Experimental Biotechnology
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Module No # 04
Lecture No # 20
Gel Filtration Chromatography (Part 1)

(Video Starts: 00:24)
(Video Ends: 01:00)

Hello everybody this is Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And in this course so far we were discussing about the chromatography and in the beginning we have discussed about the basics of chromatography and then we discuss about the (()) (01:23) systems how to operate the (()) (01:26) systems? And then previous 2 lectures we have discuss about the ion exchange chromatography followed by the hydrophobic ion exchange chromatography. Now in today's lecture we are going to discuss about the gel filtration chromatography.

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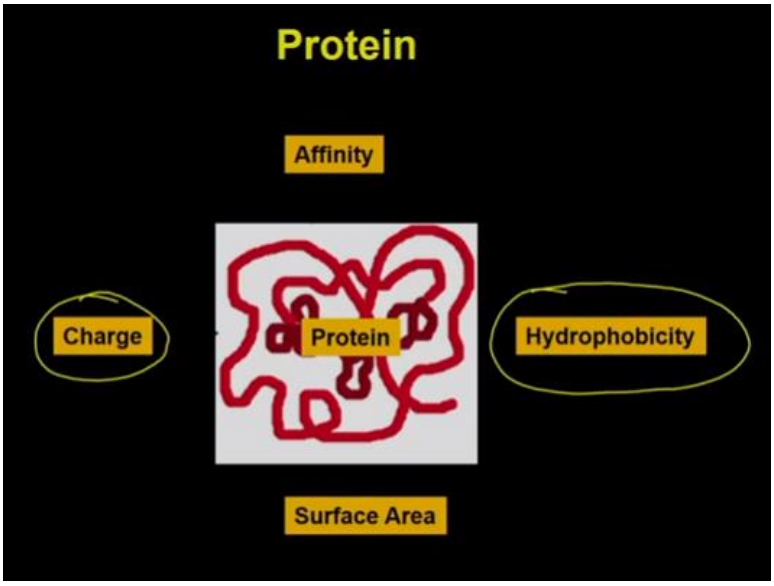

So if you recall we have discuss about this the protein has a multiple properties which can be exploited. So far we have discuss about the how to exploit the charge or the hydrophobicity region present in the protein with the help of the ion exchange chromatography as well as the hydrophobic interaction chromatography.

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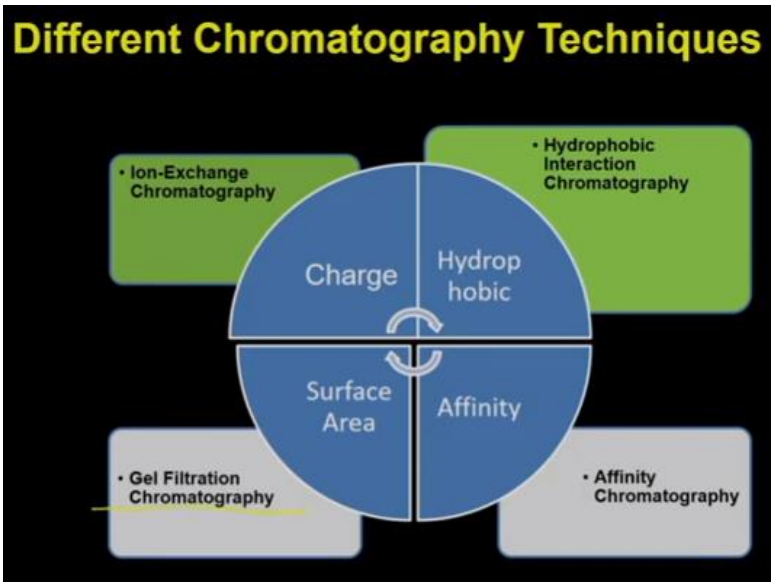

Now today we are going to discuss about the gel filtration chromatography and the gel the basic principle of the gel filtration chromatography is that where the proteins are been separated based on their size. And the in today's lecture we will discuss how we can actually be able to achieve that.

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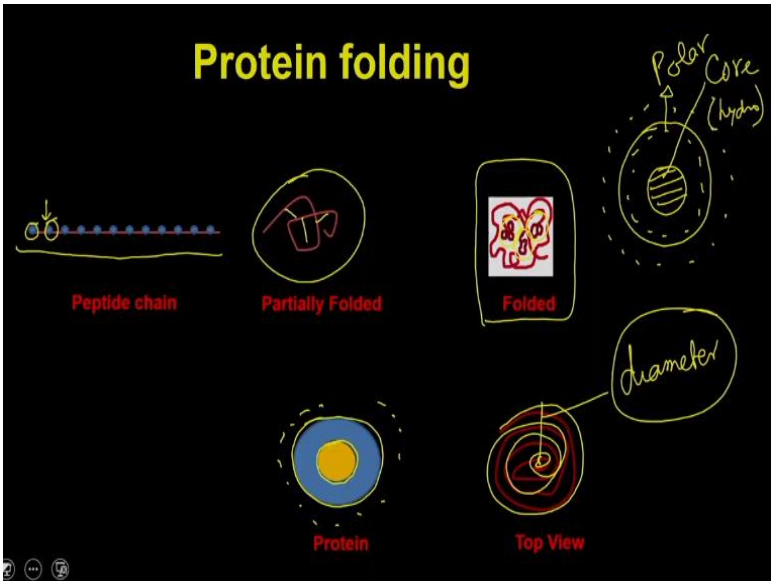

