Experimental Biochemistry
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Lecture – 23
Chromatographic Techniques - I

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We continue our discussions in Experimental Biochemistry. And in the next two lectures, we will learn about different chromatographic techniques, protein purification procedures, basics of these techniques. And in this lecture, we will cover gel filtration chromatography.

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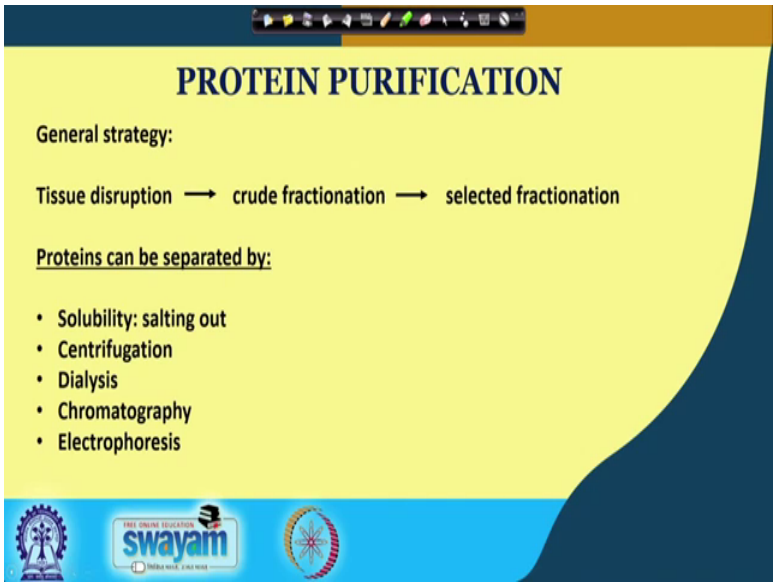

To understand about protein purification, we first need to know what or where we can get the protein from. If we were to identify or we were to isolate a protein from tissues, be it the liver tissue or even blood, there are certain techniques that we have to accomplish before we can get into the chromatographic technique where we are going to go for a further purification and isolation of the protein.

So, we start off with the tissue disruption which is going to give us a very crude fractionation, and then the selected fraction which you will see in a moment as what we mean by that. The proteins can then be separated out by salting out, centrifugation, dialysis chromatography and electrophoresis. Each of these techniques have their own unique property, their own unique characteristic. And it is important to understand which technique has to be used for protein purification and the isolation.

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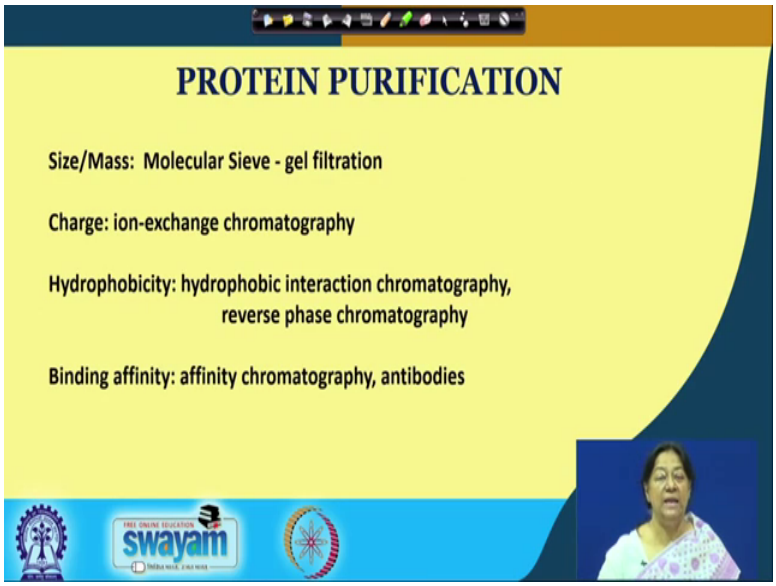

If we are going to separate the chromatographic technique that we are going to be considering has to be an property of the protein or property of the particular material we want to isolate. So, whether we are going to do this based on the size or the mass of the protein, we are going to use a general molecular sieve methodology known as gel filtration chromatography. If we are going to exploit the charge on the protein, we are going to be using ion exchange chromatography.

If we are going to exploit the hydrophobic character of the protein, we can go for hydrophobic interaction chromatography or reverse phase chromatography. And finally, if we looking at affinity chromatography, we will look at what is called the specific binding affinity of the protein to be isolated. So, in general we look at these specific techniques and depending upon which property we can exploit, we will choose a method accordingly.

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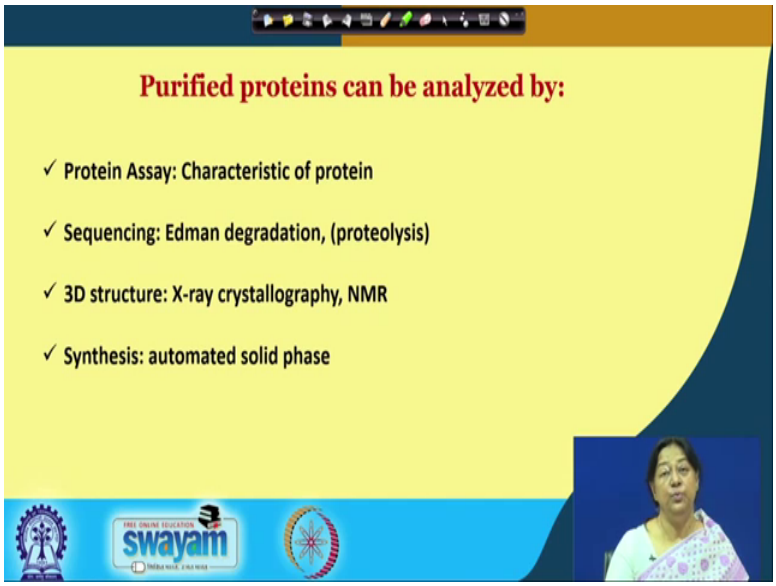

So, what we want to do then is after we have isolated the protein, after we have obtained our protein of interest, we will go for different assay methods. A protein assay to understand whether we have actually isolated the protein of interest, this is convenient if you have an enzyme because then you can do a specific enzymatic assay to see whether you have isolated the correct enzyme or the enzyme of interest. Particular sequencing to see whether we have the amino acid sequence of the specific protein. The 3D structure which involves elaborate methodology in terms of X-ray crystallography and NMR. And of course, we can look at automated solid phase synthesis.

