Genetic Engineering: Theory and Applications
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Module 8 - Isolation and Purification of Product Part-2
Lecture 24 – Gel Filtration Chromatography (Part-II)

Hello everybody. This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And so far, what we have discussed? We have discussed about different processes like, what you should follow and we should not follow while you are doing the gel filtration chromatography.

To explain you this in more detail, we have prepared a demo session where we are going to take you to my lab and where one of my student is going to show you how to pack this column, how to pack the gel filtration column, how to determine the molecular weight using different types of approaches, what we are going to discuss in subsequent slides and how you can actually exploit or detect the different types of problems in the gel filtration column.

Such as, how to detect the air bubbles, how to detect the air channels, and all other kinds of problems. And what, if you are getting some kind of problems, how to troubleshoot those problems in the column packaging itself?

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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 biomolecules. For example, if you want to separate based on size or shape, it is a kind of gel filtration chromatography which suits the most.

If you want to separate the molecules based on their charge, then you can go for the ion exchange chromatography. So these are various methods are available. But in this video, we are mainly focussing on the gel filtration or size exclusion chromatography. What is gel filtration chromatography? There are 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. But in this video, we are showing Sephadex G-75, we are using this in the stationary phase.

In this matrix, it contains beads that have small pores. So, if you want to separate a mixture of molecules starting from 1 Kda to suppose 200 Kda, so the small molecule which is 1 Kda, it will permeate through or diffuse into the pores in the beads. And the bigger molecule having 200 Kda, it will exclude 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 in separation of proteins, peptides or oligomeric peptides. So, in this video we will show you, how to pack the column first and what are the buffers required. In

gel filtration chromatography, there are two kinds of columns available, one is pre-packed columns, another one is column material which can be used for the packing of the column. Here we will show you how to pack a column.

And also we have prepacked column. At the bottom of the column there should be a centred filter in prepacked columns, which will forbid going of the beads through outlet. So, here if you see in the prepacked columns there is some centered filter at the bottom of the column. In both the ways, outlet and inlet, both contains the centred filters. Inlet which is mainly from buffer inlet, if there is any particles which will obstruct the flow, they will be separated on the top. And also the outlet one which can be used for preventing passage of the beads through outlet. So, here we do not have exactly centred filter, but we can use a piece of cotton.

So, just put the cotton. So, now we inserted the cotton. These are the beads we are going to use for the packing of the column. These are the Sephadex G-75 beads. So, there are different materials available; Sucralose, Sephadex, Sepharose. These are all derivatives of carbohydrate materials. So, here what we will do, this is the overlaid solid beads. So, you have to take the beads, a complete pack and soak it into water or the buffer. So, these are swollen one. Now, after inserting the centred filter or column, you have to wash column properly with the liquid distilled water.

So, if anything it contains, like dust or any other contaminating particles, it will remove. Now, we directly pour the beads on top of the column. If you observe closely, you can see the settling of the columns, settling of the beads. As, we can see, the beads are settling slowly. So, after complete settling of the beads, then we will put some filtered kind of thing or some piece of cotton on top of the beads. Then we will load the sample. As we can see, now the packing is over. Now, what we have to do is, we have to equilibrate the column with the 0.05 of phosphate buffer.

So, we will just add phosphate buffer and drain the any unbound solvent. So, here after the equilibration of the column, we will load the sample. After column packing, we have to check the efficiency of the column. So, for every column the parameters, V naught that is void volume, and V t total volume and elusion volume, it differs. So, for checking of the column efficiency, we have to use 0.2 percentage of total volume of the column. Acetone we will load. So, we have to observe the elution at 280 nanometres. From this we can calculate number of theoretical plates N.

So, the maximum number of theoretical plate means the more the efficiency. For calculation of any unknown proteins molecular weight, we should know what is the V t, V naught and V e. V t is the total volume of the column, means the buffer occupied in the spaces of beads and also the buffer in between the beads. So, that will be total volume. The void volume is the buffer in between the beads. Elution volume is way we elute. Suppose we are eluting protein, so at what volume it is acting, that is called as elution volume.

So, for estimating the void volume, we can use blue dextran. So, first we have to cover top with the piece of cotton, then we will load the blue dextran. After sample loading, we will start collecting the buffer in blue dextran completely eluted. That will give the void volume. So, we have loaded the blue dextran, we will add the buffer, then we will elute. So, as we see, the blue dextran is passing down, so we have to replenish the buffer continuously and we start collecting the eluted volume.

So, after complete elution of the blue dextran, we have to measure the volume and that volume give us void volume. Now, blue dextran is completely eluted, it is around 18 to 19 ml. So, now we got void volume and what about the total volume? Total volume consists of the total packed volume of the beads. So, it is around 25 ml of beads are there. So, that means total volume is 25 ml. So, with these values after eluting the protein, suppose if you are using some unknown protein, you have to calculate with for known proteins, then you have to construct a calibration curve between the partition coefficient, which is calculated with elusion volume subtracted with void volume divided by total volume subtracted void volume.

That will give partition coefficient. And on x axis you have to take non-molecular weight. Once you plot, you will observe some co-relation. Based on that you can calculate unknown protein's molecular weight.

