Enzyme Science and Technology
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Module - V
Enzyme Production (Part 3: Purification)
Lecture - 26
Chromatography (Part-II)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing? We were discussing about different properties of the enzyme. And in this context, in the current module, we are discussing about how you can be able to purify the over expressed protein or enzyme in a particular cell.

And in this context so far what we have discussed? We have discussed about the disruption of the host cells. So, that you can be able to release the enzymes into the solution and subsequent to that, we have also discussed about the you know the ion exchange chromatography and as well as the hydrophobic interaction chromatic chromatography. So, that you can be able to use them for the protein production.

Now, since the enzyme is providing the various types of opportunities, right. You can as you can see that we have discussed about that the enzymes are also having the surface you know the so surface area. So, that surface area also can be utilized for the separation of the proteins and as well as the enzymes are having the exclusive features and that can also be exploited into the affinity chromatography.

So, in today's lecture, we are going to discuss about the how you can be able to exploit the surface area of a protein in terms of the gel filtration chromatography and how you can be able to use the affinity chromatography to purify the proteins.

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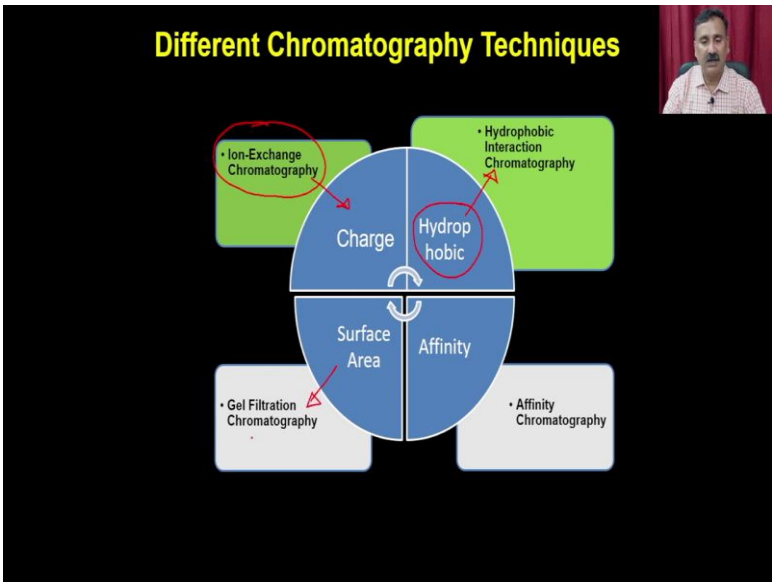

So, far what we have discussed, we have discussed about the ion exchange chromatography which is actually going to exploit the presence of the charge onto the protein structures. And then we also discussed how the hydrophobic patches what are present on the protein can be exploited in the hydrophobic interaction chromatography.

Now, in today's lecture, we are going to start the discussion about the Gel Filtration Chromatography. Now, before we discuss about the gel filtration chromatography, we have to discuss or we have to understand about the protein folding process.

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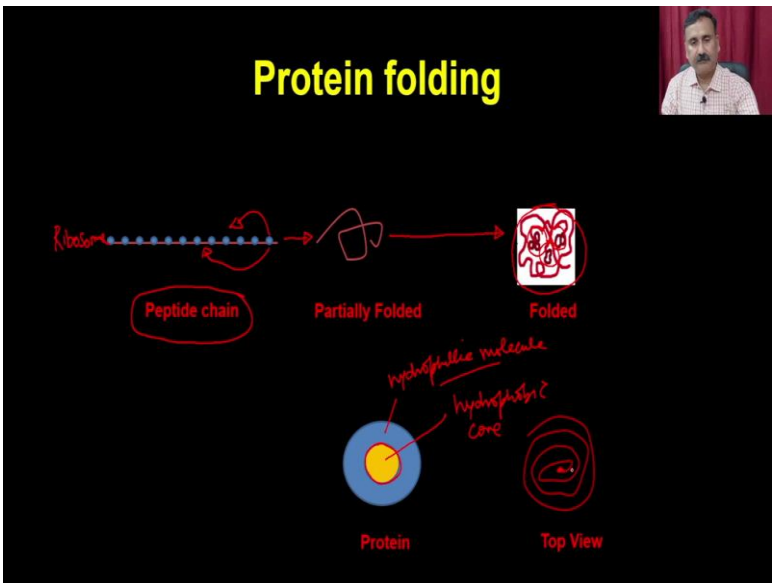

So, you know that the proteins are actually going to be produced from the ribosome. So, you can imagine that we have a ribosome on this side and from this side; the protein chain is going to come out.

So, it is actually going to be formed in a somehow in a form of see peptide chain. And once the peptide chain is going to come out from the ribosome, it is going to be get folded according to the interaction between the different types of amino acids. And based on these molecular interactions amino acids are first going to be partially folded and then this partially folded protein is actually going to acquire the additional interactions and that is how it is actually going to be completely folded.

And what you see is in this particular folded protein that it has arranged the hydrophobic residues in the center of the protein and as well as the hydrophilic residues outside, ok. So, if you see this protein from the top right, what you will see is that around the particular center the amino acids are being arranged. And that is how it is actually all the globular proteins are actually going to form the balls of the different sizes.

And a in and in a each ball what you will see is that the hydrophobic core is going to be present in the center. So, this is actually the hydrophobic core hydrophobic core which is surrounded by the hydrophilic molecules, ok. And as I said you know the when you are actually having the gel filtration when you are going to see the arrangement of amino acid around the particular center, the proteins are actually going to form the balls of the different diameters or the RG values.

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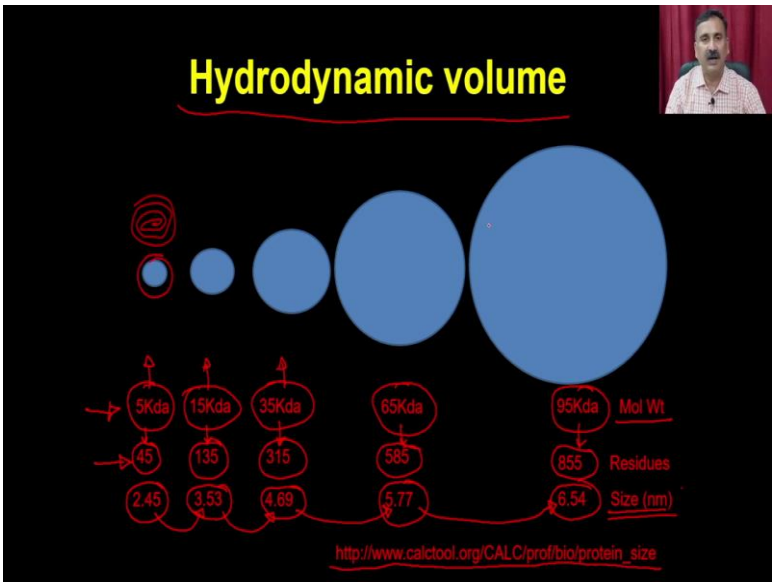

So, if I show you how the sizes are actually going to vary. So, I have taken a examples of the different proteins like this. So, I have taken a examples of 5 Kda molecules 15 Kda, 35 Kda, 65 Kda, 95 Kda. So, these are the molecular weight I have taken. Now, what you see here is that these are the number of residues.

So, in a 5 Kda protein you can have the 45 residues, in 15 Kda you have the 135 residues, in 35 Kda you have 315 residues, 55 Kda 585 and for 95 it is going to be 855 residues which means these many number of amino acids.

Now, what you see is that the radius of radius of these proteins or these balls are 2.45 nanometer in the case of the 5 Kda proteins, 3.53 in the case of the 15 Kda proteins and 4.69 nanometers in the case of the 35 Kda and 5.77 and as well as 6.54 in the case of the 65 and 95 Kda respectively.

What you see in this and all these calculations are being based on the tool what you can use to calculate the size of a protein. So, if you see if you put amino acid sequence in this particular tool or if you put the molecular weight of that protein, it is actually going to give you the sizes of the protein which means it is actually going to give you the size of that particular ball, ok.

So, what you see is that it is not proportional it is not like 5 Kda versus 15 Kda. So, size is actually going to be 3 times. So, it is not 3 times, but it will definitely going to be

increased in a particular ratio, ok. And that is how you can actually be able to exploit this feature in a chromatography which is called as the gel filtration chromatography.

Because as the size will go up it is actually going to increase the hydrodynamic volume and that actually is going to be exploited because this ball is going to be very different from this ball. So, it they can be actually be separated with the help of the gel filtration chromatography. How the gel filtration chromatography is going to separate a small ball from the large ball if we call it is going to have the sieving effects.

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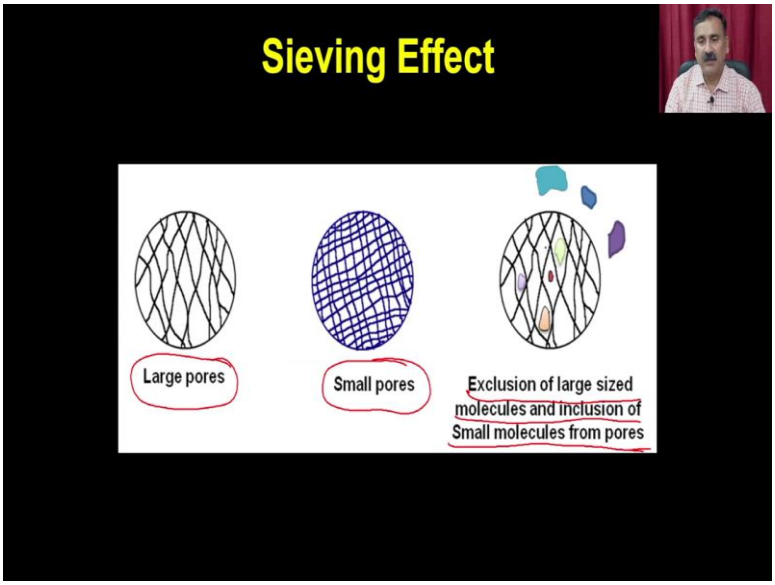

So, what is sieving effects? Sieving effect is that you are going to have the beads which are actually going to have the pores and you can have the large pores or you can actually have the small pores. So, when the molecules are going to be loaded onto the column what will happen is that either they will enter into the pore or they are actually going to be excluded from the pore.

So, exclusion of the large size molecule and inclusion of the small molecule molecules. So, the pore is actually going to be result into the separation of these molecules onto the with the help of the sieving effect which means it is actually going to filter out the molecules in a reverse orientations.

Mostly when you do the sieving it is actually going to retain the large molecule and its going to filter out the small molecule. But here you are going to have the reverse sieving

effect where it is actually going to exclude the large molecule, but it is actually going to retain the small molecules.

