Biochemical Engineering
Prof. Dr. Rintu Banerjee
Department of Agricultural and Food Engineering
Asst. Prof. Dr. Saikat Chakraborty
Department of Chemical Engineering
Indian Institute of Technology, Kharagpur

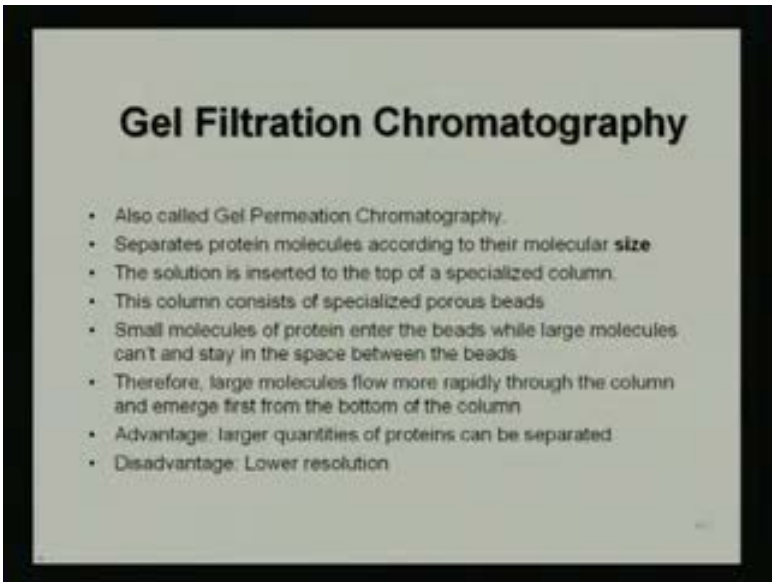
Lecture No. # 40
Strategies for Biomolecules Separation (Contd.)

Students, so in continuation of my last class where I told you about the ion exchange chromatography and I told that the separation is entirely based on the charge of the molecule. So, we yesterday we have discussed about the proteinaceous molecule and I have also mentioned you that protein is a charge particle, so based on the charge which is retain on the surface of the protein molecule we are going for the separation. That means we are if our targeted protein has got negatively charged, we are taking a positively charged matrix where the negatively charge molecule is coming and getting and it retains in the column. So, this is the separation and we are eluting our targeted protein which is retained inside the column and we are eluting with a suitable elevate or the mobile phase.

Now, the separation of the biological macromolecules can also be done with some other properties or the other characteristics of the biologicals. So, today we will be discussing on the G F C that is gel filtration chromatography where the bases of separation is based on the size of the macromolecules. Now, yesterday while telling you the strategy of separation of biologicals, I have told you along with my targeted proteins there are very many contaminated proteins which are present and I told you we have already learned that proteins are of different size, different shape and it has got different behavioral properties.

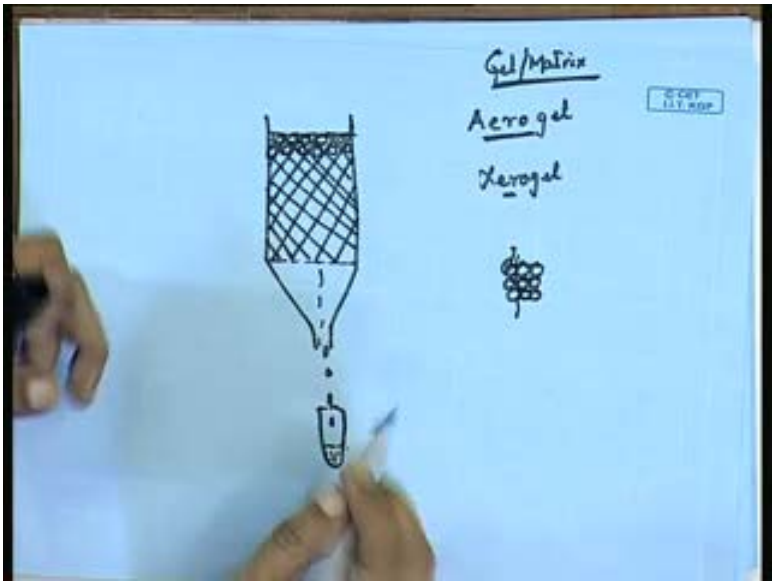
So, when a size of the protein is small obviously the molecular weight of that particular protein is low and when a protein is a huge, big size then its molecular weight is very high. So, based on the size of the protein the separation when we are going for the separation of macromolecules, we adopt the chromatographic technique which is called the gel permeation chromatography or gel filtration chromatography.

(Refer Slide Time: 03:02)



Now, gel filtration chromatography is to separate the proteinaceous molecule according to the molecular size. The solution is inserted to the top of the specialized columns and this column consists of specialized porous beads. Now, when yesterday I told you that gel the matrix molecule which is they are which we are packing inside the column is the stationary phase and this matrix molecules can be of two types. One is called Aerogel.

(Refer Slide Time: 03:49)



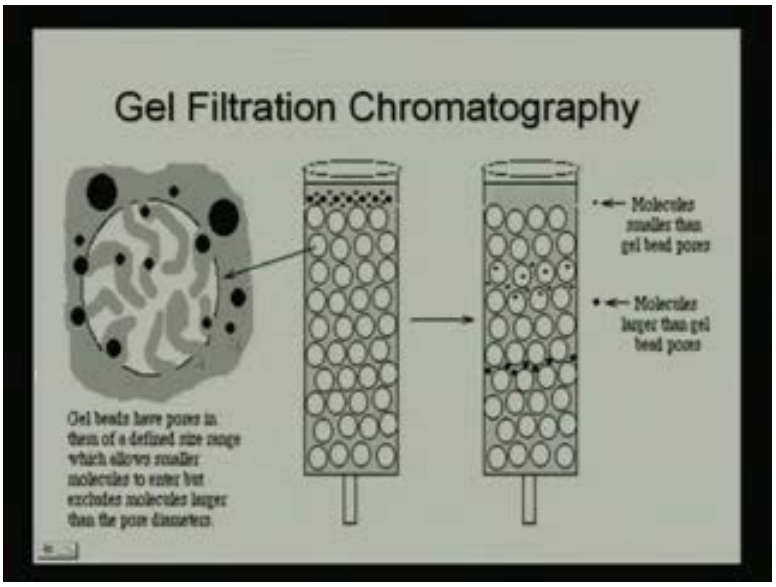
That is it is there within the liquid phase, another is another type of matrix molecules are there which we are calling it as Xerogel. Xerogel is the powdered form that means it is now then we are getting this Xerogel, it is in a powder form and before packing it inside the column we have to swell it and after appropriate swelling we are packing this particular gel to this column and here when we are getting this packing there should not be any swelling or shrinkage during the operation of or during the separation of biological macromolecules.

So, here so we are just taking this particular gel which has got a specific pore within it that means it is porous in nature. I will be coming to the different characteristics of this particular matrix molecule gradually. So, the small molecules of the protein enters the bead while large molecules cannot enter and they stay in the interspatial space of the two beads. So, this these are the matrix molecule and these molecules are tacked within the column matrix. So, the when this beads are there they are just packed, the small molecules can enter inside the beads but, bigger molecule cannot. So, what they are doing, they are just passing through the interspatial space of the two beads and they are just coming out. So, what is the result the biggest molecule will be coming first and the smallest molecule will be coming last.

So, based on this size we can separate the macromolecules and when the separation is of this type we are calling it as gel filtration chromatography, that means here the technique is just like filtration. So, the small molecules are entering from one bead to other the other and ultimately it is coming out of the column and it is we are just collecting it in different fractions.

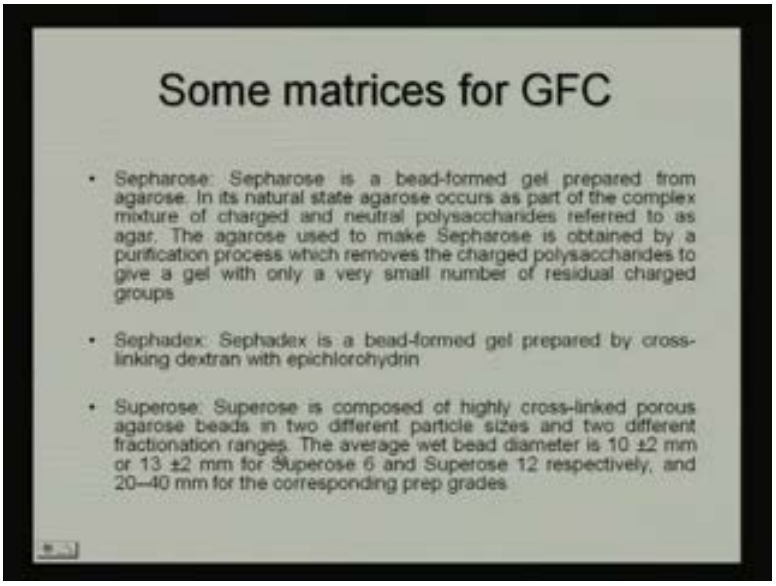
So, these are otherwise this fractionation of the macromolecules or the different mixture of molecules. So, small molecules of the protein, enters the bead while the large molecule cannot and stay in the space between the beads. Therefore, large molecules flow more rapidly through the column and emerge first from the bottom of the column. What is the advantage? Advantage is that a larger quantities of protein can be separated through this type of chromatographic technique and the disadvantage of this particular process is that lower resolution is there, we are not getting a very high resolution through GFC that is gel filtration chromatography.

(Refer Slide Time: 07:47)



Now, here you see the whatever picture I gave you this is the bead and here the different macromolecules based on their size is entering, the biggest one cannot enter but, the smaller and the smallest one can enter inside the bead. So, these beads are loaded inside this column and when they are loaded inside the column based on its size the bigger molecules are coming fast followed by the smallest one.

