Fundamentals of Protein Chemistry
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Module - 04
Protein isolation and characterization
Lecture - 17
Protein Purification

In the 2nd lecture of module 4 which deals with protein isolation and characterization, we will be looking at protein purification protocols. In the previous lecture, we looked at salting out centrifugation and got an idea about sedimentation techniques.

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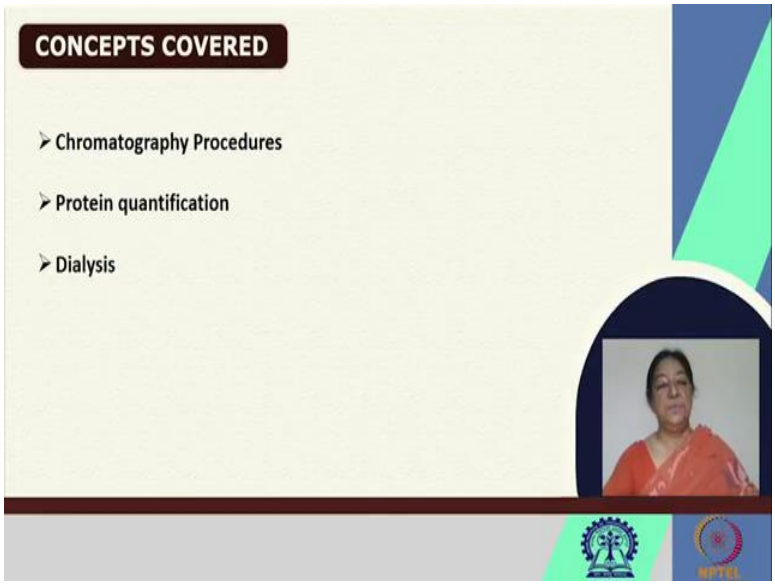

In this lecture we will be looking at specific aspects of chromatography procedures, protein quantification and finally dialysis. Our interest is our protein; the specific tissue or the microorganism from which we are going to isolate this protein.

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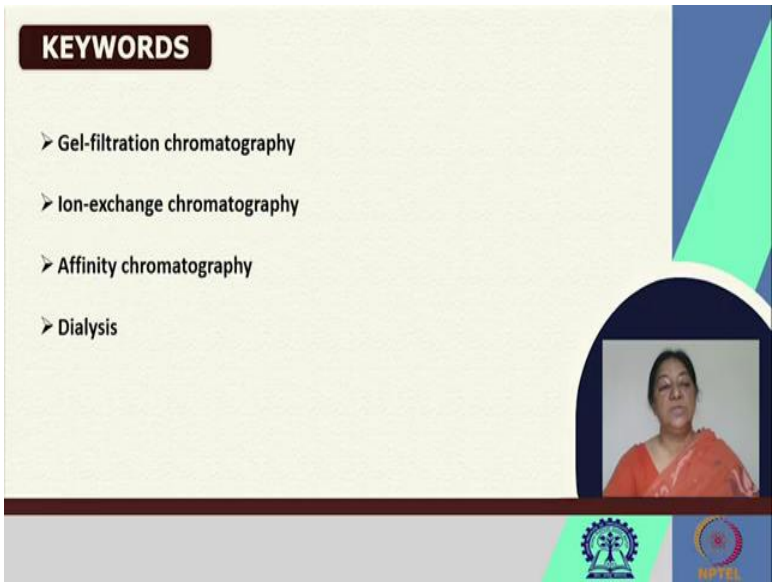

We have a crude fraction of the protein and our next idea is to look at this crude fraction and then following specific protocol, exploiting a specific property of the molecule, we are going to go for specific procedures to get to our final protein.

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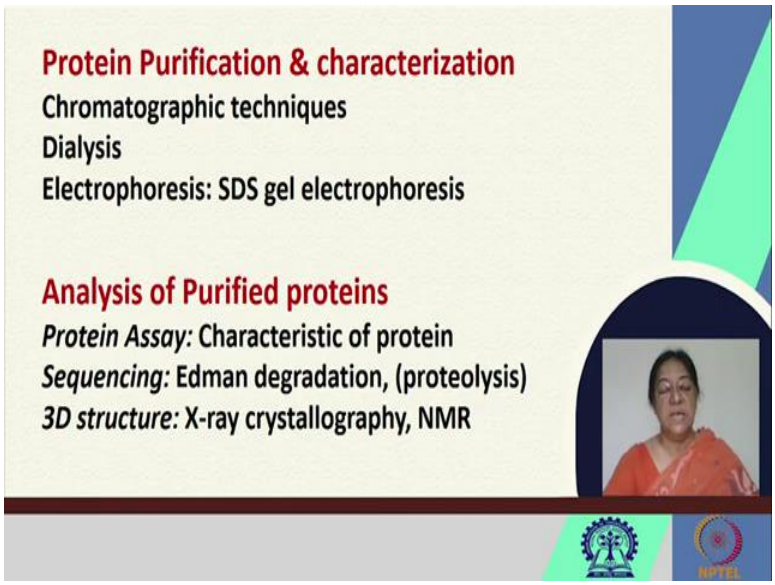

In these we will be looking at chromatographic techniques, dialysis followed by electrophoresis, which will be discussed in our biophysical methods lectures; the following three lectures of this module. When we analyze the purified proteins, we look at a protein assay that is characteristic of the protein.