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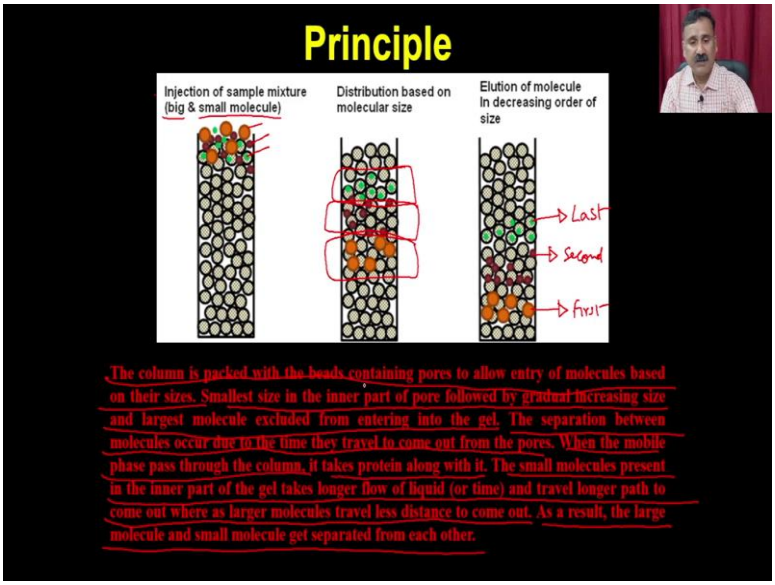

Now, what you see here is that in a gel filtration chromatography column what we have done is we have various type of molecules. So, we have a big molecule and a small molecules. And so, this is the big molecule and this is the middle size molecule and this is the small size molecule, ok. And what we have done is we have loaded a column and we have prepared a column and then we have loaded all these three molecules.

So, what will happen is that all these three molecules are actually going to complete for the pores what is present in these beads. And as a result, they are actually going to be that distributed and as I said you know large molecules are going to be excluded from the column; whereas, the middle size molecules are going to be separated from the small molecules.

So, what you see here is that in this particular region the small molecules are sitting whereas, the large molecules are sitting in this region and the large and the largest molecules are sitting in this. So, as a result what will happen is that the largest molecules are actually going to be eluted first, the middle size molecules are going to be eluted second and the very small molecules are going to be separated last.

And this is actually the principle of gel filtration chromatography. That it is actually going to distribute the molecule based on their sizes and as a result of that it is going to first elutes the large molecules then it is actually going to elute a small molecules. The column is packed with a beads containing pores to allow entry of the molecule based on their sizes.

The smallest size is the inner part of the pore followed by the gradual increasing size and large molecule excluded from entering into the gel. The separation between the molecule occur due to the time they travel to come out from the pores. When the mobile phase passes through the column it takes protein along with it.

The small molecules present in the inner part of the gel takes longer flow of liquid and travel longer path to come out whereas, larger molecule travel less distance to come out. As a result the large molecule and small molecule will get separated from each other.

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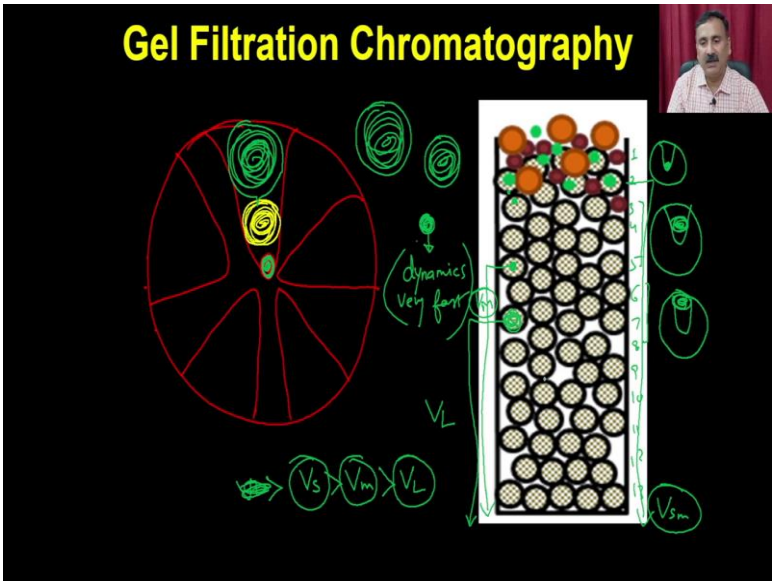

To understand this better we have I hold I am going to show you a bead, how the bead is look like. So, in a small bead you want to have is you are actually going to have the pores like this. So, these are the pores on the gel filtration beads. These pores can be different types. So, you can actually be able to have the flexibility of choosing the you know the beads of a different sizes.

So, what will happen is that in this pore the small size particles are actually going to sit at the bottom of this bead, ok. The small this is a small size right, and the middle size particles are going to sit in the middle of this particular beads, right. So, they are going to sit in the middle of this and whereas, the large particle are actually going to be excluded or they will be sitting at the top of this particular beads, ok.

So, they are going to sit on top of this. So, what happen is that when you are loading a mixture the mixture is actually competing with each other to getting filled into these funnels, ok. So, it if the you so, you have the multiple rows, right. For example, you have 1 row, 2nd row, 3rd row, 4th row, 5th row, 6th, 7, 8, 9, 10, 11, 12, 13. So, you have thirteen row of beads.

So, what happen is in the first row of the beads all the molecules are actually going to compete. So, you have three molecules, you have the large molecules, you have the middle size molecules and you also have the small size molecules. Now, when these three molecules are competing with each other they are actually you know they should run fast right, and you know that the small size molecule is very small.

So, it is actually and it also lighter in terms of the particle. So, it is actually the dynamics of this molecule is going to be very fast, right. So, it is actually going to run very fast, right. So, it is like that if you want to enter into a cinema hall to capture the seats in your room, right.

The smallest size part boy is actually going to enter and it will actually going to run very fast and that is how he is actually going to be able to capture the seats in the first row, ok. So, this is what exactly going to happen. In the first guy the smallest guy is actually going to go and capture the initial rows, right. And that is how it is actually going to filled into this particulars inner part of the cavity, ok.

So, you can imagine that if I have this the inner part of the cavity the smallest particle is going to sit. Now, by the time these middle size particle and the large size particle are actually going to try to sit in the same funnel or same bead then actually going to pushed out, ok. And that is how they will actually going to sit in the layer number 3, 4 or 5.

So, in this layer the middle size particle because now the smaller size particles are already been exhausted. So, what will happen is that the middle size particles are actually

going to sit and in the 3, 4, 5, right. Similarly, in the 6 and 7 you are going to have the large size particle which is actually going to sit at the top of this particular bead.

So, now what you have is you have the large size particle sitting here, middle size particle sitting here and the small size particle sitting in the top two layers, ok. Now, what will happen is the amount of the length what these molecules are going to run. So, for the large one it is actually going to run this.

So, this is for the V L which is for the large size particle. For the middle one it is actually has to run this much, right. So, it is actually going to be the V M or middle one, illusion volume for the middle one and the for the smallest one it has to run all the way from here to here.

This means and this is going to be the small one, ok. So, this means. So, V S is actually going to be bigger than V M and V M is going to be bigger than the V L which means this is going to be the smallest, this is going to be the middle size and this is going to be the smallest one. Which means if I if I will show you how they will be getting eluted onto the chromatogram it will be like this.

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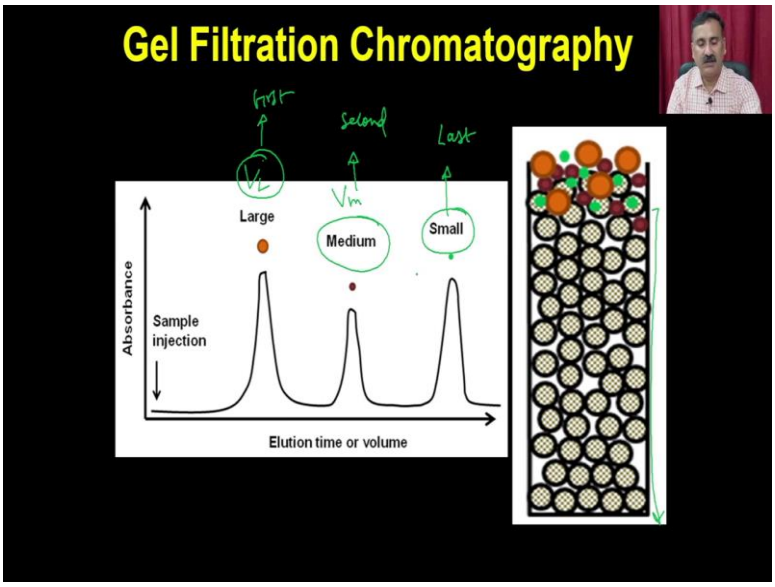

So, we remember that the V L is smallest. So, this means the V L is actually going to be eluted. First middle size the V M is actually going to be middle size. So, it is actually going to be eluted second and the small one is actually going to have to travel from all

the way from here to here and that is why it is actually going to take the largest distance, right.

So, it has to be largest. So, it has going to be the last particle which is going to be come out from the column. Using these information's you can be able to calculate the distribution coefficient.

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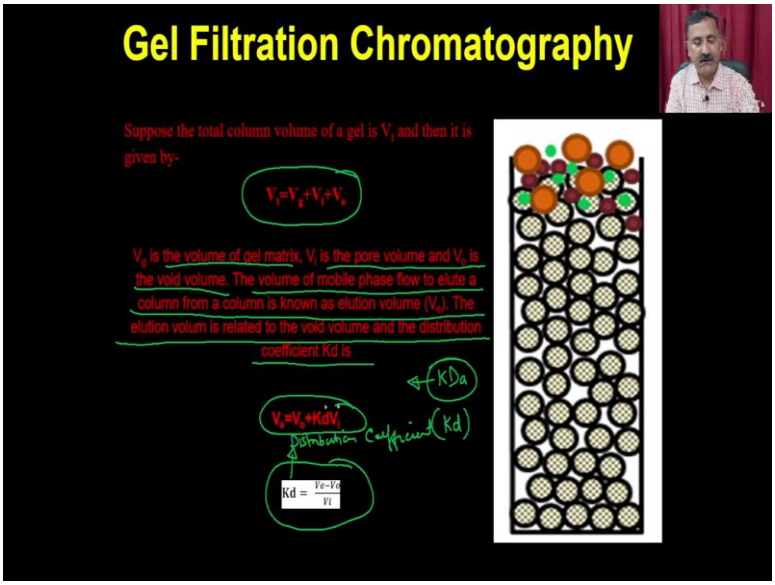

So, for the calculating of the distribution coefficient what you have to do is you have to you have to determine the some of the parameters. For example, you have to determine the V t which is the total volume of the gel then you also have to calculate the wide volume and you also have to calculate the illusion volume.