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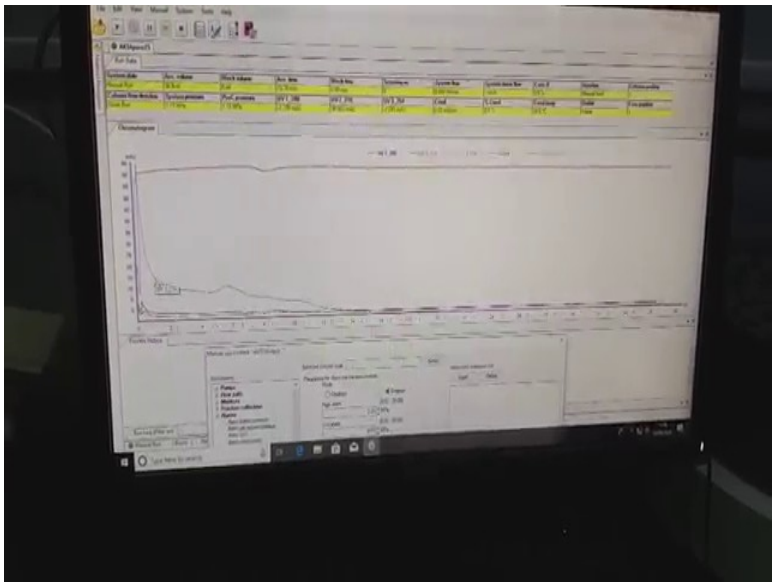