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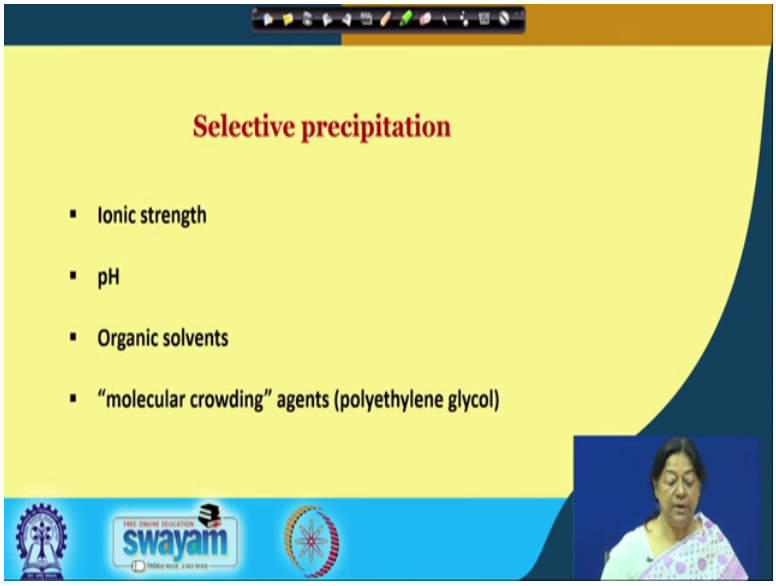

For selected precipitation of a protein, there again properties that we will look at ionic strength, pH, organic solvents, and molecular crowding methods agents such as polyethylene glycol.

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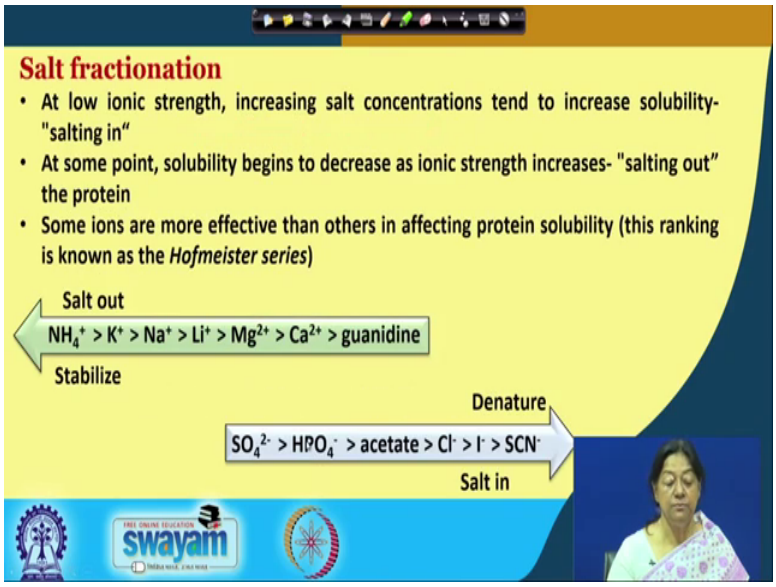

When we look at salt fractionation what we want now is we want to precipitate our protein of interest. At low ionic strength, what happens, increasing the salt concentrations will increase the solubility. At some point, the solubility is going to decrease as the ionic strength increases which is known as salting out, because the preferential aggregation of the proteins then occurs. There are some ions that are more effective than others in affecting the protein solubility as a whole. So, this ranking as has been given here in terms of salting out, and in terms of denaturing or salting in is termed as the Hofmeister series.

As you can see the highest salting out here is the ammonium ion and the greatest salty here is the sulphate ion. So, you will see in many cases ammonium sulphate is used for the salting out procedure. Now, there are different characteristics or different methodologies, different weights that you have to use depending on your protein of interest. And these specific methodologies are available in many tables in the back of experimental biochemistry books, and there are protocols that help you understand how much of the ammonium sulphate salt what concentration you are to use for the salting out method.

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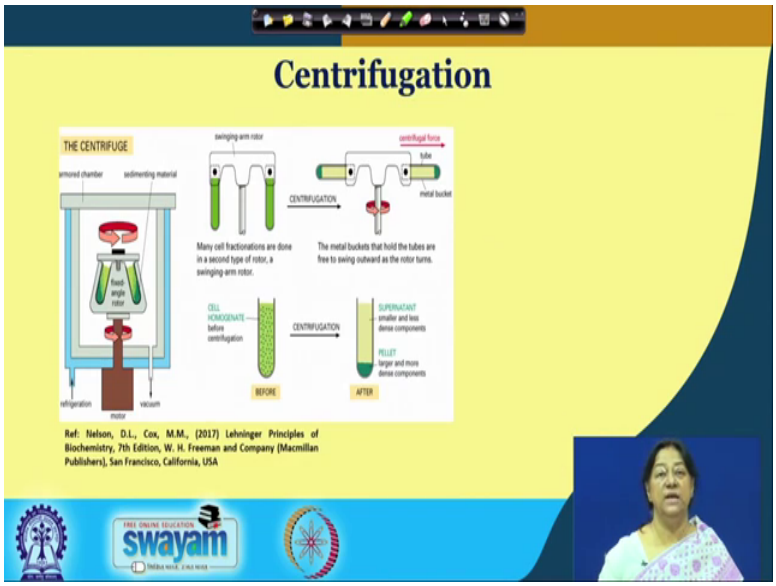

In the centrifugation procedure which you will be also being seeing in a demonstration, we are looking there are different thing different machines that are available. One is one that has a fixed angle rotator where you can see the sockets in your rotor have fixed angles, where the precipitation is at an angle. There is a swinging arm rotor which on rotation keeps the test tubes or your centrifuge tubes horizontal. The advantage of using a swinging arm rotor where the centrifugal fugal force rotates it in such a fashion that these swing out to a horizontal setup during the rotation what happens is we get the cell homogenate that before centrifugation was many self fractionation mixed up together here. But after the centrifugation, we have a pellet where the dense components are at the in the pellet, and the smaller and less dense components are present in the supernatant.