And utilizing these informations you can be able to calculate the Kd values which is actually the V e minus V o divided by V I. And using these information you can be able to calculate the distribution coefficients, ok. Many time the students get confused between the Kd what we use normally for as a unit for the molecular weight of the protein versus the coefficient, right.

So, it is actually a coefficient the distribution coefficient, ok. In the case of the you know in in the case of the KDa for the protein it is always been small K followed by the capital D and a whereas, in this case it is actually Kd. So, this is different from the KDa what you are using for the protein for the protein molecular weight.

So, Vg is the total volume of the gel, V I is the pore volume and V o is the void volume. So, volume of mobile phase flow to elute a column from a column is known as elution volume. The elution volume is related to the void volume and the distribution coefficient as the V e is V o plus Kd V I.

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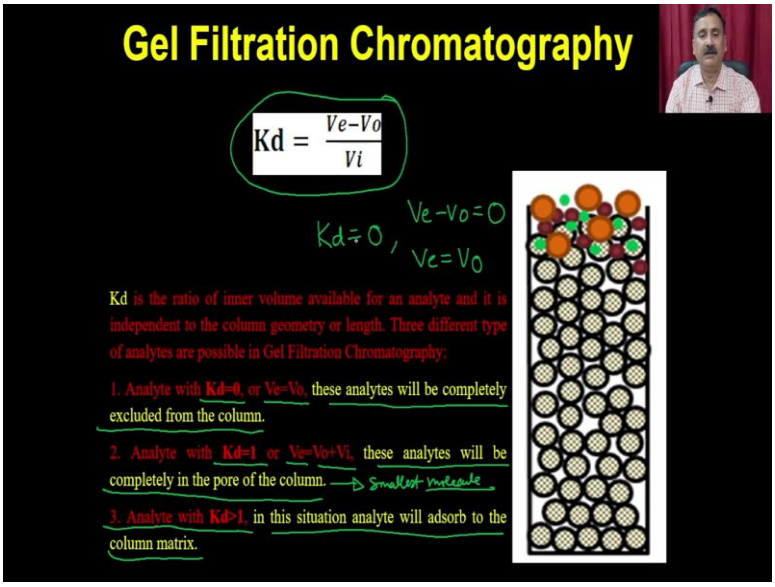

So, using this you can be able to calculate the distribution coefficients and depending on the distribution coefficient the molecules could be of the analytes could be of three types. Either you can have the Kd is equal to 0. So, this means the Kd is equal to 0, right. If the Kd is equal to 0 which means V e minus V o is actually going to be also 0, right. So, this and which is means the V e is actually equal to V o, right.

This means the V e is actual V o and these analytes will be completely excluded from the column which means if the Kd is 0 the V e is equal to V o and these analytes will be completely excluded from the column. Similarly, if you have the Kd is equal to 1 which means the V e is equal to V o plus V I and these analytes will be completely in the pore of the column which means these are the smallest molecules or smallest analyte which you can be able to analyze in this particular column.

And now if you have the Kd which is above to 1 in this situation the analyte will absorb on to the column matrix. Now, you can use the different types of matrix for the gel filtration chromatography, right.

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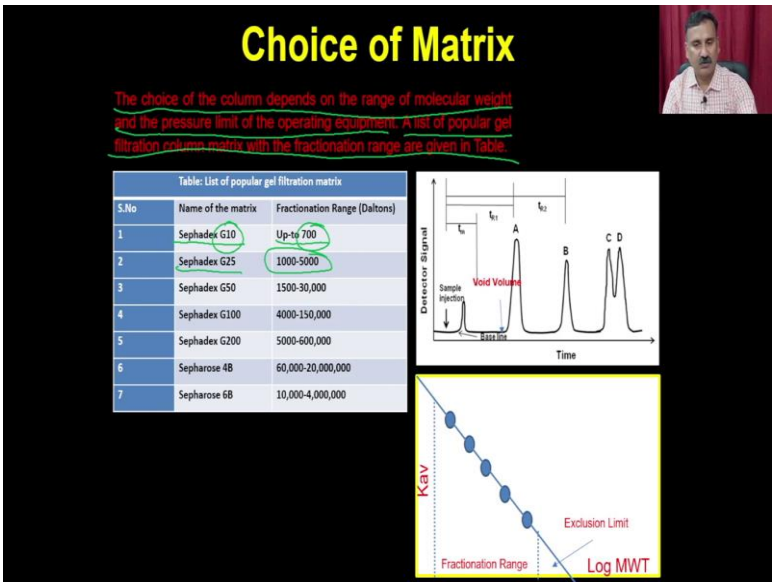

So, the choice of the column matrix depends on the range of the molecular weight and the pressure limit of the operating equipment a list of the popular filtration is a is given in the table, ok.

So, you have the gel column matrix and you also have this fractionation range. So, for example, G10 the fractionation range is 0 to 700 Daltons then you have G25 which has fractionation range between the 1000 to 5000 and so on. And you can actually be able to use the fractionation range as a criteria to choose the matrix of your choice.

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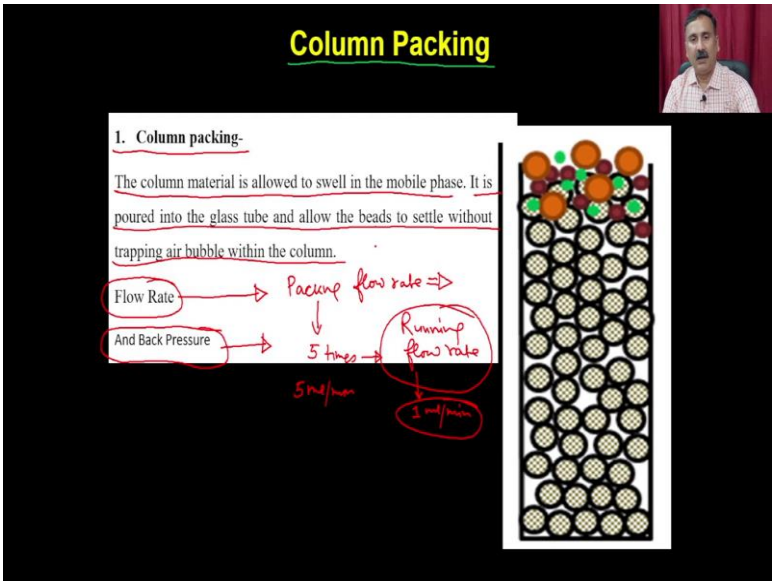

Now, if I have to run a gel filtration chromatography, I have to pack the column, right. So, column packing is where you are actually going to you have to do lot of precautions. So, first is you have to first prepare the column material. So, you are going to take the powder you have to dissolve it into the buffer of your choice where you are planning to pack the column.

And then the column material is allowed to swell in the mobile phase. It is poured into the glass tube and allow the beads to settle without trapping the air bubble within the column. You have to always consider two parameters. One is the flow rate at which you are going to pack the column.

So, the simple formula for packing the flow rate is that you are going to have two flow rates. One is the packing flow rates and the other one is called as the running flow rates, ok. This means this is the; this is the flow rate on which you are actually going to run the column, right. So. So, from the general understanding is that the packing flow rate is going to be 5 times to the running flow rate, ok.

This means if I am packing a column at 5 ml per minute, I cannot run this column more than 1 ml per minute. Because if you run it for more than that it is actually going to disturb the packing of the column. And then you also have to see the back pressure. So, back pressure also should be compatible with the protein purification system what you are using.

And we have prepared a small demo clip. So, that you can be able to use and understand how you can be able to pack a column and how you can be able to run the gel filtration chromatography.

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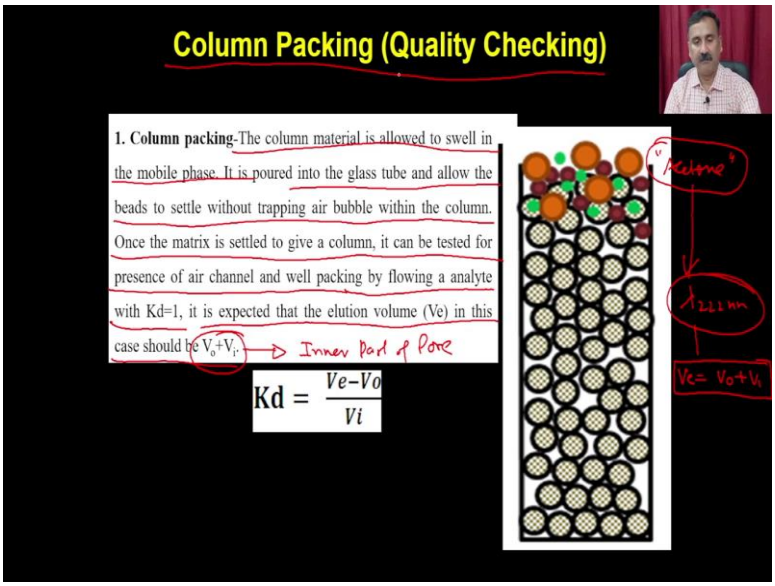

So, once you pack the column you also have to do a quality checking, right. So, the column material is allowed to swell in the mobile phase. It is poured into the glass tube and allowed the beads to settle down without trapping the air bubble within the column.

Once the matrix is settled down and column it can be tested for the presence of air channels and well packing by follow a analyte with a Kd is equal to 1. It is expected that the elution volume in this case should be V e plus V o which means it is going to be on the inner part of the inner part of the pores, right. So, what can be the smallest molecule? So, what people do is they always try to analyze the acetone, ok.

So, acetone is not it is mixable in the water, but it can be detected by taking an absorber side 220 nanometers. So, you can actually be able to know when the acetone is coming out from the column. So, if you analyze the acetone, it is actually going to take the its elution volume is equal to the V o plus V i, ok.

This means it is actually going to give you if it is less than that right, if the elution volume is different from this value, then you will say that there is a air bubbles or there are air channels which are actually being utilized by the acetone to travel faster and that is how it is actually going to tell you that whether the column packing is good or not.

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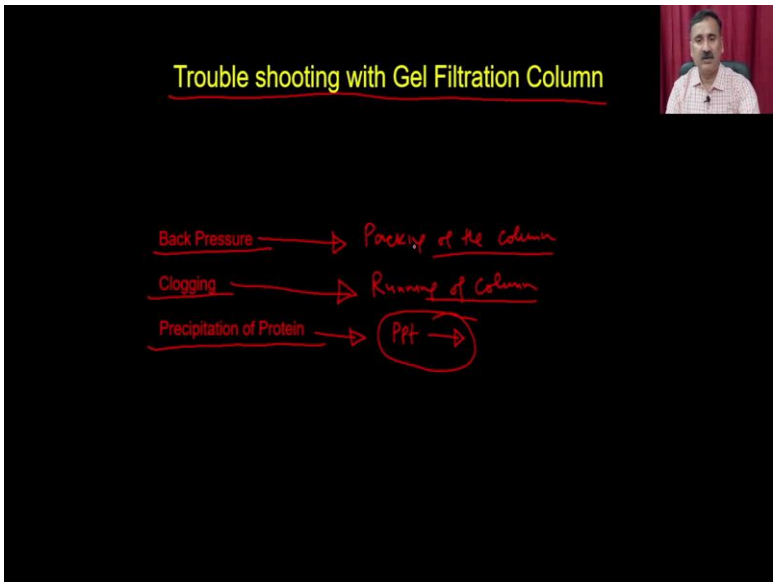

There are so many troubleshooting what you can do with gel filtration chromatography you can actually be able to do the back pressure. You can see the clogging and you also have to see the precipitation. So, back pressure is very important that you should not have you know build the back pressure into the gel filtration column. Because otherwise it is actually going to disturb the packing of the column.