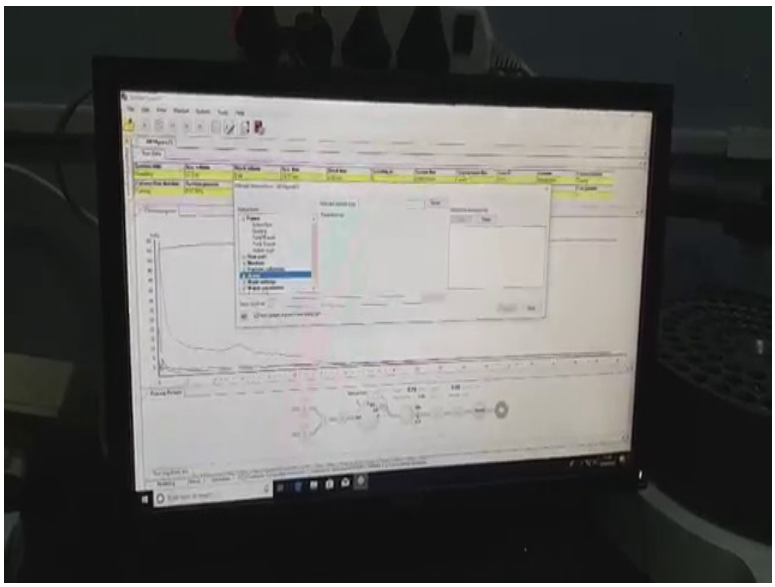

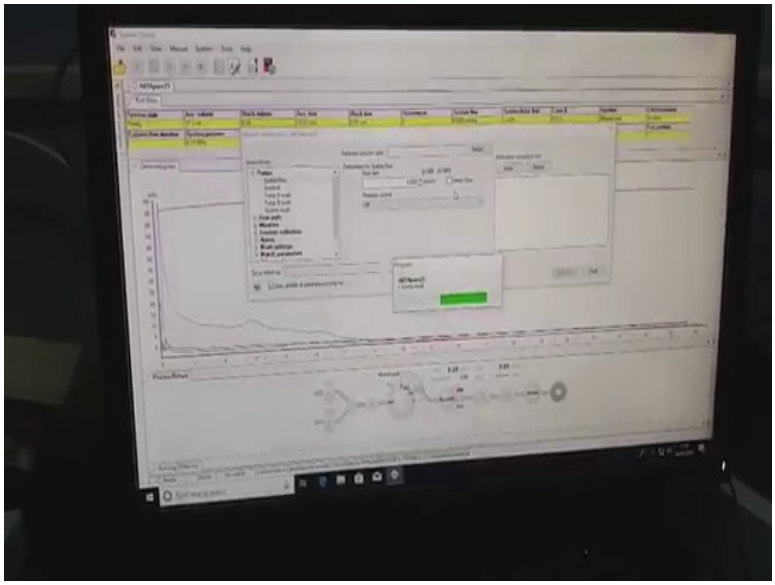

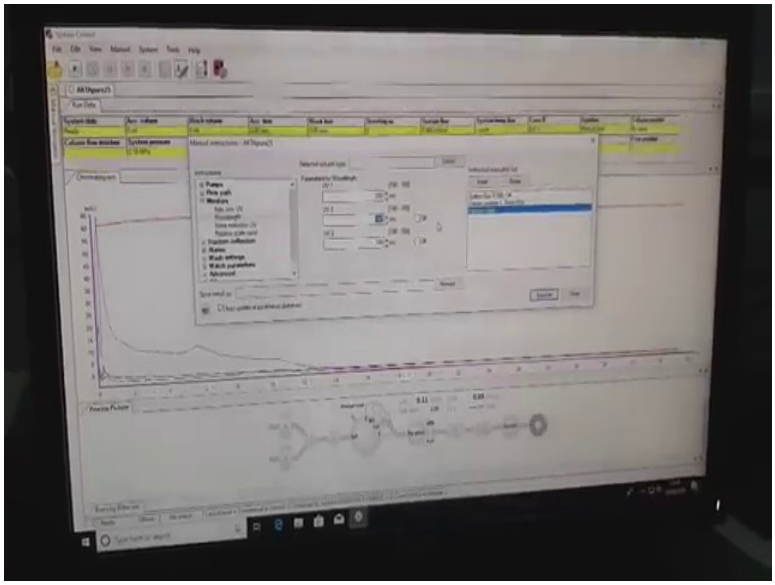

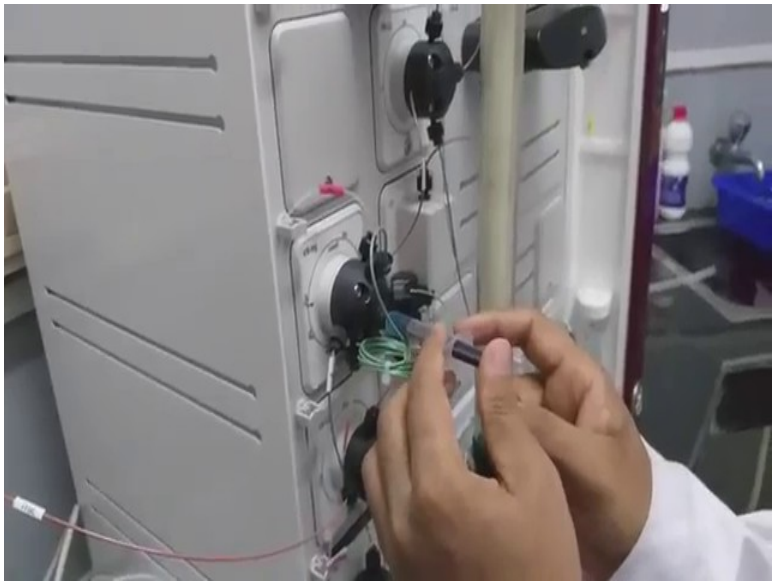

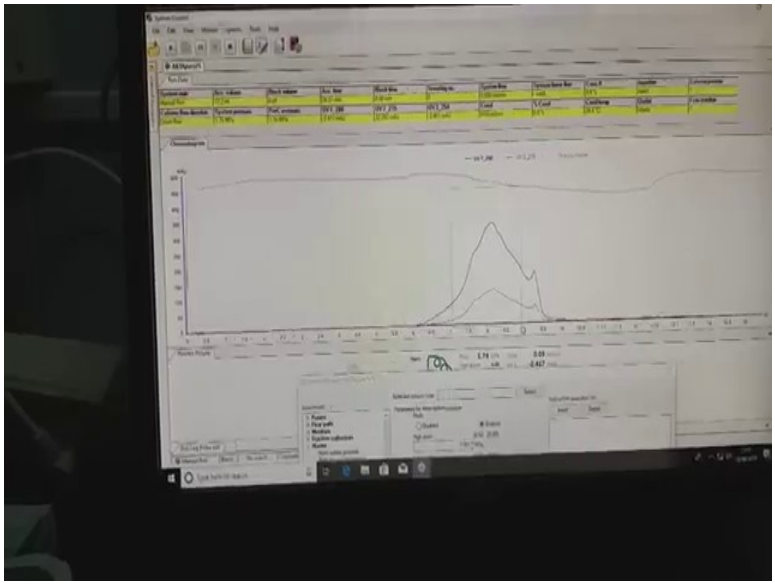

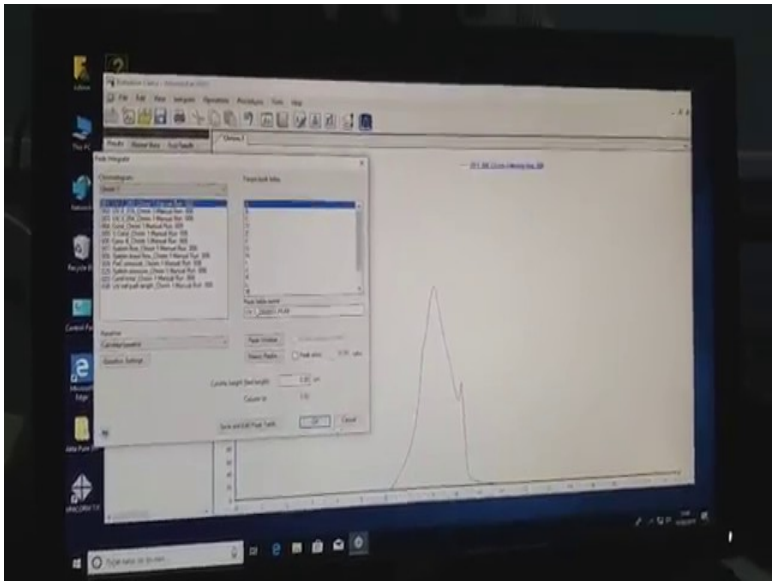

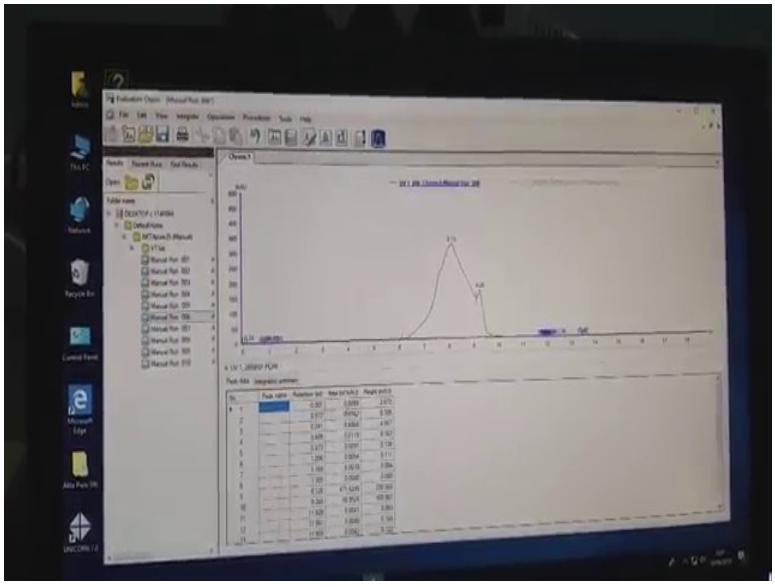

So, this is the jet filtration column attached to protein purification system. So, here it will show you, we will eject the blue dextran and BSA and show their pattern, how there are eluting. This blue dextran gives void volume of the column and also BSA gives actual elution pattern.

