Analytical Technologies in Biotechnology
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Module - 3
Chromatographic methods
Lecture - 4
Ion-exchange chromatography

In previous lecture, we have gone through the basics in chromatography. Also we have discussed about the different aspects of low pressure column chromatography and high performance liquid chromatography.

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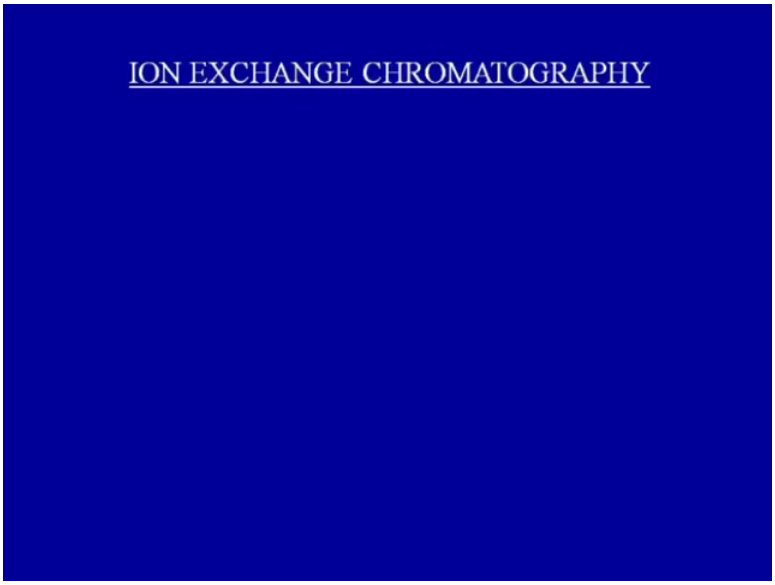

And in this lecture, we are going to discuss one of the techniques which is widely used it is ion exchange chromatography. Now, as you know many of the biological molecules are charged molecules at a particular ph. So, they carry certain charge either positive or negative at particular ph. Like for example proteins will carry positive charge below their pi value, and will carry negative charge above their pi values. And these particular properties of charge could be utilized for their separation.

So, ion exchange chromatography involves separation of macro molecules, and other molecules based on interaction between charged solute molecules and appositively charged molecules covalently linked to a chromatographic matrix. Now, ion exchange is

the most frequently used chromatographic technique for the separation and purification of proteins polypeptides, nucleic acids and polynucleotide and other molecules which are charged. Now, the reason for the success for the ion exchange chromatography is, its wide spread applicability, its high resolving power, its high capacity and the simplicity and the controllability of the method.

Before, we go into the details I would like to show you on your screen in a very simple way, the mechanism and working of the ion exchange chromatography. So, please focus on your screen. And let us understand this whole chromatography ion exchange chromatography, and then we will go into the details. So, what happens is that first thing is you have a stationary phase. And this stationary phase which is particularly charged molecule or charged ionic charged which is immobilized on to a support matrix say bead. Now, when you prepare a resin, then what happens is your resin will carry a particular charge it could be negative charge or it could be positive charge. And it is equilibrated with particular buffers.

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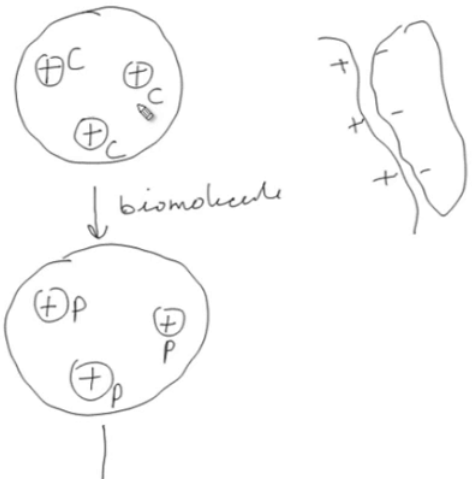

So, what is your starting condition the starting condition is if I say this your bead and this carries say particular charge, say positive charge. Then at the starting point where you have equilibrated your resin the positive charge will be will be interacting with the counter ions. Say if I say these are the counter ions which are surrounding the resin the charge on the resin. So, that is the starting position. Now second thing will be, so what

will happen how this whole separation takes place that you will add the protein or a bio molecule.

Now, when we add a bio molecule, bio molecule which are appositively charged here resin is positively charged. So, bio molecule which is negatively charged will bind to this. Now, they will replace the counter ions and they can replace the counter ions because they will have high affinity so bio molecules say protein. So, protein is bound here and protein is those protein molecules which are carrying negative charge will bind in here. So, what you have is, if I show it here it is something like this in the surface if this is positive charge on resin then the negative charge will be binding. And this might be of any molecule which are which is present in here.

Alright, so once protein is bound then you have these protein molecules if there is a mixture of protein molecules then they will bind with different affinities depending on the charge. If they have more negative charge they will bind with more affinity if less then they will bind with less affinity. Now, what you have to do, you have to elute your protein or take your protein from the matrix and that is to separate different analytes in a mixture. And this will be done on based on the affinities actually.

And here you will put high ionic strength eluent which will replace the protein with those ionic species in the mobile phase. Now, what will happen depending on as you will increase the concentration of the eluent or ionic strength like say you can add Nacl. And as you increase the concentration of Nacl depending upon their affinities or proteins they will replace it. So, if protein has higher affinity then you need higher concentration of Nacl. So, next step would be that is elution this was binding.

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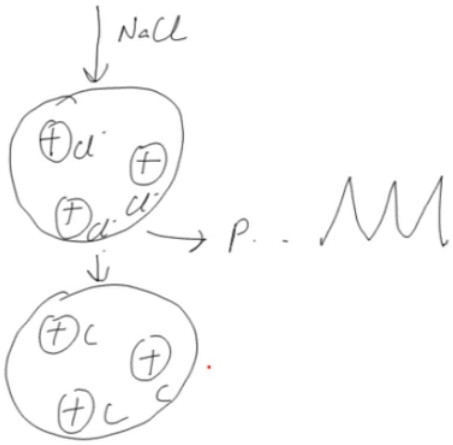

And the next step would be elution, here what we have done you have eluted it to say Nacl or some other eluent. So, what will happen this positive charge will now it will be replaced by say chloride ion. And you will get protein which is eluted from. Now, remember this protein which will be like its mixture will come out at different strengths of the salt. Once this is done, so you might get different peaks in here which are there. Once it is done then simply what you have to do regenerate. So, again the simple buffer will be put in and extra Cl or extra eluent will be washed off.

