Genetic Engineering: Theory and Applications
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Module 8- Isolation and Purification of Product Part-2
Lecture 23 - Gel Filtration Chromatography Part-1

Hello everybody! This is Dr Vishal Trivedi from the Department of Biosciences and Bioengineering IIT Guwahati and what we were discussing? We were discussing the isolation as well as the purification of the over aspects product from the cells or the host. In this context so far we have discussed the two properties of the protein which can be exploited in two different techniques.

In one of the lectures, we have discussed how you can exploit the presence of charge on the protein and that technique is called ion-exchange chromatography and in the next lecture, we have a discussion about the presence of hydrophobic patches present on the protein surfaces and how that can be exploited to purify the protein using the hydrophobic interaction chromatography.

Now, let us move onto the third property and the third property is that the proteins are having the surface area or the surface area which can be utilized to purify as well as to characterize a particular protein and the technique which is related to the surface area is known as the Gel Filtration Chromatography.

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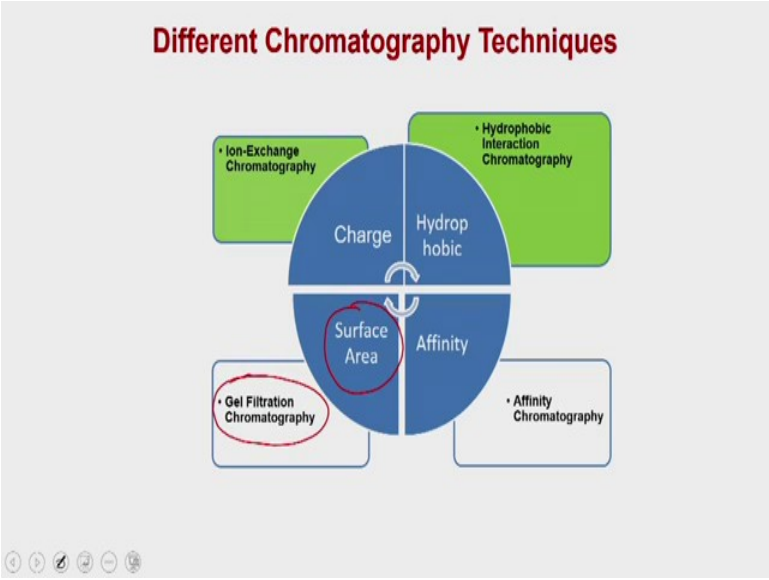

Now, before going into the details of the Gel Filtration Chromatography let us discuss how we can exploit the surface area of a protein to characterize the particular protein as well as how that can be used to purify the different protein from a complex mixture.

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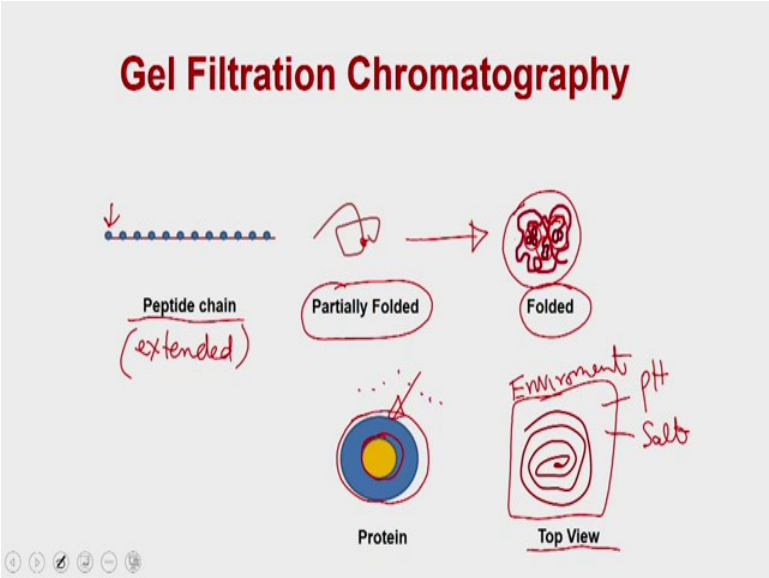

So, now you can imagine that proteins are nothing but made up of a peptide chain which is made up of different amino acid residues. So, initially, when they are being synthesized from the ribosome they have been synthesized in the linear chain and that linear chain form is called the extended form.

So, when they are being synthesized from the ribosome they are synthesized in the extended form. As soon as the chain is coming out from the ribosome, it is based on the micro-environment then it starts folding by the interaction between the residues like if the residue is present here it can make an interaction with the neighbouring residue and so on and ultimately you are going to have the partially folded protein and then that partially folded protein is going to be get converted into the fully folded protein and what you can see in a fully folded protein that the hydrophobic patches are going to be present in the centre.

Whereas the hydrophilic molecules are going to be present on the surface and by doing this whole process what protein is trying to do is it is trying to conserve the free energy and it is trying to reduce the surface area and because of that it is trying to make the structure as compact as possible. That, you can understand by this diagram. So, if you see a protein from the top what you will see that there is a centre, around this centre the proteins are arranging its amino acid residues.