So before getting into the details of the protein gel filtration chromatography it is important to understand how the protein is been synthesized in the cell. And how that synthesized protein gets folded and then it adopts the 3 dimensional confirmations. So what you can see is that the protein is been synthesized as the linear chain of peptide chain where the individual amino acids are coupled with the help of the peptide bond.

And that is how the protein is been synthesized from the ribal zone so as soon as the small stretch of the peptide comes out from the ribal zone. It is starts folding and these foldings are mostly been governed by the intra molecular interaction between the different site chain. So you know that the protein is made up of 20 different types of amino acids and all these 20 different types of amino acids have the different types of side chains.

For example you have the basic amino acids which have the positively charged side groups or you can have the acetic amino acid such as the (()) (03:50) which are actually having the acid has the side chain. And apart from that you have the polar amino acids or the non-polar amino acid and as well as the hydrophobic amino acids. So, all these amino acids are actually contributing different types of interaction.

For example the hydrophobic and hydrophobic amino acids are always been forming a interaction by the pipi interactions or been stabilized the structure by the pipi interaction whereas the positively charged residue and the negatively charged residues are forming a interaction by the either the hydrogen bonding or vendor wall interactions or the small bridges. So all these interactions initially decides are the governed the folding of these proteins and ultimately the protein will go into the properly folded to acquire a 3 dimensional confirmations.

And in the 3 dimensional confirmations what you are going to see is that the hydrophobic residues are the side chains are going to be localized within the center of this protein which is called as the hydrophobic core so this is called as the core of the protein where you are going to have the hydrophobic amino acids. Whereas the periphery is going to have the polar amino acids so this arrangement is happening because, you have the water outside and all the polar amino acids love the water.

So actually you have the hydrophobic core in the center and the hydrophilic periphery on the periphery of the proteins. And mostly the proteins are arranging their amino acid against the central access. So and that is true for most of the globular proteins so if you see a protein from the top what you will see is that it is actually forming a coiled structure like this. And where the center is been done.

So if you measure the cross sections what you will see is that the diameter of this global or protein is in directly or indirectly is going to be proportional to the size or the molecular weight of this particular protein. So this is what we are discussing that you have the hydrophobic core and the hydrophilic periphery and this hydrophilic periphery is having the water outside.

So as we discuss the amino acids are arranged along the access in the globular proteins it is always maintaining a relationship between the diameters of a protein versus the molecular weight. Let us see how it is happens?

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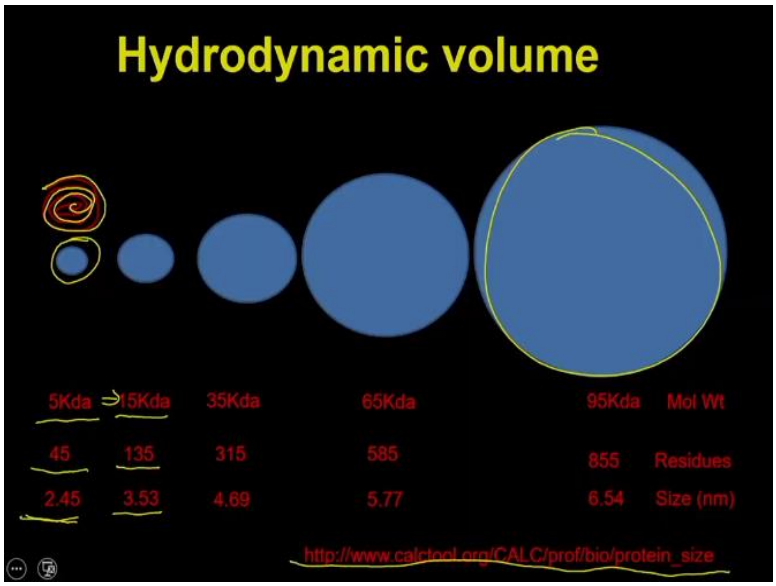

So as we discussed the protein is arranging all the amino acid along a particular axis so as you can see I have shown you the multiple examples. So what you can see is that I have taken a protein of 5 kilo Dalton and the 5 kilo Dalton means it is going to have the amino acids which are 45 in number. And if you calculate the diameter of this protein what you will see is that the diameter of this protein is 2.45, nanometer.