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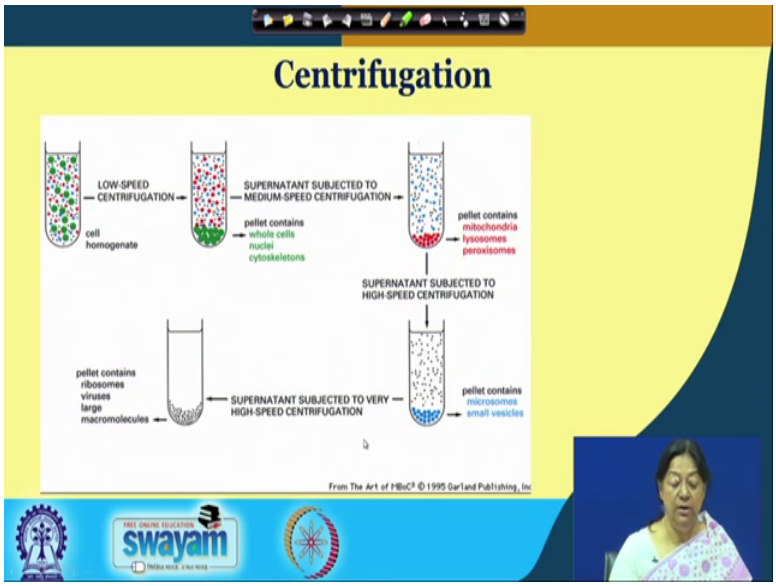

We then have to consider for low speed centrifugation what we would like to need, do we want medium sized centrifugation pellets or do we want. So, if we look at the cell homogenate, if we do a low speed centrifugation, the pellet in this case contains whole cells nuclei and cytoskeletons. If we go even further, we have mitochondria, lysosomes, peroxisomes, even further we have microsomes small vesicles; and further down we have ribosomes, viruses, and large macromolecules.

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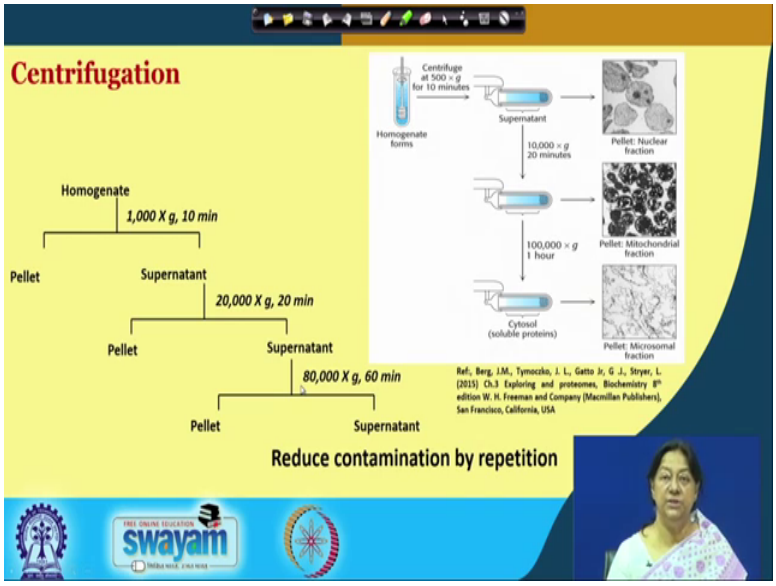

So, in this way, we can speed up the methodology depending upon exactly what we want, and we can reduce the contamination by repeated application at different speeds of centrifugation.

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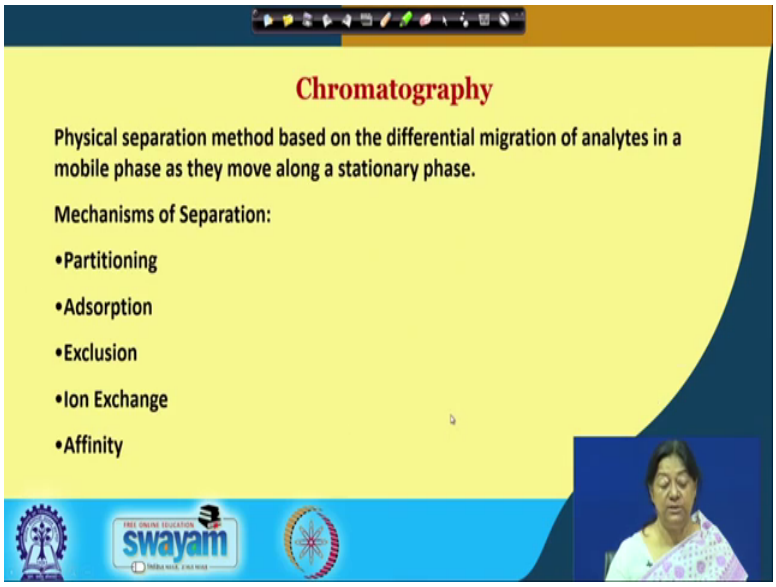

So, finally, with our supernatant of interest or if it a protein happens to be a dense molecule in the pellet, we use a physical separation method now based on the differential migration of the analytes in a mobile phase as they move along a stationary phase. We will understand what this means as we go along we have different mechanisms of separation partitioning adsorption exclusion ion exchange and affinity as was mentioned a while ago.

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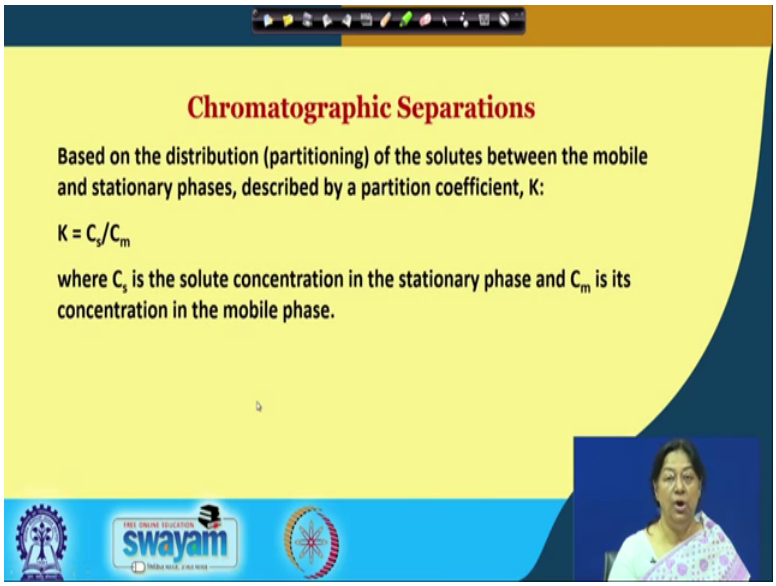

Now, based on the distribution that is the partitioning of the solutes between the mobile and the stationary phase described by a partition coefficient. Now, what do we mean by a mobile phase and what do we mean by a stationary phase. We will have a solute concentration in the stationary phase, and a concentration in the mobile phase.

