Proteomics: Principles and Techniques
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Lecture No. # 06
Protein Purification and Peptide Isolation
Using Chromatography

Welcome to the proteomics course. Today, we will talk about protein purification and peptide isolation using chromatography methods. The chromatography techniques have always been considered prominent for the separation enzymes and proteins. Protein purification by conventional chromatography is usually achieved by combining chromatography methods such as gel filtration, ion exchange and affinity chromatography. From complex proteome, it is challenging to purify a protein in the single chromatography gesture.

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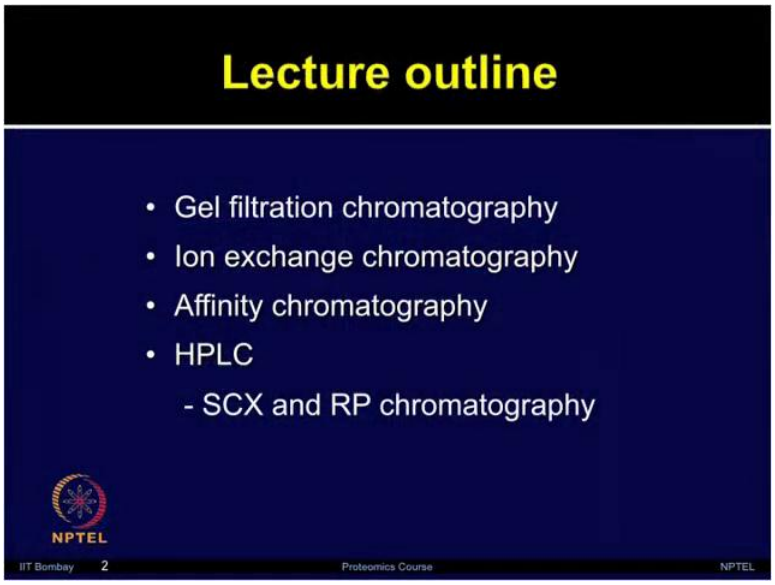

Therefore, sequential free fractionation disturbs involving different modes and types of chromatography methods are becoming necessary for proteom level analysis. It is the lecture outline, we will talk about gel filtration chromatography, ion exchange chromatography, affinity chromatography; these methods will be discussed enlight of protein purification, and then will talk about few methods such as a strong cation

exchange and reverse phase chromatography for peptide isolation using high performance liquid chromatography methods.

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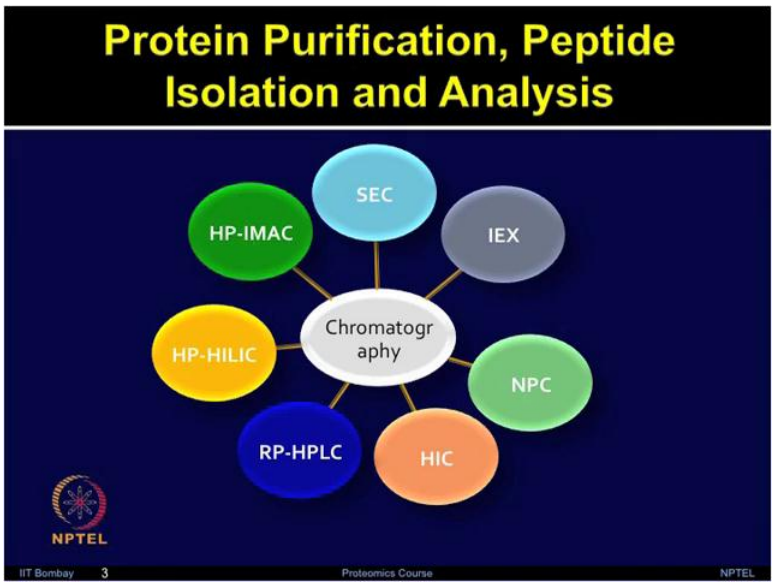

Protein purification peptide isolation and analysis; it can be performed by many methods, but there are certain chromatography methods which are commonly used such as, size exclusion chromatography SEC, ion exchange chromatography IEX, immobilized metal ion chromatography IMAC, normal phase chromatography NPC, reverse phase HPLC or RP-HPLC, hydrophobic interaction chromatography HIC and hydrophilic interaction chromatography HILIC; these are only few chromatography methods.

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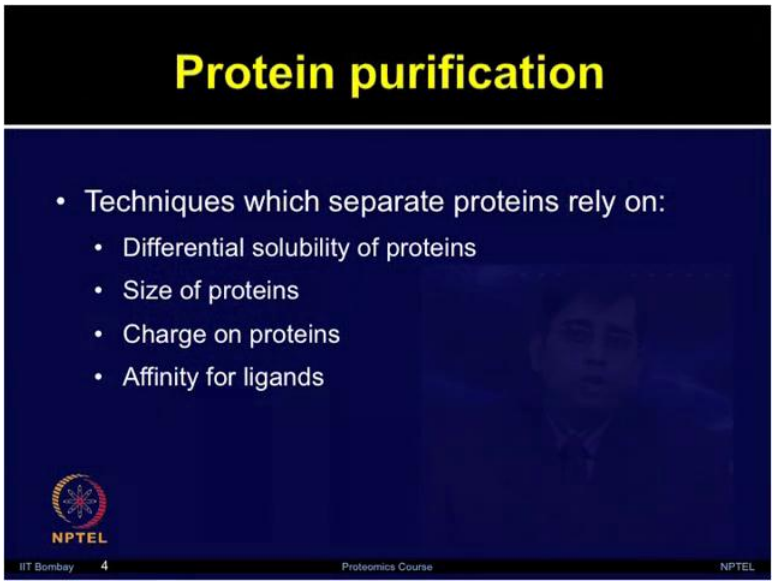

But there are many more which are also use for different type of applications. So, if you are invest to purify the protein, there are various techniques, which can separate proteins and they rely on different type of principles such as, differential solubility of the proteins, size of protein, charge on a given protein and affinity for various ligands.

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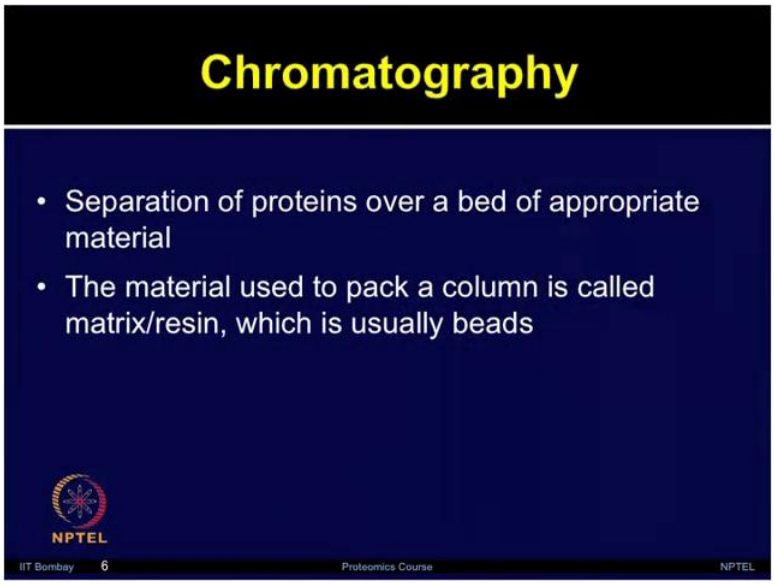

So, let us talk about what is chromatography. So, chromatographic methods involve separation of proteins over a bed of appropriate material. These materials are usually packed in a given column and known as matrix or resin. So these matrix and regions they are usually beats with or without attach chemical groups. Now, the binding and interaction of proteins with column matrix is an important feature of chromatography.