Similarly if I increase the size to the 15 kilo Dalton what you will see is that the number of amino acids will gone up to the 135 and that size is also gone by the 3.53 nanometer. Similarly what you see is that this is a small size protein and this is a large size protein and all these sizes are increasing as you are increasing the molecular weight. Which means for all the globular proteins the size of a protein is indirectly related to its molecular weight.

You can actually follow this link and be able to calculate the size of a particular protein have you out the amino acids sequence you could be able to calculate the size of that particular protein.

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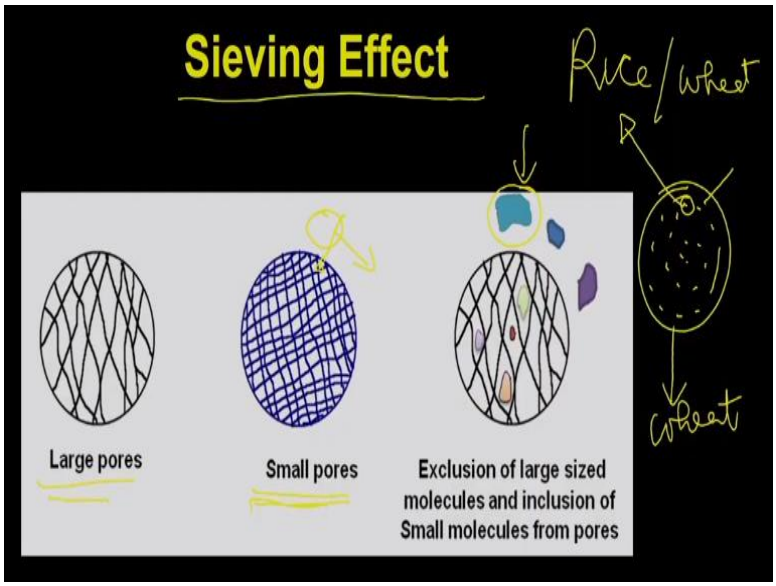

So now what is the how the gel filtration chromatography is work? Is you can imagine that I have the beads of the different sizes and what I am going to do is if I have the beads of the different sizes and if I want to separate them. For example if you have the rice and wheat with you so rice is small in size and wheat is large in size. So what you are going to do is? You are going to take the seeds or you are going to filter through the small pores and in that process what will happen is?

So if I have the picture of rice and the wheat so what I will do is I will take the molecular sieve or I will take a sieve and I will choose the sieve in such a ways that if the diameter is going to be good enough for the rice. So once I will see what will happen is that the wheat will going to be remain on this and the rice will pass through this pores and that is how the rice is going to be collected into the lower chamber and the wheat is going to be collected on the top chamber.

So that is way you are going to separate the 2 molecule which are actually different in the sizes similarly the same principle can be used to distinguish or to purify the different types of proteins. So as you have seen that we have the proteins of the different sizes starting from the 1.53 nanometer to 20, nanometer. So what you can do is you can take a sieve and if you start filtering

the protein through that sieve ultimately what will happen is that the larger molecules are going to be remain on top and the small molecule will pass through.

But in the case of gel filtration so that is what is going to happen so you have the sieve of the large pores or the small pores. So when you pass through the molecules that will happen is that the large molecules are not going to enter into these pores. So compare to the sieving effect you have the reverse sieving effect where the small molecule because in this case the small is passing through the pores.

Whereas the large is retaining on to the sieve where as in this case when you taking a bead which contains the large pores versus the small pores. And when you are passing through the proteins of different sizes the small proteins are entering into this pores whereas the large proteins are been excluded from the pores and that is how you are actually going to separate.

So what will happen is that as soon as that happen the large molecules are going to be comes out from the column first and the small molecules are going to be come later and that will continue, because you are not going to have the pores of one particular diameter you can have the pores of the different diameter. And that is how you can be able to have that sieving effect to see a separation of the molecule based on the sizes.

So let us understand this in a more elaborated way that the once you have the small pores the pores are good enough to retain the small sizes but they are not good enough to retain the large sizes. So what happens is the large sizes are going to be remain is going to be excluded from these beads. So that is how the small large sizes are going to come out first and the small sizes are going to be retained in the beads and they will come on the later size.