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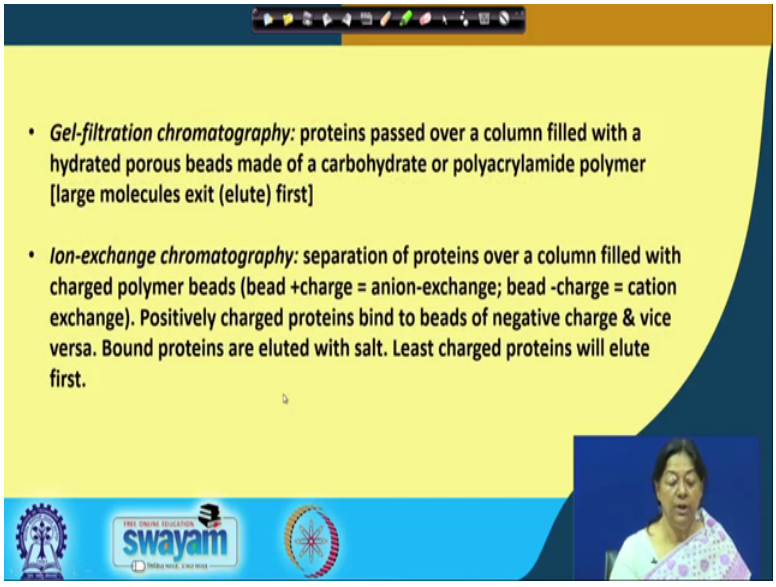

In gel filtration chromatography as was mentioned earlier, the proteins passed over a pass over column filled with the hydrated porous beads that are made of a carbohydrate or a poly acrylamide polymer. So, this these beads made up of this polymer is what is known

as the stationary phase. In the ion exchange chromatography, the polymer beads have a specific charge to them they have either can be an anion exchange or a cation exchange. Now, when we look at a positively charged protein, they will bind to beads of negative charge and vice versa.

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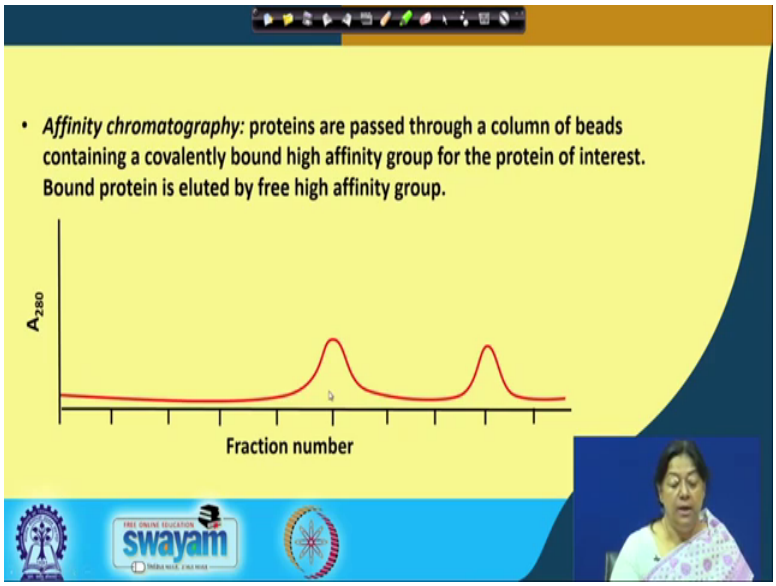

In affinity chromatography, the proteins are passed through a column of beads that contain a covalently bound high affinity group to which the protein is attracted the protein binds and it can be then eluted that has come off the column depending on our interest. Now, when we do this fractionation or this chromatographic technique, we will be collecting what are called fractions say 3 ml fractions or 2 ml fractions depending upon how you want your separation to occur.

And to understand whether we have a protein in the test tube of interest we will collect the specific fractions, and measure the absorbance at 28 nanometers with the knowledge that the protein of interest or any protein that is present in the fraction will show up or will have a specific absorbance associated with it. So, in this case we have this fraction that has a protein in it, we have this fraction that has a protein in it.

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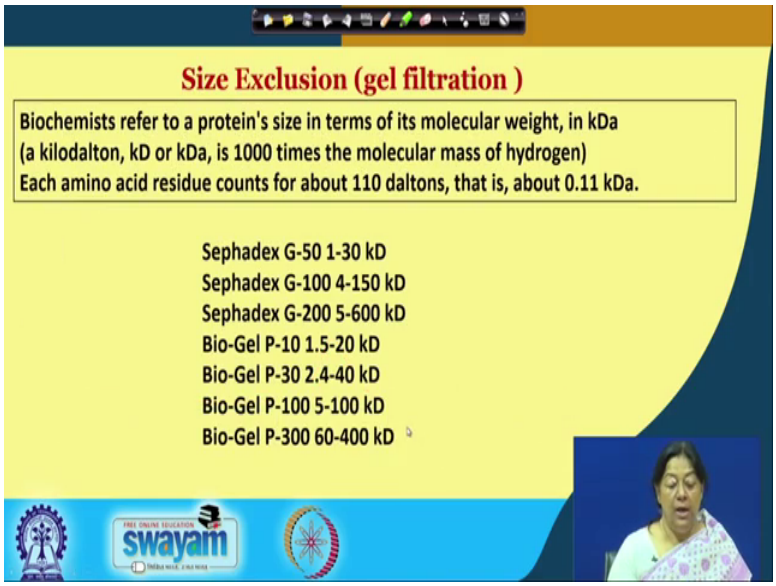

Now, when we look at the protein size we call it the molecular weight. And the molecular weight is referred to specifically as kilo daltons for convenience which is 1000 times the molecular mass of hydrogen. And these are different specific company, specific polymer resins that can separate proteins of different molecular weight ranges. So, we have sephadex G-50 that will go to 1 to 30 kilo dalton and so on and so forth.