And clogging is also very important because sometime what you do is when you do not filter the protein then you load it onto the column it is actually going to clog the column and then it is actually going to have a problem in terms of providing the back pressure and also the running of the column.

And there are so many things what you can do if you want to avoid that you can actually filter the pure sample and then only you inject and in some time, what happen is that there the protein is actually getting precipitated, right. So, protein is in solution, but when you load this protein onto the column it get precipitated.

So, precipitated protein is actually going to be eluted and it is going to be you know it is going to be contaminate your column. So, in those cases you have to either you have two choices either you wash the column with a harsh you know high salt concentration protein as high salt concentration buffer or urea containing buffer. So, that the precipitated protein is going to be removed or you have to dislodge the column wash the beads and then you can use the beads for repackings.

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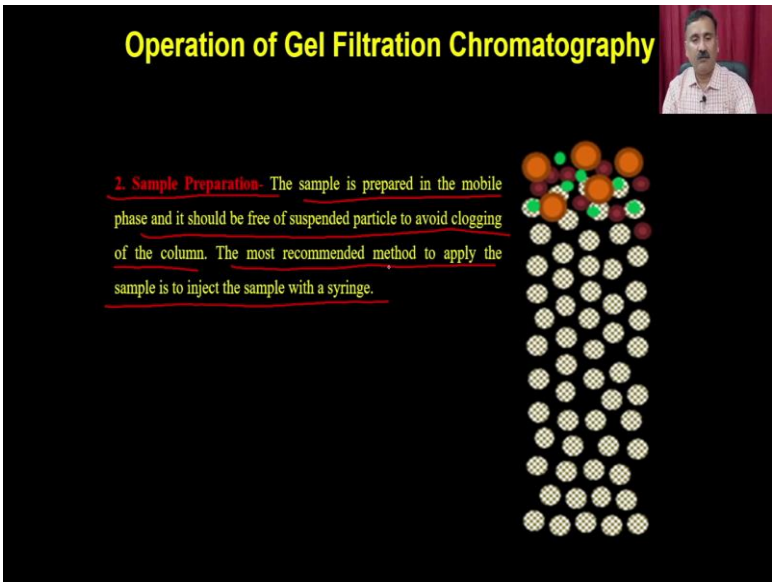

So, how you can run the gel filtration chromatography? So, you can actually first have to prepare the sample, the sample is prepared. So, you first you have to pack a column right, and in the step 2 you are going to prepare the sample. So, sample is prepared in the mobile phase and it should be free of suspended particle to avoid the clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

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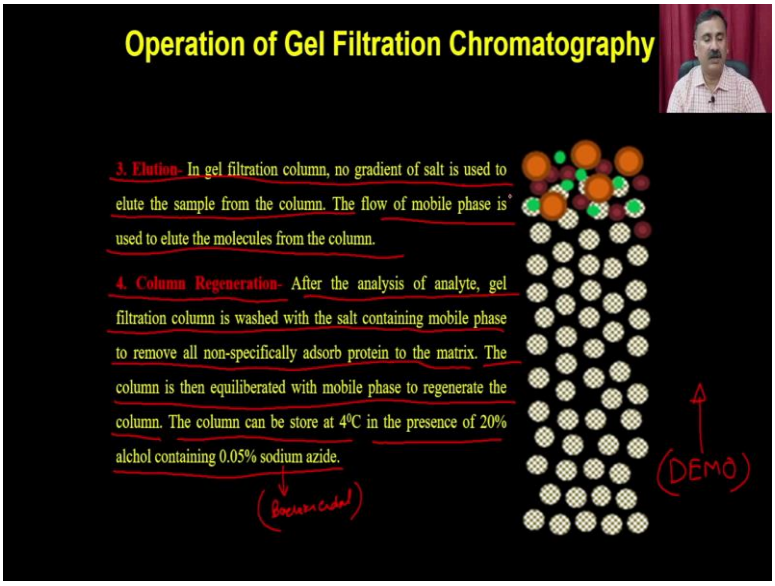

Then what you have to do is you have to do the elution. So, in gel filtration no gradient of salt is used to elute the sample from the column, the flow of mobile phase is used to elute the molecules from the column. And then once the gel filtration is over you can actually be able to do the column regeneration.

So, after the analysis of the analyte the gel filtration column is washed with a salt containing mobile phase to remove the all non-specifically absorb protein into the matrix. The column is then equilibrated with the mobile phase to generate the column right regenerate the column.

The column can be stored at 4 degree in the presence of 20 percent alcohol containing 0.05 percent sodium azide. You know that sodium azide is bactericidal. So, it is actually will not allow the bacteria to grow because most of these columns are made up of the sugary beads, right. They are made up of the sephadex or agarose and agarose is nothing, but a sugar. So, they are very susceptible for the damage.

So, we are actually prepared a very small demo clip and in this demo clip what we have done is we are actually going to explain you how you can be able to pack the column, how you can be able to you know test the quality checking and all that and then how you can be able to resolve the proteins and determine the Kd values and all that. So, in this demo the students have actually shown you all these operations of the gel filtration chromatography.

Hi everyone myself Sooram Banesh research scholar in Department of Biosciences and Bioengineering at IIT Guwahati. In this video we will demonstrate how to perform gel filtration chromatography or size exclusion chromatography. There are various methods are available in chromatography to separate different types of bio molecules.

For example, if you want to separate based on size or shape it is called a gel filtration chromatography which suits the most. If you want to separate the molecules based on the charge then you can go for the ion exchange chromatography. So, these are various methods are available. But in this video, we are mainly focusing on the gel filtration or size exclusion chromatography.

What is gel filtration chromatography? There is two phases in this process one is stationary phase another one is mobile phase. Stationary phase mainly a matrix cross

liquid matrix. For example, we can use dextran or another name of dextran is sephadex. This is highly cross liquid glucose molecules or we can use agarose this is also cross liquid or we can use polyacrylamides.

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But in this video, we are showing sephadex G75 this is the stationary phase we are using. So, in this matrix it contains beads which have a small pores. So, if you want to separate a picture of molecules starting from 1 Kda to suppose a 200 Kda. So, the small molecule which is 1 Kda it will permeates through or diffuse into the pores in the beads and the bigger molecule having a 200 Kda it will excluded from the retaining in that portion of pores.

So, it will elute first and the smaller molecule will retain there and we have to give sufficient buffer to elute that one. So, this is the overall concept of the gel filtration chromatography. It can be widely used as used in separation of proteins, peptides or oligonucleotides.

So, in this video we will show you how to pack the column first and what are the buffers required and how to we will demonstrate eluting two different molecules one is the larger size and another one is the smaller size. So, let us start the packing. So, we can use for this chromatography a purate or a column.

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So, this is a one of the column we are we are using in this experiment. So, the resolution of the molecules if you are using 10 Kda protein 20, 30, 40, 50 in this case you need to increase the length of the column that will improve the resolution of the molecules.

Otherwise, if you are using the small like this much length then the resolution is improper. You can get you know one fraction another protein you will get. So, here we are using this it is sufficient to elute one small size and another one large sized molecules. So, before using that there is no support on the bottom of the column. So, there is a direct contact if we open this one then completely whatever the solution or beads are there it will flow through.

So, for to prevent this one what we have to do is we have to block this area with the cotton a small piece of clean cotton. So, I am going to first put some cotton in the bottom of this one then we will load the beads.

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So, now we block the bottom region. Now we have to load the beads. Beads preparation is very simple this is the packet completely pour into a 500 ml of water. So, the beads will solved.

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And give the at least 100 percent swelling. So, this is already overnight swollen beads. So, we can use directly we need not to wait for another 24 hours. So. So, I am going to keep this one. So, before loading the beads, I am going to wash the column properly.

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So, that there is no other particles will present inside the column and also to soak the cotton whichever we inserted in this one. Now, we washed the column properly. So, the cotton also, the bottom one cotton also soaked properly. So, the next thing is we have to add buffer equilibrate the column. So, generally in all gel filtration experiments, we will use 0.05 molar sodium phosphate buffer.

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So, this is already prepared sodium phosphate buffer, pH 7.4. So, I will just I will add as this. I am going to open the bolt.

Once the soaking in the sodium phosphate buffer is over, we have to keep at least one fourth of buffer in column itself. Then we will load the beads. You cannot load complete beads directly because it may disturb the column packing. So, we will ask, we will load the beads and let it settle by influence of gravity.

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So, these are the beads. Just I am going to add the beads. So, while the settling is going on you can open the column.

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The flow should be less otherwise the diameter of the this opening outlet is very high. So, completely, it will come down. So, it will give cracks in the column packing. So, we are releasing the buffer very slowly. Beads are settling as we can see. The beads are settling properly on the bottom of the column.

Now, as you can see, we packed the column properly. So, now we have to, the next step is we have to load the sample. Before loading the sample, we have to remember that we cannot directly load the sample as it will disturb the column packing. So, what we have to do is, we have to put a small piece of paper on top of the beads. So, on top of that, we can load our sample. So, I am going to keep this one. So, next step is, we have to load the sample.

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So, here loading sample, it is a better practice to load with the beads rather than sample alone. So, what I am going to do is, this is our sample which contains one is 2000 Kda blue dextran. This is one of the glucose poly, this is a polysaccharide and other molecule is one of the colored compound which is a 0.5 Kda.

So, by principle, blue dextran elutes first which contains blue color and the small molecule it elutes the later onwards.

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So, we will load the sample. I will add the beads to the sample.

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And this sample I am going to load and tap out you know. Now, let this settle, then we will keep replenishing with the phosphate buffer.

So, what happens? The blue colored compound, it elutes first and the yellow one, it stays sometime, retained by the particulated gaps, then it will (Refer Time: 37:30) upon addition of extra buffer. As we can see, we have loaded two components. One is the high

molecular weight blue dextran; another one is the fluorescein which is low molecular weight. So, as we can see, blue dextran, this corresponding to blue dextran.

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So, it is eluting fast comparing to the fluorescein which is yellow in color or in orange color. So, you have to elute it completely by using the buffer. When the buffer is comes to here, comes to an end, you have to replenish with the buffer and you have to collect the fraction as you can clearly see this blue dextran band, it is eluting out.