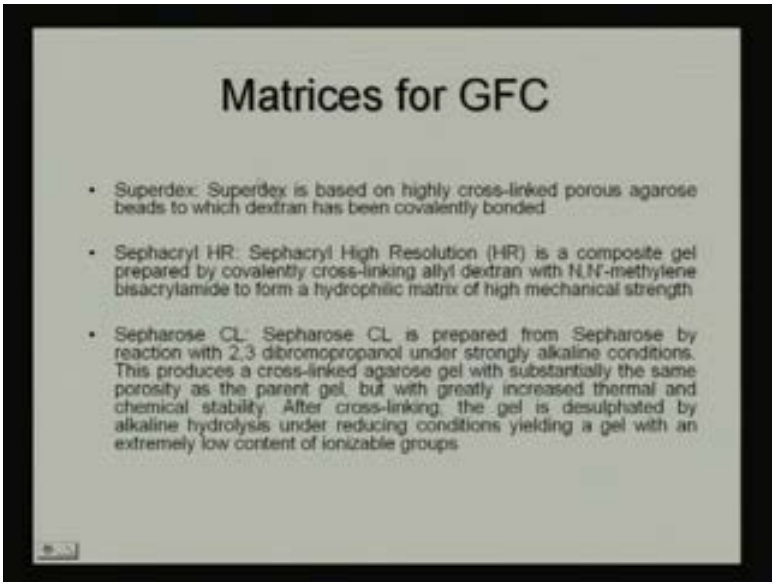
(Refer Slide Time: 08:27)



And in this way we can get an approximate idea about the size of our targeted macromolecules. Some matrixes which are used in gel filtration chromatography are Sepharose. Sepharose is a bead formed gel prepared from agarose. In its natural state agarose occurs as part of the complex mixture of charged and neutral polysaccharides referred to as agar. The agarose used to make sepharose is obtained by a purification process which removes the charged polysaccharide to keep a gel with only a very small number of residual charged groups. So, these are the sepharose molecules. So, when we are just going for the selection of this particular sepharose with the polysaccharide agarose and cross linking is going on, we are getting this sepharose molecule.

Sephadex is another matrix molecule which is also a beaded form of gel which is cross linking with the dextran molecule by the technique with of this cross linking where epichlorohydrin is used that is the chemicals which is helping in cross linking process. Superose is also another cross linked porous beads with two different particle size and two different fractionation range and in this way we are getting with a by with a variation of the percentage of cross linking, we can form different grades of beads.

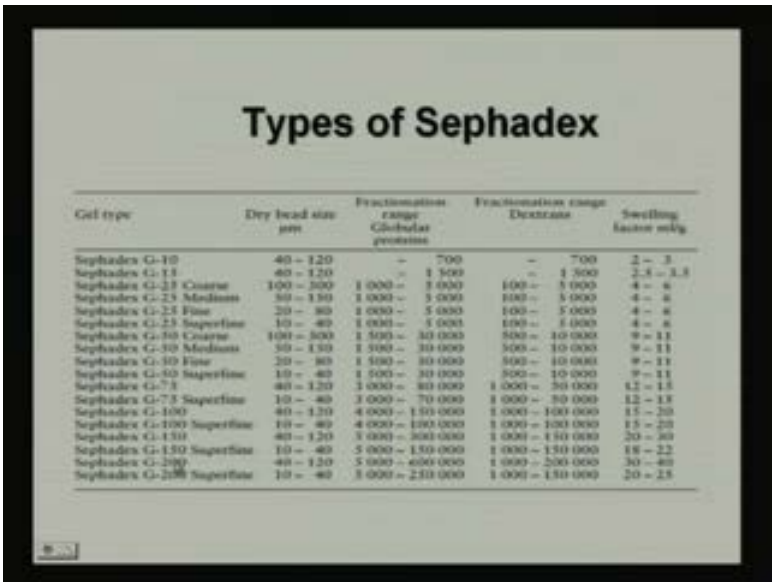
(Refer Slide Time: 10:43)



So, in this way we can go for different other this Superdex, Sephacryl, Sepharose C L. So, these are some of this particular matrix which are either cross linked with the dextran

molecule or it is cross linked with the agarose molecule.

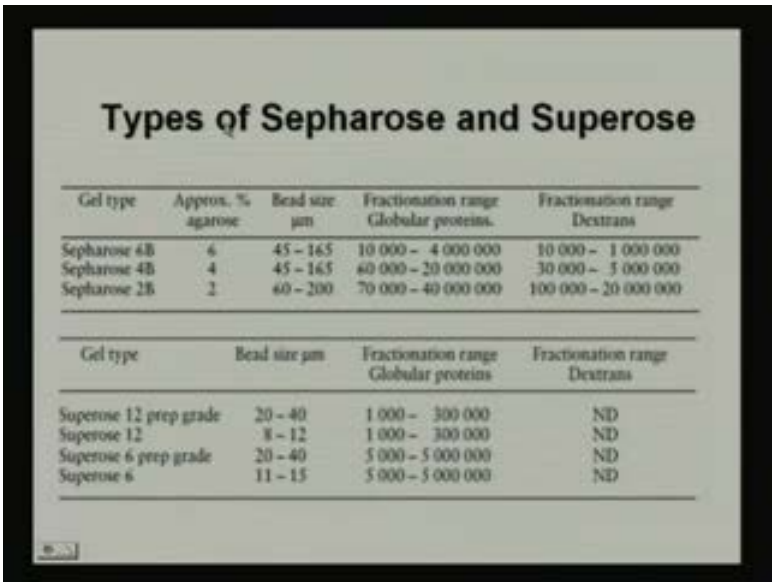
(Refer Slide Time: 11:06)



So, these are some of the commercial beads which are available in today's market. So, sephadex G 10, sephadex G 15, sephadex G 25, sephadex G 50, sephadex G 75, G 100, 150, G 200 are some of the category. And, from this number we can select the range that which protein that means how big that protein size will be to use this particular bead for separation of our targeted macromolecules.

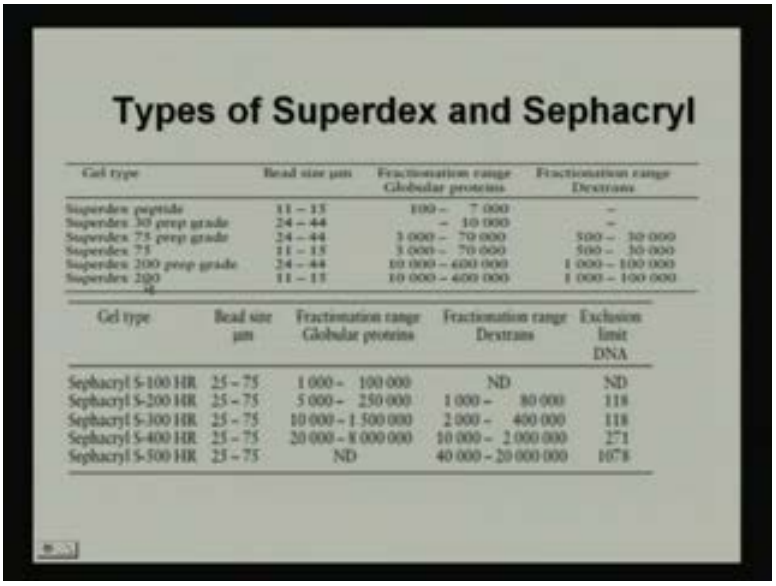
Say for example, if we are taking G 200 as one of this matrix, here the fractionation range of this particular this protein molecule can vary from 5,000 to 2,50,000 and here the fractionation range of this dextran because sephadex is a dextran derivatized beads so, here this dextran molecules are of 1,000 to 1,50,000. Similarly, if we are taking sephadex G 100 super 5 here the bead size of this particular group varies from 10 to 40 micro meter, that is the diameter of the beads and here the protein which will be those protein which are between 4,000 to 1,00,000 can easily enter inside the beads of this range and this way we are just selecting and these are all standardized matrix molecule available in today's market. So, these are some of the matrix molecule. So, I told you sephadex is the dextran derivatized bead.

(Refer Slide Time: 13:23)



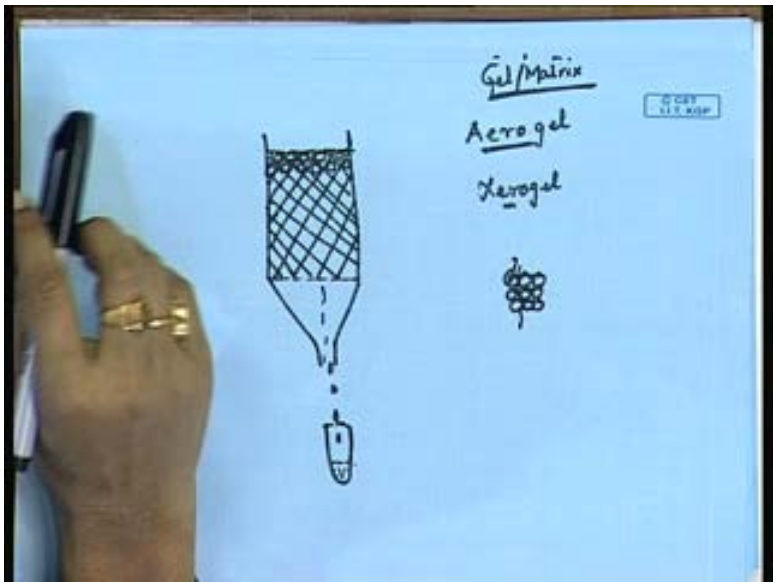
Similarly, with some other beads like sepharose or superose these are some of this beads where there this beads are agarose derivatized and here also we had the known globular proteined range along with this particular groups. So, here it is not agarose derivatized, I am sorry it is the superose 6 B, superose 2 B, superose 4 B everything are the dextran derivatized beads. And sephacryl those type of beads are agarose derivatized beads.