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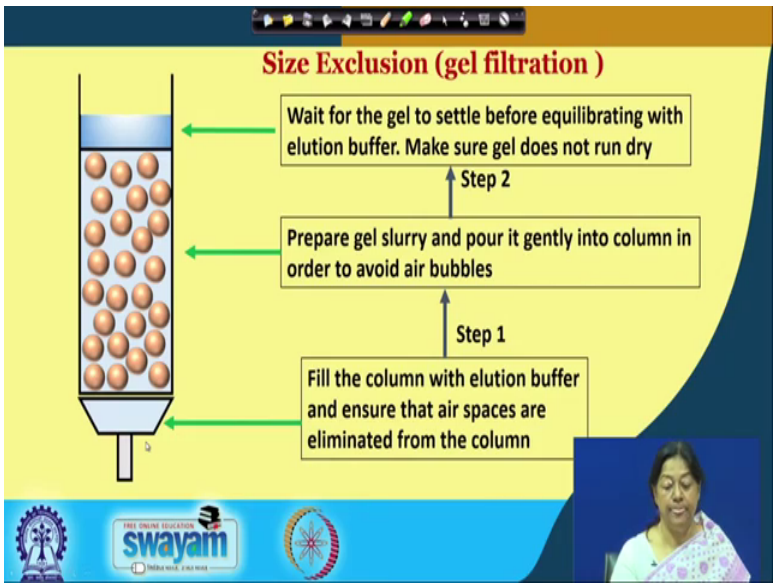

In size exclusion as we mentioned this is our column. And you can see that these beads are the stationary phase. Now, we have to wait for this gel slurry as it is called to settle

down before we can start what is called an elution. So, we wash the column with a buffer solution till we have a very low absorbance value with the full knowledge that there is nothing but the buffer present in our column here. It is also important to avoid any air bubbles in the column.

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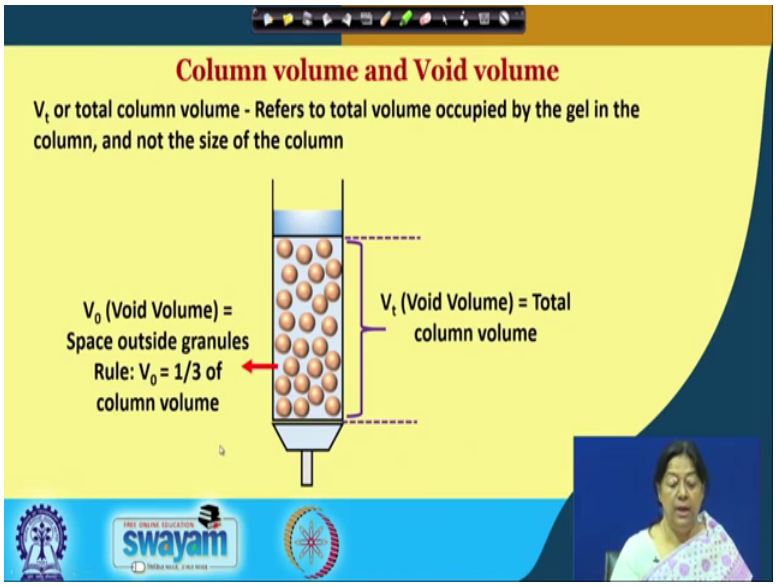

Now, we need to know the total volume that is the void volume of the column, the total column volume and the volume that is the space outside the granules.

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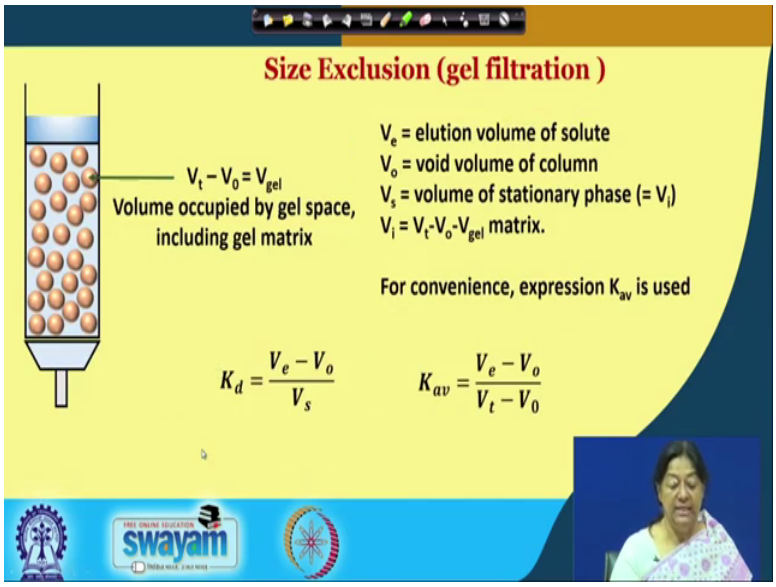

Now, when we look at this particular set up, we will see that there is therefore an area or a volume that is occupied by the gel matrix or the beads. So, we have an elution volume of the solvent known as the V e; we have a void volume of the column; we have a total volume of the column. And based on that, we can find out what is called a specific distribution; this distribution is given by this K average which is the elution volume V 0 is the void volume, V t is the total volume; V 0 is the void volume. We will see what this looks like in a moment.

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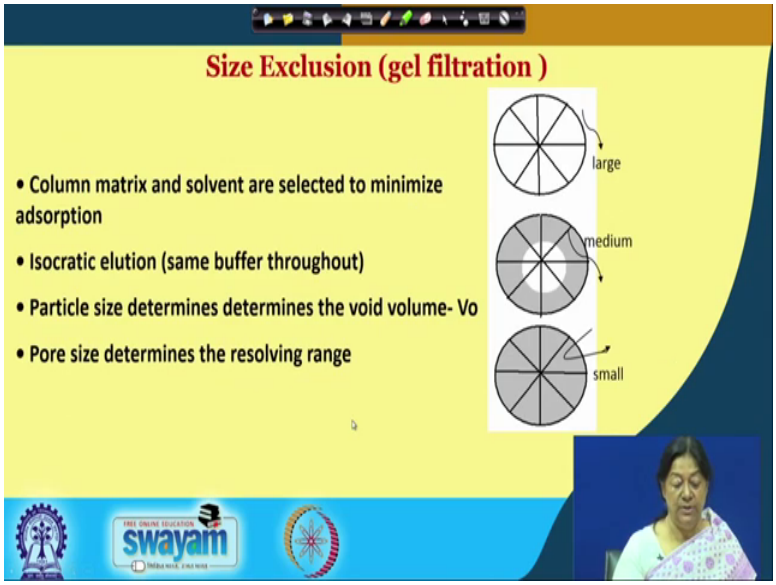

So, now if we have a column matrix and we have a distribution of the column of the material coming out of the column, then if this is our polymer bead matrix, and what is going to happen is any large molecule will not be able to enter the network of the beads. A medium sized molecule may enter it a bit, and a small size molecule will enter the network of the beads.