And as a result, the protein is going to form a globular structure and in this process what we do and how this arrange the amino acid is based on the environment which is present outside. Which means the local environment if you have a and most of the cases the environment like the pH, the salt and all other factors which are present in the micro-environment is going to induce or it is going to affect the packaging of this protein molecules in such a way but at the end, it is going to arrange along with the centre.

And that is why would you like to characterize a protein you can characterize simply by knowing the radius of gyration or the diameter of that particular ball and what you can see in a fully folded protein that the protein will arrange the hydrophobic molecules in the centre and the hydrophilic molecule around it and that is required because the micro-environment in most of the protein cases is hydrophilic, which was, which means that you have water outside and because of that all the polar, as well as the charge residues, are being arranged outside and all the hydrophobic amino acids which do not like the water or the aqueous environment are getting focused into the centre of the protein and by doing this process of folding a protein around a particular centre the protein is trying to conserve the energy as well as it is trying to reduce or make the structure as compact as possible.

And in this process what happened is most of the globular protein are making the ball-like structures and if you compare these balls you could be able to characterize these proteins as well as you can be able to purify it. Let us understand this in a more elaborated example.

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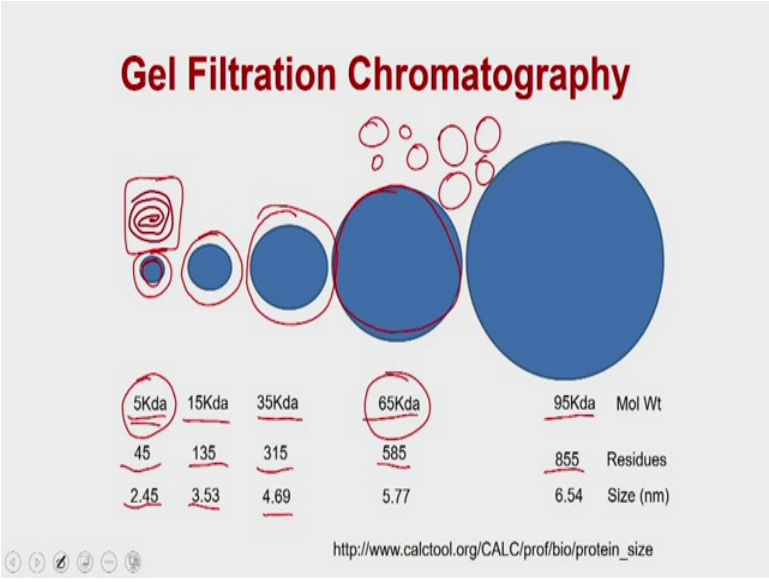

So, now we have synthesized a protein and what you see here is I have given an example of the protein which are of different molecular weight. So, it starts from the 5 kilos Dalton, 15 kilo Dalton, 35 kilo Dalton, 65 kilo Dalton and 95 kilo Dalton. What have I done is I have shown you the number of amino acid residues which are associated with most of these proteins. So, what you see is for the 5 kDa protein you have the number of residues which are 45, for 15 Kda it is 135 and 35, it is 315 and so on.

And these are the rough estimates and now based on this number of residues I have also calculated the size of this or radius of gyration of these proteins. So, what you see is that the protein which is of the 5 kilos Dalton is having a radius of gyration of 2.545 nanometres whereas in the case of 15 kilos Dalton it is a 3.53 and 4.69.

So, what you see here is that as you are increasing the size of the protein it actually increasing its radius of gyration which means as you go from a smaller protein to a larger protein and all this is applicable only for the globular protein that the diameter of that particular ball is increasing which means if you consider that only the globular proteins are present in a complex mixture, you are actually having nothing but a combination of different balls of different sizes which means you have a protein of different sizes or different balls are present in the micro-environment and all these balls can be separated simply by reducing or simply by filtering these protein particles through a different pore sizes, which means if you in a general term what you can do is if you want to separate a protein of 5 kilo Dalton from the 65 kilo Dalton, so you can see that 65 kilo Dalton is of this diameter compared to the 5 kilo Dalton which of this diameter and if you have a mixture of this protein and this protein what you can do is you can simply take the filters of two different diameters.

The diameters which are corresponding to the 5.77 nanometres and the diameter which is corresponding to the 2.45 nanometre and if you use these two folders and if you filter this protein and this protein what will happen is these two proteins are going to be separated from each other and this is what exactly the Gel Filtration is going to do. It is having the funnels of different pore sizes and based on that it is filtering these molecules.