And fine you will regenerate the column which will be as the starting position. And so this resin could then be reused for another purification. So, this was a very simple way we I tried to explain the mechanism of the ion exchange chromatography. Now, let us little bit see how the working of the chromatography is done. So, what is done here is that you have a column.

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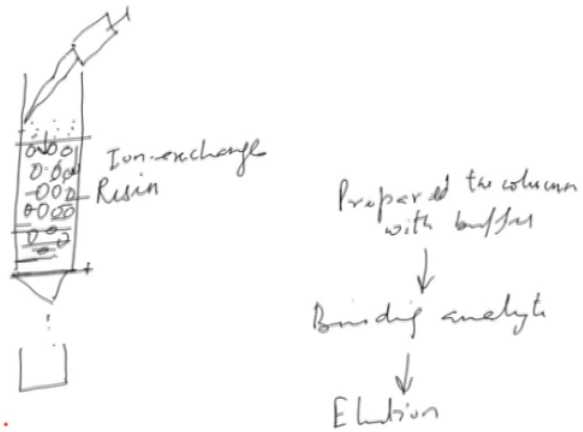

And this is a stationary phase in here filled in here and which is say up to here this is the stationary phase. Now, in ion exchange column this contains resin which we can say ion exchange resin here. And it carries a particular charge. Alright, and like I said it is in the form of beads actually it is like these are beads which are which contain the stationary phase this is support matrix.

Alright, now what you will do is there are two ways like peristaltic pump as we were discussing in ear earlier lectures. You can do it by peristaltic pump or you can do it manually. Load the samples if I have to manually load the sample then I can load the manually the sample with a micro pipette. So, this micro pipette I can load the sample and this sample could be in ion exchange.

You can have any volume of sample as this is a displacement kind of chromatography, where the ions or ionic species will bind to the appositively charged ion on the resin. Now, even what you can do is that you can put say in the sample you can put the resin itself and let it bind in a beaker. So, that is also possible that in a beaker you can bind it then you can load it on to the column that can also be done. So, what happens is once you have prepared your resin and you have loaded your sample your sample will get in.

And as sample gets in what will happen it will as it gets in and gets in contact with the resin the charges will be saturated. So, if resin is positively charged negative charge by a molecule will bind with and slowly saturated. And as these beads which are in the starting gets saturated the bio molecule will move in and it will bind to others. So, it depends on the capacity of this stationary phase as how many ionic stationary phase it has. Once it is bound then what is done that you have to wash it so that unbound very weakly binding things could be.

So, you have a knob here which could be opened and once flow through could be collected, flow through will contain bio molecules which are not bound to the resin, because they are of the same charge. And this could be then separated by another chromatography ion exchange only where it has appositively charged stationary phase. Now, once you have done then you will do the washing. So, first is you have prepared the column by equilibrating it with right buffer. Then second thing is you are binding your analyte that is appositively charged and then you will elute.

So, after binding then elution has to be done that will be done by certain ionic species which can replace your analyte. And as you increase the concentration depending on the affinities, so what will happen if you have different affinities as you utilize I can use 50 mille molar Nacl, I can use then 100 mille molar, 200 mille molar and I can go 2.5 or one molar Nacl.

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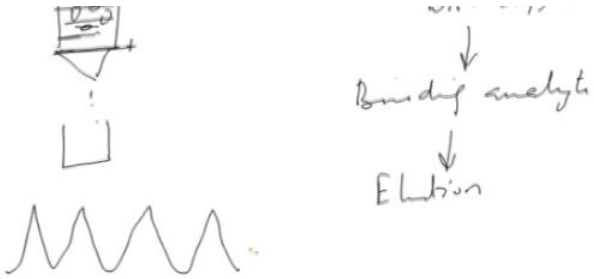

And depending on the affinities you will get the peaks here which will be separated according to the affinities. So, less affinity will come first and higher affinity peak will come later on. Now, once you have done with this you can regenerate the column as how I have explained and again reuse for another purification step. So, this is how the whole working of the column is done. Let us get into details actually this was just to make you understand how this whole thing works.

Now, let us get in the details of our discussion. Now, theory of ion exchange says that separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Now, most ion exchange experiments are performed in five main stages. And these steps are as I have explained also the first stage is equilibration in which the ion exchanger is brought to a starting state in terms of ph 9h strength. So, this allows the binding of the desired solute molecule. So, the exchanger groups are associated at this time with exchangeable counter ions. Now, these are usually simple anions or cations it could be like H OH minus or CL minus or Na plus.

So, Sodium chloride or hydroxyl or H plus ions could be the exchanger ions. Now, second stage is sample application and adsorption in which solute molecules carrying the appropriate charge will displace the counter ions and bind reversibly to the gel. So, unbound substances can be washed out from the exchanger bed using starting buffer. So, that is called extensive washing. Now, once that is done in the third stage elution will take place. So, what is done the substances is removed from the column by changing to elution conditions which are unfavorable for the ionic bonding of the solute molecules or bio molecules.

Now, this normally involves increasing the ionic strength of the eluting buffer or changing its ph. The fourth and the fifth stages are the removal from the column of substances not eluted under the previous experimental conditions that you can use higher concentration of eluent and then equilibration with the starting buffer for the next purification. So, these are the stages which I also explained in a very simple way that you have to go through when you are performing column chromatography. And this also shown in figure here.

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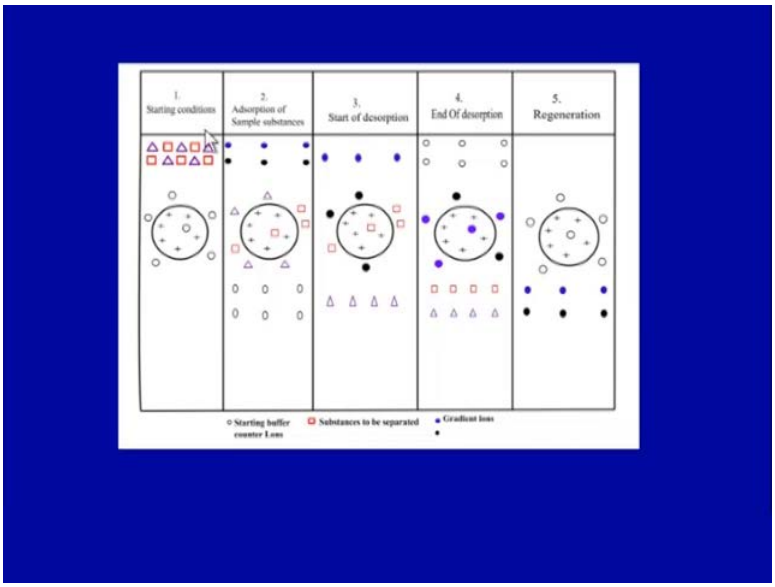

Which is like first is where counter ions are bound in starting. And then your these molecules which needs to be separated are bound to the column and one of those will bind as per the charge. Then your gradient times will be utilized like Nacl or some other which will elute as per the affinities. And you have to may be it could be increasing salt concentration is used. And then once this is done it will be regenerated. That is how it is a very simple mechanism of ion exchange chromatography.

