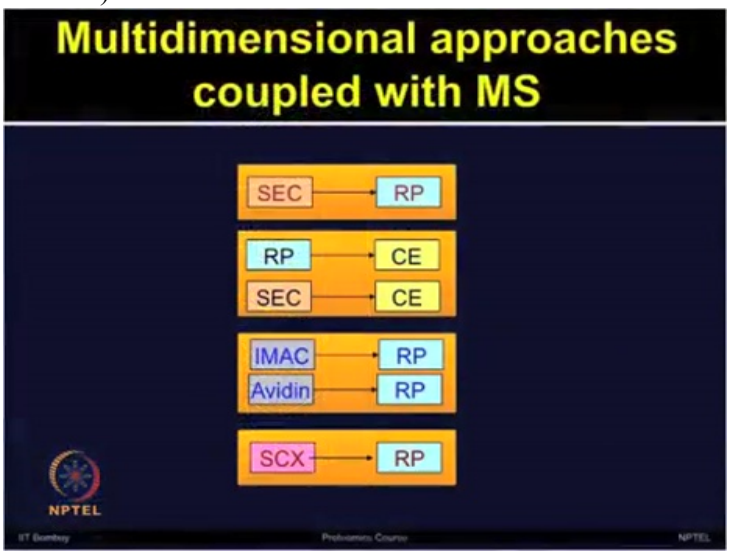

Now when application is to separate complex proteome and analyze using mass spectrometry then one has to select what type of chromatography method is applicable for their sample type.

Now multi-dimensional approaches are usually coupled prior to the mass spec analysis.

And as we have discussed there are certain chromatographic methods which are commonly available and one has to make an educated choice of selecting what type of chromatography methods can be used for first dimension and what can be used for second dimension separation.

So when size exclusion chromatography has been used followed by the reverse phase, to check the compatibility of first and second dimension separation based on the size in the SEC and the charge …

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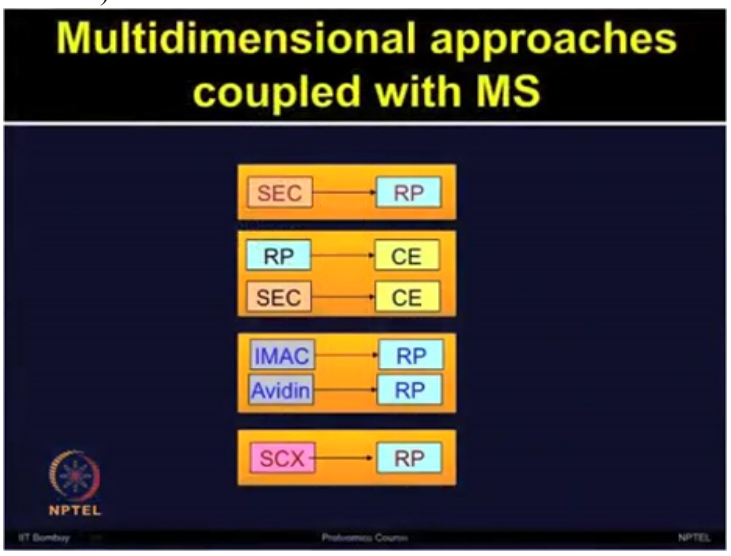

… various type of combination methods have been tried out

So let me first give you abbreviations here and then what properties they separate proteins and peptides based on and then I think we can talk about how these combinations can be applied.

So we have talked about size exclusion chromatography which separates proteins based on the size or the molecular weight, Reversed phase based on the hydrophobicity and then we have capillary electrophoresis which separates based on the charge, IMAC is affinity based interaction, Reversed phase again hydrophobicity, Strong cation exchange is based on the charge.

So these are different methods which we have discussed. Now what are we looking at how the combinations of these can be applied for proteomic investigations.

So when SEC followed by Reversed Phase has been tried the poor resolution of peptides in SEC occurs.

The Reversed Phase followed by capillary electrophoresis or SEC followed by capillary electrophoresis is limited with the loading capacity.

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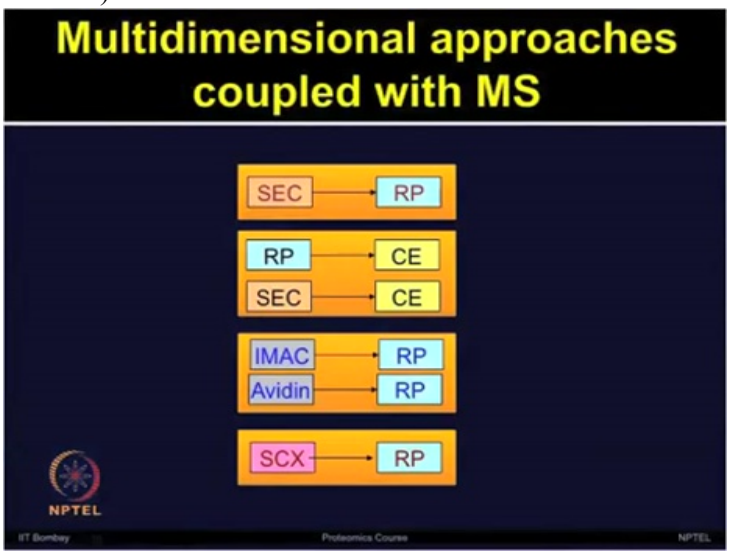

Now, third combination of using affinity chromatography as the first separation followed by Reversed Phase has been used. But what is most popular is the Strong cation exchange separation in first dimension followed by the Reversed phase chromatography in the second dimension.

That has resulted into the best result for separating the peptides and it has been used for various proteomic analysis.

So in summary, in the last few years there has been an increased effort to develop the technologies which are capable of analyzing protein expression at the proteome scale.

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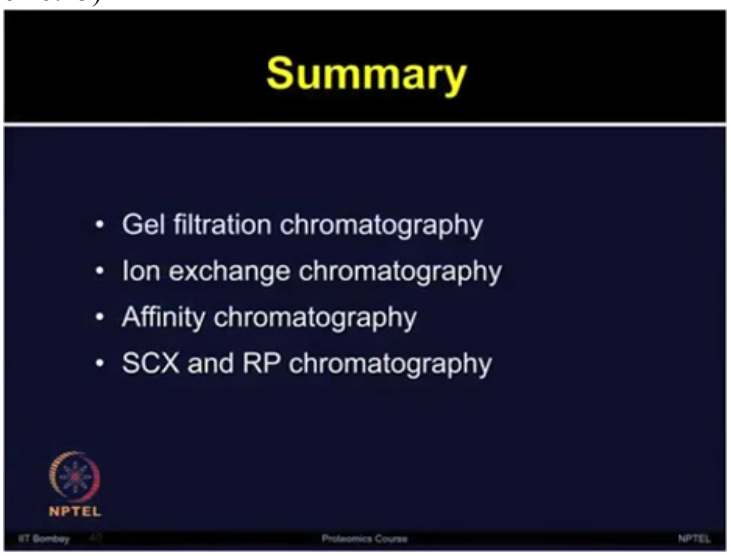

Some of the chromatographic methods such as gel filtration, ion exchange and affinity chromatography methods were discussed. The Strong Cation exchange and the Reversed phase High performance Liquid Chromatography are fundamental tools for the isolation and analysis of peptides.

The nano-liquid chromatography which makes use of C18 capillary columns has gained popularity for the proteomic studies due their ability to achieve finer separation.

So in summary we have talked about principle of different type of chromatography methods commonly being employed for proteins and peptides and we looked at some of these chromatographic methods in more detail.

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