(Refer Slide Time: 14:13)



So, superdex and sephacryl these are also this superdex and sephacryl these are the dextran derivatized beads of known molecular weight having the fixed specification of the beads. So, these are some of this particular matrix molecules which can be used for the separation of the proteinaceous molecule.

(Refer Slide Time: 14:47)



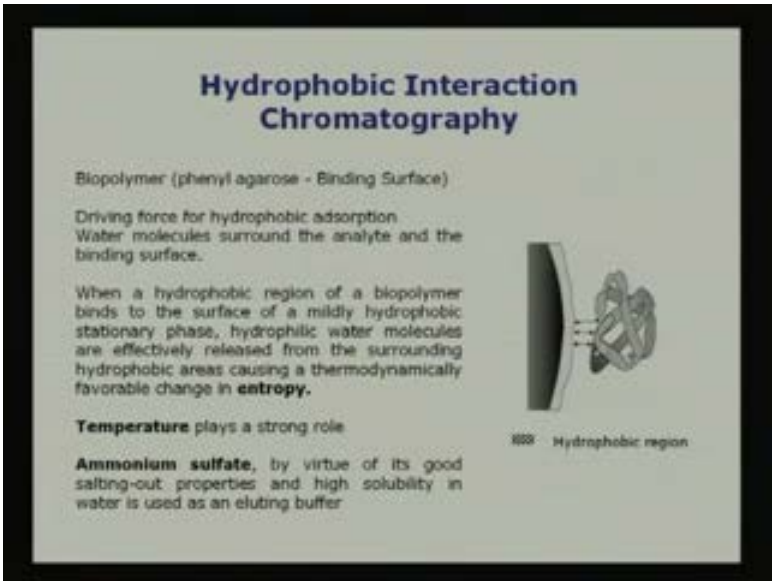
And when we are selecting this type of molecule we are just using the neutral type of either water or buffer type of mobile phase where our protein can retain its activity and this flow is taking place in a very very slow movement.

So, that it can follow the principle of filtration. So, through this beads from one bead to another to another and this way the filtration of this smaller, smallest molecule to the biggest molecules are taking place and it takes a longer time to cross the beads and based and they are getting sufficient time to get themselves arrange within the column while moving from top to bottom and then they are getting collected. So, we can get the approximate molecular weight. Now, if someone can question that when the separation is taking place then sometimes gravity, gravitational force may also play a significant role.

So, what to do in that case? So, in that case we can pass the proteinaceous molecule from bottom of this column and we can take the fractions from the top to avoid the

gravitational force. So, it can also be done and this process can be done continuously till this fractionations are coming. And, this way based on the size of the macromolecules we can get the targeted protein out of this particular column.

(Refer Slide Time: 16:47)



Now, coming to the another type of separation technique, where we are just separating the macromolecules based on its particular characteristics that is the hydrophobicity of any macromolecules.

Now, when we are talking about the hydrophobicity that means I have already told you during my first class that amino acids can be categorized into four different groups, one group is the non-polar amino acid. So, if in a protein molecule the percentage of non-polar amino acids are there then this hydrophobicity is hydro they are hydrophobic in nature, those particular amino acids has got the tendency that one amino acid can self associate with another and they form the aggregates. So, this way this aggregation is taking place and this association is called hydrophobic interaction.

Now, the scientist they wanted to exploit this particular behavioral properties of this group of amino acids. So, now in a protein molecule if any such amino acids are present. So, they have their own properties characteristics that they are hydrophobic in nature that

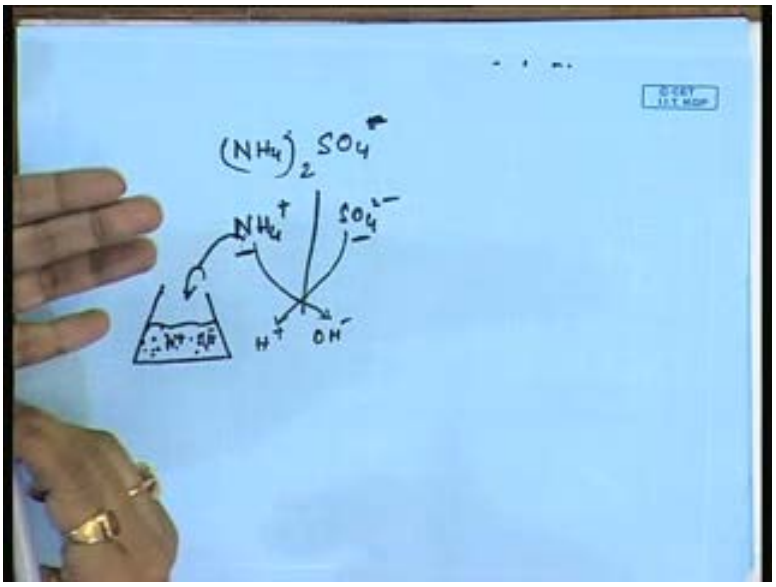
means aqueous phase is not at all a suitable media for them to get expose to the outer environment. So, what is seen that most of the hydrophobic amino acids are burette inside. They are just coming and where and it is getting protected that means when this 3D structure of the protein is there, it is getting folded in such a way that hydrophobic molecules are trying to go inside and coverage is with a hydrophilic amino acids which are present in the molecules. So, when such type of association is there with one hydrophobic amino acid with another hydrophobic amino acid and when they self associate it is called hydrophobic interactions.

So, the phenyl agarose is one of this matrix molecule where which can be used for hydrophobic interaction chromatography the driving force for hydrophobic adsorption is there within the molecules itself. The water molecule surround the analyte and the binding surface when a hydrophobic region of a biopolymer binds to the surface of a mildly hydrophobic stationary phase, hydrophilic water molecules are effectively released from the surrounding hydrophobic areas causing a thermodynamically favorable change in entropy and which is very very important as far as this type of association is there. Temperature is also playing a significant important role as far as this hydrophobic interaction is there.

Now, ammonium sulphate by virtue of its good salting out properties it has got high solubility in water which is used as the eluting buffer of this particular interaction.

Now, this particular hydrophobic interaction is just opposite to that of this ion exchange chromatography. Now, in case of ion exchange chromatography separation is based on the charge, net charge of the macromolecule. In case of hydrophobic interaction chromatography it is based on the hydrophobicity of the macromolecules. So, here when we are enhancing we want to increase the binding force between one molecule to another molecule. What we are doing? We are adding some salt to this particular environment. What the salt they are doing?

(Refer Slide Time: 21:45)



Now, we know that ammonium sulphate is one of this salting out this a precipitant, precipitating agent. So, ammonium sulphate in ionized condition we are getting N H 4 plus and S O 4 minus. So, in its ionized form when it is coming in contact with this water when we are just taking this water along with this soluble protein and if we are adding this ammonium sulphate to this, so first it is going and water in the ionized form is H plus and O H minus. So, this ions are coming and getting binded with this.

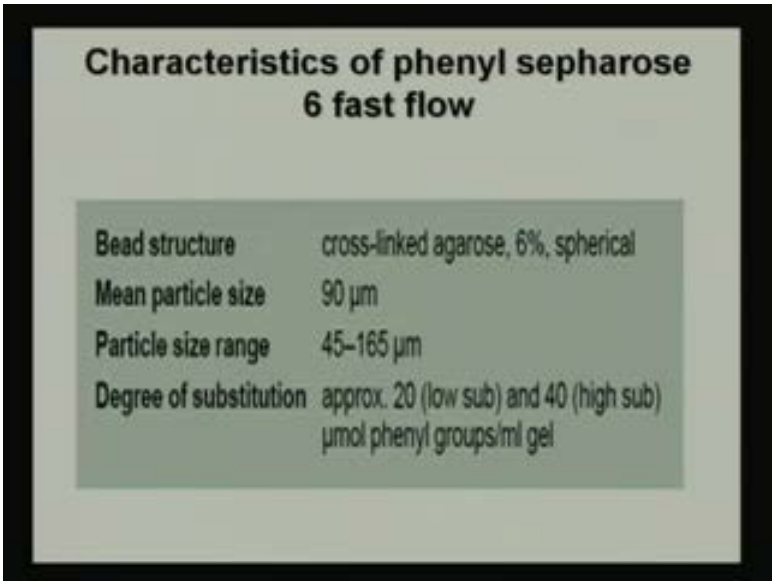
So, as a result what is happening? It is binding, it is just saturating, this H plus and O H minus ion and after sometime the proteins which are there along with this particular the water is become this environment is now gradually this water molecules are now getting associated with this N H 4 plus and S O 4 minus and as a result the hydrophobicity of that particular environment is getting change and the proteins which got binded with this water molecule is gradually getting precipitated. And based on the strength of binding that protein start precipitating and we can get, we can isolate this particular protein molecule. Here, in case of hydrophobic interaction chromatography the scientist they are using the same principle.

Now, here what we are doing. We are using some salt because salt is from this particular this mechanism we have learned that this ammonium sulphate is increasing the

hydrophobicity of that particular environment. So, more the hydrophobic environment, more stronger will be the binding. So, what we are doing? We are adding some salt for stronger binding because we know that hydrophobic interaction is not that strong binding. So, here to enhance this particular, in this binding force we are adding salt and while elution what we are doing? We are just withdrawing the salt and when we are withdrawing the salt the dissociate the strength of binding becomes weak, weaker and weakest and molecule starts coming out from the column.