It is a very simple very versatile technique to be used. Now, ion exchanger consist of an in soluble matrix to which charge groups have been covalently bound the charge groups are associated with mobile counter ions. Now, these counter ions can be reversibly exchanged with the other ions of the same charge without altering the matrix. The matrix may be based on may be inorganic compounds, synthetic, resins, polysaccharides etcetera. And the commonly used matrixes could be like polystyrenes, cellulose, argons, and dextrin and so on.

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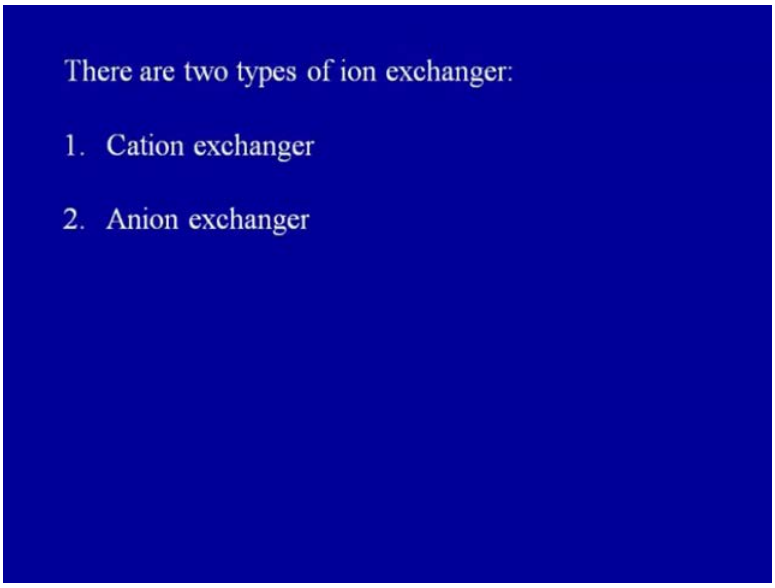

Now, in ion exchange chromatography there are two kinds of ion exchangers. One is called cation exchanger and this depends on the charge they carry. So, the cation exchanger and anion exchanger. Now, cation exchangers are the ones which posses negative charge as they are exchanging the cations. As they by themselves posses negative charge groups and will attract positively charged cations.

These are also called acetic ion exchangers because their negative charge results from ionization of acetic groups. Then there are anion exchangers and they posses positively charged groups and will attract negative charged anions. These are also called basic anion exchangers as their positive charges result from the association of protons with basic groups.

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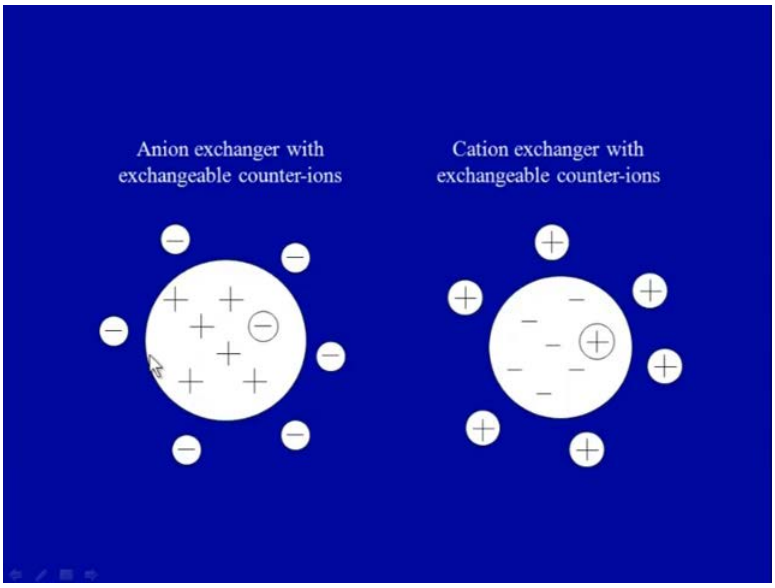

So, if you see these look something like that this anion exchanger and this cation exchanger. So, anion exchanger will exchange anions and cation exchanger will exchange cations, itself they are charged accordingly. This is positively charged and cation exchanger is negatively charged. Now, there are lots of examples of commonly used ion exchangers like you can see in this table.

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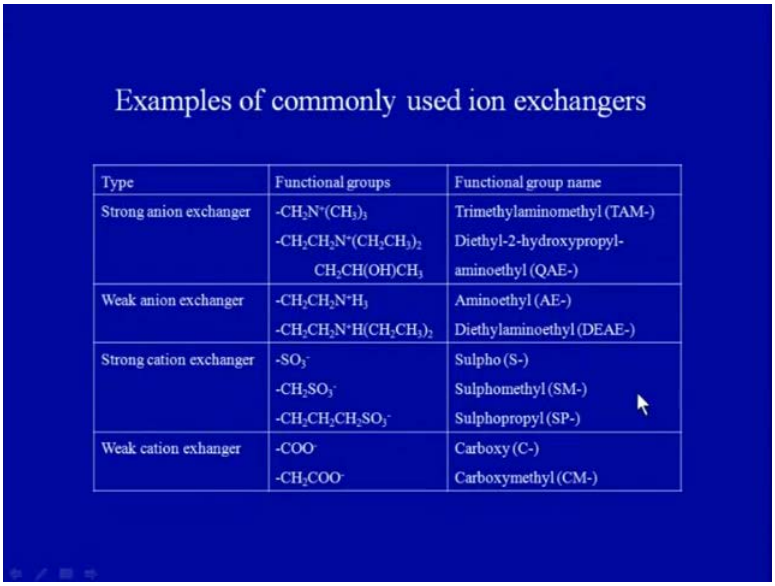

There are strong anion exchangers, weak anion exchanger. Some examples are of strong anion exchangers are trimethylaminomethyl which is also called tam. There is diethyl 2 hydroxylpropyl these are strong anion exchanger, if you say weak anion exchanger then aminoethyl and diethylaminoethyl DEAE. These are weak anion exchangers. Then there are strong cation exchangers like sulpho groups are strong cation exchangers which is sulphomethyl or sulphopropyl.