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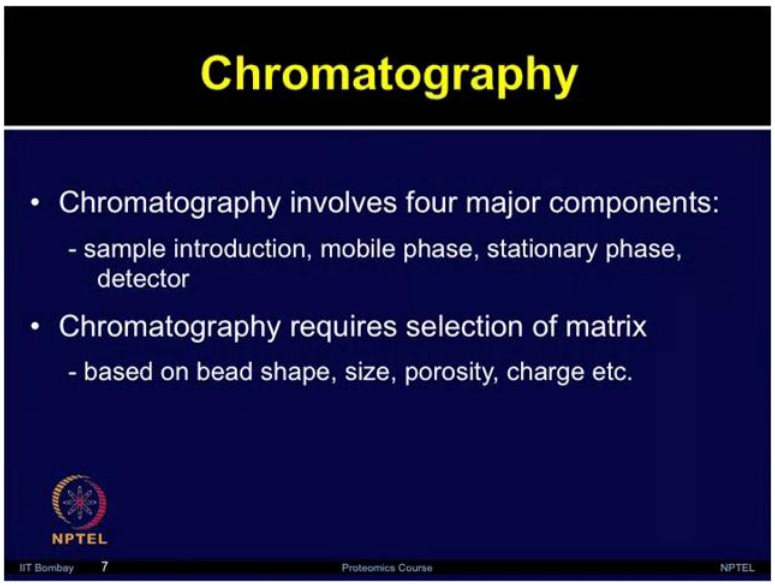

The chromatographic technique involves four major components; an inlet for sample introduction, the mobile phase, a stationary phase and a detector. Each type of chromatography requires a very educated and informs choice of matrix considering the bead shape, size and porosity. In addition to the functional group type the charge, distribution and density as well as elution conditions such as the pH, the ionic strength and gradient shape.

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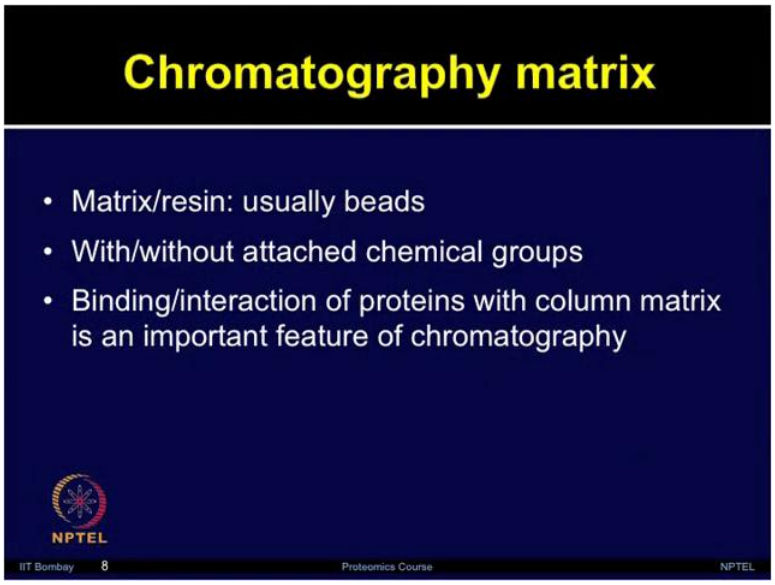

Just we talked the chromatography involves the selection of right matrix. These matrix are very important for binding and interaction of proteins, and they can determine the kind of achievement, which we want to achieve from these type of chromatographic methods.

So, let us move on to more specific chromatographic techniques such as gel filtration chromatography. The gel filtration chromatography, it separates proteins on the bases of difference in size. It is used to separate a protein of interest from protein mixture that are larger or a smaller in size. If proteins are having similar sizes, then the gel filtration or size exclusion chromatography, it is not an appropriate choice for during the protein purification. So, this method is also use for various proteomic applications, when there is need to remove even contaminants such as salts and low molecular size detergents.

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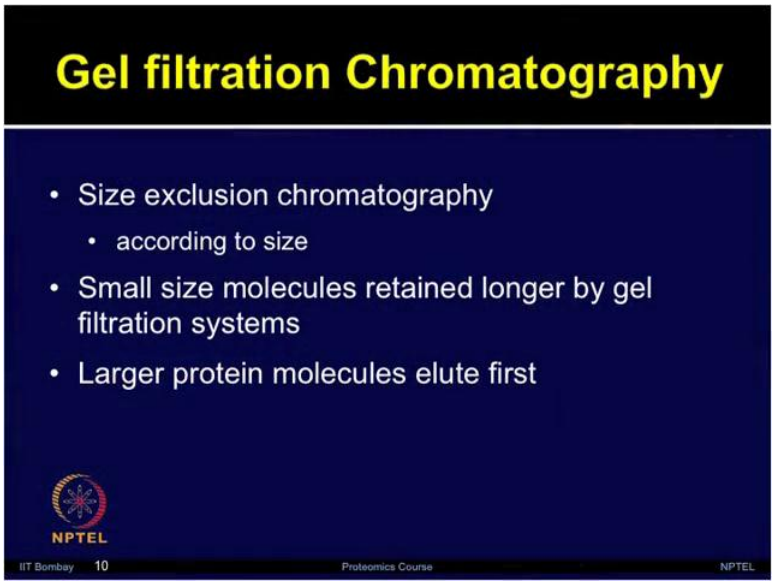

So, gel filtration chromatographic is also known as size exclusion chromatographic; it means, the molecules are separated according to the given size, the small molecules or even if you are separating small molecules such as salt, those will be retained longer by the gel filtration system, and larger molecules such as proteins will elute first, because they cannot enter inside the porous these beads, and they can be used to separate proteins based on the size.

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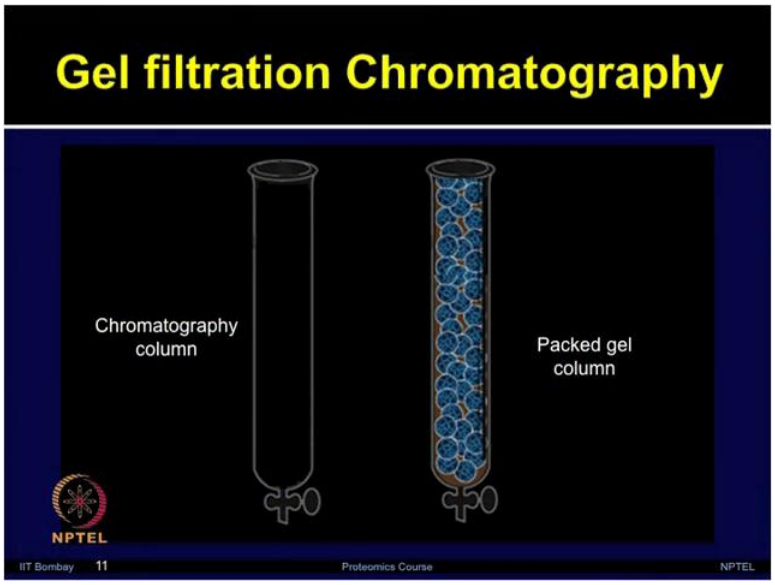

The gel filtration column is composed of porous beads which are made from polyacrylamide, dextram or agarose. Now, these columns are packed with the hydrated porous gel matrix.