So, if you run few more proteins with known molecular weight, we will get the calculation curve, with that we can calculate unknown protein's molecular weight. So, this column is equilibrated, as you can see here. When we introduce it into buffer after the 20 percent ethanol and water also, so we can see blue line corresponds to 280 nanometre which is relevant to protein 1.

So, we can see, there is an initial spike but gradually the curve flattened. So, that means there is no contaminates 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 show the pattern they are eluting.

So, here we will end the programme and we will start the new programme. System flow will give 0.5 ml per minute; insert flow path, column position at 1 and the downward flow; insert monitors, we need 3 different wavelengths, 215 for peptide 1, 250 for nucleic acid, and 280 or aromatic amino acids. And we have to set the alarms also. Insert this 3, and this one will govern complete system pressure 3, and this one, 0, 1.

So, we will inject the protein now. Then we will see how it is mixing. This is the port where we are going to inject the sample. So, once we will inject this one and execute, inject. So, this is the pattern of injected components. This one corresponds to the blue dextran 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. Once this is finished, we have to run another one column of buffer to remove any other proteins. And after that, we will keep it in water so to remove any kind of salts if present. Then we will keep at the 20 percent ethanol, we will run at least 1 column volume.

So, directly we can use to preserve the column. After that, we have to permit the 20 percent ethanol complete system so that there is no contamination of practical work if you left for few days also. So, this is all about the gel filtration chromatography. So, we will show you how to analyse the result. Once the gel filtration is over, you have to analyse the result. So, this is the

software we will use for the evaluation purpose. So we have to open the chromatogram which you want to analyse. So already opened, this is the chromatogram we run recently. So we have to analyze the peaks, so peak integrate option is there, so just say which one you want to analyse, 280 nanometre one or 215. We have only 281, so that is let us say analysed. As we can see, it give 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 connecting through instrument. So, I hope this will be help to improve your research.

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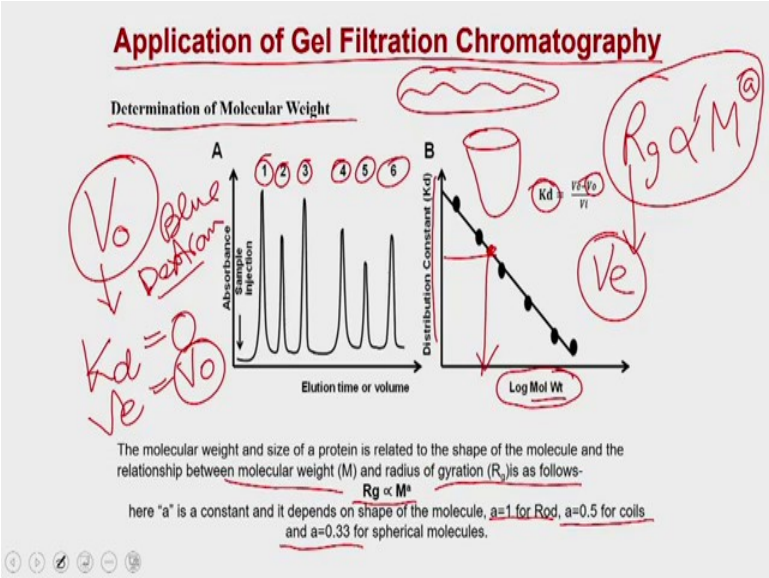

Now, let us see the application of gel filtration chromatography. Apart from purification, gel filtration chromatography is being extensively used for the determination of molecular weight. As you remember we have just discussed that, the molecular weight is related to the number of residues that this particular protein is going to have. And the number of residues are also directly related to the radius of gyration, which means the radius of gyration is directly proportional to the molecular weight. This the formula, the molecular weight and the radius of gyration is irreversibly related which means R g is directly proportional to the M a, so R g is directly proportional to M a and where a is a constant which depends on the shape of the molecule, a is 1 for rod, a is 0.5 for coils and a is 0.33 for spherical molecules.

Which means if you plot a graph between radius of gyration versus the molecular weight, and radius of gyration is directly proportional to the elution volume or V e. Which means V e is

going to be inversely proportional to the molecular weight. So, to determine the molecular weight, what you are going to do is you can calibrate this column simply by running the proteins of different molecular weight and then you plot this. So, that will give you a calibration curve between the log molecular weight and the distribution coefficient, K d of all these individual proteins which you are using for calibration.

And all these proteins are globular in nature, that is why they are going to follow the basic principle of gel filtration. Then what you do is you suppose you do the exercise and calculate the V e. And then you calculate the K d value of your unknown protein. Suppose this is your unknown protein, that actually will give you the log molecular weight. And then you can calculate from this log molecular weight, you can be able to calculate the molecular weight of that particular protein.

Now, in this particular experiment or in this particular type of exercise, what you do not know is about the void volume. So, the void volume, how to calculate the void volume? Now, as we said, for detecting the air channels, you have to have a molecule which actually has a K d value equal to 1. Whereas, to calculate the V o, which means the void volume, you have to have a molecule which is the K d value of 0. Which means, in that case only, elution volume is equivalent to the void volume.

How to get a molecule which has a K d value equal to 0? Now, to answer this question you can understand that most of these gel filtration chromatography columns are only working with the globular proteins which means they are not going to work with the fibrous proteins.