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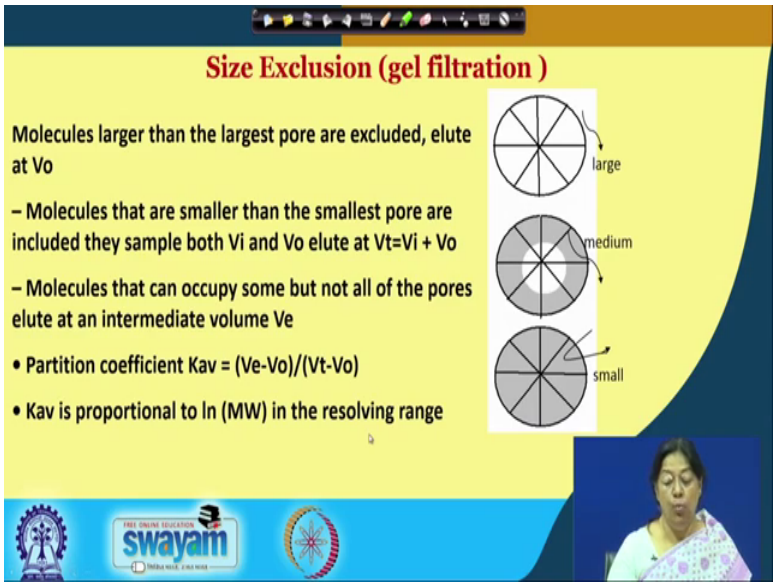

What will happen is the molecules that are larger than the largest pore size will be eluted first that means they will come off the column first. Molecules of medium size will come out in an intermediate fashion in the middle. And molecules that are small will occupy the whole bead network like the sample that we see here.

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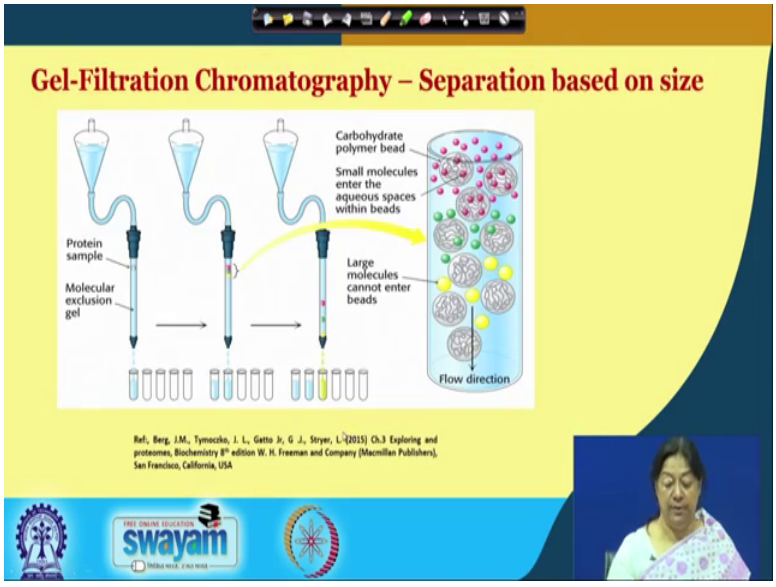

So, this is our column. This is are the carbohydrate polymer beads as you can see as the network. The larger molecules that are yellow colored here do not enter; the medium colored ones that are green here will enter partially, but the smaller ones enter. So, as

these are washed out with the flow of the buffer that is poured into the column, what is going to happen we will have a separation. And the separation will be based on what initially we have our protein sample, there is a gradual separation according here based on the size of the molecules. So, the yellow molecules will come out first followed by the green followed by the pink.

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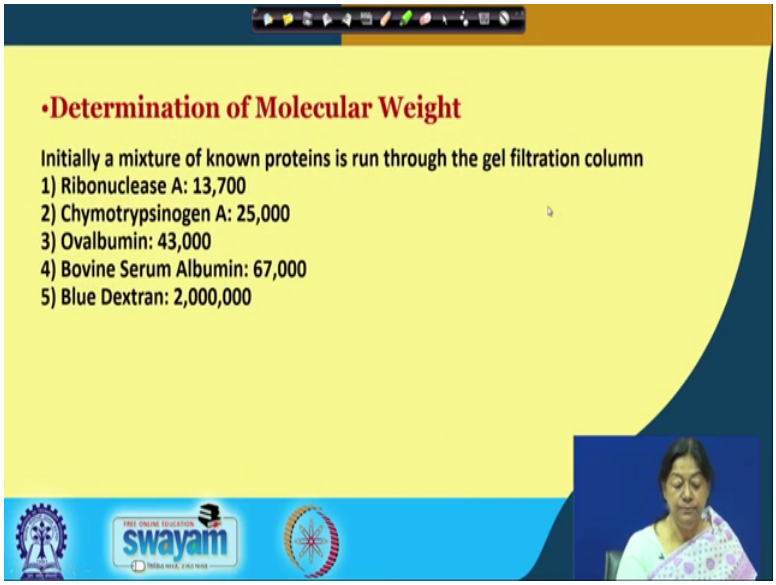

To determine the molecular weight of our proteins, we have a mixture of proteins of known molecular weight that are run through the gel and a calibration is done.

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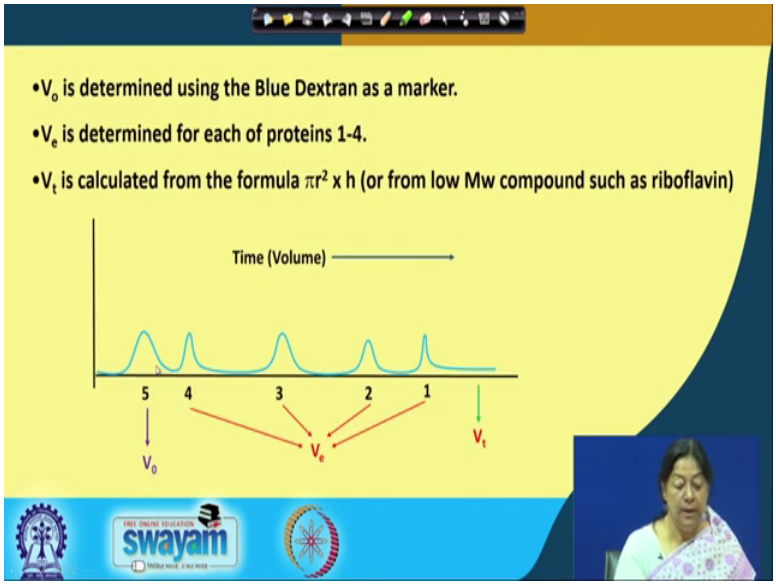

So, if we use V 0, it is determined using Blue Dextran, and you understand that Blue Dextran is very large molecule that is not going to enter the beads. V e is determined for each of the volume proteins 1 to 4 the middle set of proteins. And V t is going to be either the pi r squared h that is the total volume of our column. So, now, we will have the larger molecule come out first, the intermediate columns and the smallest one come out last.