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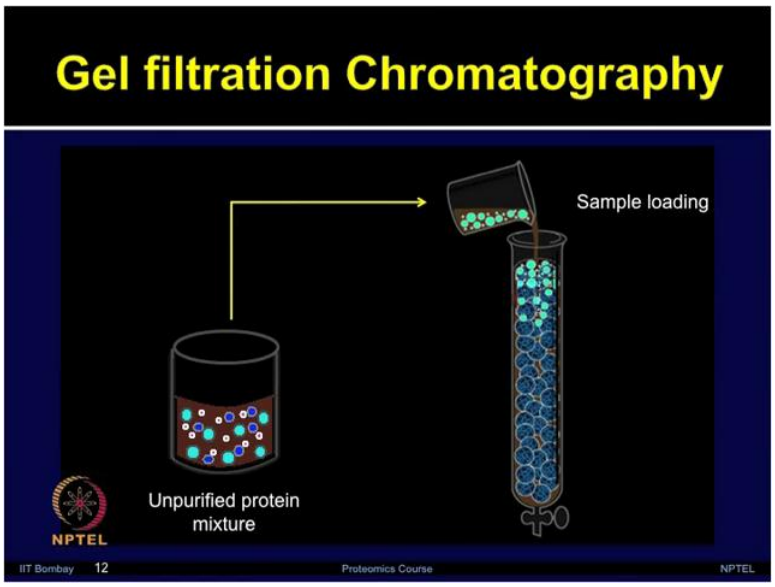

The protein sample which contains a mixture of unpurified proteins of different size then

loaded on these columns.

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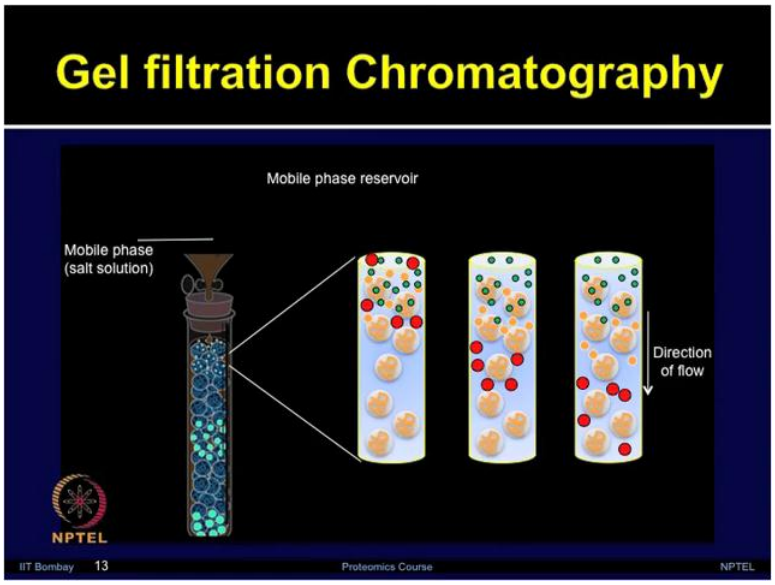

Now, when protein sample is applied to the column, the small proteins passes from the ports of the beads while the large proteins are excluded; therefore, this technique is also known as molecular exclusion chromatography. The beads of different port sizes or increases retention time by adjusting the increase column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

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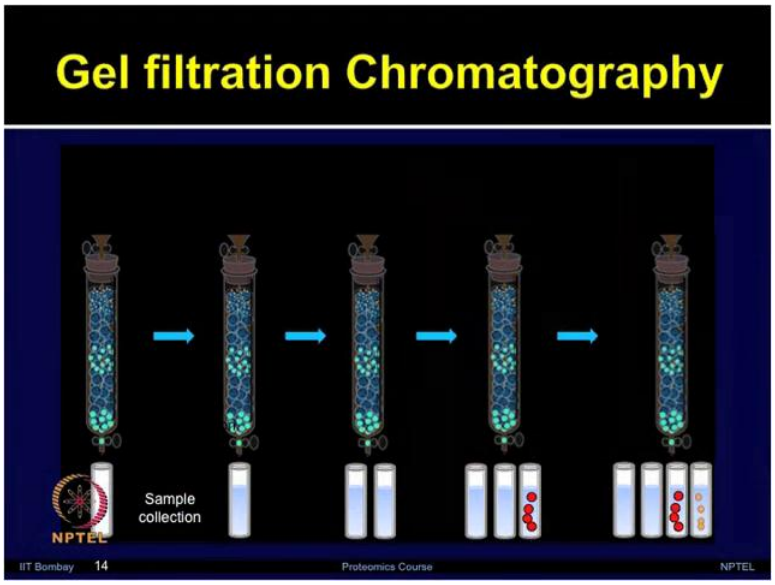

After the these steps the fractions are collected and analyzed for the protein content. So, you can expect that larger proteins will elute first and then, the small molecules will be eluted later. So, gel filtration chromatography is useful for removal of even contaminants, doing desalting steps as well as for buffer exchange. However, its drawbacks are at low capacity; broad distribution of port size and a small sample volume to be analyzed.

So, let me describe the gel chromatographic technique in following animation. Let us first get the definition of few components. Size exclusion gel matrix the matrix filling the gel filtration column consist of a highly hydrated polymeric material commonly dextram, agarose or polyacrylamide. The protein mixture is a mixture of unpurified proteins of different sizes, which is apply it on top of the column. Mobile phase the proteins are eluted out of the column by using a suitable mobile phase that carries protein out for elusion. For gel filtration a salt solution of appropriate a strength is commonly used so that it will not have any effect on properties of the proteins being purified. Solvent or buffer systems are often used in other types of chromatography. Effluent sample fractions the solution leaving the column is collected in suitably size fractions. Initial fraction will contain only the mobile phase while later of fractions will have purified proteins. Let me give you an analogy of gel filtration with sieve. This process is very similar to separation

of a small particulate matter from food grains using a sieve. The larger grains remain behind the sieve while smaller sand or a stone particles pass through them and are removed. In keeping with this, the gel filtration technique is also commonly referred as molecular thief.

Now, let me show you how this process works. So, first the (( )) which is suitable for the required protein separation. This packed gel matrix is then loaded with the protein sample containing a mixture of unpurified proteins of different sizes. The column will then eluted with a salt solution of appropriate concentration. Large proteins that cannot inter the ports of the gel move down through the interstitial spaces at a faster rate and are eluted first. These smaller proteins move in and out of the ports thereby taking longer time to be remove from the column.

The fractions of appropriate size should be collected and analyzed for their protein content. The largest proteins eluting out first will be present in the initial fractions, while a smaller proteins which elute out later will be present in the later fractions. Once all these fractions are collected then, it can be analyzed for their protein content by using a UV visible a spectrophotometer. Now, analyze the protein content by using UV visible spectrophotometer at 280 nanometers. Once all the absorbance values are recorded, a graph of eluant volume versus protein concentration can be plotted for the highest molecular weight is in the beginning and lowest molecular weight towards the end.

Let us now move on to next chromatographic method which is ion exchange chromatographic. This is one of the most versatile chromatographic separation method which relies on differences between number of charges and distribution of charged groups in define pH and solvent conditions.

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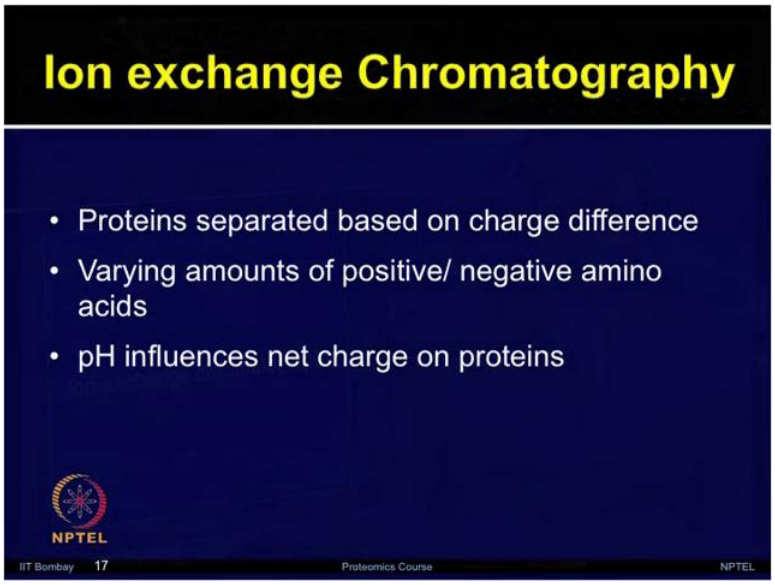

In ion exchange chromatography, the proteins are separated based on charge difference. The proteins with over all negative charge will interact with positive charges or the vice versa. So, by varying the amount of positive and negative amino acids and even pH can influence the net charge on proteins.