Likewise, carboxy weak cation exchangers include carboxy exchangers like which contains carboxy group or carboxymethyl groups. So, these are some examples there could be many more of weak and strong anion and cation exchangers. Now, when we say strong or weak it refers to the extent of variation of ionization with ph and not really the strength of the binding. Strong ion exchangers are completely ionized over a wide ph whereas with weak ion exchanger the degree of dissociation and thus exchange capacity varies much more markedly with ph.

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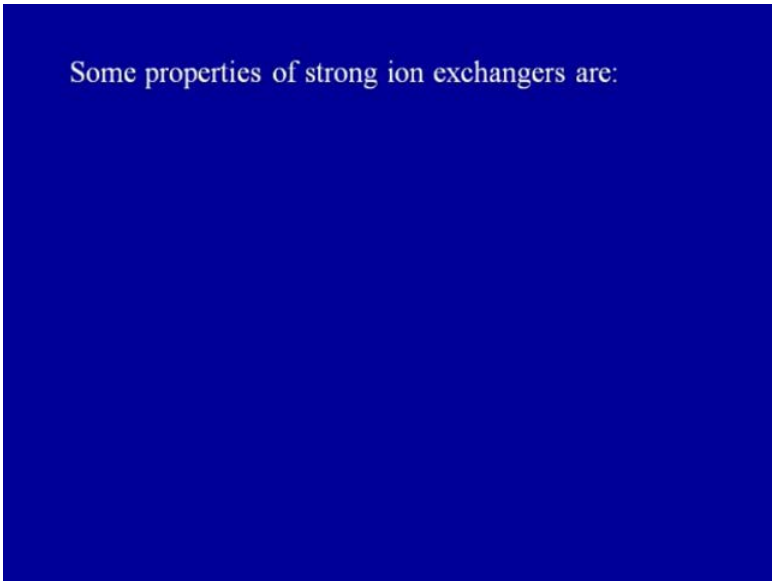

If you say some properties of what are the properties of strong or weak ion exchangers. Strong ion exchangers the sample load loading capacity does not decrease at high or low ph values. They are due to loss of charge from the ion exchanger. So, they remain ionized at a wide range of ph very simple mechanisms of interaction adjust between the ion exchanger and the solute. Ion exchange experiments are more controllable, since the charge characteristics of the media do not change with change in ph.

And this makes strong exchanger ideal for working with data derived from electro forestation titration curves etcetera. Another important characteristic is capacity. Now, the capacity of an ion exchanger is a quantitative measure of its ability to take up exchangeable counter ions and is therefore, of major importance. Now, capacity may be expressed as total ionic capacity or available capacity or dynamic capacity. Now, total ionic capacity is the number of charged substituent groups per gram dry ion exchanger or per ml swollen gel.

Total capacity can be measured by titration with a strong acid or base. Then there is actual amount of protein which can be bound to an ion exchanger under defined experimental condition and that is referred to as available capacity for the gel. Now, if the if the defined capacity or defined conditions include the flow rate at which, the gel was operated then the amount bound is referred to as dynamic capacity for the ion exchanger. So, when flow rate is included it becomes dynamic capacity for the ion exchanger.

Available and dynamic capacities will depend on many factors and which includes say properties of the protein. Now, properties of the protein will include say the molecular size and charge or ph relationships. Then properties of the ion exchanger will also affect this capacity like exclusion limit of the matrix type and number of the charge substituent etcetera, then chosen experimental conditions like ph, ionic strength of the buffer, nature of the counter ion, flow rate temperature.

All these factors will certainly affect the available and dynamic capacities of a particular experiment or particular resin. Another important factor is porosity of matrix. Now, the porosity of the matrix is an important feature because the charge groups are both inside and outside the matrix. And matrix is support material. So, because of this the matrix also acts as a molecular seal because matrix a flow has to be maintained through the matrix. So, matrix is not an itself is a mesh like a structure and there is a porosity here and when you immobilize these ions they will be all over the matrix.

They could be totally exposed or they could be somewhat inside. Now, large molecules may be unable to penetrate the pores it might happen that certain ions or certain charged groups may not be approachable by the large molecules, because they will not be able to reach while the small molecules will be able to reach. So, capacity will be affected by the

porosity also. Many times when we are selecting ion exchange matrix you will also look for the porosity.

And it is like when we say CMC 25 or some C 50. It refers to the porosity and accordingly you can choose the matrix for a particular size of the sample to be purified. Mesh size is also important which determines porosity like ion exchangers will come in a variety of size called mesh size. Now, final mesh size means an increase surface to volume ratio and therefore, increased capacity and decreased time for exchange to occur for a given volume of the exchanger. On the other hand, fine mesh means slow flow rate which can increase diffusional spreading.

So, there a whole lot of different mesh size is available and accordingly one has to choose that mesh size. As we have explained before mechanism of ion exchange is taught to be composed of 5 distinct steps. And this is like different from the procedure we have discussed earlier. One is the first step is the diffusion of the ion to the exchanger surface. Now, this occurs very quickly in homogeneous solution as you put your sample the diffusion occurs.

Then diffusion ion through the matrix the structure of the exchanger to the exchanged site actually where it has to bind. And this is dependent upon the degree of cross linking of the exchanger and the concentration of the solution. This process controls the rate of whole ion exchange process and then exchange of ions at the exchanger site. This is thought to occur instantaneously and to be an equilibrium process.