So, there are two different types of protein. One is globular protein, which are ball like proteins, which actually maintains all the residues along with the centre. Whereas there are fibrous proteins, which are actually not following this particular rule. One of the classical examples of globular protein is haemoglobin. The haemoglobin, the molecule which actually carry the oxygen from one part of the body to another part. The classical example of the fibrous protein is the hair which we have. The purpose of the globular protein and the fibrous proteins are very different.

The globular proteins are mostly the protein which are actually taking part in the enzymatic reactions or all sort of metabolic reactions. Whereas, most of the fibrous proteins are part of the structural proteins such as, the collagen or the fibrous keratin, or all other protein which are forming this, like the protein which is making the nails.

So, if you take any protein which is of fibrous in nature, which what is meant by fibrous in nature is that, it is going to be of an extended confirmation. Suppose you take your hair, the hair are of fibre like structure, that is why the name is known as fibrous protein. So, if you take a structure which is fibrous in nature, that fibrous structure is not going to enter into this particular bead, irrespective of the size. So, one of the easiest fibrous structure is, instead of taking a protein, what you can do is, you can take a sugar change. You can take a sugar polymer.

And sugar polymers are also present in the extended confirmation, so they will not be going to enter into the beads. One of the classical example of the sugar what people use is the dextran. So, you can use the dextran and because you want to detect this particular dextran, what people do is, they just stain this particular dextran with blue dye, and that is why the name is blue dextran.

So, if you load the blue dextran onto the column, the blue dextran is going to be excluded from the column because it is in the extended conformation, and it will exhibit the K d value which is equivalent to 0. Which means the V e is going to be your V o, and in that case you can just get the elution volume of the blue dextran, and that is going to be the void volume of this particular column, under that particular condition. Which means, if you change the pH, if you change the temperature, if you change the packaging, both, for void volume as well as the K d values everything is going to be changed.

So, all these things are relative whether it is void volume or K d values. So, as soon as you change the packaging and all other conditions, all these parameters are going to be changed. Which means, the calibration curve is also not going to be hold for that particular column. You also have to draw the again new calibration curve if you would like to determine the molecular weight. So, as long as the column is maintaining the particular type of packaging, you can keep using this calibration curve. But if you change the conditions, you have to re-draw the calibration curve to precisely calculate the molecular weights.

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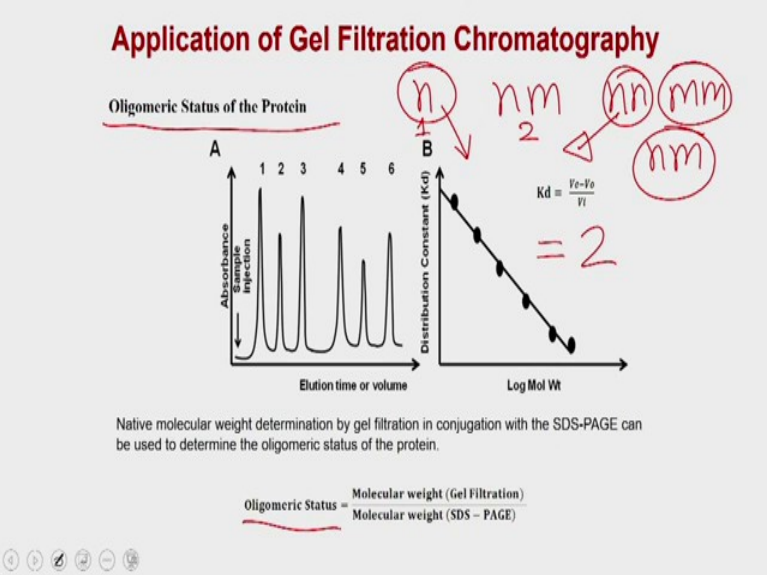

Now, once you calculated the molecular weight, you could also be able to calculate the oligomeric status of the protein. What is mean by oligomeric status is that either it is one monomer, which means one particular chain is going to be part of the protein or the two chains are going to be attached to each other.

So, in this case, the oligomeric status is monomeric. Whereas in this case, the oligomeric status could be the homodimeric status or the heterodimeric status, which means it could be either nn or mm or it could be nm. So, this is the heterodimer, these two are called as the homodimer. Similarly, it could be tetramer, it could be pentamer. So, how many sub-units are associating with each other and giving you the final protein is called as the oligomeric status of that particular protein. So, once you calculate the molecular weight of this protein utilizing the matter just now what we have discussed, then what you can do is you can also calculate the molecular weight using the SDS-page.

So, SDS-page is going to give you the denaturating conditions which means in the case of the monomeric protein, it will give you the molecular weight of n. Whereas the gel filtration is going to give you the molecular weight of nn, which means if you divide the nn from the n, it will say that the oligomeric status is 2 which means dimer.

So, the molecular weights which you are getting from the gel filtration if you divide that by the molecular weight which you are getting from the SDS-page, that will give you the oligomeric status.

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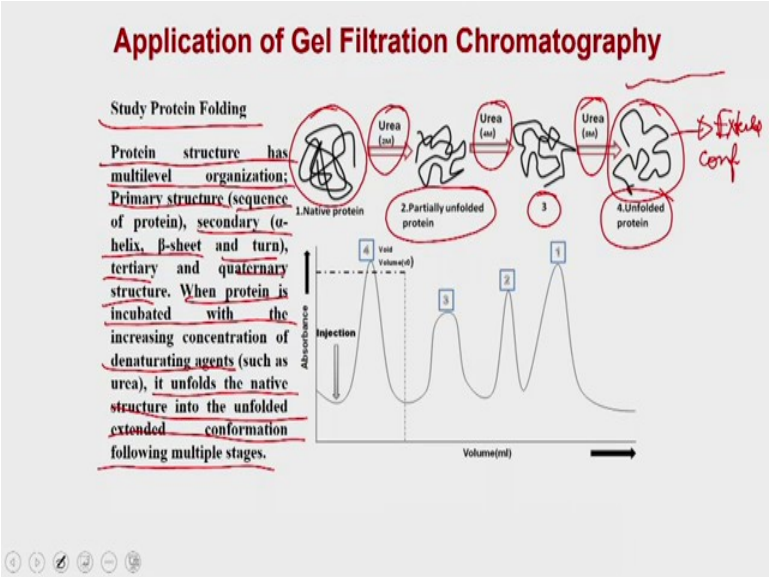