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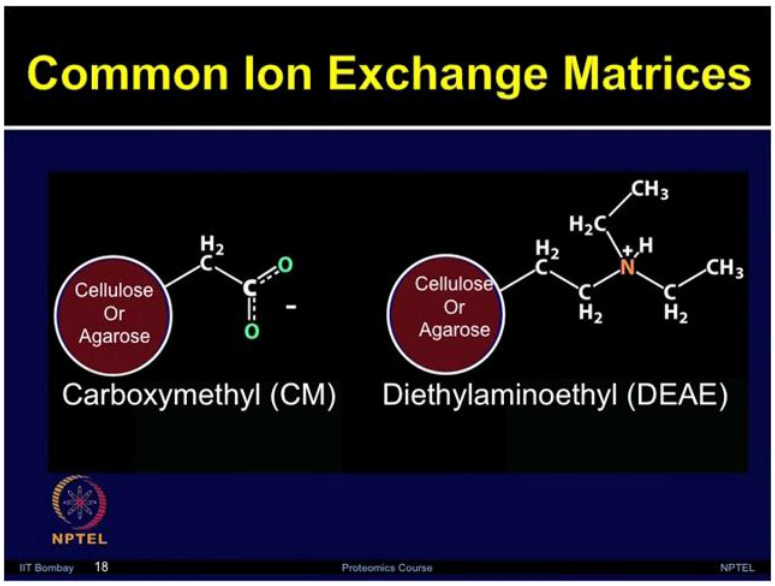

So, in this slide some of the common ion exchange matrices are shown like carboxymethyl CM and diethylaminoethyl DEAE. So, when a desired protein is positively charge, the cation exchange chromatography should be used. When a desired protein is negatively charge, the anion exchange chromatography method should be used.

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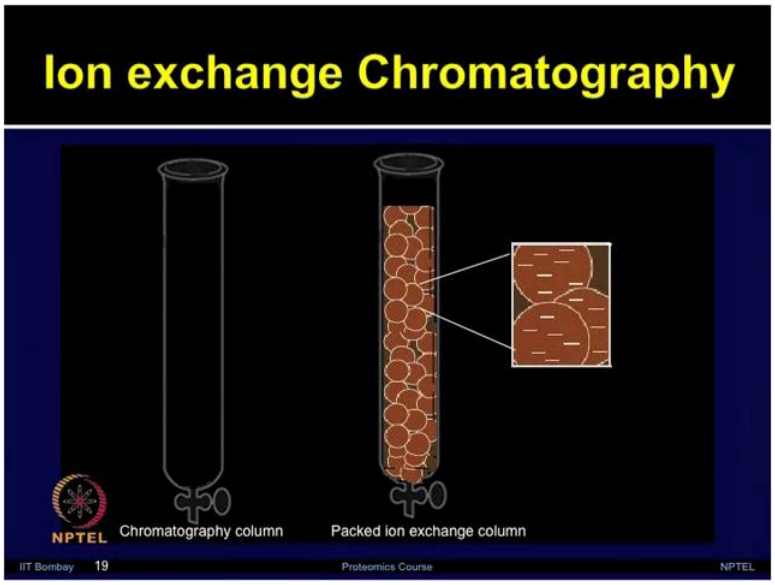

So now, when exchange chromatography the column is packed with a resin whether it is cation or anion exchanger depending upon the charge of the protein that needs to be bound to the column and purified.

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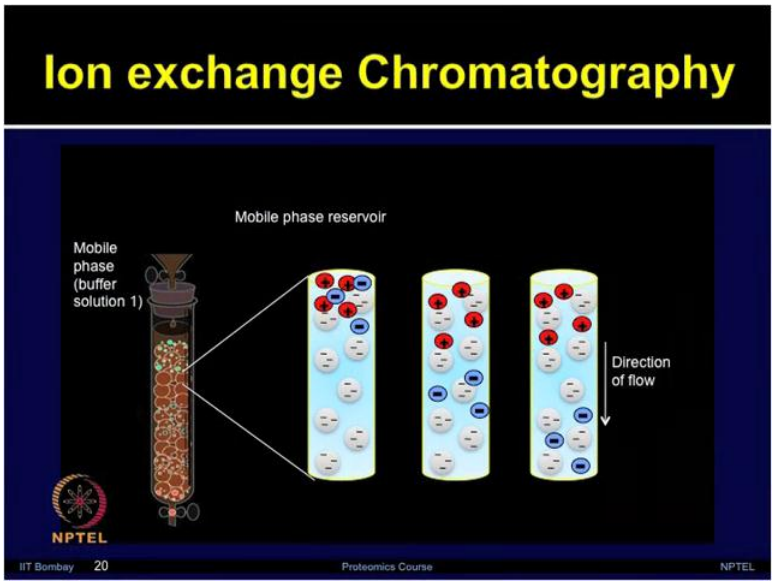

So, proteins are adsorbs to the ion exchange column and then, it can be desorbed by increasing the salt or altering the pH of the buffer which can change the charge on protein. So, various anionic buffers such as acetate and phosphate are used for cation exchange and cationic buffers such as stress chloride or ethanolamine are used for the anion exchange.

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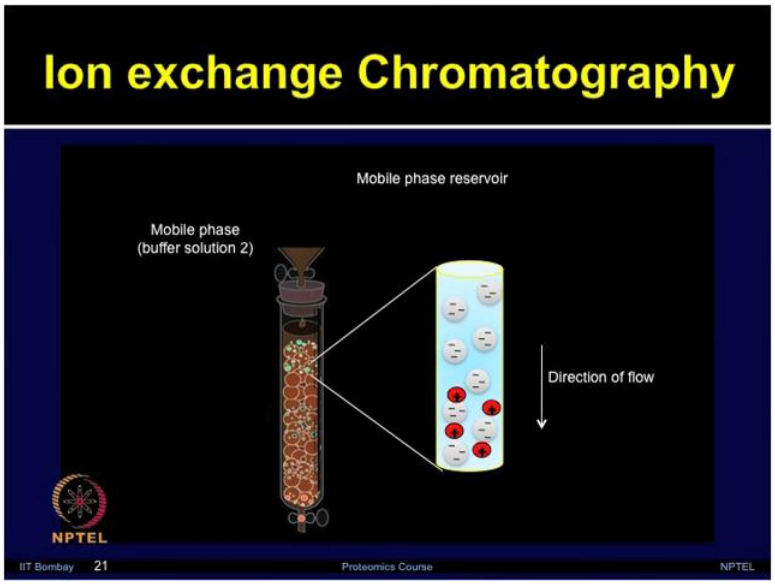

Now, the buffer solution exchange so that the net pH of the protein of interest can be modified at no longer binds to the ion exchange resin. Therefore, the bound protein can be eluted out as shown in this slide.