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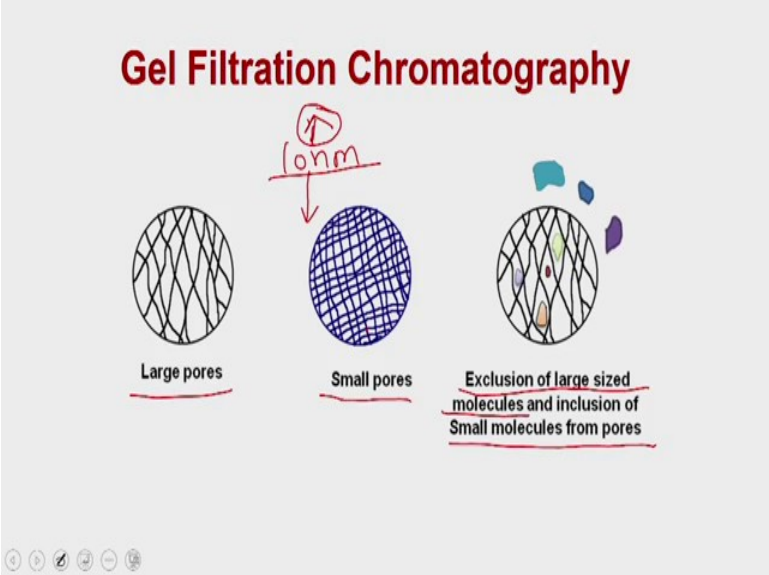

So, in a typical gel filtration what you have is you have the beads which are having the large pore size, you have the beads which are of smaller pore size and in general when you put the molecules through this the large size molecules are going to be excluded whereas the small molecules are going to be included. This is exactly the reverse. In most of the cases when you are doing a filtration the small molecules are getting passed through that diameter whereas the larger molecules are going to be retained.

Whereas in the case of gel filtration chromatography you are going to exclude the larger molecules and you are going to retain the smaller molecule into those pores, that is why the gel filtration chromatography is also called as the reverse sieving techniques or it does not follow the filtration principle. In the normal filtration principle what you have is suppose I am using a filter of 10 nanometres.

So, what that means is that the particles which are lower to this are going to filter out whereas the particles which are larger to this are going to be retained onto the filter. Whereas in this case if you have a bead of 10 nanometres which means you are going to exclude or you going to reject all the molecules which are actually of lager size to 10 nanometres whereas you are going to retain all the molecules which are of the lower size. That is why the Gel Filtration Chromatography follows the reverse principle of sieving.

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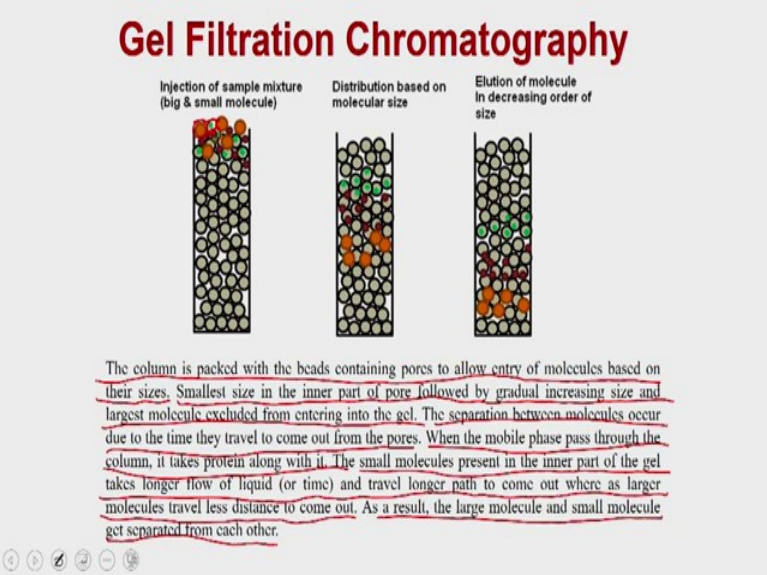

Now, let us understand how the gel filtration chromatography work. So, in this particular chromatography, the column is packed with the beads containing the pores of different sizes and that is going to resolve or going to allow the entry of molecule based on their sizes. So, you can imagine that you have injected a complex mixture of the protein molecules which are of different diameters of like orange, green, red and so on.

So, all these molecules are going to distribute within these beads and once it distributes within the beads because these beads are not the normal beads there these beads are going to have the pores, So, they will going distribute between the space which is present inside the pore and space which is present outside the pore. So, if the molecule is of a smaller diameter it will get filled into those pores whereas the larger molecule is going to be excluded from these pores.

So, the smallest size in the inner part of the pore followed by the gradually increasing size and the largest molecule excluded from entering into that gel. This means here also the molecules are going to be distributed from the outside buffer into the buffer which is present inside the pores. So, it will get exchanged with the buffer which is present inside the pores and the smallest molecule is going to sit at the bottom of the pore whereas the larger molecules or larger molecules are going to sit on top of the small molecules.

And the largest molecule which will not enter into the pore is going to be excluded from these pores and that will remain into the buffer phase. The separation between the molecules occurs due to the time they travel to come out from the pore. When the mobile phase passes

through the column it takes protein along with the smaller molecule present in the inner part of the gel takes longer time to come out and travel longer part to come out whereas the larger molecule travel less distance to come out. As a result, a large molecule and small molecule gets separated from each other.