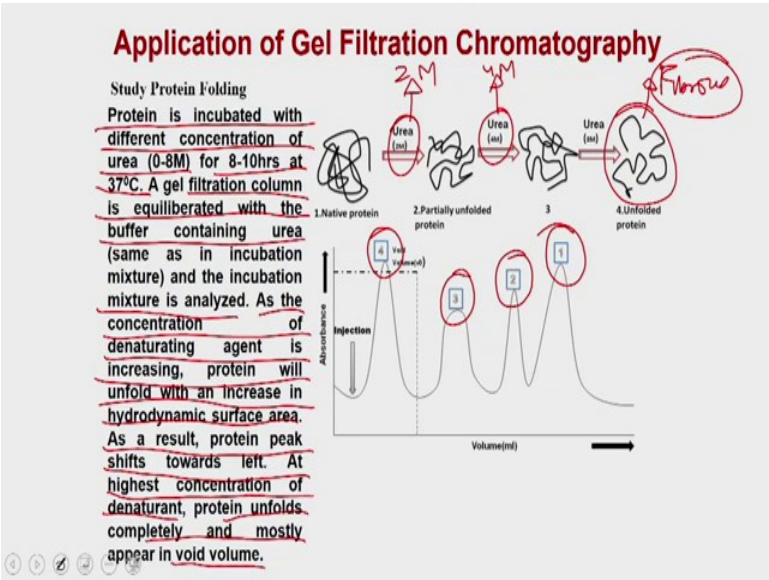

Now the next is you can study the protein folding by the gel filtration. You know that, once it is generated as a small extended confirmation, it gets into the native confirmation, which gets folded and then it rearrange all the amino acids. If you put them under the denaturating condition such as the urea, so if you put it in the 2-molar urea, it is going to be a partially folded protein. If you put it under the 4-molar urea, it is going to be unfolded further. But if you put it under the 8-molar urea, it is going to be completely unfolded. Which means this is going to be the extended confirmation, this is going to be extended which means this will behave like a fibrous protein.

This is going to be behave like a globular protein. And we are only talking about the globular protein. So, the protein structure has the multi-level organisation; primary structure which is

the sequence of the protein, secondary structure which means alpha-helix and beta-sheets and turn and the tertiary and the quaternary structure. When the protein is incubated with the increasing concentration of denaturation agent such as urea or (())(30:00), it unfolds the native structure into the unfolded extended conformation following the different types of steps.

Sometimes, the proteins are getting unfolded completely, sometimes they are getting unfolded domain-wise. So, that is why there is no universal law that protein unfolding is going to follow a particular pattern. So, once if you would like to study that, the protein is incubated with the different concentration of urea which is from 0 to 8 Molar for 8-10 hours at 37 degree Celsius. A gel filtration column if you would like to use, gel filtration column is also equilibrated with the buffer containing the urea which is of same concentration. Which means, for this particular concentration you are going to run the gel filtration buffer which contains the 2-molar urea, in this one it is going to contain the 4-molar urea.

So, the sample also you are going to prepare. In these particular denaturating conditions, the column is also going to run in the same buffer. And then the mixture is analysed as the concentration of denaturating agent is increasing, the protein will unfold with an increase in hydrodynamic surface area. Which means, if the protein is in a native confirmation, it is going to make a compact ball. As soon as you are putting under the denaturating conditions, it is getting unfolded. And that is why that diameter of that particular ball is increasing, and ultimately it is going to form an extended confirmation.

As a result, the protein peak shifts towards the left. Which means, this is the peak for the native confirmations and as the size of the peak will go, the protein will come out more and more onto the left side. At highest concentration of denaturant, the protein unfolds completely and mostly appear in the void volume.

That is what we said. This is an extended confirmation, present just like as a fibrous protein. So, this particular protein is going to be come out into the void volume. Whereas these two are the intermediate stages which are corresponding to the partially folded proteins. And this is the native proteins.

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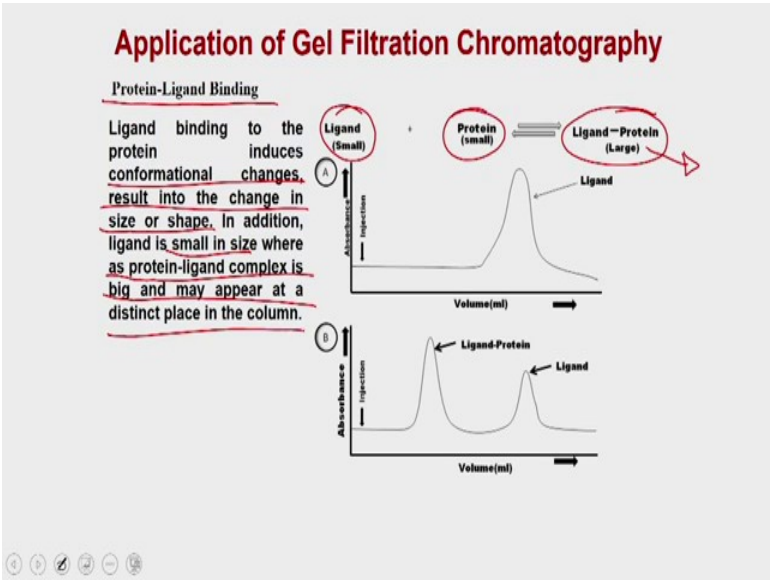