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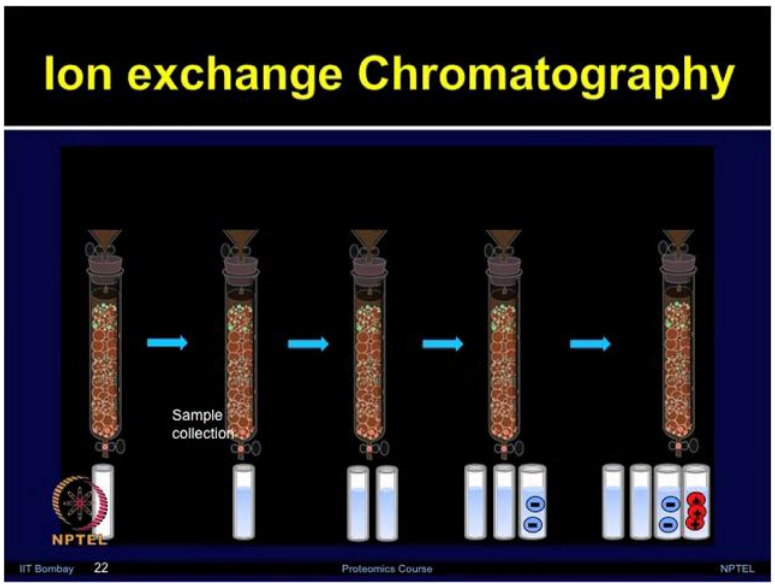

So, if you have negatively charged protein which gets eluted first will be present in the

initial fractions, while the positively charged protein that bound to the column will be eluted in the later fraction or it will be vice versa.

So let me describe, how ion exchange chromatography works step by step in following animation. The charged stationary phase; the column stationary phase consists of a positively or negatively charged polymeric matrix which will bind molecules of the opposite charge. Commonly used ion exchangers include negatively charged carboxymethyl cellulose or CM cellulose which is a cation exchanger and positively charge DEA cellulose which is an anion exchanger.

The protein mixture unpurified protein mixture which consists of proteins of different net charges are 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. Mobile phase the proteins are eluted out of the columns 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 brief description of the components, let me show you the process in animation.

The column is packed with a suitable cation or anion exchange resin depending upon the charge of the protein that needs to be bound to the column and purified. An ion exchange column is then loaded with the impure protein mixture consisting of 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, while the positively charged 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 bind the ion exchange resin. Therefore, the bound protein also gets eluted out of the column in this manner. The fraction of appropriate size must be collected and analyzed for their protein contents. The negatively charged proteins which get eluted first will be present in the initial fractions, while the positively charged protein 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. So, analyze the contents these fractions for their protein content by

using a UV visible spectrophotometer at 280 nanometers. A graph of eluent volume versus protein concentration can then be plotted and this particular example the negative charge large molecules coming first and then positively charged fractions are coming later. Let us now talk about another very important method which is affinity chromatography which is a desirable method for protein purification to the homogeneity. Due to the selectivity of immune recognition, it is possible to purify a protein in a single step under the favorable conditions, and affinity chromatography is one of the method of choice for protein purification.

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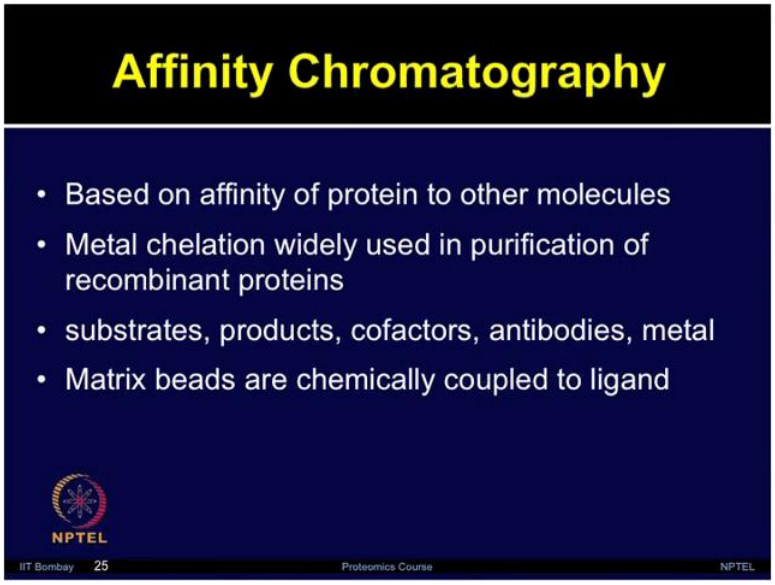

So, affinity chromatography is based on affinity of proteins to its ligands or other molecules. The metal chelation is widely used in purification of recombinant proteins. Various substrates, products, cofactors, antibody[ies or metals these can show the affinity for given protein, and this is used for a strategy to purify the proteins based on its affinity. The matrix beads are chemically coupled to these ligands.

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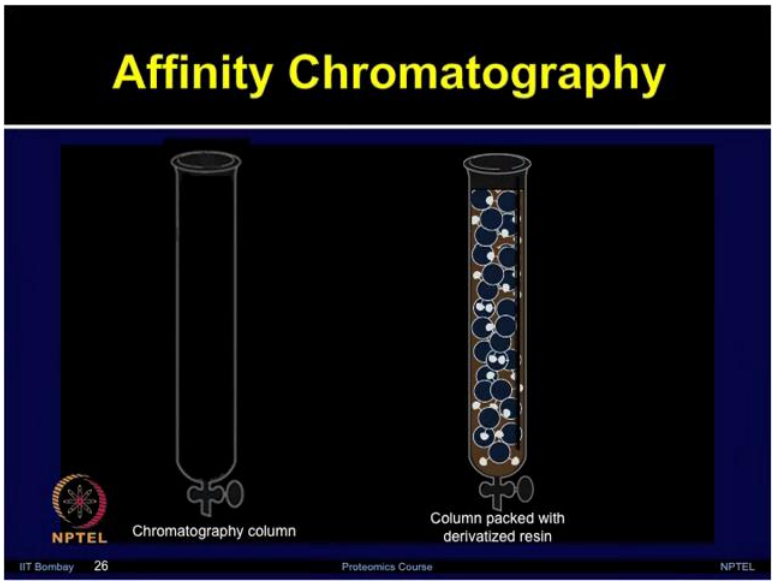

So in affinity chromatography, the column is packed with a resin, which is covalently coupled to the ligand specific to the protein of interest.

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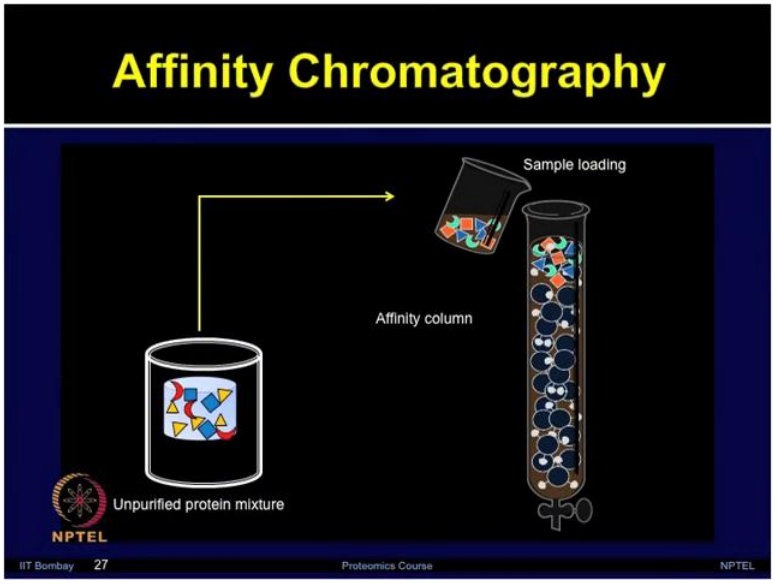

The protein mixture is passed over the derivatized affinity column; the protein of interest binds through a specific interaction, while all other proteins which do not interact will

not bind.