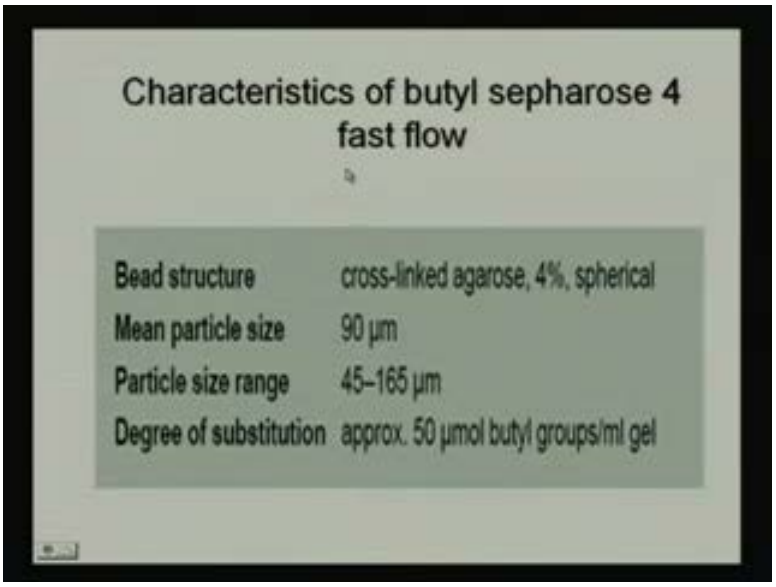
So, here if we are taking this phenyl agarose as one of the column. So, during binding we are adding salt and during elution we are withdrawing the salt. It is just reverse to that of this ion exchange chromatography. In ion exchange chromatography what we did? We first added our protein molecules to the column and while elution we used N a C l that salt and it drag the molecule out of this column and we isolated, we just separated our targeted protein. Here, it is just opposite to that.

(Refer Slide Time: 25:27)



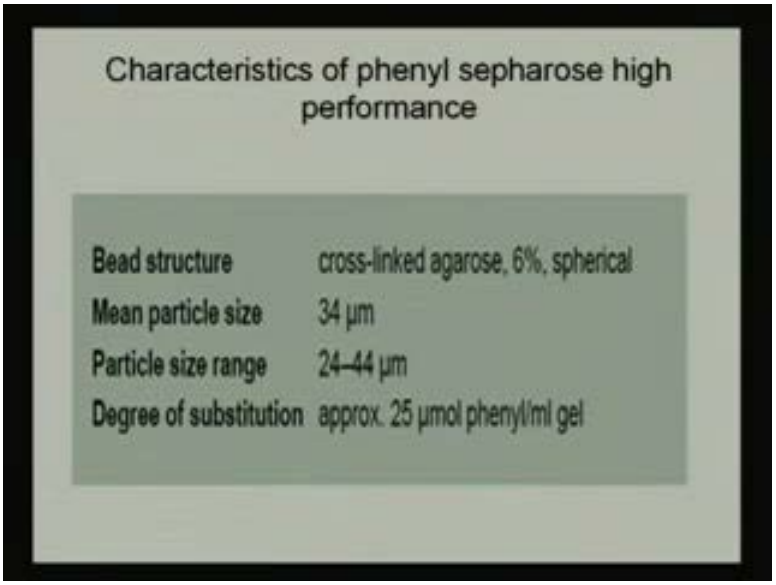
Now, there are different types of matrix molecules which can be used are phenyl sepharose 6 fast flow, but bead structure, the particle size everything that particle range and degree of substitution the every details are there which are commercially available.

(Refer Slide Time: 25:52)



Not only this, phenyl sepharose butyl sepharose are also another matrix which are very popular as far as hydrophobic interaction is concerned.

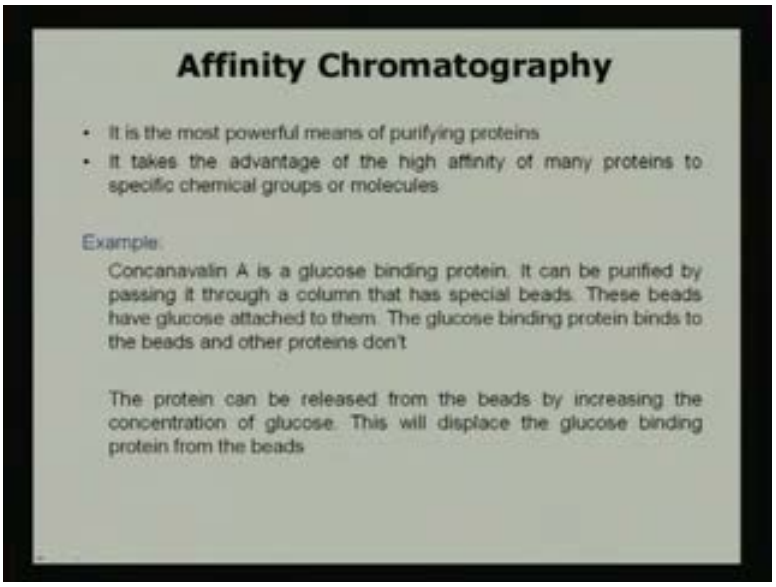
(Refer Slide Time: 26:08)



Now, when we are going for this type of particular selection of the matrix molecule, matrix molecules. We are just going for the selection of the matrix for suitable protein

separation that means hydrophobic, which type of hydrophobic molecules are better separated the resolution is improved. So, based on that we are separating the macromolecules based on its particular biological characteristics that is the hydrophobicity.

(Refer Slide Time: 27:05)



Now, as I have told you earlier that affinity chromatography is a big umbrella and here when we are talking about this affinity chromatography it is the most powerful means of purifying proteinaceous molecule. It takes the advantage of the high affinity of many proteins to specific chemical groups or molecules and if we want to give this example just few minutes back while when I was talking about this hydrophobicity it is also a type of affinity chromatography.

Now, during my last initial few classes when I discussed about this macromolecules, I have already mentioned about some of the complex macromolecules like glycoproteins. So, this type of molecules can easily be separated with this affinity chromatography. Concanavalin A is one of such example. Concanavalin A is a glucose binding protein. It can be purified by passing it through a column that has special beads. These beads has glucose attached with them. The glucose binding with the protein and they bind with the proteins that matrix molecules or the beaded molecules and other proteins which do not

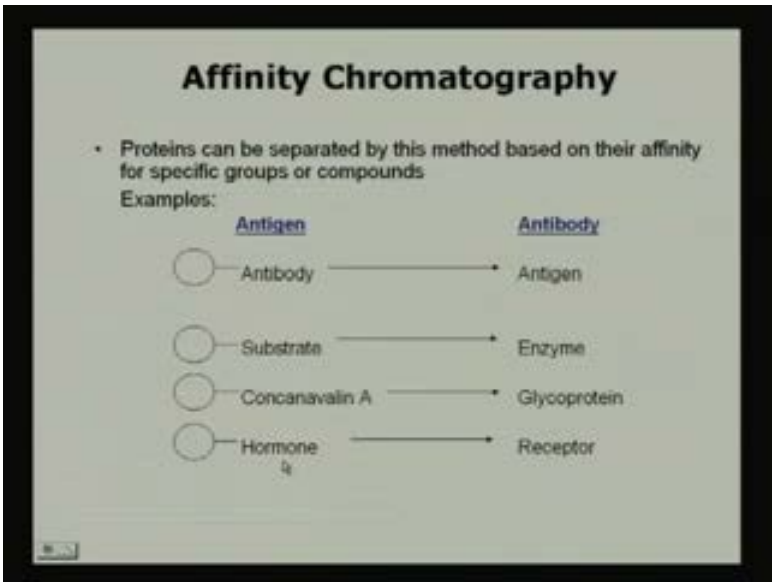
have this affinity they simply pass out through this column and the targeted molecule which has got glucose retained to that particular column and this way based on the this the particular biologicals based on the glucose moieties whether it is present or not, we can separate our molecules.

So, if we know that our targeted molecule has got affinity towards glucose this particular, it has got glucose. So, Concanavalin A can be used as a matrix molecule because it has got very specific binding property affinity towards glucose moieties and it will just bind. So, these are the biological properties. Similar, just earlier chromatographic technique what I have discussed is the hydrophobicity. So, hydrophobic hydrophobic molecules are associated. So, it is this self binding this association is there and we are just making that environment little more favorable, so that the binding will be more stronger and separation will be very that resolution will be very good. So, that is the understanding that is the selection process for this type of matrix for particular biological separation.

Now, the protein can be released from the beads. So, those proteins which got retain to the column is to be now taken out of this column. So, how we can release those binded protein? So, this proteins can be released from the beads by increasing the concentration of glucose. This will displace the glucose binding protein from the beads. So, if we are just now we know that our protein has got glucose moieties, so, it got binded with this.

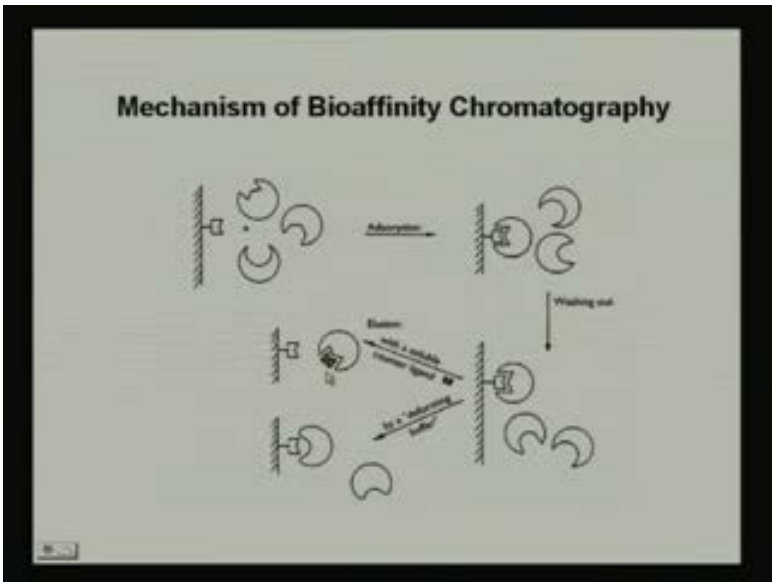
Now, while elution we will start passing this glucose to that. So, what will happen the our protein will now start coming because glucose has got more affinity towards this particular glucose binding concanavalin a, so it will start displacing our protein molecule and it will go and block that particular glucose binding site. So, our protein will start coming out of that particular column.