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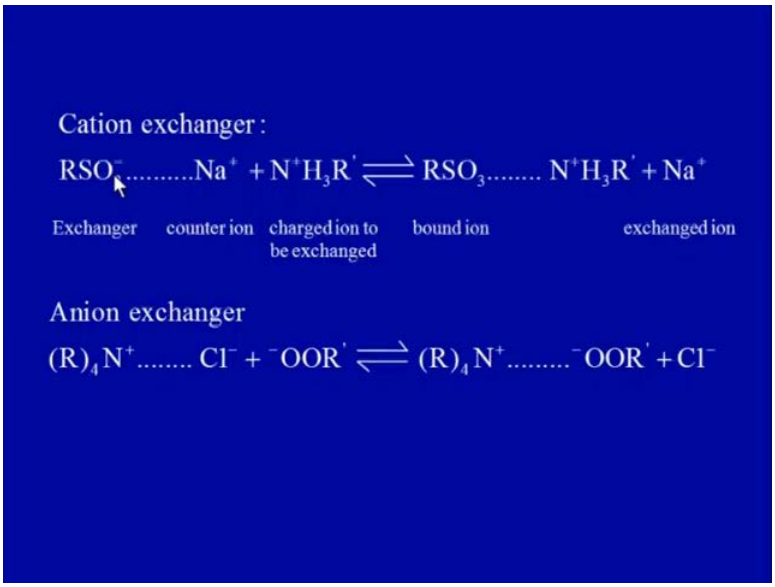

So, what happens like when you have a particular ion resin which is kind of interacting with the counter ion and then an analyte comes or a protein comes when it will be replaced a counter ion as in here. And like in anion exchanger similarly, the analyte will replace the counter ion as it has higher affinity. The more highly charged ionized molecules to be exchanged the tighter it binds to the and the less readily it is displaced by the ions. So, this depicts the affinity of a particular analyte for the exchanger.

Then the diffusion of the exchanged ion through the exchanger to the surface and the selective desorption by the eluent and the diffusion of the molecules into the external element. So, all these steps happen one by one and this is how the mechanism is unfolds of the ion exchange chromatography. And the selective desorption of the bound ion is achieved by it could be simply ph or the concentration of a particular ion that is ionic concentration could be used.

So, this was mechanism of ion exchange chromatography. Now, selection of conditions for ion exchange purification depends on many factors. A wide variety of ion exchangers and adsorption conditions are available, and one has to choose according to what kind of experiment they are doing what is their analyte like say ph stability, what is the charges at particular ph all this things has to be taken care and likewise buffers and eluent has to be selected.

Now, the factors involved in making each choice are described here. So, if you say choice of matrix, so we are discussing those factors. For choice of matrix for low and medium pressure line exchange, you can use say cellulose which can be fibrous, micron granular or beaded it could you can use argons, textron, polyvinyl a lot of other matrices are available for high pressure ion exchange. You need strong ion exchanger matrices like silica based or other resins which are have been made for high pressure chromatography.

These matrices mainly differ in their particle size and ph range and those could be selected accordingly. Lot of commercial resins is available for high performance liquid chromatography. Selection of functional group is another important factor. For example, an anionic or cationic group has to be selected. Now, if the material to be bound to the column has a single charge then either positive or negative. So, then choice is very clear. Now, problem comes in ampoteric materials like proteins which might have both positive and negative charge. So, ph stability has to be considered here if a protein is more stable above its isoeletric point then anion exchanger should be chosen and if it is stable below isoeletric point then a cation exchanger should be chosen. As the charge of the protein will be accordingly be at it is above or below its pi value.

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For a weaker ionize substances which requires very low or high for ionization, strong ion exchanger is used. How about for certain other substances weak ion exchanger are preferred, because strong exchangers are often capable of distorting a molecule so much that the molecule will might denature. So, accordingly one has to choose and see the conditions, if it is a very weakly ionized substance certainly strong ion exchanger needs to be taken.

Now, the most commonly used functionalities are weak ionizanic groups and these are in common use these are weak exchangers like diethyl aminoethyl DEAE commonly known as. This group is usually used in anion exchanger to purify negatively charged proteins, while the carboxy methyl is another commonly used or frequently used cation exchanger for recovery of positively charged groups. So, depending on what kind of analyte you have one of the exchanger either cation or anion. And most of the time weak exchangers are utilized.

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Now, buffers used in ion exchange chromatography again you have to be careful in here for successful ion exchange chromatography. Now, in ion exchange chromatography buffers are chosen in order to minimize ph fluctuations and maximize the adsorbent capacity. Now, two important factors or rules has to be taken care of one is that is charged form of buffer should not interfere with the ion exchange process and we will discuss that.

And then again another factor is temperature which will affect the pk value of the buffer, and such that its buffering capacity will be different in purification carried out in the cold conditions. For example, twist buffer will have if you set the ph in say room temperature and you carry it to the cold room conditions then certainly change in ph buffer will take place and that might hamper your protein purification. The commonly used buffer this is a table gives you some of the commonly used buffer with cation and anion exchange chromatographs.

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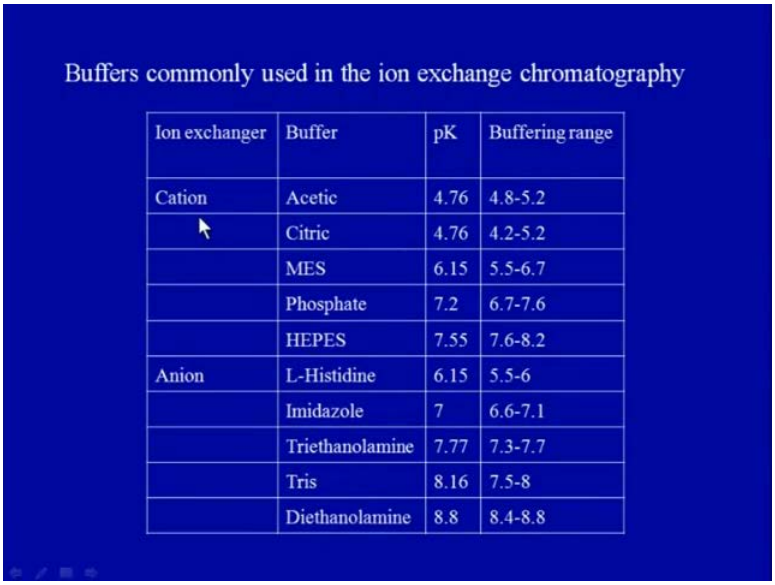

For example with cation exchange you can see acetic, citric, MES, phosphate, hEPES buffers are used with cation exchangers while with anion exchanger you can say l-histidine, imadazole, triethonolamine, tris and all these buffers are used with anion exchangers. So, you have to worry about that which kind of buffer which will not interfere with the particular kind of type of exchanger needs to be used.