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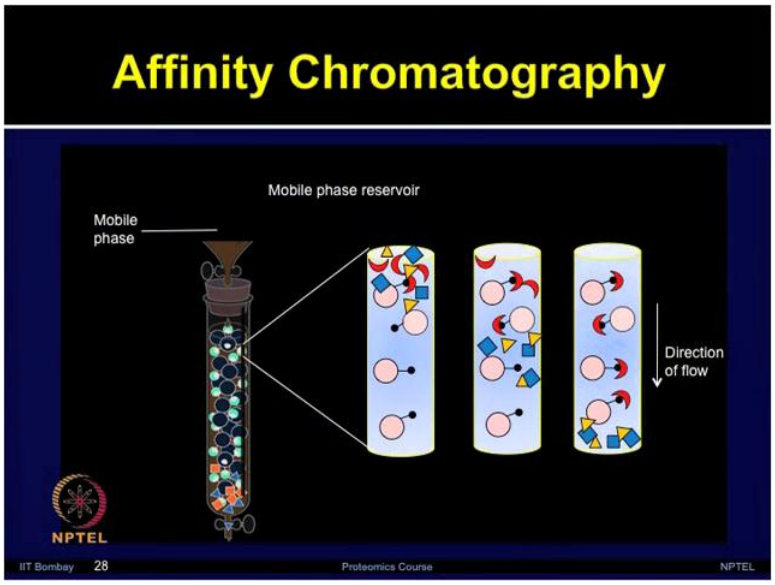

Now, the column is washed with a suitable mobile phase to remove the unbound protein and protein of interest which has higher affinity for the ligand remains bound to the derivatized column matrix, and it is not remove during the washing step. Now, these proteins of interest can be desorbed by applying excess ligand in the solution. So, due to the specific interaction, the affinity chromatography achieves very high degree of protein purification.

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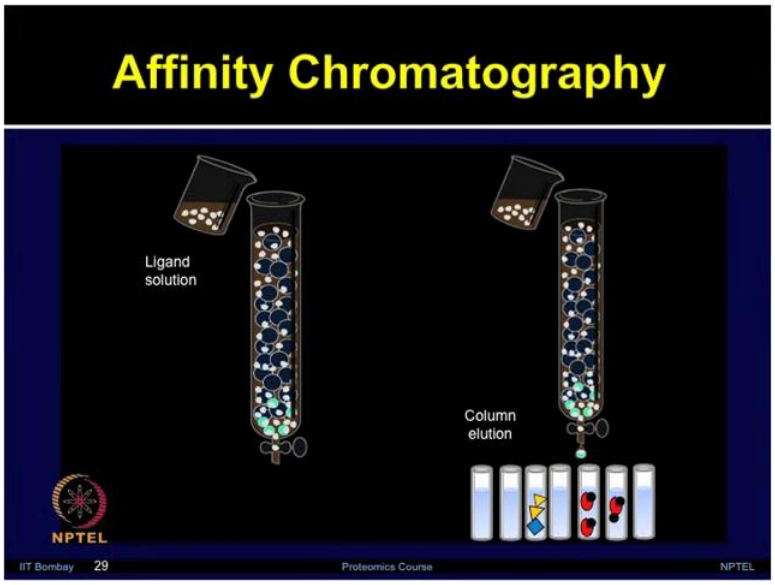

It is not limited by the sample volume, which is the case of gel filtration chromatography then because of it is superiority in achieving the pure protein, it is usually consider as the final step for the protein purification.

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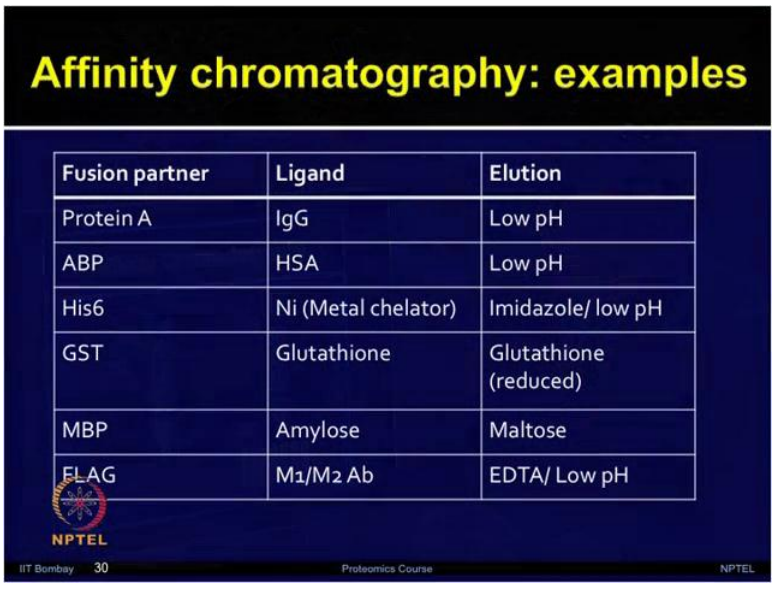

There are various examples where affinity chromatography can be used. Especially, the

antigen antibody pairs they are commonly used for bio affinity pairs. The matrix containing protein A which is use for the IgG purification. So in this case, the protein A recognizes the (( )) region of IgG, and this interaction is being used for protein purification using affinity chromatography. Another strategy is concanavalin A protein binds to the glucose molecule.

So, by adding a concentrated solution of glucose, the glucose can displays the column where these molecules are attached on the binding site of concanavalin A, but these are only few examples. There are many other examples where different type of fusion partners and ligands are used for affinity chromatography method. As shown in this slide, the protein A binds with IgG and the proteins can be eluted by lowering the pH; ABP binds with HSA and elusion is again with the low pH; His6 histidine tag binds with nickel NTA columns the metal chelators and imidazole or low pH condition can be use for elusion; GST glutathione as transferece binds with glutathione and reduce glutathione is use for elusion. The maltose binding protein MBP binds with amylose and it can be eluted with maltose. Then flag proteins can be bound to M1 or M2 antibodies and EDTA or low pH elusion can be use for eluting the proteins.

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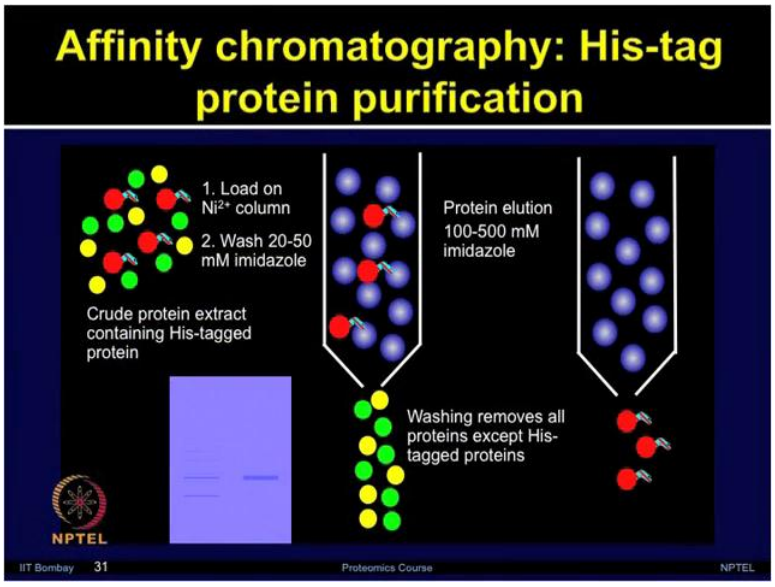

There are various other examples, but these are common strategies being employed for

protein purification. So, we just discussed there are various strategies by adding a tag or by applying some affinity interaction the proteins can be purified. The genetic engineering methods have made it possible to create diffusion protein, which shows a strong affinity between the fuse protein and the ligand.