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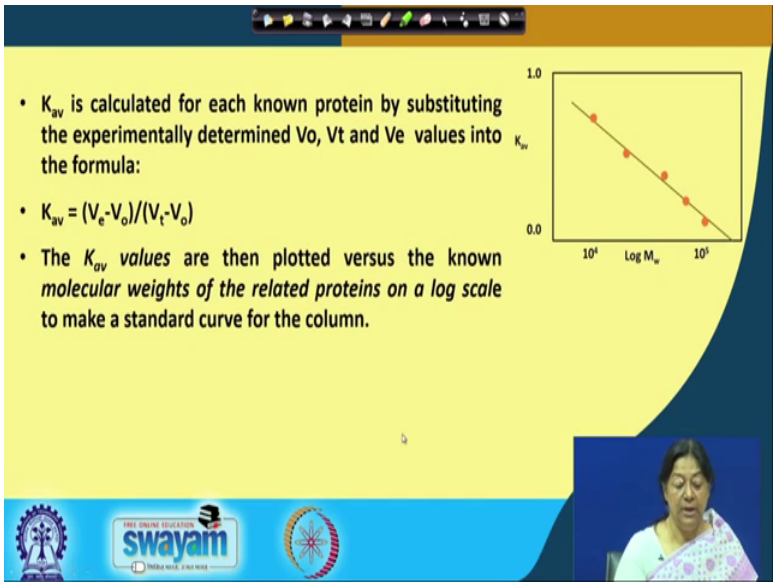

Then we calculate the K average as was found out the distribution is calculated for each known protein by substituting the experimental values here. And we have a plot of K av versus the log molecular weight. Now, for any unknown protein when you are when it is loaded into the column, there in the same column the same flow speed and the same elution conditions, then we will have a specific elution volume for the unknown protein, a knowledge of the K av will tell us what the log of the molecular weight of the unknown protein is.

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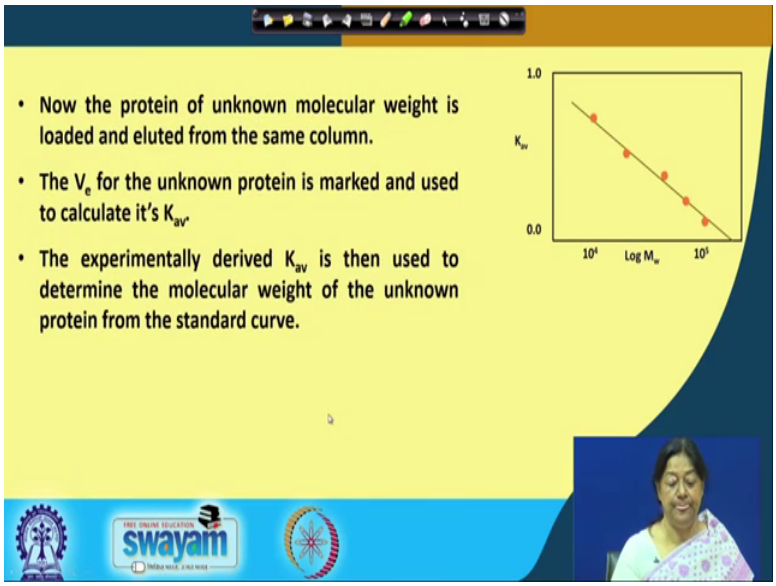

So, the protein of unknown molecular weight is loaded and eluted from the same column, and then we can calculate the molecular weight from the standard curve.

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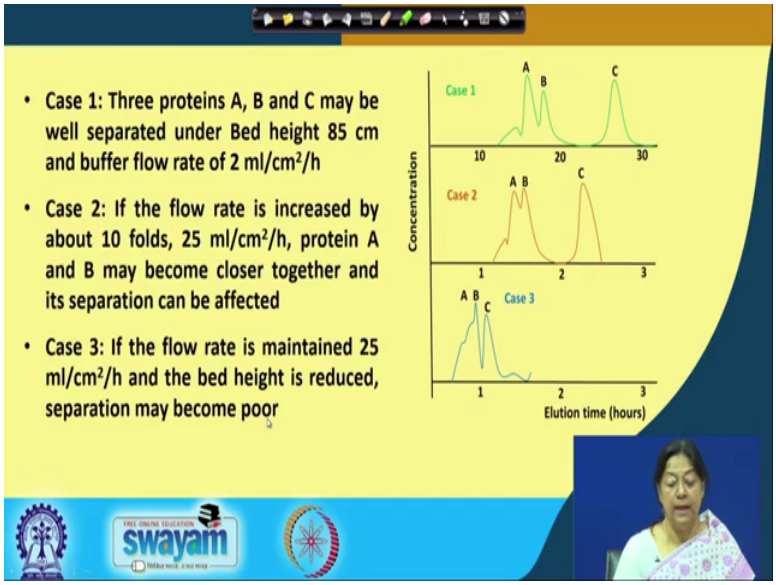

Now, how would we have specific conditions in gel filtration chromatography dependent on the size of the column and the flow rate? For example, in case one, there are three proteins here A, B and C. They are well separated we can say because we see distinctly three peaks. The bed height in this case is 85 centimeter with a buffer flow rate that is washing off the proteins from the column at 2 milliliter per centimeter square per hour.

Now, if the flow rate is increased by 10 folds, then what happens is we are forcing the proteins to come out earlier and faster, and the separation as you can see is not as good and A and B are not distinctly separated from a one, one another. So, the separation is affected.

Now, if we keep the flow rate at 25, but we reduce the bed height, then again we see that since we do not have a long enough bed height, we do not have a good enough separation. So, the optimum is to have a long column with a small flow rate for better separation of the proteins.