(Refer Slide Time: 31:32)



So, this is the simple affinity. Based on this, I can tell you that proteins can be separated by this method based on their affinity for specific groups or compounds. So, if we have got this antigen and if we have the antibody then antigen and antibody they can associate, they can bind because of the there affinity. If we have the substrate and if we have this enzyme, targeted enzyme suppose protein is there, proteus is there. So, protein and proteus will come and it will just bind. Concanavalin A, any type of glycoprotein, any type of glucose containing protein if that is there glucose moieties, if those protein which has got this moieties will come and bind with this particular matrix molecule. If we have hormone and if we have hormone receptor it will come and bind with the hormone receptors. So, these are the simple principle of affinity chromatography. This affinity is nothing but, it is the inherent biological properties of the biological macromolecules.

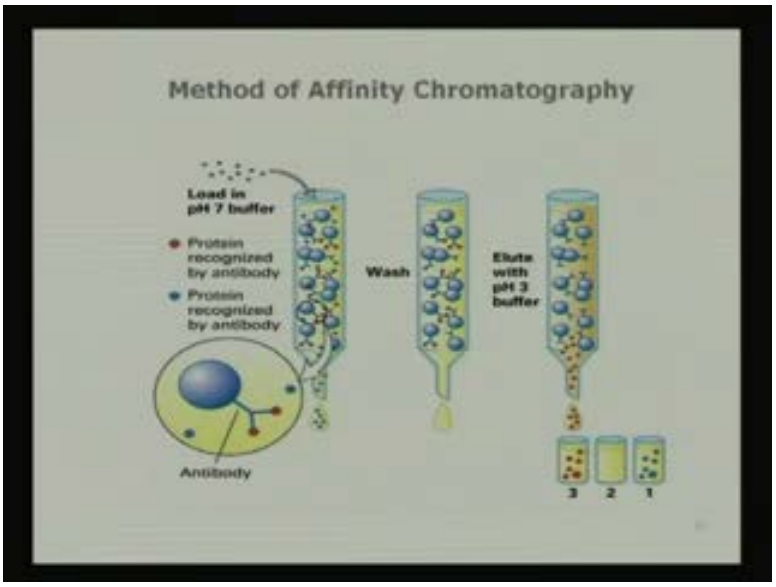
(Refer Slide Time: 32:53)



This is the mechanism of bioaffinity chromatography. See this is the ligand, this is the matrix and ligand molecule is attached to this. And this ligand has got the affinity for a particular molecule. Now, here I have got a mixture of molecules. We want to separate our targeted protein from that mixture. So, here you see different types of proteins are there but, a particular protein which is having the counterpart will come and get associated with the ligand. And, other molecules it will be just passing out, washing out of this column. So, this binded protein is retained and other proteins are coming out of this column.

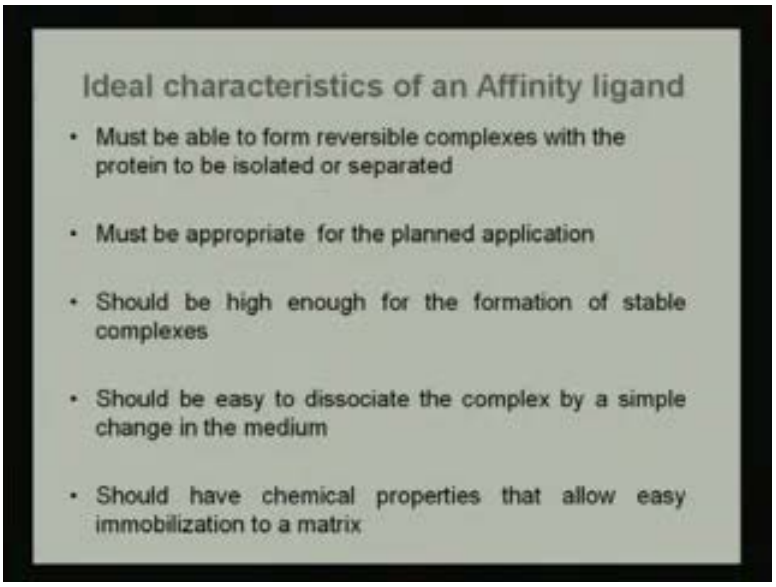
Now, what we want to do? We want to now elute, we want to now drag this particular molecule out because our objective is to separate our targeted protein. So, my targeted protein is now retained in the column. So, we can go for elution with the counter ligand techniques and with a suitable media mobile phase we can elute our particular macromolecule from this particular ligand and this way this bioaffinity biological affinity which is an inherent properties of the biologicals macromolecules or micromolecules are getting exploited by the scientist.

(Refer Slide Time: 34:44)



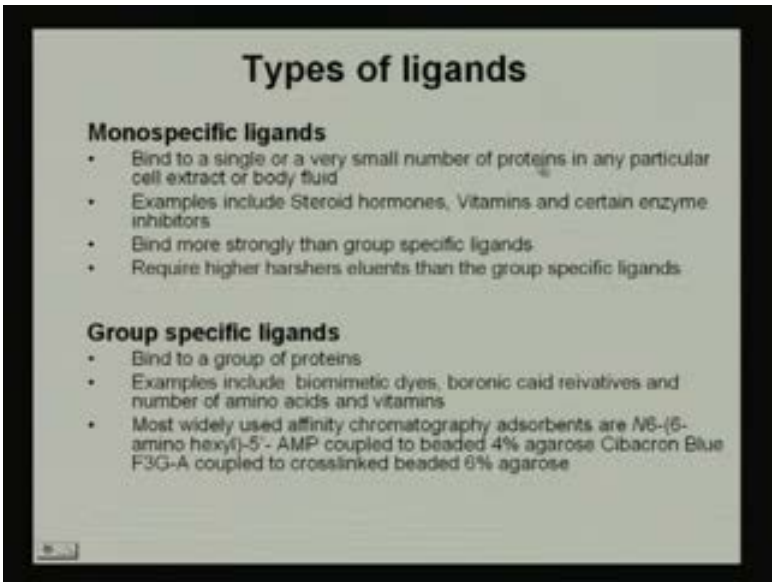
This is the method of affinity chromatography. See this is the antigen and antibody this particular binding is there. So, we are just loading it and as soon as this binding is over this antigen is there and antibody is passing through and first what is happening? The after this binding, we are just washing the unbound proteins or this molecules which are there is coming out of this column and then we are just eluting with a suitable mobile phase with a suitable condition and we are getting in different fraction, different molecules. So, this way this affinity chromatography if the selection of ligand and counter ligand is appropriate, we can get absolute purity within a single step of operations and that is the reason why this chromatographic technique is so popular and why the scientist they are running after this type of chromatographic technique because within a single step we can go for the homogeneous purity, sometimes if the selection is appropriate.

(Refer Slide Time: 36:11)



Now, here I have used one particular terminology which is called the ligand. Now, this ideal characteristics of this ligand is that it must be able to form reversible complexes with the protein to be isolated or separated. It must be appropriate for a planned application, it should be high enough for the formation of stable complexes, it should be easy to dissociate the complex by a simple change in the medium, it should have a chemical properties that allow easy immobilization to the matrix.

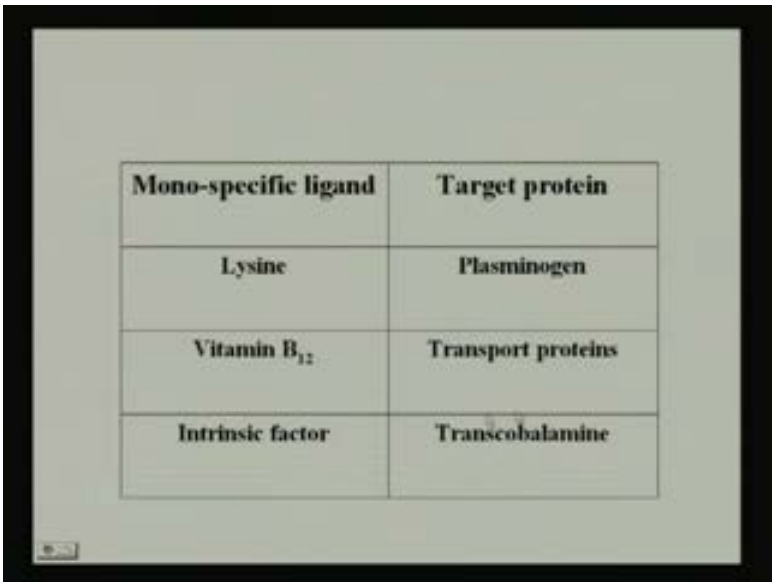
(Refer Slide Time: 36:52)



So, if in analyze this further classify this ligands then ligands can be of two types. One is called mono specific ligand, that means it has got single specificity and another group is called group specific ligand. So, in mono specific ligand we can bind the single or a very small number of protein in any particular cell extract or body fluid. The examples include steroid hormone, vitamins and certain enzyme inhibitors which bind more strongly than the groups specific ligand and it requires highly, higher, harsher eluent than the group specific ligands. In case of group specific ligand they bind to a particular group of protein example includes the biomimetic dyes, boronic acid and so on they are some amino acids and vitamins etcetera are some of the example of this group specific ligand.