So, you have to collect this and check for purity. Suppose if you are separating three different proteins, one is 70 Kda, another one is the 100 Kda, another one is the 300 Kda. So, according to this principle of gel filtration chromatography and their size and shape, 300 Kda protein elutes first, you have to collect the fractions from initial fractions, that gives you 300 Kda protein and 100 Kda comes later and at last 70 Kda protein elutes.

But, after collecting, how do you know these proteins are their corresponding molecular weights? So, what you have to do is, you have to collect all the fractions and you can verify through other polyacrylamide gel electrophoresis or you can check the UV visible spectrometer and based on the protein absorption spectra.

So, we can clearly see it is eluting out. With this, we understand that gel filtration chromatography is a very advantageous technique for separation of proteins, peptides and oligonucleotides. And also, during this experiment, we have to remember few more

points and follow the cautiously. The buffer need to be prepared and it is it should not, it will be free from the any air bubbles.

So, we can prepare the buffer and keep it in ultrasonic path. So, it will which will remove the remaining air bubbles. While packing up the column also ensure that there is no air bubbles in between the beads that will decrease the efficiency of the separation or resolution of the proteins. And also, one more thing, while packing the column, you should not directly add the beads; you just have to keep some of the buffer and then add the buffers.

And also once the separation is over, you have to wash the column with sufficient amount of buffer and water and also point to normal sodium hydroxide to remove any suspended particles or any proteins. So, after that, you can store it in the 20 percent ethanol for further use. You can use as many times as you want till the beads are a beads retain the activity.

So, this is the; this is all about the gel filtration chromatography. This is not only manually or gravity based on, you can also use it in the different instruments like a pro a PLC or protein other protein purification systems. So, this is all about the gel filtration chromatography. Thanks for watching.

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So, this is the gel filtration column attached to protein purification system. So, here I can show you we will inject the blue dextran and BSA show the show their pattern how they are eluting. This blue dextran gives void volume of the column and also BSA given HL elution pattern. So, if you run few more proteins with non molecular weight will get the calibration curve with that, we can calculate unknown proteins molecular weight.

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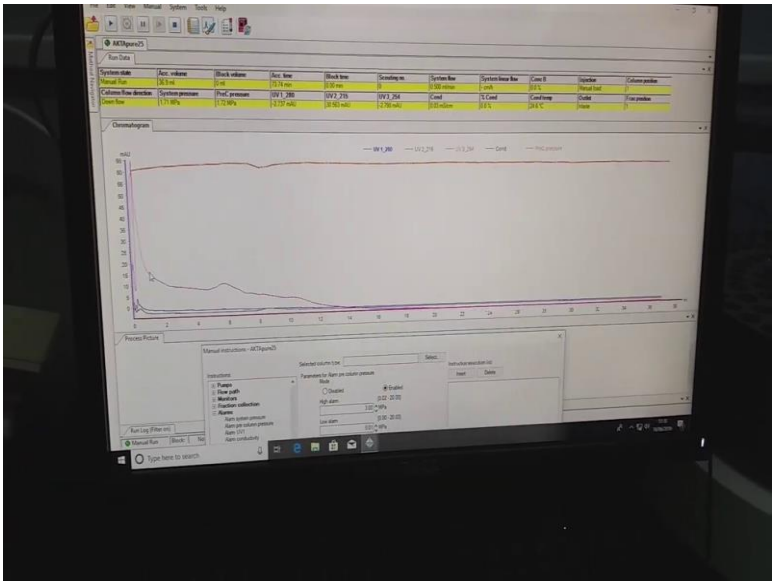

So, this column is equilibrated as we can see here when we introduced into buffer after removing the 20 percent ethanol and water also. So, we can see this is this one corresponds to blue line corresponds to 280 nanometer which is relevant to protein one. So, we can see we have there is a initial spike, but gradually it the line the curve flat flattened.

So, that means, there is no contaminants and now the column is ready to inject the protein. So, what we will do? We will inject the protein and we will show how to inject protein also. Then we will the show the pattern they are eluting. So, here we will end the program.

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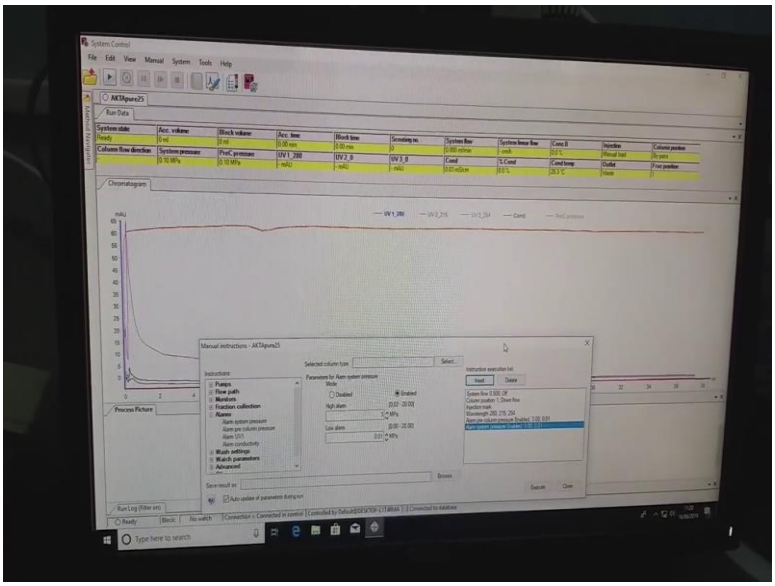

So, we will start the new program. System flow will be 0.5 ml per minute insert flow path column position at 1 and downward flow insert monitors we need 3 different wavelengths 215 for peptide bond 254 for nucleic acid and 280 for aromatic amino acids.

And we have to set the alarms also. We will set this 3 and this one 0 1 complete system pressure 3 and this one 0 1. So, we will inject the protein now then we will see how it works.

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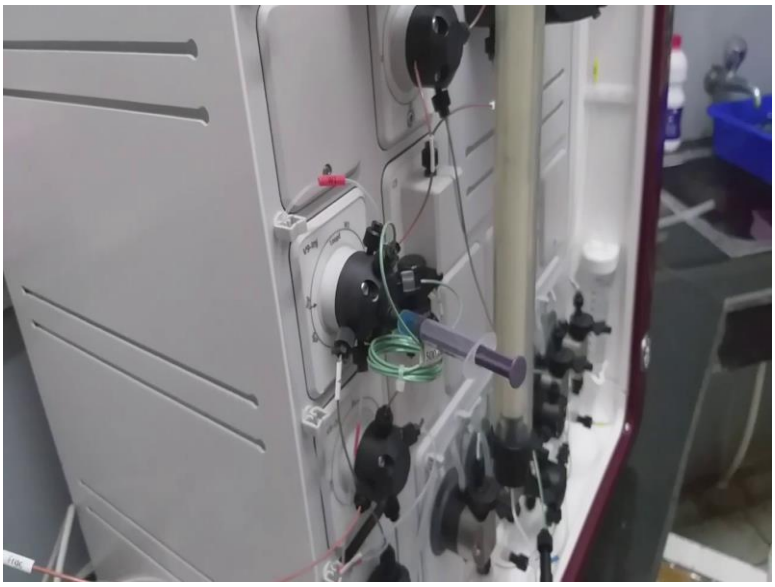

This is the port where we are going to inject the sample. So, once we will inject this one and execute inject.

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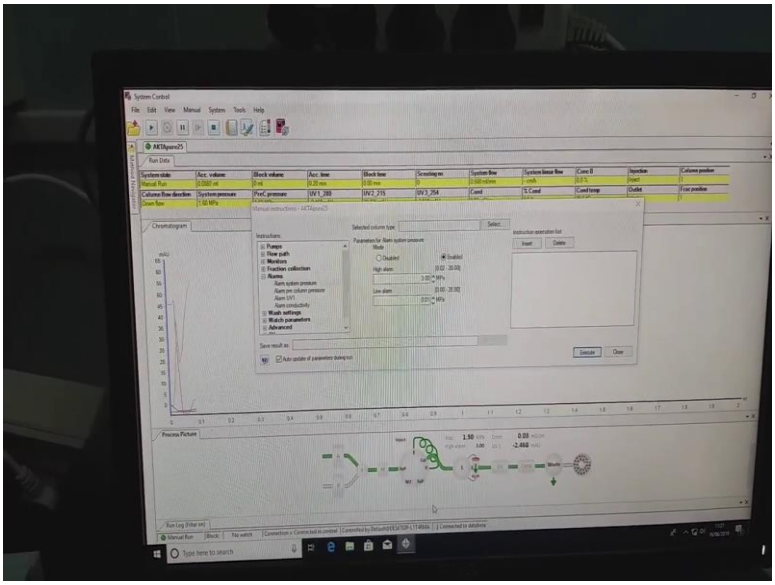

So, this is a pattern of injected components.

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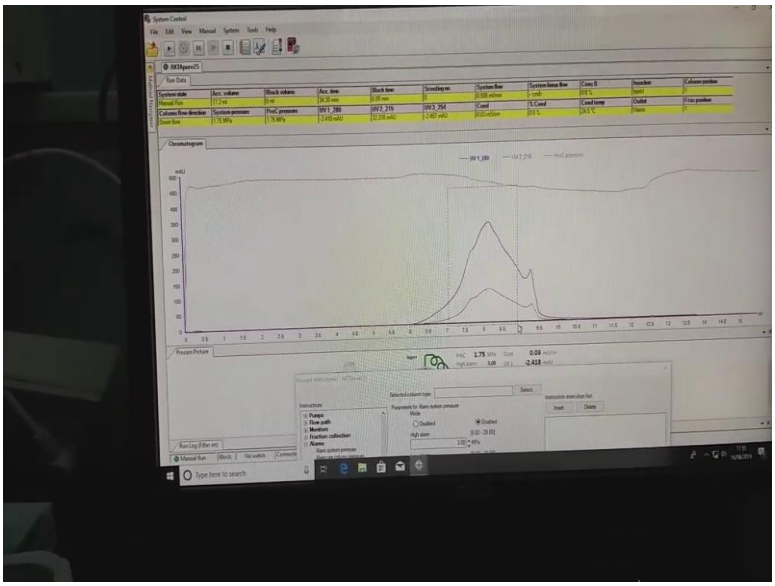

This one corresponds to the blue dextran.