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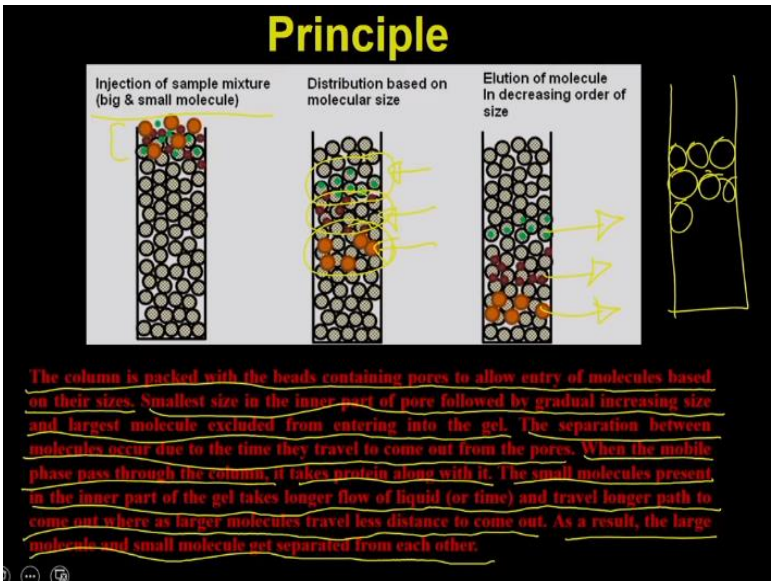

So this is what you will understand so this is the just to explain you the basic principle so in the gel filtration what you have is the column is packed with the beads containing the pores allow the entry of the molecule based on their sizes. A smallest size in the pore inner part of the pore is followed by the gradual increasing size and the largest molecule excluded from entering into the gel. The separation between the molecule; occurs due to the time travel to come out from the pores.

When the mobile phase passes through the column it takes protein along with it the small molecule present in the inner part of the gel takes longer flow of liquid or and travel longer path to come out whereas the larger molecule travel less distance to come out. As a result the large molecules and the small molecule get separated from each other. So suppose so in the gel filtration what you are doing is you are taking a column and you are filling this column with the beads these beads are actually having the pores.

So what will happen is if you are injecting a mixture of the a small as well as the big molecules the small molecules are entering into the beads at the different positions for example in this case you see that the green is been entered into the beads at this position red is entered into the this position and the orange is actually the big enough. So it is been excluded or it is actually entering into a later portion of the column.

So in the beginning the molecules are been present on to the top of the column once they travel throughout the column they get distributed as per their molecular sizes and get filled into the different area of the beads. And then once you flow the mobile phase the proteins comes out will travel throughout the mobile phase and that is how the orange will come first the red will come later on and the green will come to at that end. And that is how all these molecules are going to be separated from each other.

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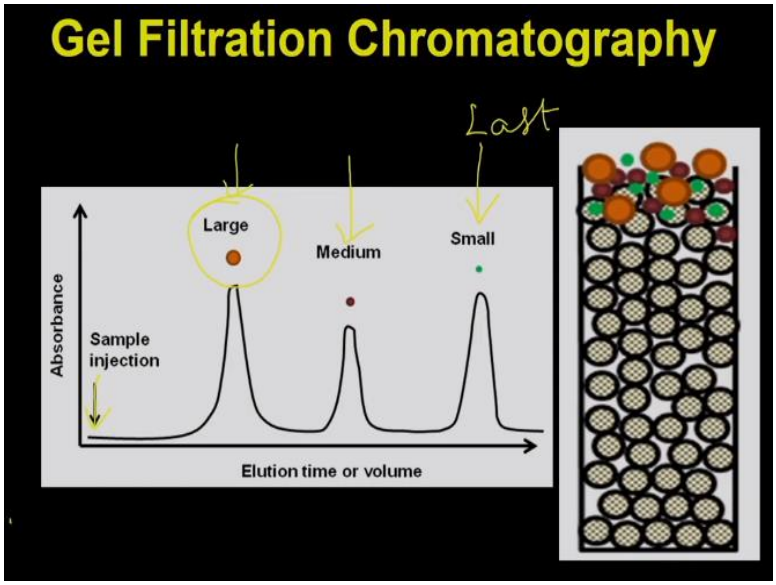

So now to understand this how these separation phenomena works let me show you the typical bead how the beads of a gel filtration chromatography look like. So in a typical gel filtration chromatography the bead is having a cone shape pores from all the sides something like this okay. So this is the portion where is the narrowest diameter and this is the largest diameter. So when you are loading these kinds of beads with the different types of molecules what will happen is?

The molecules are entering into these beads or these sports and the smallest molecule is entering and sitting into the bottom of the pore, then the molecule bigger to this is sitting on top of this the molecule bigger to this is sitting on top of this and the largest molecule is sitting on top of this. Whereas the molecule which is bigger to this is been excluded from the column now when you loading the samples okay this does not occurs in every bead what is present in the column.

Because there is a competition between the different molecules work is been loaded on to the column. So when you load the molecules these molecules are been separated or there is a competitions. So what will happen is see the green is the smallest molecule and the red is the middle size whereas the orange is the largest molecule. So what happen is because the orange is the largest molecule it as it takes more time to travel throughout the column.

And as a result all these molecules are competating for entering into the pores. So what happen is the green is entering into the pore first and capturing the pores or capturing the pores which are present on to the first layer. Whereas the red which is actually of the middle size is competing well compared to the orange one but it is not competating well compared to the green one. So what will happen is the green ones binding to the different set of layers of the pores different set of layers in the column.