So for example as shown in this slide, the affinity of histidine tag with nickel NTA column, now because of interaction of histidine tag with nickel, the proteins which contain histidine tag will bound the these regions of nickel NTA. Now, after washing with the mild imidazole such as 20 to 50 milli molar unbound residues will come out, but by increasing the concentration such as 100 to 500 milli molar of imidazole the proteins can be eluted out. The effectiveness of protein purification should be further assessed on SDA space gel, where one need to see whether one pure band is shown or even some contaminating bands are also seen. So, one case is shown on left side where contaminating bands are seen whereas, in the other case only a pure band can be seen.

So, let us discuss the affinity chromatography methods step by step in following animation. Let me give a definition of few components. The derivatized stationary phase the stationary phase raisin in affinity chromatography consists of a covalently bound ligand that will a specifically bind the protein of interest by interacting it. The protein mixture it is unpurified protein mixture which consists of proteins having different properties and interaction specificity for the ligand bound to the column matrix. Mobile phase following the sample loading the unbound proteins are washed out of the column using suitable mobile phase depending on protein of interest; this is could be either water or sometimes salt solution. The ligand solution that solution is passed through the column to elute the bound protein of interest since, it contains the same ligand that is bound to the column matrix. It is capable of eluting the proteins by interacting with it. The effluent sample fractions the solution leaving the column is collected and suitably sized fraction for further analysis. The unbound proteins are eluted from the column first followed by the bound fractions, which are removed after washing with the ligand solution.

Now, let us see the process in the following animation. The column is first packed with a suitable raisin that has been covalently coupled to the specific to the protein of interest.

This derivitzed affinity column is then loaded with the protein mixture containing various proteins having different properties and interaction specificity. The column is washed with a suitable mobile phase to remove all the unbound proteins. The protein of interest which has higher affinity for the ligand remains bound to the derivatized column matrix and it is not removed during the washing. The fractions collected during sample washing can be analyzed, assessed and discarded if they are not required. After the column has been washed thoroughly, the protein of interest is eluted by a passing a ligand solution which binds to the matrix bound protein and removes it from the column. The fractions are then analyzed for their protein content using a UV visible spectrophotometer at 280 nanometers. A graph of eluent volume versus protein concentration can then be plotted.

Let us now talk about 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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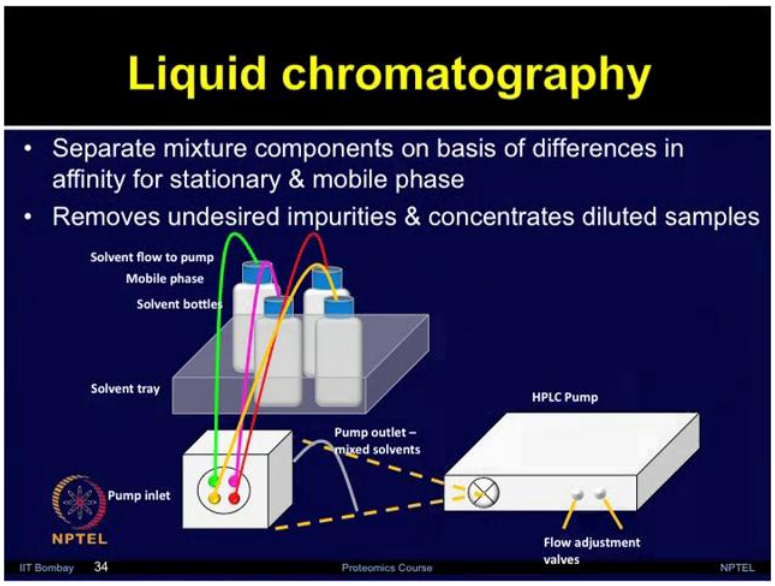

So, what is liquid chromatography? The liquid chromatography separates mixture components on the basis of differences in the affinity of stationary and mobile phase. It

can remove the undesired impurities; therefore, for various type of mass spectrometer base application, it increases the reproducibility and robustness for analysis of peptides and proteins. It also concentrates the diluted samples and therefore, it increases the sensitivity detection of the low level proteins by using when you are applying the different type of proteomic technologies. It is mostly used for separating the peptide mixture; one of the diagram is shown here which is a typical liquid chromatography setup which consists of solvent bottles, degasifiers, (( )) coronary pumps, the sample injector, column and detectors. Different solvents can be placed in the solvent bottles depending upon the purification requirements.

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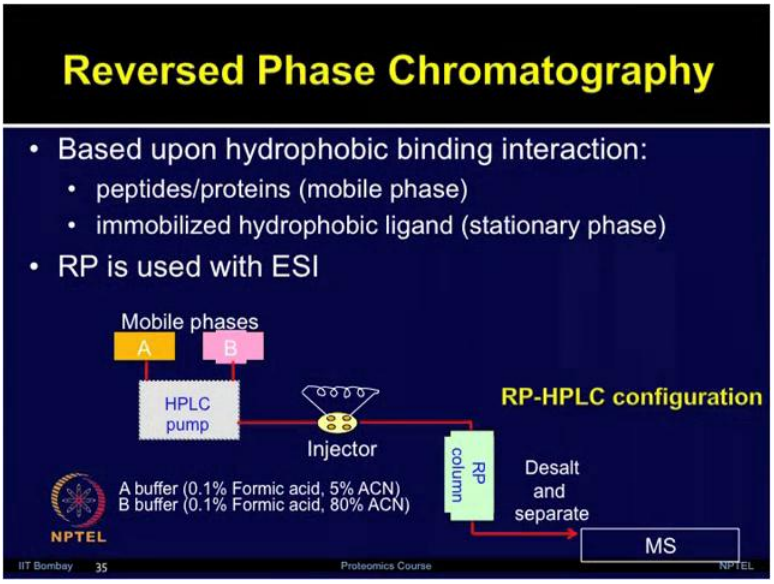

Let us first talk about reverse phased RP chromatography. Reverse phase chromatography is based upon the hydrophobic binding interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase. In reverse phase, the stationary phase consists of the long aliphatic carbon chains which is highly hydrophobic in nature. Molecules are bound on the column by means of hydrophobic interactions and are eluted out when the solvent polarity is modified. The reverse phase chromatography is mostly used with electrospray ionization in mass spec due to its compatibility of its acidic aqueous and polar mobile with ESI. It is also used for disserting the peptides before injecting for electrospray ionization.

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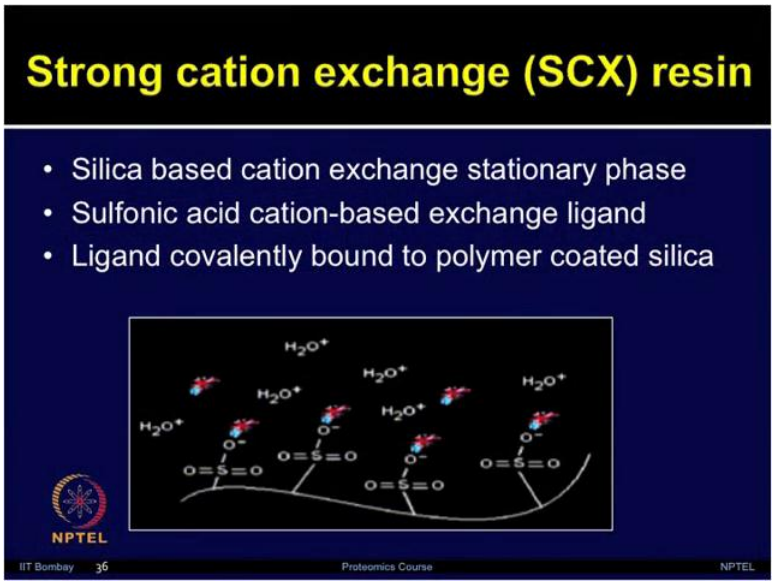

Let us now talk about a strong cation exchange or SCX chromatography. SCX consist of stationary phase matrix which is made up of negatively charged sulfonic acid groups which bind the oppositely charged peptide molecules. These molecules can be eluted out using a positively charged mobile phase which binds the analyte molecules more firmly.