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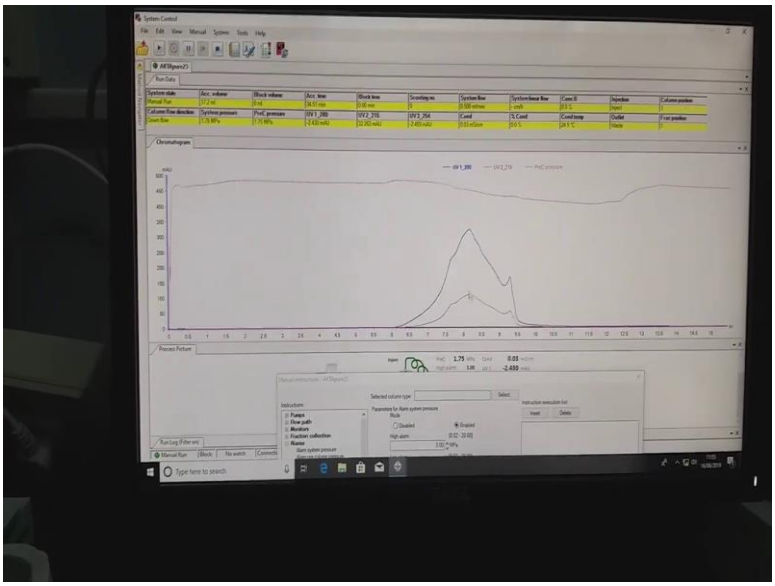

And it gives the void volume of 8 ml as we can see here it corresponds to 8 ml. So, there is no proper resolution between BSA and the blue dextran. This corresponds to 8 ml which is blue dextran and this one is the 9.2 to 9.5 this corresponds to the BSA.

So, once this is finished, we have to run another one column of in buffer to remove any other proteins and after that we will keep it in a keep it in water. So, to remove any kind of salts if present then we will keep it the 20 percent ethanol we will run at least one column volume. So, that directly we can use to preserve the column. After that we have to purge with the 20 percent ethanol complete system.

So, that the there is no contamination and practical growth if you left for few days also. So, this is all about gel filtration chromatography. So, we will show you how to analyze the result. So, once the gel filtration (Refer Time: 47:25) is over we have to analyze the results.

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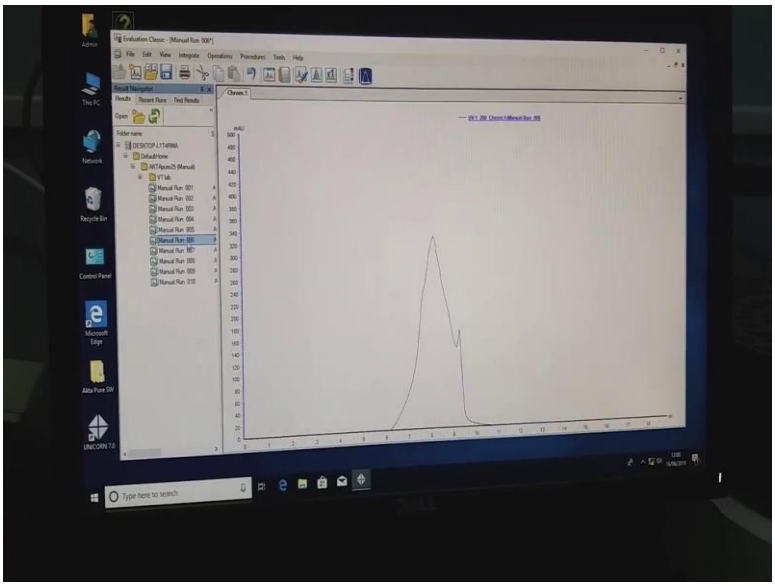

So, this is the software we will use for the evaluation purpose. So, we have to open the chromatogram which you want to analyze. So, we already opened this is the chromatogram we run recently. So, we have to analyze peaks. So, peak integrate option is there.

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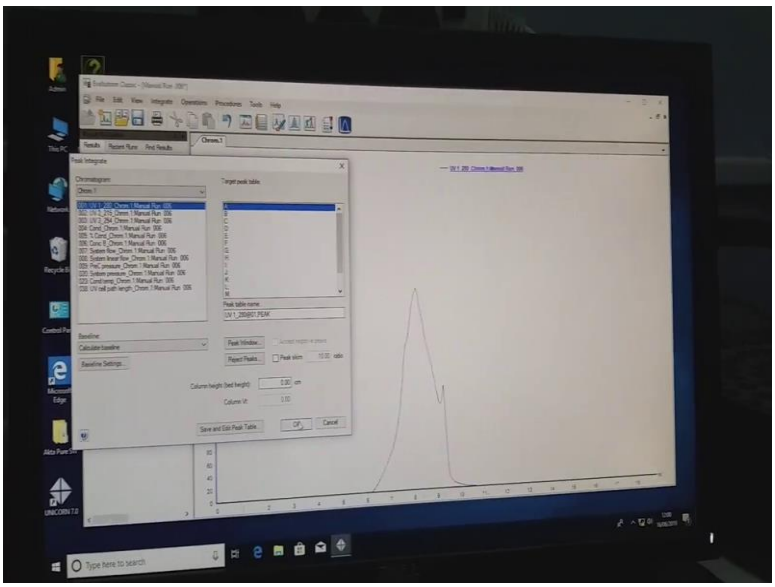

So, just say which one you want to analyze UV 280 nanometer 1 or 215 we have only 281. So, that is let us say analyze.

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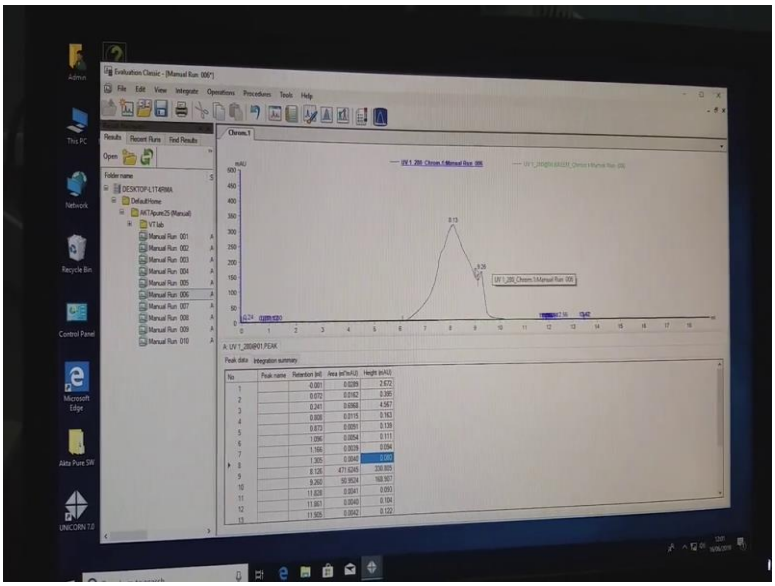

So, as we can see it gave the retention volumes of the peaks and also the area and height of the peak. These values can be used for constructing calibration curve this one belongs to blue dextran and this one is for BSA. So, in a summary in this video we showed how to run a gel filtration, we showed manually how to pack the column with the beads and also connecting through instrument.

So, hope this will help for your research to improve your research. Thanks for watching. So, this is all about the gel filtration chromatography where we have discussed about the many properties of the we have discussed about the principle of the technique and then we also have discussed about the how you can be able to exploit the technique for purification on the proteins and how you can be able to perform the gel filtration chromatography.

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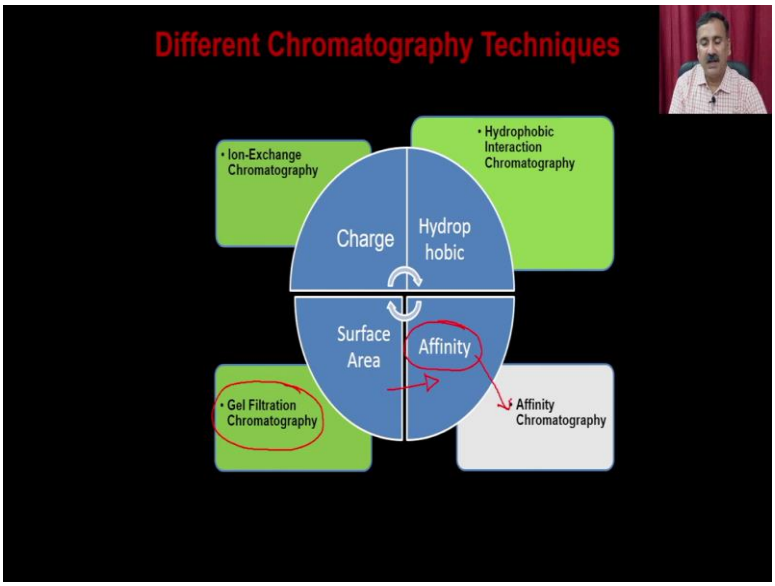

Now, once we are done with gel filtration chromatography we can go to the next chromatography technique which is based on the exclusive features, but its present onto the column and this is called as the affinity chromatography.

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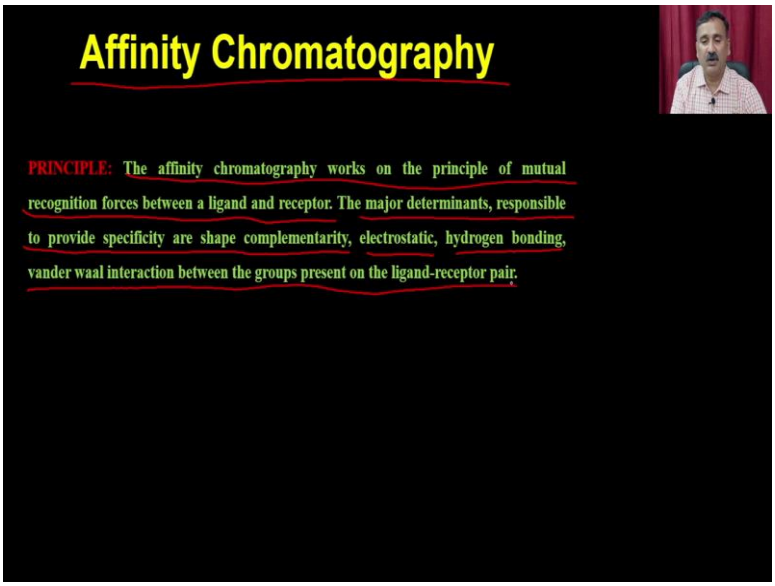

Affinity chromatography, as a name suggest the affinity chromatography is works on the principle of mutual recognition forces between a ligand and the receptor.

The major determinants responsible to provide the specificity are shape complementarity, electrostatic, hydrogen bonding and Van der Waal interaction between the group which are present onto the ligand receptor pair.