Now, selection of adsorption and elution ph is an important factor and for protein adsorption on the matrix ph about 1 ph unit above or below of the pi of the target protein is chosen. While for protein elution 0.5 of a ph unit from its pi at an ionic strength of point 5 molar Nacl conditions are chosen. And these things need to be standardized these are not hurdle fast rule here. When you do your experiment one has to standardize that how much salt concentration needs to be taken for elution or if it is done through ph conditions what changes in ph has to done.

Now, selection of adsorption and elution ionic strength like I said needs to be standardized needs to be taken care of and one has to go through the experiment to do that. Some guidelines on the adsorption step, the highest ionic strength which will allow protein binding is used, while on elution step the lowest ionic strength is recommended. So, reason for this is as follows if you have a buffer then you should use a buffer of high highest ionic strength when you are binding the protein and when you are eluting then you have to use the low ionic strength buffer.

Only thing you have to take care that your protein remains stable in both conditions. If say at the low ionic strength it is not stable then you have to avoid that. Now, if the ionic strength is too low on absorption, then the protein will bind too tightly and effectively elution will be made difficult. So, that is why one has to use high ionic strength. Then keeping the high ionic strength as high as possible on adsorption minimize the binding of unwanted contaminates that is very important that non-specific or unwanted contaminates will be avoided if high ionic strength is utilized.

Conversely keeping the ionic strength as low as possible on elution minimizes the elution of bound contaminants. So, you can get a better purification. Now, the strategy outlined above simplifies the elution step and the salt concentration say of 20 to 50 mille molar is usually used during the adsorption, and say up to 0.5 molar Nacl is used during elution process.

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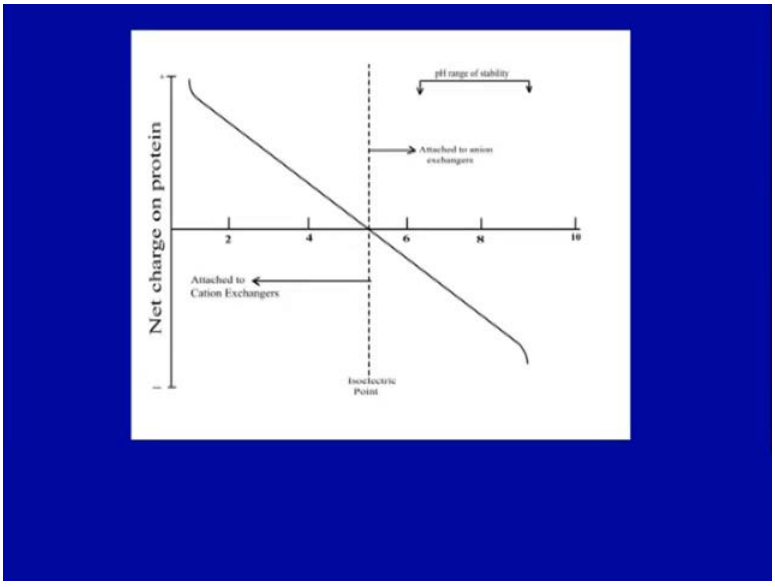

This graph here figure shows that you can use something like say this is your pi value, and your ph range of stability for a protein is certain ph range. And that could be utilized for that could be utilized, and this could be seen and particular ph of stability could be used for purification. So, the next charge of a protein as a function of ph and showing the ph range for stability and for binding two cation and to anion exchanger. You can say it will bind accordingly as per its ph either it is ph is above or below.

So, this protein would be coroneted graph on an ion exchange chromatography using a buffer adjustable to ph 6.3 to 8.4. So, accordingly it has to be like, if it is anionic protein is anionic that particular ph that is stable then it has to be purified one anion exchanger and like here 6.3 to 8.4 is the right ph that needs to be utilized.

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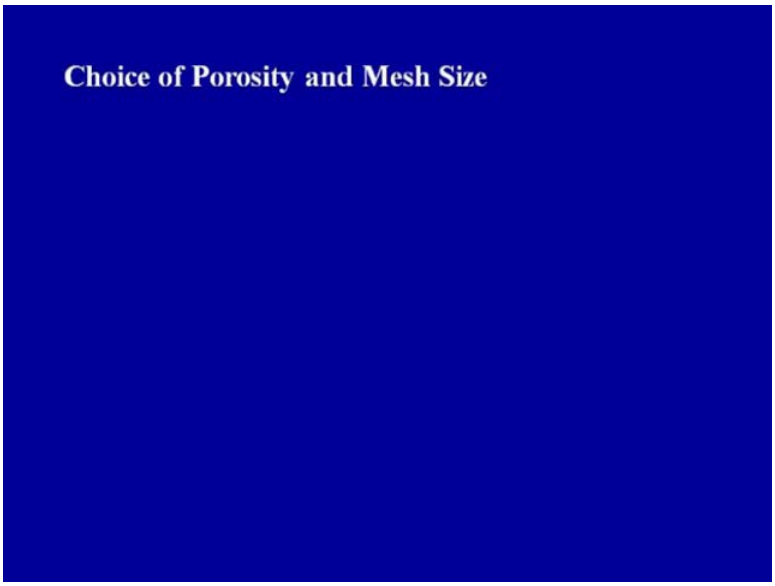

Now, choice of porosity and mesh size where we were talking about, now small molecules are best separated on matrixes with small pore size, whereas macro molecule will need large pore size. Like I was discussing many commercial resins they tell that by certain specifications that what could be the mesh size and porosity. Accordingly if it is a macro molecule we require to use a larger pore size if it is a smaller molecule, then a smaller pore size could be utilized.

Selecting a mesh size is difficult and determined empirically as a small mesh size improves resolution but decreases flow rate. So, accordingly one has to choose the conditions. So, this was like a all different factors we have discussed regarding when we are attempting to do an exchange chromatography and what are the factors we have to take care. We have to accordingly design your ion exchange chromatography experiment. Now, if we see the simple procedure as I was explaining earlier in a very simple way.