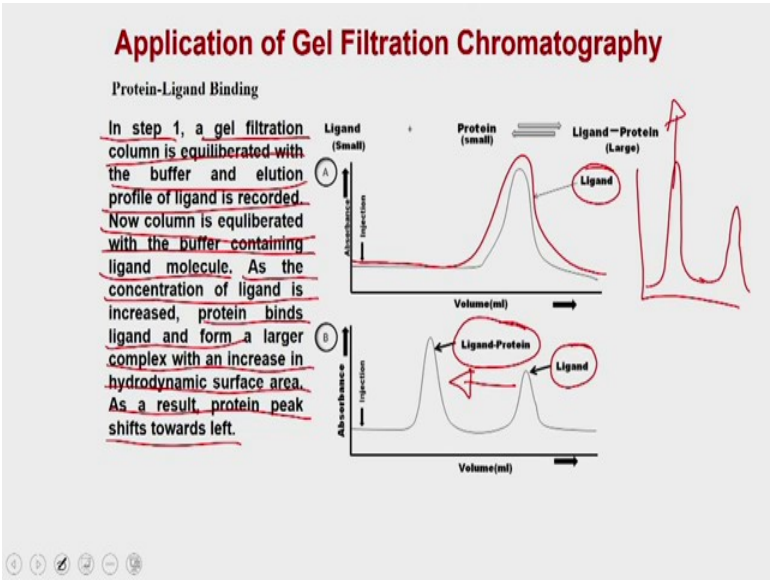

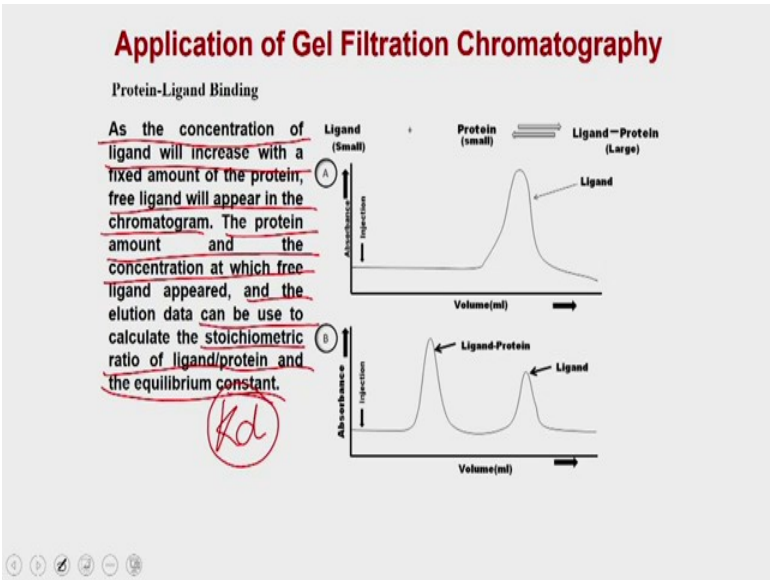

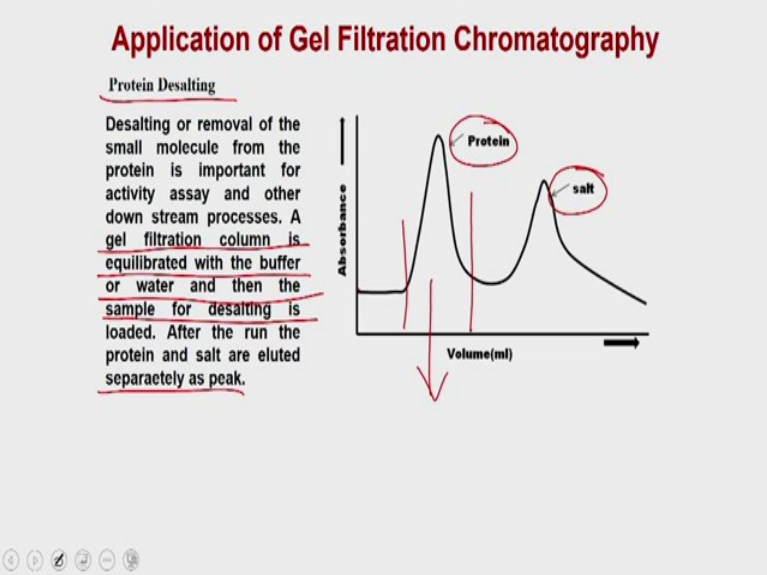

Now, you can also study the protein-ligand interactions. So, ligand-binding to the protein induces conformational changes, results into the change in size or shape. In addition, ligand is small in size. So, if you analyse the ligands, it is going to elute at a different size. If you study the proteins, it is also going to elute at a different size.

So, the ligand which small in size, the protein which is also small in size, if you put them together and if they make a complex, that complex is going to be of large hetrodynamic surface area. Whereas, the protein-ligand complex is big and may appear at a distinct place in the column.

So, in the step one what you do is a gel filtration column is equilibrated with the buffer and the elution profile of ligand is recorded. So, in the first step what you do is you equilibrate the column and then you study the elution of the ligand only. Then what you do is now column is equilibrated with the buffer containing the ligand molecules and you can use the different concentration of the ligand.

So, as the concentration of the ligand is increased, the protein binds to these ligands and they are going to start forming the complexes of different sizes. And with the increase, so they will form the complexes, it increase in hydrodynamic surface area. As a result, the protein peak shifts towards the left.