So, what is mudPIT or multi dimensional protein identification technology? So, mudPIT method combines cation exchange and reverse phase HPLC chromatographic separation of tryptic peptides for the proteom based analysis.

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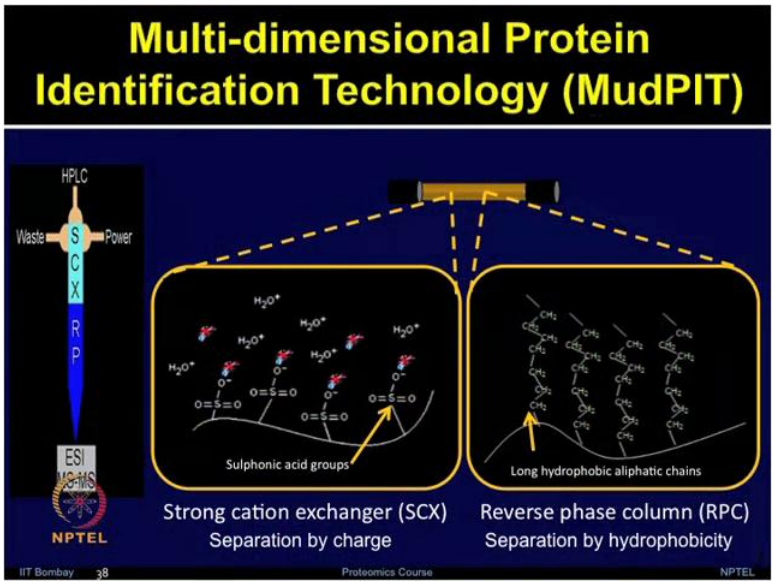

So, we have already discussed two separate methods SCX and RP. So, strong cation exchange is based on the electrostatic attraction between the negatively sulphonic acid and positive peptide, and the illusion can be caused by addition of positively charged mobile phase. The reverse phase shown on the right side is based on the hydrophobic interations between analyte and the stationary base. The illusion 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 the reverse phased HPLC followed by mass spectrometry. Alternatively, both reverse phase and strong cation exchange raisins can be packed into a single column and by introducing buffers in the series, the multi dimensional separation can be achieved.

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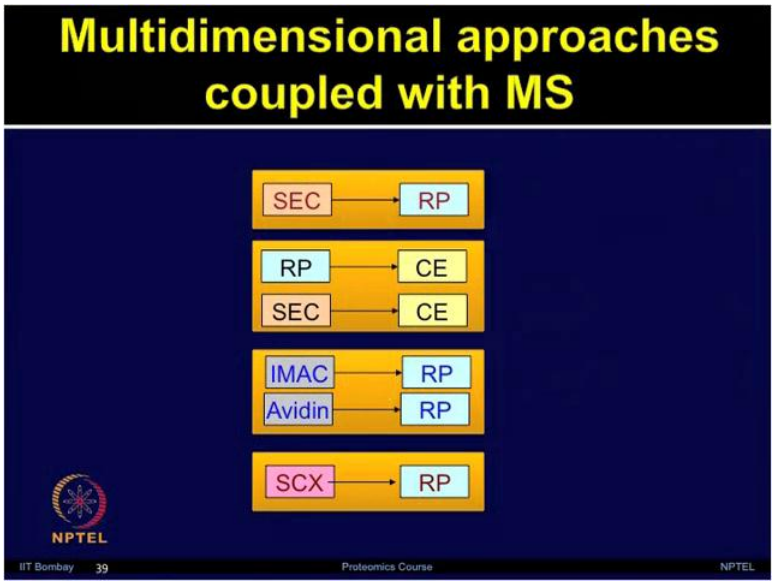

Now, when application is to separate the complex protein and analyse using mass spectrometry then, one has to select what type of chromatography method is applicable for their sample type. Now, multi dimensional approaches are usually coupled prior to the mass spec analysis and as we have discussed, there are certain chromatography methods which are commonly available, and one has to make an educated trials 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. So, to check the compatibility of first and second dimension separation based on the size in the SEC and the charge various types of combination methods have been tried out. So, let me first give you the abbreviations here, and then what property 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; reverse phased based on hydrophobicity, then we have capillary electrophoresis which separate ways on the charge; IMAC is affinity based interaction; reverse phase again hydrophobicity; strong cation exchange based on the charge. So, these are different methods which we have discussed.

Now, what we are looking at how the combinations of these can be applied for proteomic investigations. So, when SEC followed by reverse phase has been tried, the poor resolution of peptides in SEC occurs. The reverse phase followed by capillary electrophoresis are SEC followed by capillary electrophoresis it is limited with the loading capacity. Now, third combination of using affinity chromatography as the first separation followed by reverse phase has been used, but what is most popular is the a strong cation exchange separation in the first dimension followed by the reverse 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.

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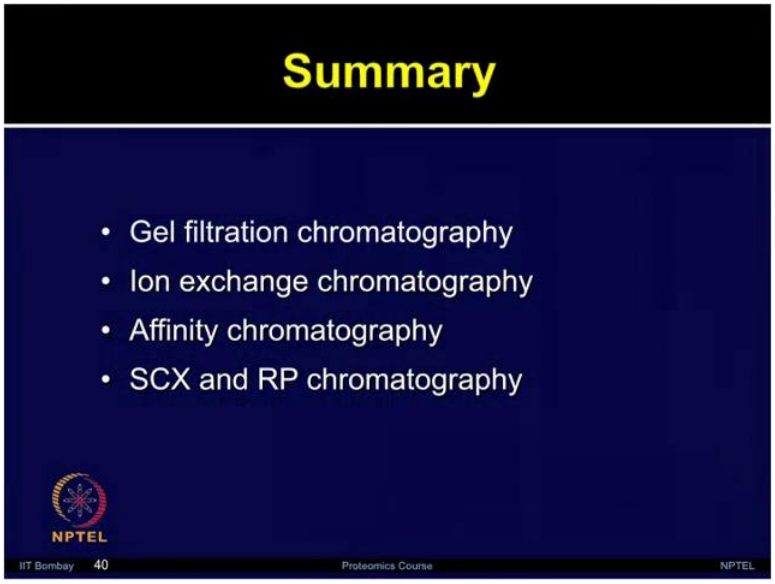

So in summary, in the last few years there has been an increased effort to develop the technologies which are capable of analyzing protein expression at the proteomic scale. Some of the chromatographic methods such as gel filtration, ion exchange and affinity chromatography methods were discussed. The strong cation exchange and reverse phased, high performance liquid chromatography are fundamental tools for the isolation and analysis of peptides. The nano liquid chromatography which makes use of c 18 capillary columns has gain popularity for the proteomic studies due to their ability to achieve finite separation. So in summary, we have talked about principle of different type of chromatography method commonly being employed for the proteins and peptides, and

we looked at some of these chromatographic methods in more detail thank you.

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