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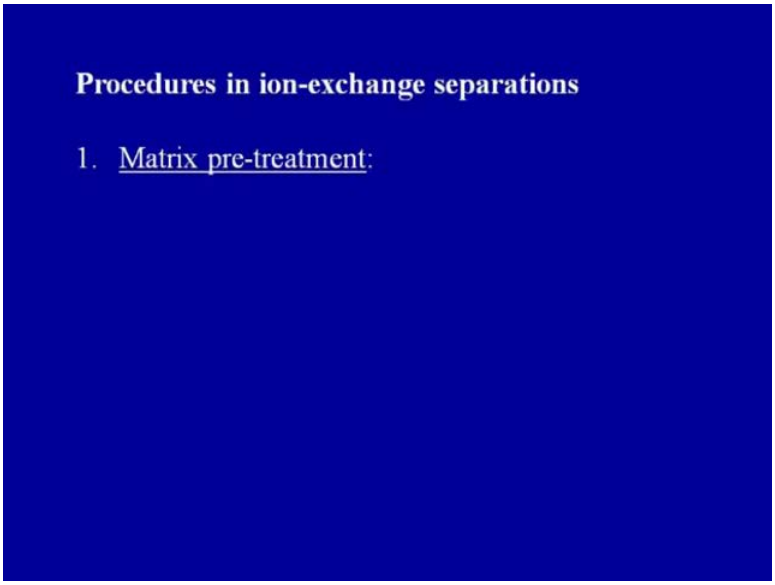

The procedure in ion exchange separation is quite straight forward. The first step to be done is you have to select the matrix. Once you have selected the matrix the pre treatment of matrix has to be done. Now, remember matrix will be available either in say powder form or in some solvent say 30 percent ethanol. So, the matrix has to be in both ways matrix has to be swollen it has to be put in proper buffer which needs to be equilibrated or you can start with water then you can put it in buffer.

So, what will be done, like as you put the matrix in the buffer or suitable buffer or water. Mostly you can start with water, but then you should exchange it to a suitable buffer in a beaker. So, once you do that certain fines which are really not cannot be used and they will be unnecessarily increase in the volume can be removed by decanting. Then you will allow though in ion exchange swelling is not so important, but you will allow it to exchange with the buffer.

And then it will wash it and equilibrate it to the proper buffer and then if say it has to be converted to correct counter ion that will be done. So, matrix pre-treatment is the first stage of ion exchange separation. Once you have done that then matrix has to be loaded on to the column. You can also load your sample before loading it on to the on to the column and there are various methods of adsorption, so different adsorption methods could be. One could be batch adsorption.

Now, batch adsorption is carried out in free solution, but it has a lower efficiency like I said you can do it in a beaker it is ideally suited for initial treatment of large volumes of sample. Say, if you have a liters of sample and what you can do is suitable amount of matrix could be directly put into the pre-treated matrix could be directly put into this large volume of the sample. And you can gently stir the sample and the protein or the analyte will bind to the stationary phase.

Now, this does not suffer from problems of bad swelling and shrinkage now. Once it is done then elution can be carried out either in batch mode or ion exchange slurry may be packed into a column and then eluted. Like in a batch mode say once you have bound the analyte to the stationary phase, then sample can be decanted and then elution bound buffer could be first you can wash and then elution buffer could be put in the beaker. And batch elution you can directly use 0.5 molar Nacl.

And let it be there with a little gentle stir and then whole analyte will be eluted into that buffer like say 0.5 molar Nacl and that could be decanted and be used for further applications. So, this is batch mode or what you can do is rather than eluting in batch mode you can pack whole thing in the column and then elute accordingly. In column adsorption rather than batch adsorption you can also do it in column adsorption. So, in batch adsorption a protein is purified by adsorption of contaminants which allows the desired protein to pass through the column without binding. Now, no concentration of protein results in here in column binding.

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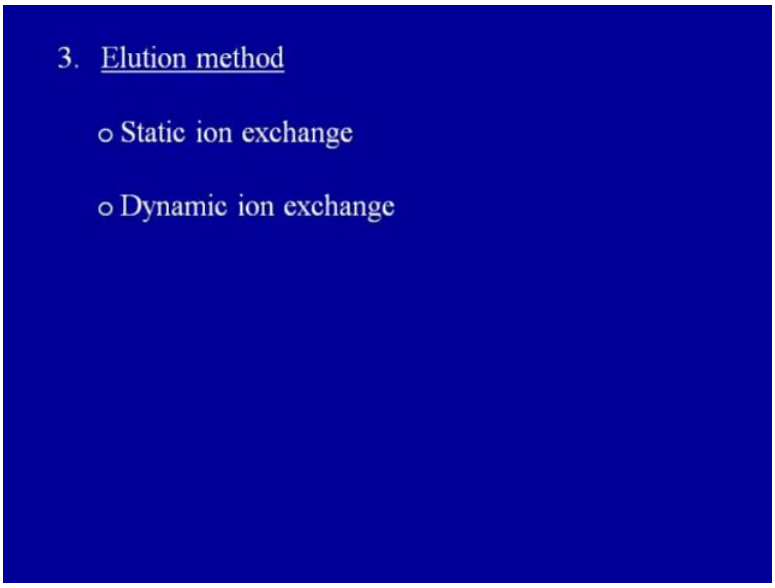

Rather than batch mode you can also do it by elution method. Now, here in elution method it could be static ion exchange or dynamic ion exchange. Now static ion exchange protein is fully adjured to the bed, and completely eluted by displacement into a mobile phase using a small volume of strong eluent like I said you can take 0.5 molar or 1 molar Nacl and you can elute the protein.

Now, this is useful for concentration of protein from a large volume of a sample because what you are doing is you are eluting though it is a strong eluent, but you are eluting it in smaller volume. So, what you get is much concentrated solution of the protein. In dynamic ion exchange separation of protein is achieved by their relative speed of migration through the column. So, what is done all the sample components will migrate, but they will be separate depending on their relative equilibrium distribution or distribution coefficient or we can say relative affinities to stationary phase.

So, what will happen as you increase the ionic concentration the molecules with different affinities will be eluted. And as I showed you earlier they will be eluted in different fractions and those fractions can be collected in fraction collector. And they could be observed on spectrophotometer or if your chromatograph contains UV monitor then they could be observed in form of different peaks.