So, you can see, when you are analysing the ligand, it is making a separate peak. But when you are resolving the proteins onto a column which also contains the ligands, the protein is forming a complex with the ligand, and that is how the ligand peak is shifting towards the protein side.

And you can actually calculate the dissociation constants simply, because after some time it is not going to migrate and that is the place where it is going to saturate. So, the number of ligand molecules which are going to bind this particular protein is fixed. So, suppose you are resolving a 1 micro molar of protein, and the stoichiometry of interaction between the protein and ligand is 1 is to 10.

Then it is going to interact up to 10 micro molar with the ligand. But once you increase that particular concentration and you go to 20 micro molar, 30 micro molar or 50 micro molar, the remaining amount is going to be form the separate peaks as a ligand. So, in that case, when you resolve that, what will happen is, the protein ligand complex is going to appear.

Then you will see that, the ligand is reappearing in that particular condition. That means that, now this is the stage at which the protein is fully saturated with the ligand. And if you use these particular values, you could be able to calculate the dissociation constant of the ligand protein interactions.

So, as the concentration will increase with the fixed amount of the protein, the free ligand will appear in the chromatogram. The protein amount and the concentration at which the free ligand appeared and the elution data can be used to calculate the stoichiometric ratio of ligand protein and the equilibrium constant, which means you can be able to calculate the dissociation constant K d.

The protein, you can also use for protein desalting. So, you know that sometime the protein is being purified by, for example, if you are purifying a protein using the ion exchange chromatography, you are actually putting lot of salt and that salt is you are using for elutions. Now, in a subsequent step suppose you would like to do the enzymatic reaction and that salt is going to interfere, or suppose you want to use this protein for any other application and this salt is going to interfere into your analysis, then you have to remove this salt.

And there are multiple ways in which you can remove this salt. One of the ways is that you can put the dialysis of this particular protein against the buffer, if you do the dialysis the salt is going to be exchanged along with the water and that is how the water will come in and the salt is going to be removed.

But the dialysis is a very-very small step, so in case some proteins are prone for degradation or some proteins are getting inactivated, if you are going to continue this process for 10 hours or 12 hours. In those cases, we are also using the gel filtration chromatography to remove the

salt. Because salt is nothing but the small molecules compared to the protein which is of large molecules. So, if you inject the protein onto a gel filtration column, the protein is going to form a separate peak and the salt is going to form the separate peaks.

Because salt is small in size, it will going to come and it will sit into the bottom of the beads whereas the protein will go and bind to the other sides. So, because of that a gel filtration column is equilibrated with the buffer or water and then the sample for desalting is loaded.

After the run, the proteins and salts are eluted separately as a peak. So, if you will elute the protein will come out first, and the salt is going to come the later on. So, you can collect this protein's peak amount, and that is how your protein is going to be separated from the salt. And this protein can be used for downstream applications.

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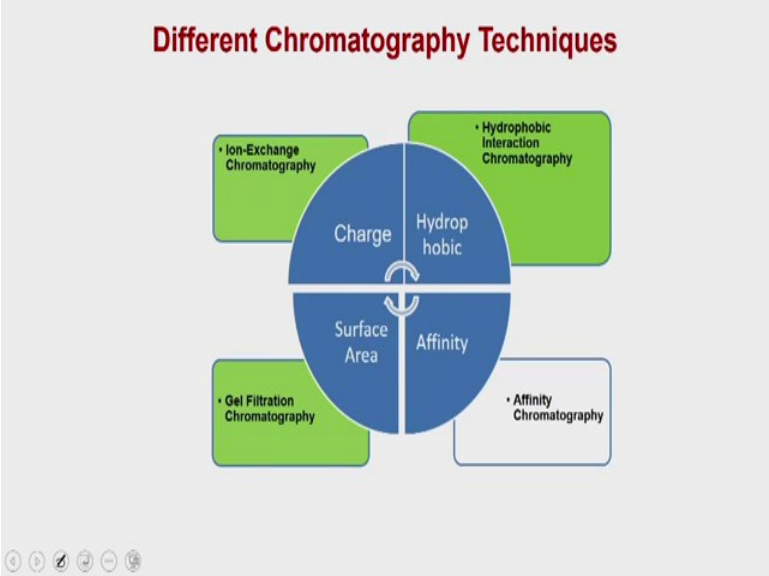

So, now with this we have already discussed about the ion-exchange chromatography, we have discussed about hydrophobic interaction chromatography and we have discussed about the gel filtration chromatography.

And so far, we have discussed about all these three chromatography techniques, which people are very often using for protein purification as well as the digestion of the proteins. In general, people are running the ion exchange chromatography first, followed by the hydrophobic interaction chromatography and followed by the gel filtration chromatography.

The reason is that, the ion exchange chromatography requires the protein to present without salt whereas the hydrophobic interaction chromatography requires the high salt concentration. So, the fraction which you elute from the ion exchange chromatography already contains salt.

This means you can directly load those fractions without going through a process of dialysis or desalting into the hydrophobic interaction chromatography.

And once the sample which you are going to get from the hydrophobic interaction chromatography is going to be of low salt, because if you remember the elution conditions for the hydrophobic interaction chromatography is that we are decreasing the salt. So, your sample or the fraction which actually will contain your protein are going to have the low salt conditions. And then you can directly load those low salt proteins onto the gel filtration column to purify or characterize.

Now, in the subsequent lecture we are going to discuss about the affinity chromatography, and we are going to discuss about how to generate the affinity columns and we are going to take up different examples of affinity chromatography. So, with this I would like to conclude our lecture here. In the subsequent lecture we are going to discuss about the affinity chromatography. Thank you.