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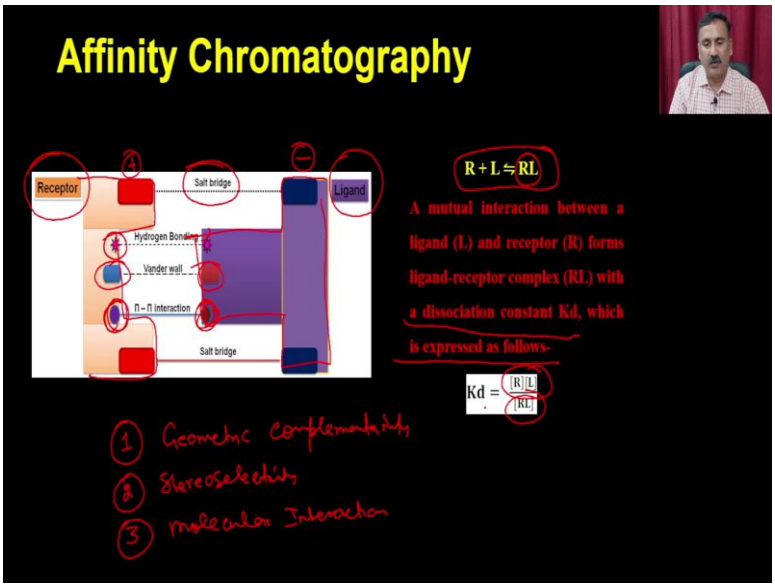

So, you can imagine that I have a ligand receptor, right. So, this is the receptor and this is the ligand. Now, these ligand receptors are getting recognized by the multiple interactions and multiple criteria. First the criteria is that the ligand should be 3 dimensionally compatible with the receptor, ok. So, if the receptor has the cavity like this the ligand is also should have the complementarity.

So, the first thing what you are going to have is you are going to have the geometric complementarity, ok. Which means the 3D structure should match which means this notch is going to fit into this group. Now, apart from that it also should have the stereoselectivity ok, which means the ligand should be of stereoselective.

So, that it should have the made up of the L amino acids and the receptor also should recognize the ligands made up of the L amino acids. Then the third is the molecular interactions. So, within the molecular interactions you can imagine that the interaction what represent on the ligand should be compatible with the receptor.

For example, you have the positive charge on this then it should have the negative charge onto the ligand and this should be at the same point. So, when this ligand will go and fit

into this particular receptor this positive is actually going to interact with the negative and that is how it is actually going to have the salt-bridge interactions.

Similarly, you can have the hydrogen donor and acceptor. So, it could be that you that the receptor has the hydrogen donor. So, the ligand should have the hydrogen acceptor and that is how they should actually have a hydrogen bonding. Similarly, you can have the you know groups which are actually going to be involved in the Van der Waal interactions and you can also have the hydrophobic residues which are actually going to be interact with the hydrophobic residues present on the receptor with the pi interaction.

So, these interactions are very important to hold the hold the receptor and the ligand for the fruitful outcomes. So, in a mutual interaction between the ligand and receptor to form ligand receptor complex the dissociation constant that Kd which is expressed as Kd is R L by R L, right.

So, R plus L is forming a reversible complex with the receptor and that is how it is actually going to give you a complex of R L and you can be able to use this equation to calculate the dissociation constant and this is actually the basic principle of the affinity chromatography.

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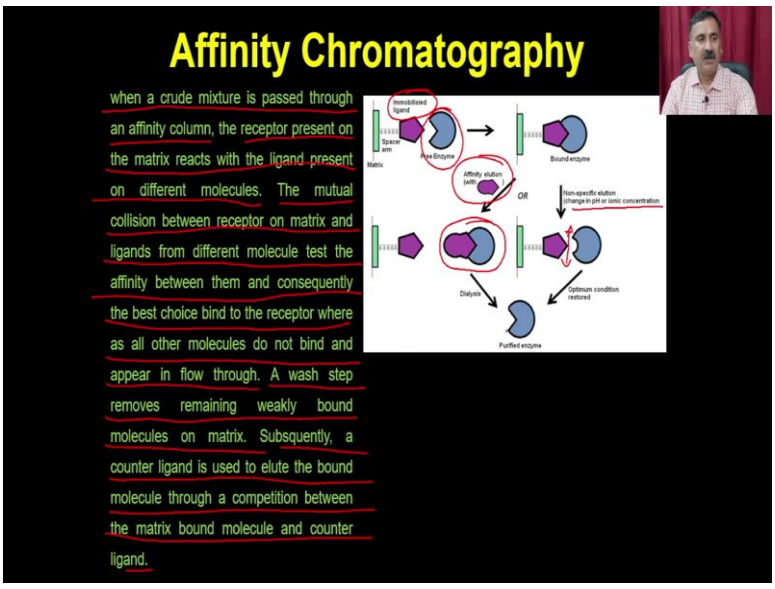

So, what happened in the affinity chromatography is that when a crude mixture is passed through an affinity column the receptor present on the matrix react with the ligand

present on the different molecules, right. The mutual collision between the receptor on the matrix and the ligand on different molecule test the affinity between them and consequently the best choice binds to the receptor whereas, all other molecules do not bind and appear in a flow through.

A wash step removes the remaining weakly bound molecules on the matrix subsequently a counter ligand is used to elute the bound molecule through a competition between the matrix bound molecule and a counter ligands which is what exactly happened right, in a chromatography what you have is you have a immobilized ligand what is present on the matrix.

And when it reacts with the free enzyme it actually reacts with many type of ligand this free enzyme is actually go and bind to this particular ligand and that is how it is actually going to form the enzyme ligand complex.

Now, at this stage you can have the multiple options either you use the competitor right, you can put the similar kind of ligands and that is how it is actually going to destroy its actually going to compete for the enzyme bound enzyme which is bound to the matrix versus the free ligand. And that is how it is the enzyme will go and bind to the free ligand and that is how it is actually going to be removed.

The option is that you can actually be do the non specific elution where you can change the pH or the ionic strength and that is how it is actually going to break the interaction between the matrix bound ligand to the enzyme and that is how it is actually going to you know elute out the purified enzyme.

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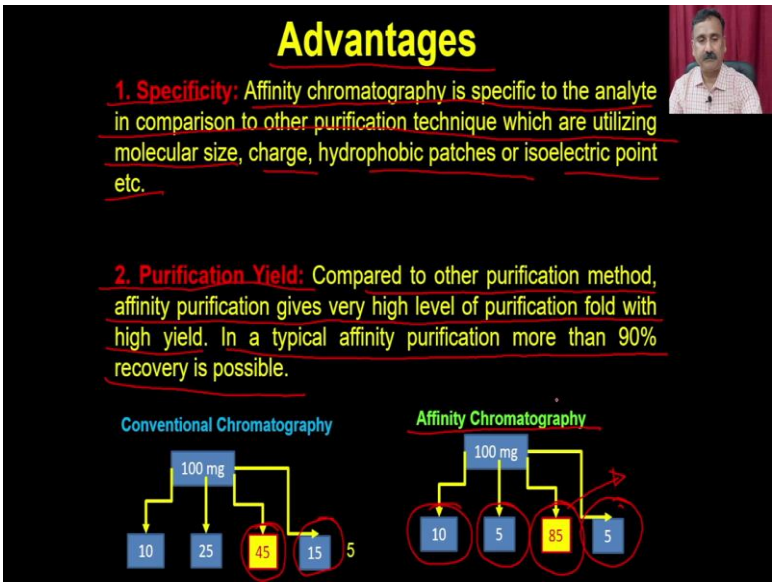

What are the advantages of the gel filtration the affinity chromatography? So, what are the advantage of the affinity chromatography? First is specificity. Affinity chromatography is specific to the analyte in comparison to the other purification techniques which are utilizing the molecular size, charge, hydrophobic patches or the isoelectric point.

Then the second point is the purification yield compared to the other purification method the affinity purification gives very high level of purification fold with the high yield. In a typical purification affinity purification, more than 90 percent recovery is possible. So, this is the conventional chromatography.

So, if you started with the 100 milligrams of protein it could be possible that after the first you know purification you can actually have the 10 milligrams in one fraction 25 milligrams in third fraction 45 milligram is what your protein is and the 15 milligram what is going to be in the fourth column.

Similarly, in the case of affinity chromatography what will happen is that it is going to have the 10 milligram (Refer Time: 55:16) wastage in the first column, first fraction the 5 milligram in the second fraction and more than 85 percent is going to be in the your purified fraction.

So, that is how your recovery is going to be very high compared to the compared to the conventional chromatography. Because conventional chromatography relies on the basic properties rather than affinity chromatography is rely on the exclusive properties. Then the third is reproducible.

Affinity purification is reproducible and it gives the consistent result from one purification to other as long as it is independent to the presence of contaminating its species.

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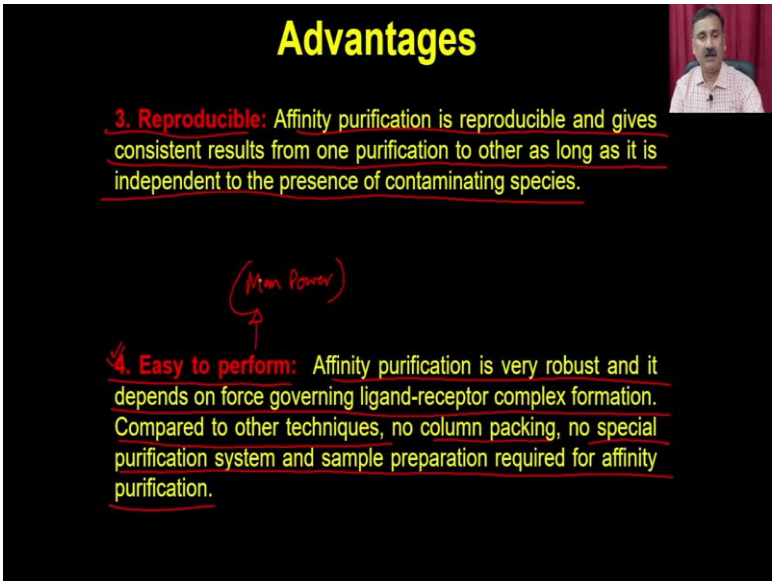

Then it is easy to perform. Affinity chromatography is a very robust and it depends on the force governing the ligand receptor complex formation compared to the other technique no column packing, no special purification system and sample preparation required for the affinity chromatography.

And. So, this is very very important because it actually saves your time for training a manpower and that is how anybody can be able to do affinity chromatography without much training.