But at a different, positions within the pore and ultimately the orange ones are also getting distributed and then they are binding to the different positions within the pore. But not on the same bead which means the green one's are binding into the initial flue layers or the red one's are binding to the subsequent few layers and orange one's are binding into this layer. So as a result what will happen is that running the mobile phase the green, red or orange are getting separated are getting travelled throughout the column.

So what will happen is the green once are travelling from the first layer and up to this okay. So imagine that if this is a column of 25 ml the green one's is probably travelling somewhere are 20ml okay because it is binding into the first layer which is and the red one's which are present in the fourth or third or fourth layer is travelling all the way up to the end and probably travelling like 17ml.

Similarly and the orange one's are binding to the third layer or the very lower size is travelling somewhere around 12 ml. So what will happen is so if you see a chromatogram so this is what exactly happens when you are injecting a sample the orange once which are actually travelling very less because the orange once are only travelling the 12ml's the red ones are travel in the 17ml and the green one are travel in the 20 ml.

So that is how they are actually going to come at a different time points so the large one's which is the orange one's are going to be come first the red one's which are of the middle size are coming at the in the middle of the column and the smallest one's are going to come at the last. And that is how the molecules of the different sizes are going to be separated from each other. So this is the basic principle of the chromatography.

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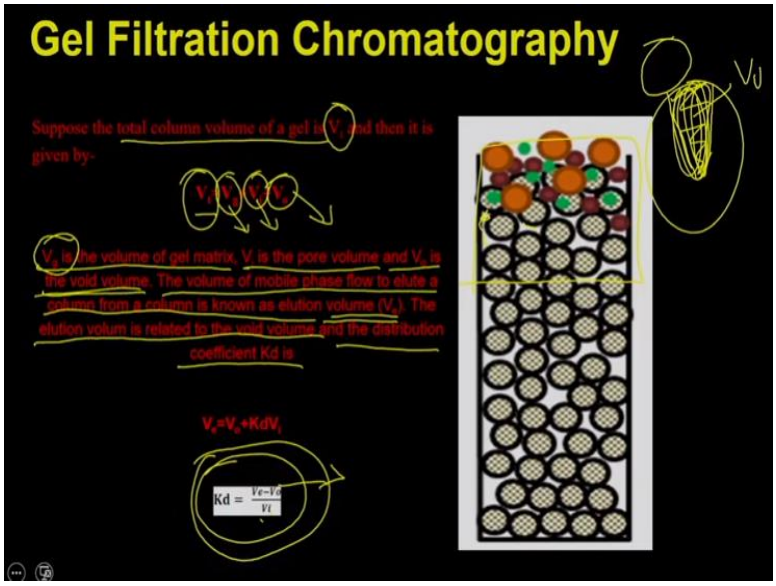

Now if you would like to exploit and utilize the gel filtration chromatography you have to understand the different types of parameters which are associated with gel filtration chromatography. So when you pack a column you are actually going to use the gel material and the total amount of gel what is going to use. Suppose it is the Vt then Vt is equivalent to the Vg, Vi and Vo okay. So Vg what is the Vg?

Vg is the volume of the gel matrix which means suppose you have taken the 10ml of the gel so you have the gel g means the beads what is the beads so this is the volume of the beads okay. Then Vi is the pore volume which means if you have a bead okay the you have the pores right so this are called as the Vi and this beads the volume of the total bead is called as the Vg and the V0 is the wide volume which means the portion of the column which is actually not going to be participate into the fascination.

Which; means if you take this column okay there are some portion of the column which is actually not going to participate into the fascination because this region is not participating into

the fascination. Which means the region which is in present in between the beads so that is called as the wide volume. Now the volume of the mobile phase to elute a analyte from the column is known as the illusion volume or the Ve.

So you have the 4 parameters Vt, Vg, Vi, Vo and Ve and if you recall the Vt is the total column Vg is the volume of the beads or the gel matrix Vi is the inner pore volume. And the Vo is the wide volume and the Ve is the illusion volume. The illusion volume is related to the wide volume and the distribution coefficient Kd. So the relationship is that the Kd is equivalent to Ve – Vo divided by the Vi and you can be able to calculate the Kd value.

So if you still recall we have discuss about the distribution coefficient in our first lecture of the chromatography where we have set that the distribution coefficients actually determines how the molecules are going to be distributed between the 2 phases. So in this case you have 2 phases one is the aqueous phase which is actually outside and other phase is the pores which are present within the these beads.

So this is the Vi so that is why the molecules are going to be distributed between the Vi and the rest of the volume and that proportion or the ratio of that proportion is only known as a the distribution coefficient. And that is how the distribution coefficient is giving in the term of Kd = Ve – Vo divided by the Vi because this is the volume for the outside volume in which the molecule is present versus this is the total pore volume.

So the concentration of the molecule within this pore versus the concentration of the molecule outside is known as the distribution coefficient. And that is very important parameter to characterize or to evaluate any protein or any molecules within the gel filtration chromatography.