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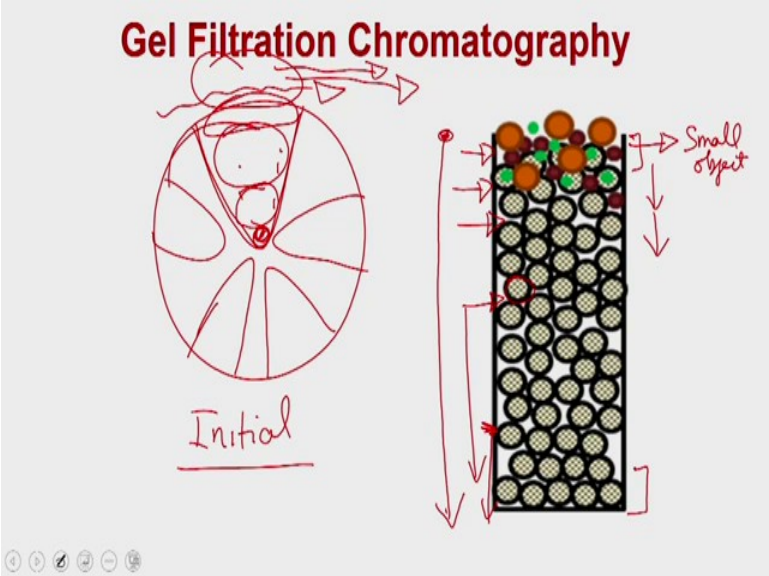

Now, let us understand so what is the kind of pore is present and how the separation is occurring in a gel filtration chromatography. So, if I show you a pore this is the gel filtration bead and the pore is present in a funnel shape pore which is present on these beads. So, you can imagine that you have a funnel shape beads which has a pore-like this from all the sides.

Now, what will happen is while the molecules are flowing on top of these beads, they will be going to enter they will going to replace the buffer what you are falling into these beads and they are going to fill. So, the smallest molecules are going to sit onto the bottom of these beads whereas the molecule which is larger to this is going to sit on top of this and the molecule larger to this is going to sit on top of this and the largest molecule is going to sit on top of this and the molecule which even larger to this is not going to enter into the pore and it will go away along with the buffers.

Now, this is the situation when you have initially injected the molecule and they are passing through the different layer. So, this kind of distribution is going to occur from the first layer to the last layer of the column, which means the first layer is going to have the smallest beads, the smallest objects. The second layer is going to have the objects which are larger to this and the third layer is going to have the objects larger to this.

So, if you understand or if you have a confusion that every bead is going to have the molecules of these molecules arranged in a pore, then that is not true because the dynamics of these molecules are very different. Because of their sizes, the dynamics of the small molecule is going to be very large, that is why when the molecules are going to be distributed from the buffer which is filled within the pore versus the aqueous environment which is present outside, in the first layer when that distribution occurs the objects are going to be filled at the bottom of the pore and by the time the next molecule will try to fill into the same pore where already existing molecules are present, it will get over and flow by the liquid and then it will be getting exchanged into the next layer.

And in the next layer, it is going to sit like this and then the third layer it is going to have this and ultimately the last layer is where the molecules are going to be excluded because they will not enter and that is why they will come out from the aqueous phase and they will going to come out from the column and they will be present in the fourth row.

So, that exchange occurs at every layer and as a result of the throughout the column you are going to have the molecules filled within these beads and that is why you can imagine that this molecule has to travel all the way, this molecule which is filled in the first layer has to travel from first of the column to the last of the column and that is why the smaller molecules are going to travel larger distances or larger volume.

Whereas the larger molecules which are being present within the beads which are very far away from the smaller object will going to travel smaller distances and that is why large molecules come first and small molecule comes later and the largest molecule which will not enter into the beads are probably present somewhere here and that is why they will come out very early on and they may come out within the wide volume.

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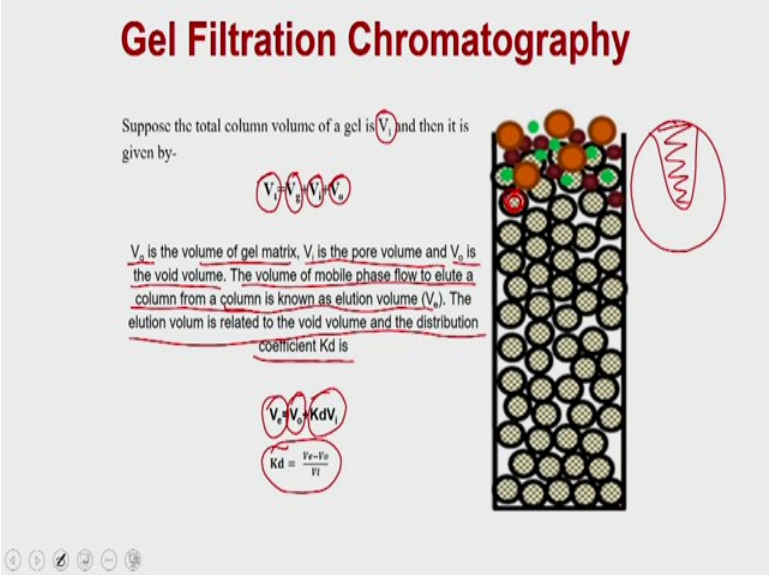

Now, suppose you are preparing a column and the total volume of the gel what you have used is called V t. Then the V t which is the total volume of the column is equivalent to V g plus V i plus V o. What is meant by the V g is the volume of the gel matrix which means the volume of these beads. V i is the pore volume which means the volume of these pores which you are going to, which is going to be taking part into the exchange and V o is the wide volume which is the volume which is being excluded.