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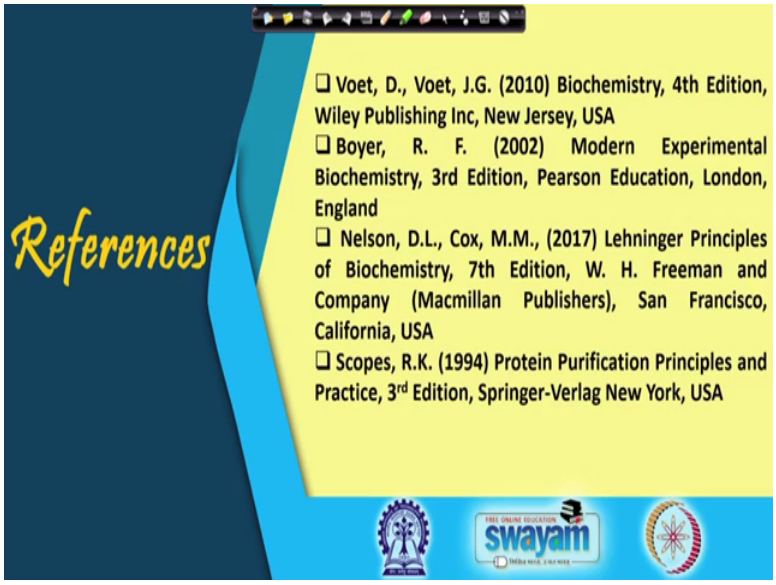

What we will study here is in these references, references based on Modern Experimental Biochemistry and Protein Separation Methods. If we just go back once to look at the specific proteins that we were looking at here where we had a Blue Dextran that was extremely large in size, bovine serum albumin smaller than that, then ovalbumin, then chymotrypsinogen, and then ribonuclease A. So, based on our understanding of what we considered based on the column separation, it would mean that the larger protein molecules will come out earlier followed by the intermediate ones, then followed by the smaller ones.

So, what do we expect in our set of proteins, we have 5 proteins here. We would expect the Blue Dextran to come out first, followed by BSA number 4, followed by number 3, followed by number 2; then followed by number 1. So, when we look at our specific v

zero which is determined use the Blue Dextran we see that number 5 has come out earlier.

So, what do we have on the y-axis, it is to be the absorbance at 280 nanometer, then here we have the specific elution volumes that tell us that this is where we have the Blue Dextran. And since we know the molecular weight we can calculate it from the specific V e values that we get for proteins 4, 3, 2, and 1. With this knowledge as we learned earlier we can find out what the K av value is. And from the K av value, we can look at what the elution volume is, what the void volume is, what the total volume is and what the void volume is again.

So, this V t minus V 0 is constant; V 0 is constant; V e is what varies from protein to protein. So, each protein will have its own elution volume, and the elution volume in gel filtration chromatography is going to depend upon the size of the protein. The size and the mass of the protein we understand that if this is large enough, it is not going to enter the polymer matrix. If it is medium sized, then it will partially enter the polymer matrix. And if it is very small, then it will surely enter the polymer matrix. And it will be delayed in its elution which means that it will take a longer time to come out of the column and then it will be have a larger elution volume.

So, based on that we will have a plot where we have the K av value plotted against the log molecular weight. Now, this is going to so we have five points here from the five proteins that we have considered. And now we consider an unknown protein. When we change our protein to the unknown protein, we have to remember that this has to be loaded and eluted from the same column to prevent any calibration mistakes, because if we do it with a this has been obtained using a specific condition.

So, we have to use the same conditions the same V 0, the same V t, and the elution volume is going to be different because our unknown protein will have a different mass, it will have a different size. So, then we calculate we obtain the V e from the fractionation. And from the formula for we find its K av value, and then the experimentally determined ka v value is used to determine the molecular weight of the unknown protein from the standard curve.

We looked at different methodologies based on how the column height, the flow rate is important in determining how well we can get a separation in our gel filtration

chromatography technique. So, what do we mean is for gel filtration, it is important to have the optimum height close to a meter 85 centimeter in this case and a low flow rate. What do we mean by a flow rate, a flow rate is the collection of the fractions as was mentioned. If we have a collection in a single test tube say around 3 milliliters per test tube, then we can look at the collections for the 2 ml, and determine the absorbance values of each of these test tubes that are our fractions.

But if we have a two large a flow rate, the problem will arise that the proteins will be coming out too fast, and they will overlap with one another in their separation. And if we have a shorter bed height, then again we do not allow a longer separation to occur which will result in again an overlapping of the proteins.

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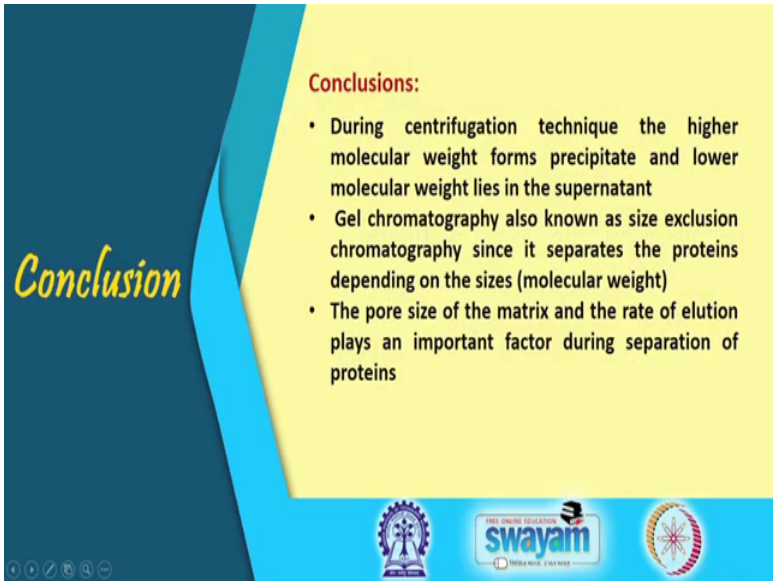

So, what we learn is in the centrifugation techniques, we have the higher molecular weight, the denser material that is forming a precipitate; and the lower molecular weight lies in the supernatant. The gel chromatography also known as size exclusion chromatography since it separates the proteins depending on the sizes that is the molecular weight. And the pore size of the matrix in this case and the rate of elution we found out plays an important factor during the separation of the proteins. So, we have to remember that when we are separating the proteins according to their size or mass, we will use a gel filtration chromatography technique. And the column height and the flow rate are important factors that we need to take into consideration.