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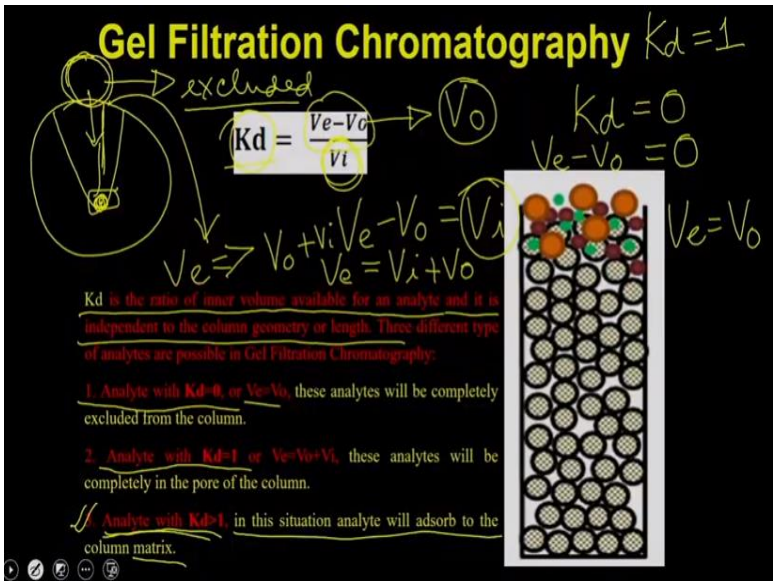

So the Kd is Ve – V0 divided by the Vi and the Kd is the ratio of the inner volume available for an analyte which means the Vi and it is independent to the column geometric or the length because if you increase the column length or if you decrease the column length you are also going to change the Vo. So if you change the Vo it is actually also so the pore volume may not change but the Ve – Vo will going to be proportionally going to be change and that is why the Kd is a ratio of the concentration of the analyte on the outer volume versus the inner volume.

And it is independent of the column geometric or the length now once you know the Kd values the molecules are going to be of 3 different categories depending on the Kd values okay. Now you can have the analytes which have the Kd value equal to 0 which means Kd is 0. So if I put the 0 here what will happen is that Ve – V0 is equivalent to 0 under what conditions you could think that the Ve – Vo could be equivalent to the 0 okay.

So let me draw the pore okay if the Kd is 0 the Ve – V0 is equivalent to 0 which means the Ve is equivalent to Vo which means the illusion volume is equivalent to the wide volume. Which means the molecule is sitting somewhere here it is not entering into the pore which means the molecule is going to be excluded from the column. So if the Kd value is equivalent to 0 Ve – Vo is going to be 0 and in those cases the Ve is going to be equivalent to the Vo which means the molecule is present into the outside to the beads or the not present into the pore.

Which means it is going to be excluded from the pore okay now imagine that you have a analyte which if the having a Kd is equivalent to 1 which means the Kd is equivalent to 1. So if I put the value what will happen is the Ve – Vo is going to be equivalent to Vi so under what condition the Ve – Vo could be the equivalent to Vi which means the Ve is equivalent to Vi + Vo okay. So under what condition you can have the illusion volume which is equivalent to the volume of the pore plus the volume of the wide volume.

Which means the molecule is sitting at the end of the pore which means he has to travel the Vi from top to this and then he has to travel outside as well. Which; means he has to travel the volume which is present within the pore and outside as well. So under what condition the only condition when the analyte is sitting at the end of the pore and it is going to travel all the way okay.

Now you have the third condition where the analyte is having Kd value which is more than 1 which means if the Kd value is more than 1 which means that the Ve is going to be bigger than the Vo + Vi. Which means the molecule is going to be struck into the column which means it is going to be absorbed onto the column and it will not come out from the column even if you flow the volume which is equivalent to the Vo + Vi.

Which means the molecule is permanently is binding to into the column and it is not coming out and that is actually happens when you are doing the ion exchange chromatography or hydrophobic interaction chromatography or affinity chromatography where the Ve is not following this kind of equations. But there the Ve is very much bigger than the 1 column volume or 2 column volumes.

It depends on the, what kind of gradient you are using in ion exchange chromatography or hydrophobic ion exchange chromatography. But in the case of gel filtration chromatography if you are running a column of gel 25ml you are going to see the illusion of each and every molecule by the end of 25ml. If the molecules are not coming out in 25ml this means they are falling into the third category where the Kd value is more than 1.

Which means; the molecules are now binding to the column and you need to you do some harsh treatments so that these molecules will come out from the column. So once if you have the

analyte which is where the Kd value is more than 1 in this situation the analyte will absorb on to the column matrix and that is actually is not a desirable situation as far as the gel filtration is concerned.