So, wide volume means the volume which is being avoided by the molecules and which will not take parts into an exchange or the distribution phenomena. The volume of the mobile phase flow to elute a substance from a column is known as the elution volume or thee. The elution volume is related to the volume and the distribution coefficient K d. So, the elution volume V e is equivalent to V o plus K d V i. So, if you rearrange the K d is equivalent to V e minus V o by V i.

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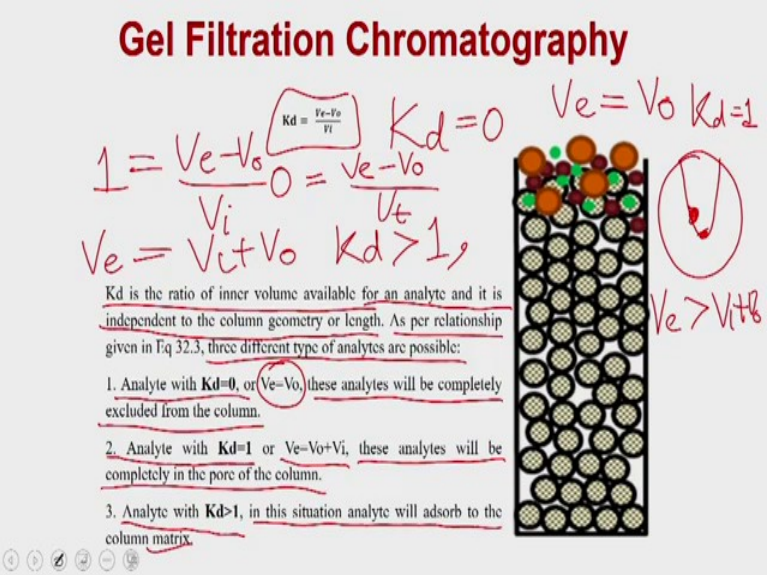

So, that is the formula for K d. So, K d is the ratio of inner volume available for an analyte, is the independent to the column geometry or the length. As per the relationship, three different types of analyses are possible in a gel filtration column. Now, imagine that you have an analyte which has a K d equivalent to 0, K d is 0. If the K d is 0 if you put the0, then what will happen is that V e minus V o divided by V i.

So, if you do the rearrangement what you will see is that V e is equivalent to V o which means that these analytes will be completely excluded from the column. So, if the K d is zero which means the molecule is not entering into the pores of these beads and because of that the V i is going to be zero and as a result, the V e is going to be equivalent to V o which is wide volume. Now, you can imagine that you have an analyte which is having the K d is equivalent to 1.

So, if the K d is 1 then it is V e minus V o divided by V I and if it is V e, so V e is equivalent to V I plus V o. Which means the V e is equivalent to V o plus V I which means these analytes will be completely in the pore of the base which means if you see the beads the molecules which are sitting at the innermost area of the beads is going to give you a K d value which is 1.

Now, if you have a K d value which is the K d is bigger than 1 which means now you can imagine that if the K d is bigger than this if the K d is bigger than 1, what that means is that the V e is going to be bigger than V I plus V o. Which means in those cases in what

conditions the V e is going to be bigger than V I plus V o? Under only one condition that the molecule which you are resolving is irreversibly binding to the beads.

So, one of the things which you have to consider while you are doing the gel filtration is that while you are doing gel filtration, the molecules are only getting exchanged between the pores versus outside area. They are not getting associated with the beads either by the non-coordinate bonds or the coordinate bonds or any kind of interaction, which means even if your protein has a charge the beads are not going to have the association with this molecule because there is a charge present.

So, that is why most of the beads what people use in the gel filtration does not have any kind of affinity or any kind of preferences or any kind of association with the molecules. But if the molecule is going to show you the K d which is bigger than 1 which means the molecule has irreversibly being bound to these beads and in those cases, you can flow the liquids but the molecule will not be going to come out.

Which means what I am saying is if the molecule will go and bind to one of the pores then even if you flow the liquid because we are not going to do any salt gradient or any kind of such kind of treatment. That is why the molecule will remain stuck to these beads and it will not come out. So, that is why it, if the K d is bigger than 1, in this situation analyte, will adsorb onto the column matrix and if that happens then you have to wash this column, you have to trip this particular molecule from the beads and then you have to repack, which means if that happens you have to repack the column and it is going to destroy the column packing.

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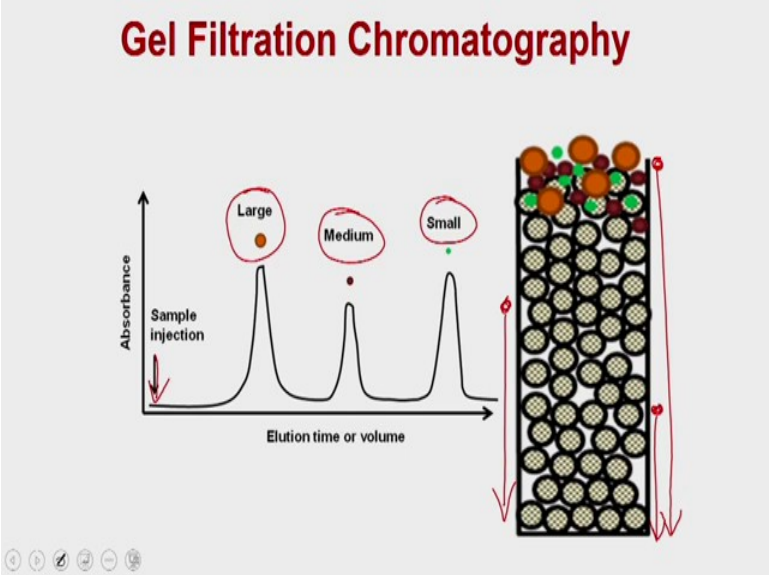