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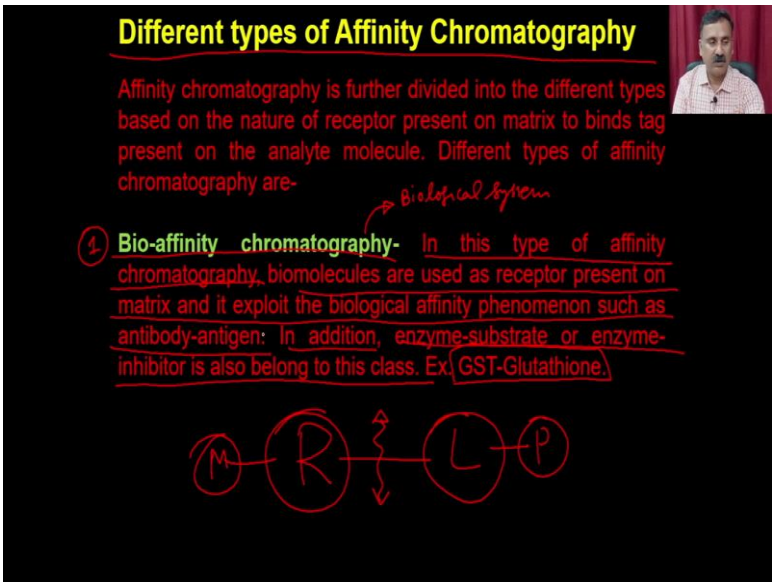

Now, once we are talk about as we have talked about the different types of affinity chromatography did you. So, you can have the bio-affinity chromatography. So, in a bio-affinity chromatography in this type of affinity chromatography bio molecules are used as a receptor present on the matrix and it exploits the biological affinity phenomena such as antibody-antigen.

In addition, the enzyme substrate or the enzyme inhibitor is also belong to this class for example, the GST and glutathione. So, in the bio-affinity chromatography you are exploiting the already existing you know the pairs in the biological system. So, that you can actually be able to use and in a every biological system you are going to have the receptor and you are going to have the ligand.

So, either of these you can actually be able to couple to the matrix and the ligand you can actually be able to couple to the protein of your interest and that is how you can be able to exploit this for in the purifying the your protein of your interest. There are examples like the enzyme substrate or enzyme inhibitor like the GST and glutathione whereas, antigen antibody is also a example of the bio-affinity chromatography.

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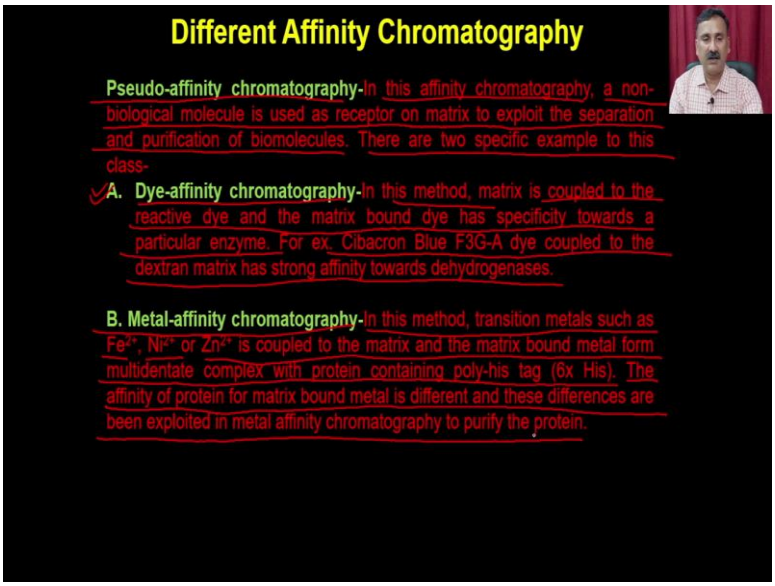

Then you have the pseudo affinity chromatography. So, in this affinity chromatography a non-biological molecule is used as a receptor on the matrix to exploit the separation and the purification of the bio molecules there are two specific examples. You can have the dye-affinity chromatography.

So, in this method the matrix is coupled to the reactive dye and the matrix bound dye has a specificity towards a particular enzyme. For example, the cibacron blue F3G-A dye coupled to the dextran matrix has a very strong affinity for the dehydrogenases. Similarly, you can have the metal affinity chromatography.

So, in this method the transition metals such as the iron nickel or zinc is coupled to the matrix and matrix bound metal form the multidentate complex with the protein containing poly histidine tag. The affinity of the protein for matrix bound metal is different and these differences are been exploited in metal affinity chromatography to purify the proteins.

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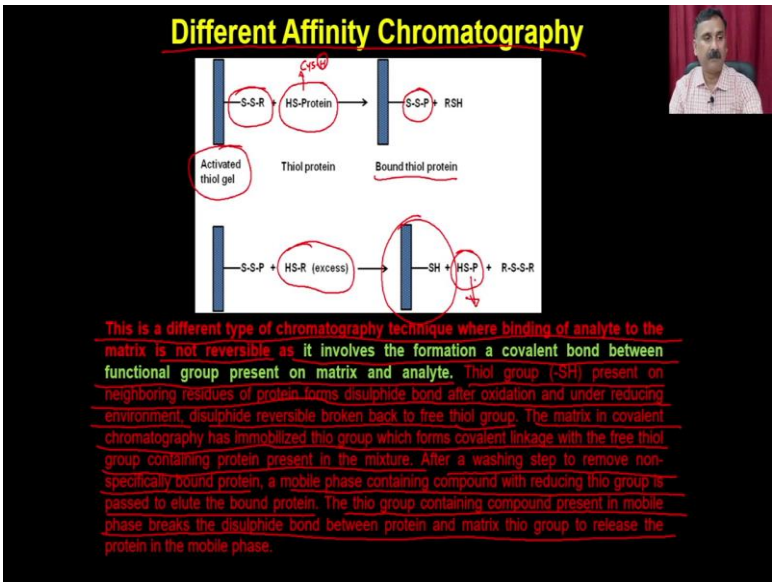

Apart from these two categories you can also have the covalent chromatography. In a covalent chromatography what you have is that where the analyte is permanently actually go and bind to the matrix. So, it is not reversible majority of the affinity chromatography or all other chromatography techniques are reversible.

But this is the different chromatography where binding of the analyte to the matrix it is not reversible as it involves the formation of a covalent bond between the functional group present on the matrix and the analyte. So, the thiol group which are present on the neighboring residue of protein forms a disulfide bond after oxidation and under reducing environment disulphide reversible brokage back to the free thiol.

The matrix in the covalent chromatography has immobilized thiol group which forms a covalent linkage with the free thiol groups containing protein present in the matrix. After a washing step to remove the non specifically bound protein a mobile phase containing compound with reducing thiol group is passed to elute the bound protein.

The thiol group containing compound present in the mobile phase breaks the disulphide bond between the protein and matrix thiol group to release the protein in the mobile phase. So, what you have is you have the activated thiol group which is like S-S-R and when you are actually adding a protein. So, in the protein you actually have the free cysteine, right.

So, if the free you have the free cysteine, then it is actually going to react and the making a disulfide linkages between the cysteine what is present on the protein with the disulfide linkages and this is going to be bound thiol. And then when you are adding the reducing equivalents right, it is actually going to reducing the column and that is how it is the we are going to release the protein into the supernatant.

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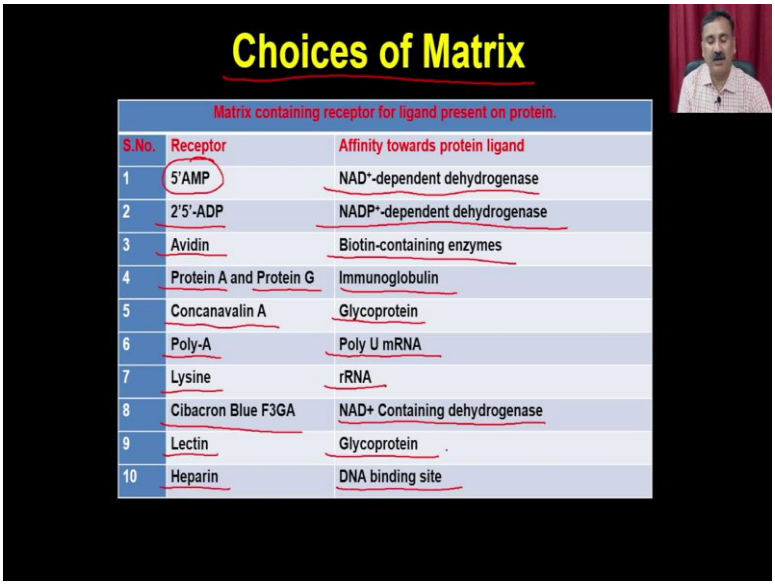

For the affinity chromatography you can actually have the different types of choices you can have the receptor like 5 prime AMP and that is going to be have a affinity for nad plus dependent dehydrogenases 2 prime 5 prime ADP that is NADP plus dependent dehydrogenase, you can have avidin which is the biotine containing enzymes then you can have the protein A or protein G that is going to have the affinity for anti bodies.

The concanavalin A which is going to have the affinity for glycoproteins, the protein A which is going to have the poly U messenger RNA, lysine which is going to have the affinity for ribosomal RNA, then you can have the cibacron blue F3G-A that is going to be NAD plus dependent dehydrogenases, then you have lectins that is for the glycoproteins and you have heparin that is for the DNA binding proteins.

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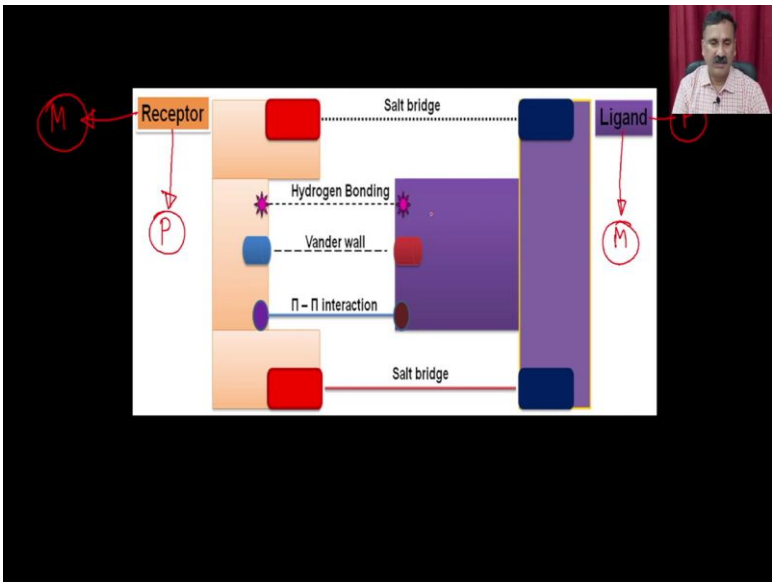

Now, if you want to do affinity chromatography you have the receptor and which is actually going to have the interaction with the ligands. Now, at this stage the receptor has 2 options you have 2 options either receptor can be you know tagged with the matrix and then you can actually be able to put the ligand onto the protein, right.

And that is how you can be able to use this pair for protein purifications or alternatively the receptor can be tagged to the protein and the ligand can be put onto the matrix. And in that case, you can actually be also be able to purify the proteins. So, this is all about the affinity chromatography and very briefly we have discussed about how the 2 mutually exclusive parameters can be used to purify the proteins with the help of the affinity chromatography.

In our subsequent lecture we are going to discuss about how you can be able to generate the receptor or the ligand and how you can be able to perform the affinity chromatography. So, with this I would like to conclude my lecture here.

Thank you.