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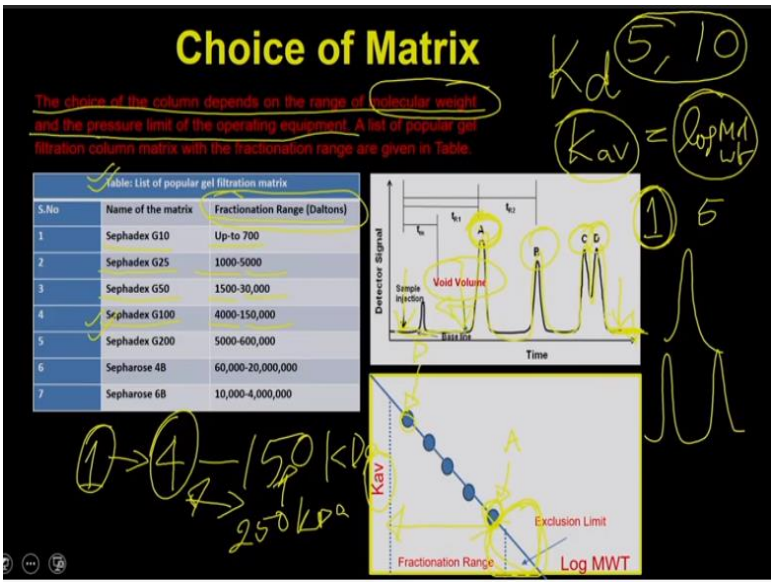

Now once you what like to perform the gel filtration chromatography you have to decide many parameters. So one of the crucial parameter is how to choose the matrix so matrix is the choice of the column depends on the range of the molecular weight and the pressure limit of the operating equipment. Which; means you have to consider 2 parameters one is the molecular weight the range in which you are interested to perform the chromatography or gel filtration chromatography.

And then what kind of pressure limit or what kind of operating chromatography system you have in your laboratory. So these are the some of the popular gel matrix are available where you have this Sephadex G10 the fractionation range for this is up to 700 Dalton whereas the Sephadex G25 which is goes from 1000 to 5000 Daltons. Then you have Sephadex 50 which goes from the 1500 to 30000 Daltons.

So now the question comes what you know by the fractionation range so if you see the illusion profile of the different types of proteins from a gel filtration column what you will see is that you have injected the sample. And then it travels and then you see a baseline thing and then you see

the proteins are been eluted from this column at regular intervals. And ultimately this is the end of the column.

And then what you see is that the place where it is called as the void volume so if you plot and calculate the Kav of this column versus the log molecular weight okay. So if you take the molecular weight of these proteins okay and calculate the distribution coefficient versus the log molecular weight what you will see is that the Kv and the log molecular weight are maintaining a linear relationship.

So this is the protein and this is the last protein what you are fascinating in this column okay so the protein which is the first protein and the last protein which means protein which is coming just after the void volume is the this one right so this is the A protein and this is your D protein okay. Now what you see is that beyond this even if you have something it is not going to be fractionated this means it is not going to be separated for a particular column.

Whereas the protein if you have the protein larger than this size is also not going to be separated because then you are reaching very close to the wide volume. So that is why the region beyond the wide volume or very close to the wide volume is called as the exclusion limit. So any protein which is bigger to this is going to be fall with the exclusion limit and that is how it decides at what range of the protein you can be able fractionate.

In this case you can fractionate from A to D which means this is your fractionation range of this particular column that the is not mean that. For example if you take this Sephadex G100 right the fractionation range is that 4000 to 150000 Dalton. Which means if you have a protein between 4 to 150 Kda that particular protein is going to be separated by the Sephadex G100 that does not mean that you cannot be able to separate the 1 Kda protein using this column.

You can be able to separate the 1 Kda protein but you will not be able to separate 1 Kda from the 4 Kda. So what is mean by the fractionation range is that is a suppose you have the pruprotein which is of the 1 Kda and 5 Kda this particular column is going to give you the single peak like A. Because all these 2 proteins are within the outside the fractionation range so 1 is also outside the fractionation range.

So what will happen is you are going to see a single peak of 1 and 5 whereas if you have 2 proteins which are of 5 and 10 you can be able to see the 2 different peaks because these are the 2 values following within the fractionation range. So what is mean by the fractionation range is that in this particular range the column is going to be efficient enough to separate the molecules efficiently.

Whereas if you go above to that or below to that will still be able to separate but you will not be able to see the 2 individual peaks. Which; means that solution of that column is going to be compromised beyond the fascination range. For example if you take 250 kilo Dalton protein okay 250 kilo Dalton protein is also going to come along with the 150 kilo Dalton protein. Because you cannot go beyond the wide volume that the protein cannot be get eluted before the wide volume. So everything will come into the within the wide volume.