So, this is what we are talking about. If you resolve the molecules of different sizes if you inject the molecule, what you will see is the large-sized molecule will come out first, the medium-sized molecule will come out later and the small-sized molecule or the smallest size of a molecule will come later which means the elution volume for the large is going to be very small.

The elution volume for the medium is going to be in between and the elution volume for the smallest one is going to be the largest and that is simply because the small molecule has to travel from from the top of the column to the bottom. Whereas the medium one has to travel the medium distance and the largest one has to travel the smallest distance, that is why they will come out on a different volume.

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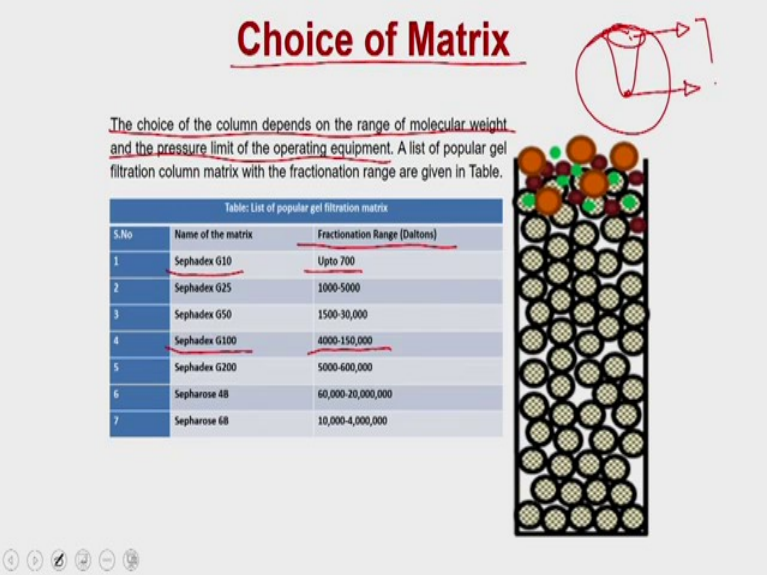

Now, let us see what are the matrix you can use for gel filtration. The choice of the column depends on the range of the molecular weight and the pressure limit of the operating equipment. If you remember when we were discussing the protein purification system we have discussed the different types of pumps what you can use. You can use the low-pressure pumps, you can use the middle-pressure pump and you can use the high-pressure pumps.

Similarly, you can also use molecular weights. So, you should always know what molecular weight is my target molecule, that is why you can use the gel filtration chromatography matrix based on the pressure limit which you are going to use and as well as the molecular weight range which you are going to resolve. For example, if you use the Sephadex G10, so G10 is a matrix which actually can fractionate from small molecule to 700 Dalton.

Whereas in the case of Sephadex G100, so you can see that it actually can resolve from 4 K da to 150 K da. So, what is meant by the fractionation range? The fractionation range means this molecule will be the largest size which this column can resolve and this is the smallest molecule which is this molecule is can resolve. So, that is what is the, that decides the fractionation range which means within this fractionation range the column is going to have the better resolution and it is going to fractionate the different molecules.

But if you take the molecules which are larger to these, for example, if you take a molecule of 180 kilos Dalton that does not mean that 180 kilos Dalton will not go to be resolved. But if you see a separation between 150 versus 180, these two molecules will come together

compared to if you are resolving the 120 and the 150. The difference between 120 and 150 is also 30 kilo Dalton.

The difference between 150 and 180 is also thirty kilos Dalton but because the column has a fractionation range between the 4 K da to 150 K da it will go into resolve 120 kDa to 150 kDa better compared to the from 150 kDa to 180 kDa because the pore size will not allow the 180 K da to be resolved from the 150 kDa. So, both molecule will sit on top of each other and when you elute they both are going to come together.