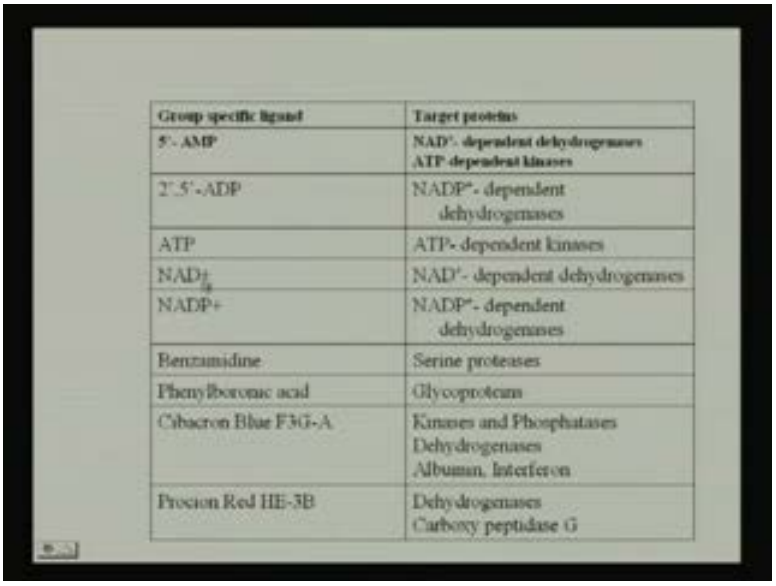
Most widely used affinity chromatography adsorbents are 6 amino hexyl 5 AMP coupled with the beaded 4 percent agarose where the cibacron blue F3G-A coupled to cross linked beaded with 6 percent agarose. So, some of these group specific ligands are procion red, cibacron blue etcetera. Those are the dibinding ligand and it has got a very white specificity for a particular group of molecules and they can based on there that affinity they can separate the particular biology.

(Refer Slide Time: 39:00)



Now, if we give some example of this mono specific ligand then lysine. If lysine is a ligand then targeted protein is the plasminogen, vitamin B 12 transport proteins, intrinsic factors it is transcobalamine.

(Refer Slide Time: 39:25)

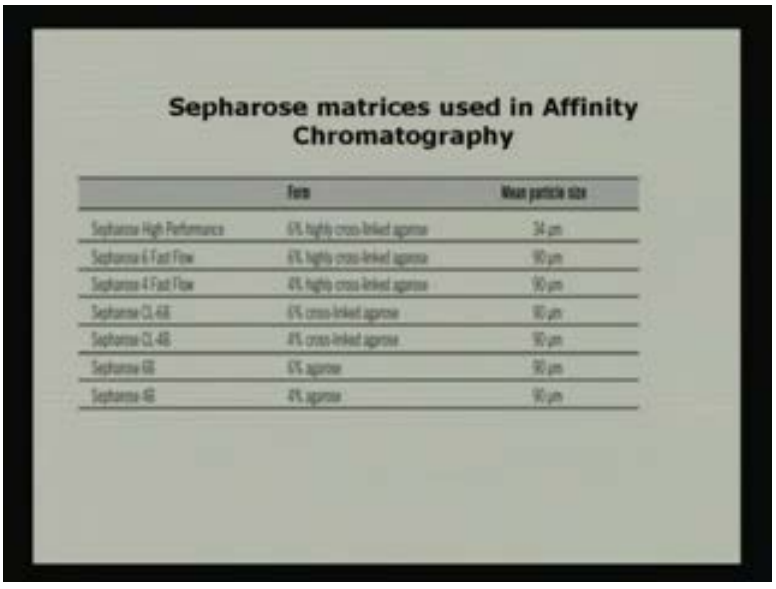


So, these are some of this example of the monospecific ligand and if we are going for the

groups specific ligand then some of the examples are your procion red, I have told cibacron blue.

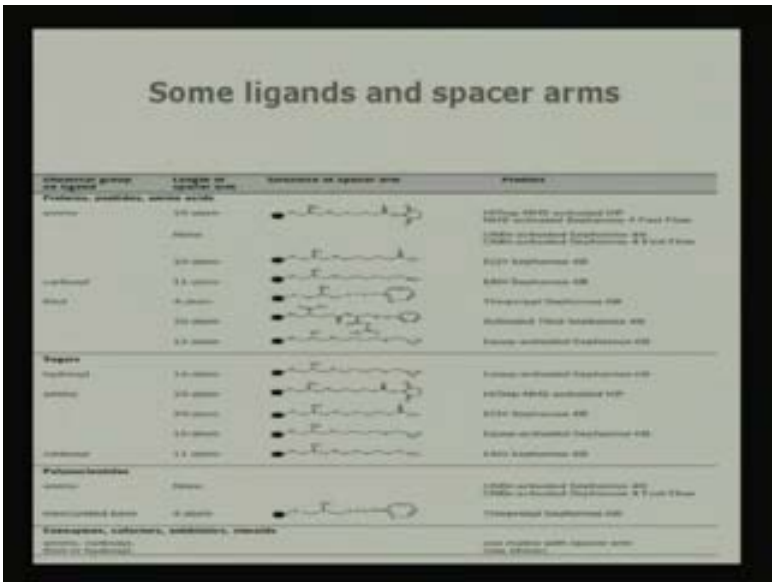
So, here any type of dehydrogenase group of enzymes or in case of cibacron blue any kinases or phosphatase group of enzymes, dehydrogenase group of enzymes interferon albumin etcetera can easily be separated through this particular group of group specific ligand. If any NAD, NADP, ATP this type of molecules are used as a ligand then the counter ligand will be for ATP ATP dependent kinases, for NAD NAD dependent dehydrogenases, for NADP NADP dependent dehydrogenases. If it is a benzamidine then it is the serine proteases, any type of proteases particularly serine proteases has got very strong affinity with this type of molecules. If we have phenylboronic acid as the group specific ligand, we can separate any type of glycoprotein from the mixture of intracellular cell extract.

(Refer Slide Time: 41:02)



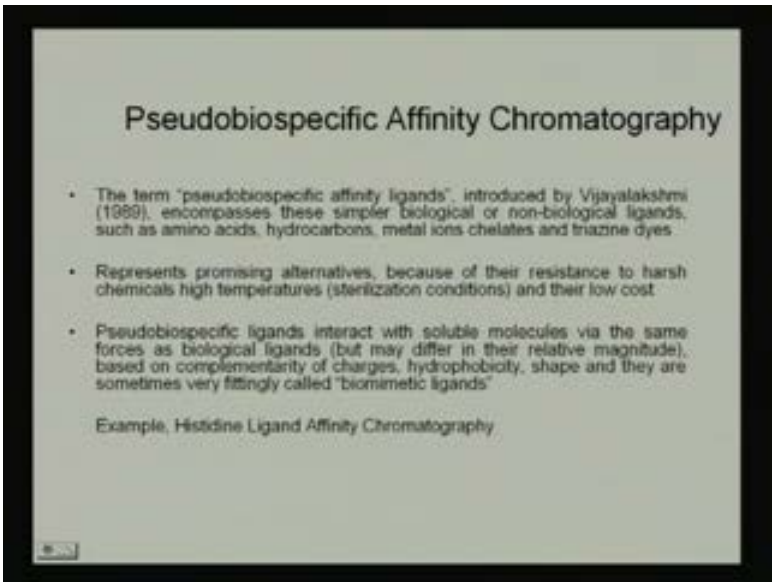
So, these are some of this mono-specific and group specific ligand. Some of this matrices are already available in the market which are generally used for this type of chromatographic application.

(Refer Slide Time: 41:13)



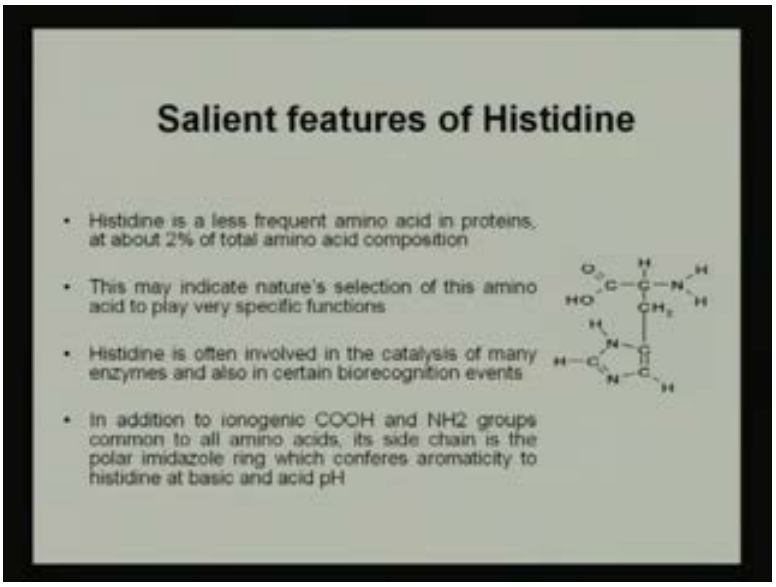
Now, to avoid the steric hindrance sometimes what we are doing? We are using some spacer arm. So, what we are doing? First, the matrix, then the spacer arm, then the ligand and counter ligand is coming and binding. So, if this globular proteins are there. Suppose, ligand is a protein, counter ligand is also protein, suppose antigen antibody. So, sometimes steric hindrance are there. So, to avoid this steric hindrance we are providing some extra space through some spacer arm. The main objective is that spacer arm should not be too long which may contribute some of the hydrophobicity of that particular medium. So, this spacer arm when we are talking, we are talking about a definite length of this particular molecules and these are some of the example of the spacer arm which is attaching to the ligand molecule.