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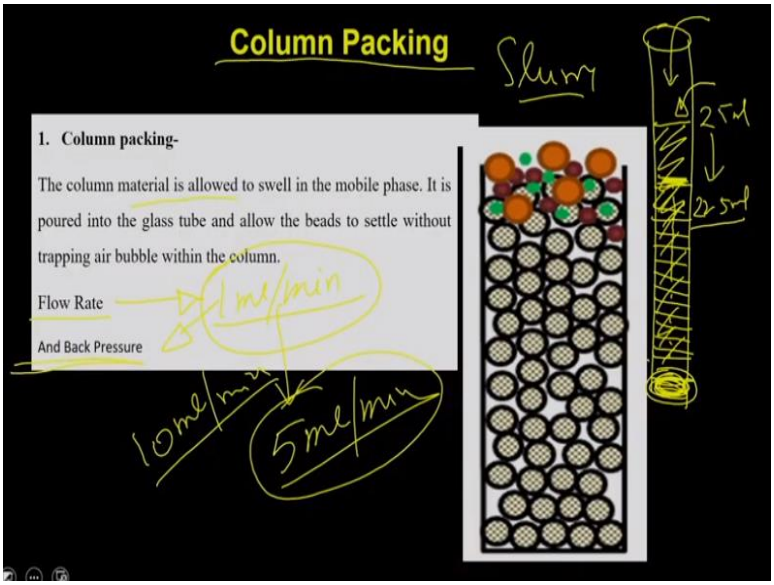

Now how to perform the gel filtration chromatography the first and the most important component is the column package. Because most of the gel filtration chromatography solely depends how well you are packing the column. So in a column packing the column is as a I think we have discussed in a ion exchange chromatography also that when you are getting the column material. Either you are getting 3 (()) (35:27) column material or you are getting the column material as the powder.

Irrespective of the conditions the first thing what you have to do is you have to take the column material and wash it properly and then you allow it to equilibrate into the buffer in which you are going to perform the gel filtration chromatography. And let it be swell for overnight so that the beads are going to take up the water because most of these bead are made up of sugar. So once you keep them in a dried powdered form they loss the water and they do not have so they will not be able to maintain or they will not be able to form the uniform beads.

So you have to allow them to take up the water so that they will swell and they will reach to equilibrium. Once you they will swell then you actually what you will do is you can take a cylindering tube or you can take the column as well then you first you clock the column with the help of the cotton or you can use the glass wool. Then what you have to do is you have to fill the tube 50% with the water okay and then you start pouring the column material which is present in the form of slurry.

So once you pour the slurry will going to settle here and then starts falling into this and then will start settling down from the bottom and that is how it is going to start forming the continuous column like this where you all the beads are going to be arranged okay. And this arrangement has to be homogenous they should be no breaking or they should be no discontinuity because if there will be discontinue to it is actually going to hamper the running of the samples.

Now ultimately what will happen is the whole column is going to be filled with these beads and your gel filtration column is going to be ready. Now if you would like to back the columns you have to take consider multiple parameters. For example the flow rate because the flow rate at which the maximally you would like to perform or you would like to run the gel filtration chromatography. The basic principle is very simple suppose I want to run this column at 1ml per minute okay.

Then what I will do is I will pack this column as 5ml per minute flow rate okay so if I connect this column to peristaltic pump or to any pump so that the flow of liquid is there. I will do the packing on 5ml per minute and then I can be able to operate this column at 1ml per minute maximally because that is the pressure or the back pressure this column can be able to withstand.

Why it is so? It is so because you do not want to disturb the packing once the packing is settled or once the material is settled it is going to form a continuous column or continuous chamber.

You do not want to disturb that chamber simply because if you increase or decrease the flow rate it is actually going to bring the air bubbles or it is actually going to disturb the packing. For example if I am going to start running this column at 10ml per minute which is actually recommended to run it at 1ml per minute okay. This means you are actually applying lot of pressure on to this column okay.

So what will happen? The column is of 25ml okay as soon as you apply lot of pressure this column will turn into 22.5ml which means 2.5ml beads are going to be compressed okay. Ultimately what is going to affect it is actually going to reduce the fascination range because earlier you were having the 1000 beads or the 1000 plates within this column and now since you compress this column you are actually changing the pattern.

So you are actually going to change all the parameter for example you are going to change the wide volume once you are going to change all other parameters. So, your relationship between the distribution co-efficient versus log molecular weight. For the previous column whatever we have done will not going to hold for this column as well. Apart from that you also have to consider what kind of back pressure you are interested because if you are running a low pressure or the middle pressure column or if you are using a low pressure or the middle pressure chromatography systems you cannot pack the column at a very high flow rate.

Otherwise that particular column will not be able to use by your chromatography system so that is also one of the, another you have to consider okay. So with this we would like to conclude our lecture here and in the subsequent lecture we are going to take you to my lab for showing you a demo how to pack the gel filtration column. And what are the different precautions you have to take when you are doing the packing and as well as what are the things you have to consider.

So that you will be able to utilize the gel filtration chromatography in your laboratory very successfully, to complete your experiments thank you.