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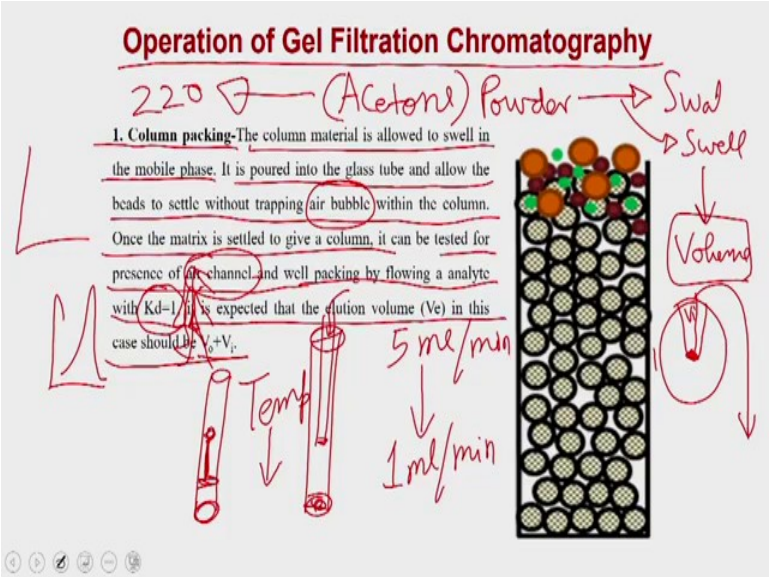

Now, let us see how to do the operation of Gel Filtration Chromatography. So, one of the crucial parameters of the gel filtration chromatography is that the column packing. So, the first event is that you have to do a column packing. So, in the column packing of gel filtration chromatography, you have to be very very careful. For example, the column material what you are going to get from the companies are already are either be present in a liquid phase or they will probably give you the column in a powder.

So, if you are getting a column in the form of powder, then this powder has to be put it into the buffer and then you have to put it in these buffers so that the column will get swelled and so if the column will get swelled, it going to increase its volume and that is how you have to use this particular column. If it is present in the liquid form then mostly the column material is always being stored in 20 per cent alcohol containing the 0.2 per cent aside. In those cases then you have to do at least three to four-time buffer exchange so that they will be no alcohol present in that column.

Now, once your beads are ready either they are being swollen from the powder or you have already got the swollen beads from the companies and you have washed and prepared it. You have to allow the column material to settle down in a mobile phase. So, mostly you have a glass column which has a filter at the bottom and you have a plunger which you can use to pack the column.

So, through this plunger, you are going to pore the material. So, it is going to pour into the glass tube and allow the beads to settle without trapping the air within the column. So, well while you are settling down you are also going to flow the water or flow the buffer through a pump. So, you can use the peristaltic pump or you can use the pump which is present in the protein purification system.

Now, you suppose what will be the relationship between the column packaging flow rate versus the flow rate at which you can operate this column. Now, suppose I am packing this column at 5 ml per minute. Now, if I am packaging this column at 5 ml per minute it is recommended that you should not use this column beyond 1 ml per minute.

Why it is so? Because if you run it beyond 1 ml per minute you probably going to affect the column packaging because if you remember the gel filtration is where the beads are arranged in the column and from the bead number layer number one the molecules are going to start fractionating. In the bead number two, the medium size molecules are going to fractionate and that is how.

So if you change the packaging if you change the amount of the interspatial or inter-bead spaces, you are going to either decrease or increase the number of layers and that is going to affect the overall fractionation of the molecules within that particular column. The other thing is that you should always try to avoid the bubble or any air bubble or any kind of air within the column and that is very important for the chromatography of gel filtration. That is very important if you would like to resolve the molecule on a gel filtration chromatography column.

Now once the matrix is settled down to give a column it can be tested for the presence of air channels and well packing by flowing analyte with K d equal to1. It is expected that the elution volume, in this case, should be V o equal to V i. So, now apart from the air bubble you also have another problem of air channels. What is meant by air channel is suppose you

have packed a column and it is a continuous layer of beads from bottom to top and in between some layer you have a channel of air.

So, how the channel of air is going to disturb the overall purification? What will happen is as soon as the molecule will reach to this particular channel they are not going to fractionate from the pores to the aqueous environment because they have a free movement of within these channels.

So, they will travel within this movement within this channel and they will not going to fractionate between the buffer what you are using versus the pores which are present on the beads and because of that they will be going to mix up with the molecules which are already present in this particular layer and as a result, they will not going to follow the principle of gel filtration chromatography which means there are not going to be get separated nicely and they will get mixed up with the other molecule and as a result, it is overall going to affect the separation or the resolution of this particular column.

Now, the question is, how you can test that there are no air channels? Now, if you would like to test that, you have to pass through a material which is of K d equal to 1 and if you remember, we have said that what is meant by K d equal to 1 is the molecule which is going to sit at the bottom of that particular pore and that should take the amount of time which is equivalent to V o equal to V o plus V I, which means it should travel from the pores which are actually the V I versus it should travel from the top to bottom.

What could this molecule be? The simplest molecule could be the water molecule because the water molecule is the simplest and the smallest molecule which can be present on the bottom of these pores but the problem with the water molecule is that water molecule you cannot monitor until it is (())(33:29) and we are not going to do (())(33:32)experiment just simply by knowing whether there is a channel or whether there is an air bubble present in this column or not.

So, to know this particular aspect you can use some molecule which is of smallest but it should be getting detectable. One such molecule is the acetone. Acetone is an organic molecule. So, its immiscibility within the water is less but it is still can be in a very very small molecule. So, the acetone will sit at the bottom of this particular well and acetone is compared to the water. Acetone absorbs very strongly at the 220 nanometre which is absorbance for the most of the aliphatic compounds. The compound which contains the

carbon-carbon bonds and you can use acetone. So, what you do is you layer the acetone, so the acetone will get filled at the bottom of this particular pore and then it will travel all the way.