(Refer Slide Time: 42:30)



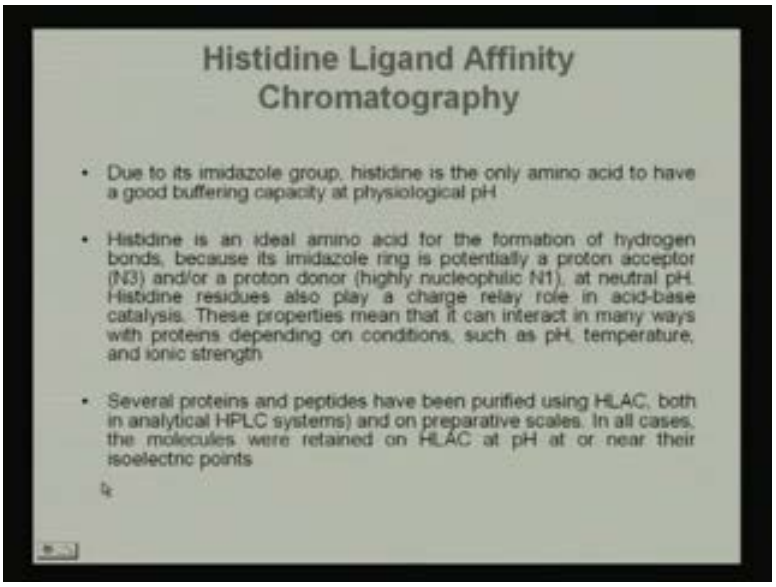
Now, when we are talking about this affinity chromatography I have already mentioned you that pseudobiospecific affinity chromatography is one of such affinity chromatography where we can separate through biomimetic properties and histidine ligand chromatography IMAC immobilized metal chelate affinity chromatography dye ligand affinity chromatography are some of the example which are coming under this pseudobiospecific affinity chromatographic technique.

(Refer Slide Time: 43:08)



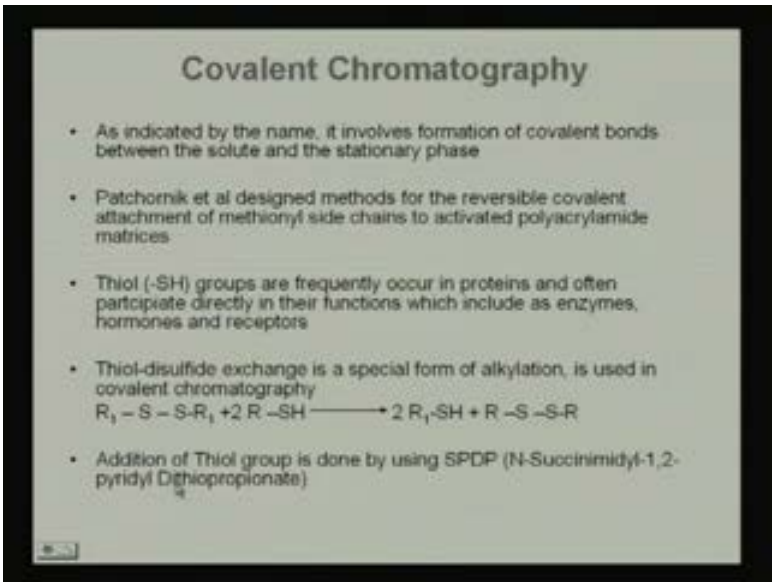
Now, because of the limited time period I will just simply go through this histidine ligand chromatography. And, the silent feature of this histidine is that histidine is less frequent amino acid in protein at about 2 percent of the total amino acid composition. This may indicate the nature selection of the amino acid to play a very specific function. Histidine is often involved in the catalysis of many enzymes and also in certain bio recognition events. In addition to ion ionogenic that C O O H and N H 2 groups which are there common to all amino acid the side chain is polar imidazole ring which confirms the aromaticity to the histidine at basic and acidic p H.

(Refer Slide Time: 44:15)



Histidine has got very many characteristics due to its imidazolium group, histidine is the only amino acid to have a good buffering capacity at physiological p H. Histidine is an ideal amino acid for the formation of hydrogen bond because of this imidazole ring is potentially a proton acceptor and or proton donor and under highly nucleophilic condition and at neutral p H histidine residues also play a charge relay role in acid base catalysis. These properties means that it can interact in many ways with the proteins depending on its condition such as p H temperature and the ionic strength, several proteins and peptides have been purified using histidine ligand affinity chromatography both in analytical that is HPLC system and on preparatory preparative scales. In all cases the molecules were retained on this histidine ligand at p H at or near to their isoelectric point.

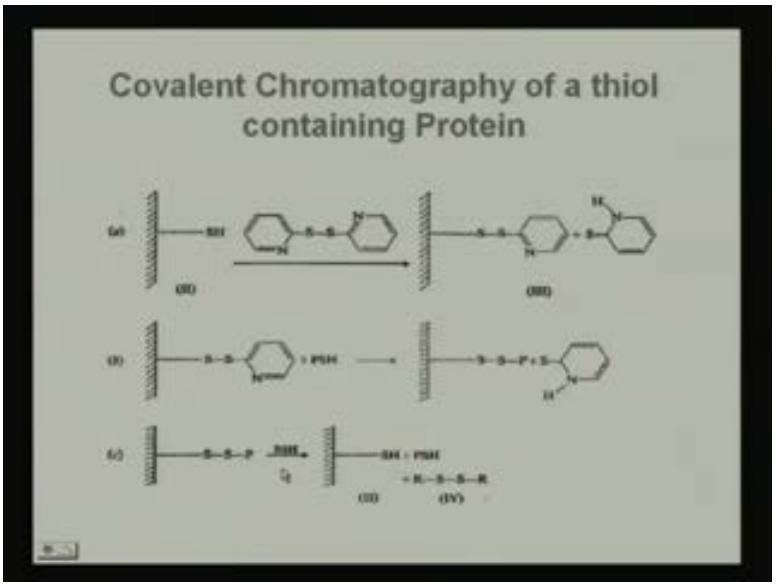
(Refer Slide Time: 45:44)



Now, these are some of this particular biological molecules. Similarly, we can go for this covalent chromatographic technique and where the bases of separation is based on the sulphur containing amino acid. Now, here this sulphur containing that means the thiol containing amino acids are the major consideration of the separation of any biological through this SH group or the sulphur thiol group present in the macromolecule. Thiol-disulfide exchange is a special form of alkylation which is used in covalent chromatography and this is the reaction you can see the addition of thiol.

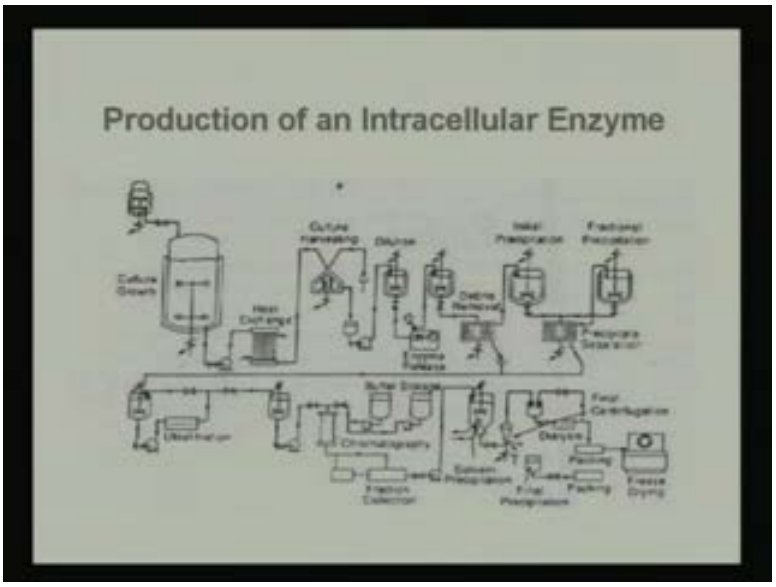
Now, suppose I do not have any thiol containing group in my protein. I can incorporate the thiol group to the my particular protein provided if I find that with this in new incorporation my protein is not getting denatured. So, this type of incorporation can also be done using this chemicals that is SPDP, that is N succinimidyl 1, 2 pyridyl dithiopropionate.

(Refer Slide Time: 47:16)



So, this is one of this chemical through which we can incorporate some of the thiol group to our matrix in this protein molecule. So, that it can come and thiol thiol this interaction, this binding will be there, covalent linkages will be there and this covalent itself is telling that it is a very strong binding, this interaction is very strong and this when we are just eluting, we are just very mild condition, we are just passing the this reducing agent to elute our targeted protein.

(Refer Slide Time: 47:53)



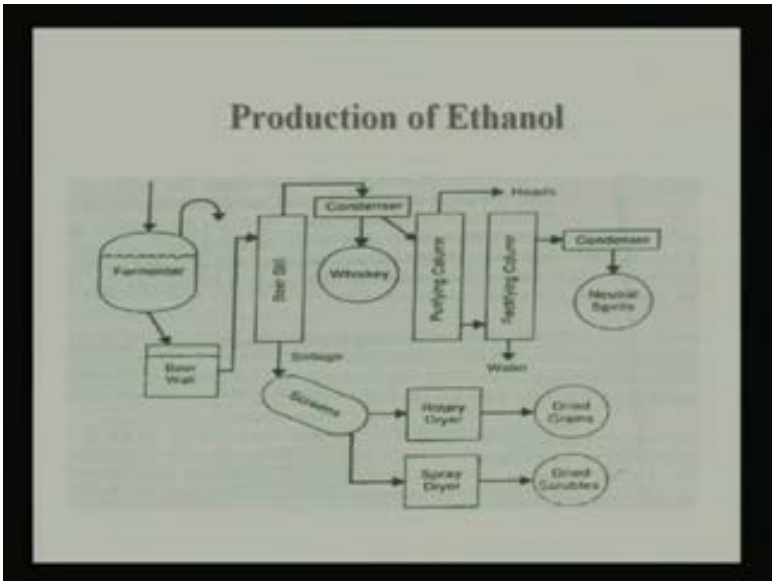
Now, if we come to some of these example of this protein separation. Now, as I had discussed in my earlier class that that upstream processing and downstream processing. So, upstream processing means here this culture is growing, fermentation is going on and after fermentation when we are just harvesting, then we are just taking this particular extract and we are going for this particular cell this here up to this fermentation is over, then we are just taking this extract and we are just going for the precipitation. This is for intracellular enzyme production. So, from biologicals to biologicals the entire strategy is different. I am giving you some of the example. So, here this one of this examples is that intracellular enzyme production.