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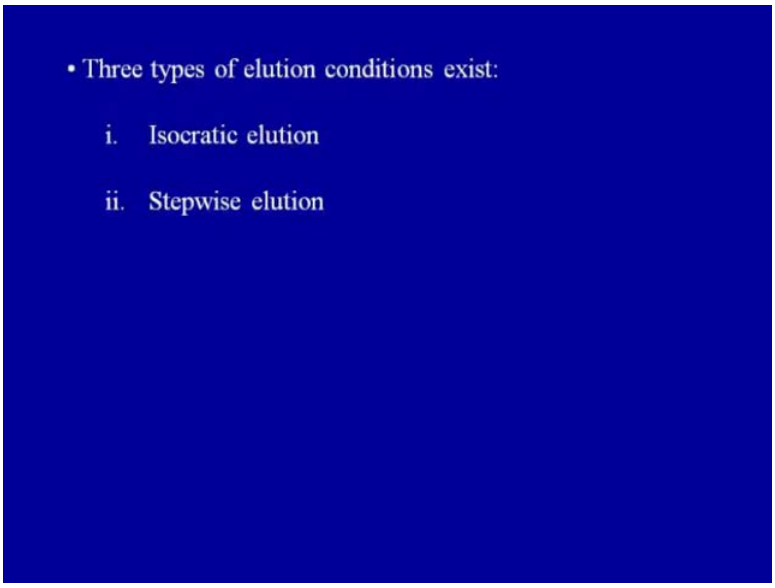

There are three types of elution conditions which could be utilized that could be isocratic elution. In isocratic elution sample volume should be between 1 percent and 5 percent of the word volume and a long column is used with a diameter to a length ratio of around 1 is to 20. So, the starting buffer is used throughout the separation.

So, its isocratic elution where you are not changing the buffer as such is a one single buffer which will be eluting it and it would be concentrated buffer or high amount of eluent might be utilized. Then there is stepwise elution. And stepwise elutions are sequential discontinuous change in ph or salt concentration will be done. Like for example, say you can change ph sequentially like you can first start with say ph 5 elute I can start with say 7. Then I could come down to 6 5 4 likewise or I can go up in that order or likewise salt concentration could be increased.

Like for example, I can start with 100 mille molar salt concentration then I can go to 150 200 250 and up to 0.5 molar Nacl. Now, column volume is determined by the exchanger capacity and the sample volume here. So, the sample should adsorb to 5 to 10 percent of the total bad capacity and the column length is usually shorter. So, here it is a stepwise elution and different fractions will contain different peaks. And those analytes having different affinities will elute accordingly more affinity one will elute later on and less affinity analyte will elute first.

You can use gradient elution also rather than stepwise elution. So, it is a continuous gradient rather than step gradient. So, the composition of the eluent that is ph or ionic concentration is changed continuously, and sample content should be 5 to 10 percent of the bad capacity ideally, and column length is usually 20 to 40 centimeter with a diameter to length ratio of not more than 1 is to 5. The volume of the buffer required is determined empirically and in too steep gradient resolution will be lost while too shallow a gradient will result in unnecessary dilution as long and long separation times.

So, accordingly one has to choose the condition. The total volume of the sample should be 5 times the bad volume. These things are you know are not hurdle fast and one has to really go through standardizing these values. So, in continuous gradient could be made by two pumps and a gradient mixture could be used where you can say start the zero molar Nacl buffer and say one molar Nacl buffer, and accordingly you can have a gradient which could be too steep or you can choose the gradient to be simple gradient or otherwise.

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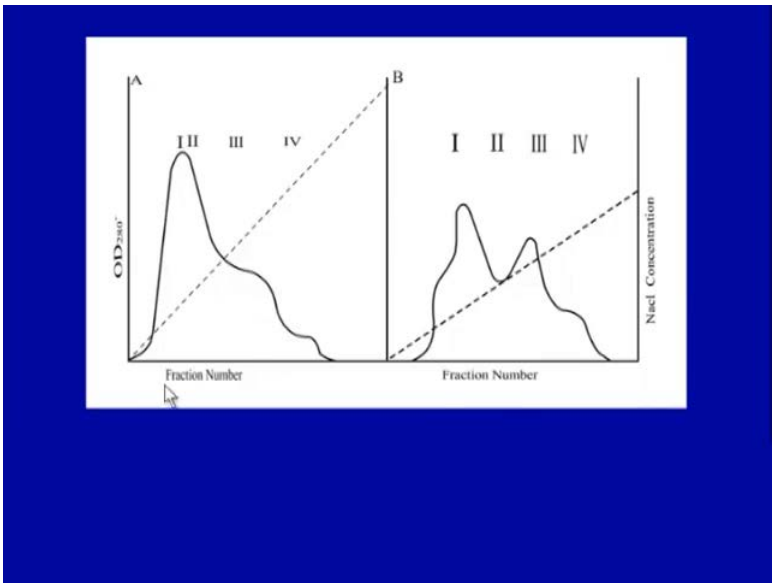

Now, as you see here the chromatographic purification has been compared for a steep gradient and not somewhat so steep gradient. And you can see that here these two peaks here one is only first peak is very clear other peaks are not very clear. But, here you can see two peaks are separating another peaks are visible. So, that is a very important that separation of 4 peaks by ion exchange chromatography is taken here, but in higher steep

gradient only one peak is very clear other peaks are not so clear, but in shallower gradient peak 2 and 3 also becomes clear and distance of 4 peaks becomes obvious.

So, these are like again as you go along doing this things general guidelines very clear, but one has to standardize its own conditions to get that best results. Finally, once you have separated your bio molecules on column by combination of say cation or anion exchange chromatography then the column could be reused many times. So, it has to be regenerated, and if you are not being right away the next purification, you can store it also.

Regeneration involves removal of tightly bound contaminants, and conversion of the support to the required counter ion form ready for equilibration and protein adsorption. Removal of tightly bound protein is achieved say using 2 molar Nacl and then followed by an alkaline wash for storage antimicrobial agents are used to avoid microbial growth. And it could be high organic solvents are recommended or Sodium acid could also be used like antimicrobial agents like Sodium acid can be used. In certain conditions where anti microbial agents cannot be used then say 30 percent ethanol or other organic solvents could be used for this purpose.

So, this completes our ion exchange chromatography section and as we have gone through this particular section I think you I hope that you are able to understand that how column ion exchanges chromatography is performed. It is a very straight forward chromatography but depending on the type of biological mole molecules to be separated depend you have to worry about lot of different factors and which include say conditions of the experiment, the stability of the analytes, the mesh size, or porosity of the column, the capacity of the column, standard conditions for running the column, kind of buffer you have to use, kind of eluent you have to use.

All these things have to be taken care of. General guidelines are very clear as it is a very straight forward if it is your analyte is positively charged, you have to use negatively charged column and vice versa. But certainly one needs to standardize all the conditions and then once, for a particular analyte we standardize the condition could be followed in your lab. And purification can be performed. In the next lecture we are going to discuss about another important chromatographic technique, and that is exclusion chromatography. Thank you.