So, once the acetone comes it will give you a peak 220 and by knowing the peak you could be able to calculate the elution volume and if the elution volume is equivalent to the V i plus V o which means you will know that the column does not have any channels, the column does not have any pores. That does not mean that it is not going to develop the channel or the pores, air bubbles. For that only most of the gel filtration column has to packed in the same temperature where you are planning to operate these columns.

Which means if you change the temperature of packaging from the running, you know the temperature is going to make the expansion of the liquids or it is going to expand the air which is present within that particular liquid. And if you change the temperature, suppose you go from 4 degrees to 37 degrees, then you are expanding the air which is present in the water in which your column is being packed and because of that this particular expansion of this air which is water-soluble air is going to come out and it is going to create the channel as well as it is going to create the bubbles.

Other than that if you would like to avoid the air bubbles or the channels you also have to use the water which is degassed. What is meant by degassed is that you can reduce the amount of dissolved oxygen and by degassing you can do simply by putting that particular liquid into the vacuum or you can also do the sonication, both are these processes are removing the oxygen from the water.

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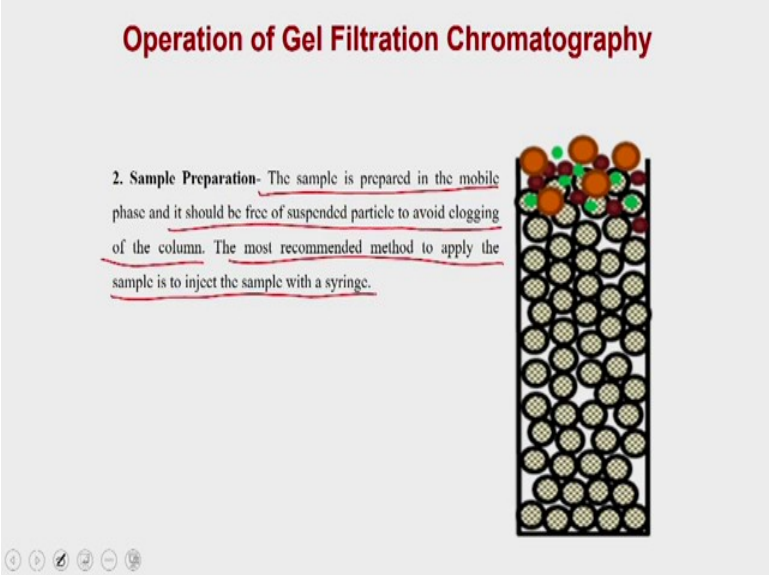

Now, once the packaging is over then you can load the column and you can prepare the sample in your mobile phase. So, whatever the condition of your mobile phase you can use and you have to ensure that it is free of suspended particles so that the column is not going to be clocked. The most recommended method to apply the sample is to inject the sample with a syringe.

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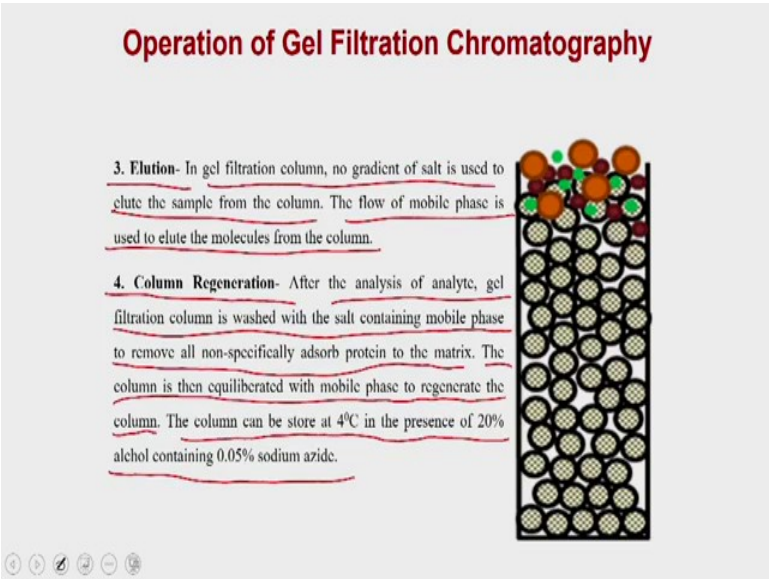

Now, once you have done you can do the elution, so compared to the ion exchange chromatography or the hydrophobic interaction chromatography in gel filtration there is no gradient of salt to use to elute the sample from the column. The flow of the mobile phase is used to elute the molecule from the column. Once you are done with the elution you have to

do regeneration. So, regeneration after that analysis of the analyte gel filtration column is washed with the salt-containing mobile phase to remove all non-specially absorbed protein to the matrix.

The column is then equilibrated with the mobile phase to regenerate the column. The column can be stored at 4 degrees in the presence of 20 per cent alcohol containing 0.5 per cent sodium aside and just like as we discussed for the ion exchange chromatography or hydrophobic interaction chromatography the column is always being stored in 20 per cent alcohol containing 0.5 per cent aside. So, you will not be going to see the growth of bacteria.

So, with these, I would like to conclude our lecture here. Thank you.