Now, my cell is containing the product. We have to harvest the cell. Now, this harvesting is over, now I have got the cell extract. Now, here we will be going for this centrifugation and the cell debris which are there is getting removed. Now, here we are just going for the initial precipitation of this particular protein and then we are just taking the fractional precipitant and we are just going for this ultra filtration with through which the selection further fractionation is taking place and then we are taking this broth to this chromatographic techniques and through this chromatographic technique further, we are just fractionating this protein and then we are further going and we are going for this solvent precipitation and finally, we are getting the enzyme which is concentrated, we are

going for the dialysis to remove the excess unwanted solvent and we are going for this fridge drying and then finally, we are going for the packaging of that particular intracellular enzyme.

So, see here each and every unit operations we have used. So, starting from your extraction, the cell disruption that homogenization, centrifugation, ultra filtration, precipitation, chromatographic techniques and then ultimately we have the final product. So, each and every unit operation is needed for purifying this type of intracellular enzyme.

(Refer Slide Time: 50:37)

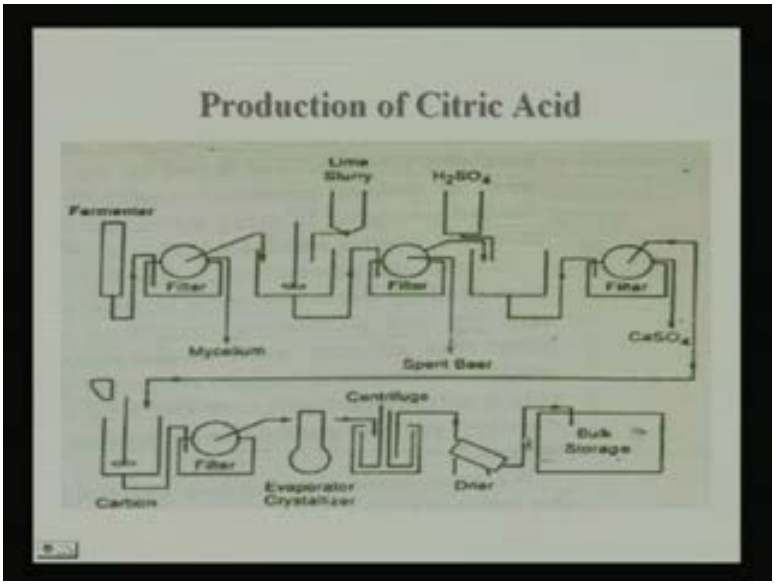


Now, if we are coming for this ethanol production. You just see the processes is entirely different from that of this intracellular enzyme. Now, here we have the fermented broth, this broth contains this water and ethanol. Now, we want to extract the ethanol from water. So, this is the beer well, we are just passing it to this beer still. Now, here this still age is there and we are just screening this and it is the rotary evaporator, rotary vacuum evaporator is there and we are just drying it and we are getting the dried grains and the soluble which are there we are just spraying it and we are getting the dried soluble.

Now, here this particular beer still which is undergoing this condensation and we are

getting the concentrated little bit, concentrated product which is called the whisky. Now, if we want to further concentrate this particular product, we are once again putting this thing to this purifying column and from there rectifying columns are there and through this rectifying column, we are just condensation is going on and we are getting the neutral spirit. So, see here no chromatography, no units separation simply distillation is taking place. So, how one step is different from other, one biological separation is different from other biological separation.

(Refer Slide Time: 52:14)

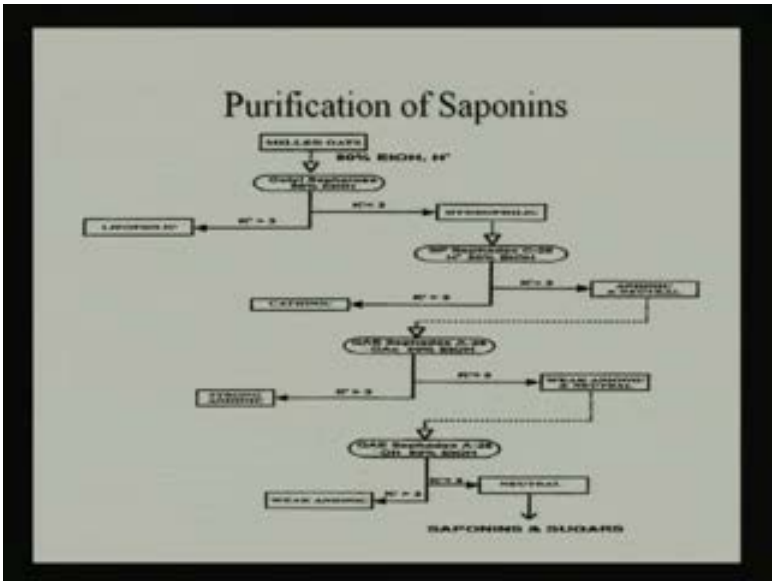


Say for example, if we are going for the citric acid production and purification. So, we had the fermenter, here this citric acid containing broth is there, so we are just going for the filtration we are separating the mycelia and this filtrate, we are taking and we are first adding to the lime slurry. Lime slurry is added to this and then we are once again going for the filtration.

Now, as soon as the filtration is over. Now, this spent beer is just removed and to this filtrate we are just adding H 2 S O 4. Now, acid is added and once again this it is undergoing the filtration process, we are getting calcium sulphate. And this broth once again coming to this activated carbons, this bed and then we are once again filtering it, we are just evaporating and crystallizing once again, centrifuging and we are getting the

dried product and we are going for the storage. Entirely different process of separation. So, I am just giving you one example you can get some idea that how one product separation is different from another product.

(Refer Slide Time: 53:38)



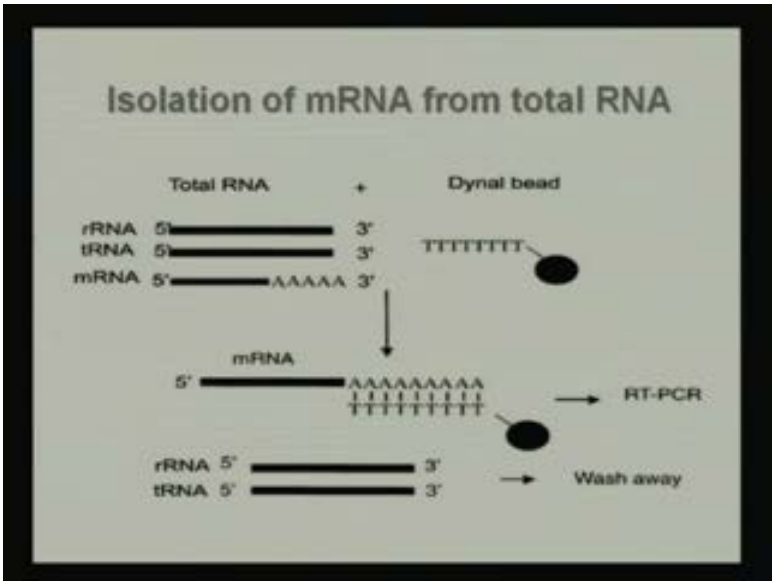
Let us take another example that purification of saponin molecules. Now, when we are going for this saponin molecules purification, we are just going for the separate, totally separate technique this adoption of the process.

Now, here you see this is the milled this grain and we are just taking this particular compound and we are just feeding it to the octyl sepharose column that is the hydrophobic interaction chromatography. We are just extracting it with fifty percent ethanol, we are separating the lipophilic fraction from the hydrophilic fraction and then we are just going for the another S p sephadex column which is 50 percent ethanol and we are once again separating it to this cataionic and anionic that neutral fraction followed by the QAE ion exchange chromatographic technique and when we are going for this QAE bead that is ion exchanger bead, we are just separating the stronger anionic and weaker anionic groups and this from the weaker anionic group when once again we are passing it to the column. We are getting the weaker anionic and neutral compound and this neutral compound is nothing but, it is the saponin molecules along with some sugar.

So, this saponins are mostly this glyco conjugates. So, here some sugar and the saponin molecules are there.

So, these are some of the techniques which we are following. Just starting from this hydrophobic amino these columns and followed by ion exchange column. We have never gone for any other techniques. What we have seen in case of citric acid or some other ethanol or some intracellular enzymes.

(Refer Slide Time: 55:47)



The beauty is that, when we are going for this mRNA isolation from the mixture of mRNA t RNA are and r RNA, we know that mRNA has got poly a tail and if we are just going for the oligo d t a tail then we are just binding this a to t binding will be there mRNA is getting separated and tRNA and rRNA mixture is there, you just simply pass it through GFC column based on the size, we can separate both molecules out of this column and with these I have tried to give you the particular behavioral characteristics of different macromolecules, different biologicals. It has got a definite characteristics and if you have some idea, some knowledge about the targeted molecule, it is very important and essential that what is the characteristics of our targeted particular biologicals. We can design the downstream processing of that particular biologicals or we can we can fix the strategy of downstream processing.

So, with this particular example I think you have got some idea that how one biologicals are different from another and why there is no hard and fast rule for separation of any particular biologicals, with a particular this, the procedure. It is you who will be deciding and if you know the characteristics of this biologicals, it will be very easy for you to fix the strategy of biological separation. I think I have tried to give you some idea about the biological macromolecules and with this particular idea, with this particular knowledge you will be able to separate any biologicals, what you want to separate in your future activities. Thank you very much.