We want to know whether we have actually isolated our protein of interest, which would mean that if it were an enzyme, we would look for an enzymatic assay; if it were a specific functional protein, we would look to see whether it is performing its function. To realize that, we have our protein of interest.

Following this we can do sequencing to look at whether we have the peptides in the amino acids in the correct sequence or following the 3D structure, which would actually tell us whether we have the particular protein of interest in its folded form.

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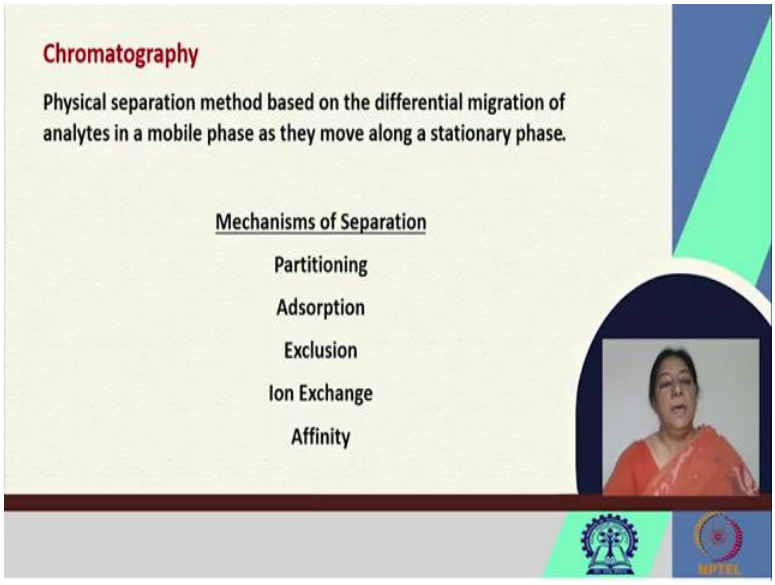

In the physical separation methods of chromatography, what we are looking at here is [refer to slide], we are looking at the differential migration of analytes in a mobile phase, as they move along a stationary phase.

Now in the mechanisms of separation that are known, there is specific partitioning of the compounds, adsorption, exclusion, ion exchange and affinity. The mechanism of separation now looks at a specific property of the protein that we need to isolate.

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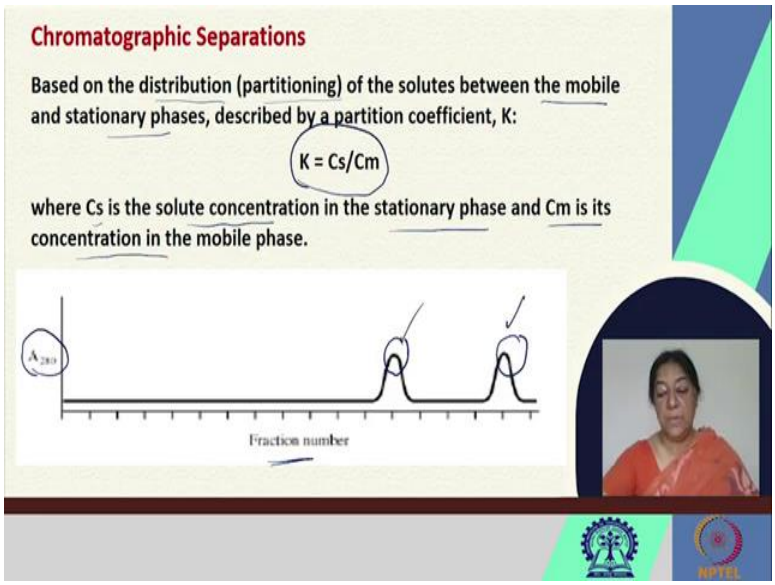

For example, the distribution that is based on the partitioning in general for any chromatography separation, looks at the mobile phase and the stationary phase that is described by a partition coefficient K. Where Cs is the solute concentration in the stationary phase and Cm is its concentration in the mobile phase. So as it is moving along the column that we will see, we will see a separation method, that is going to be exploited to isolate our protein of interest. What is marked on the y axis here [refer to slide] is the absorbance at 280 nm.

We had seen previously that in the uv absorption of proteins, absorption at 280 nm indicated the presence of the aromatic amino acid residues. Assuming that the proteins that we are going to isolate in this case do have aromatic amino acid residues present.

We have adsorption at specific fractions of our collections that we are going to be looking at in a moment, that tell us that these particular fractions are the ones that have proteins in them.

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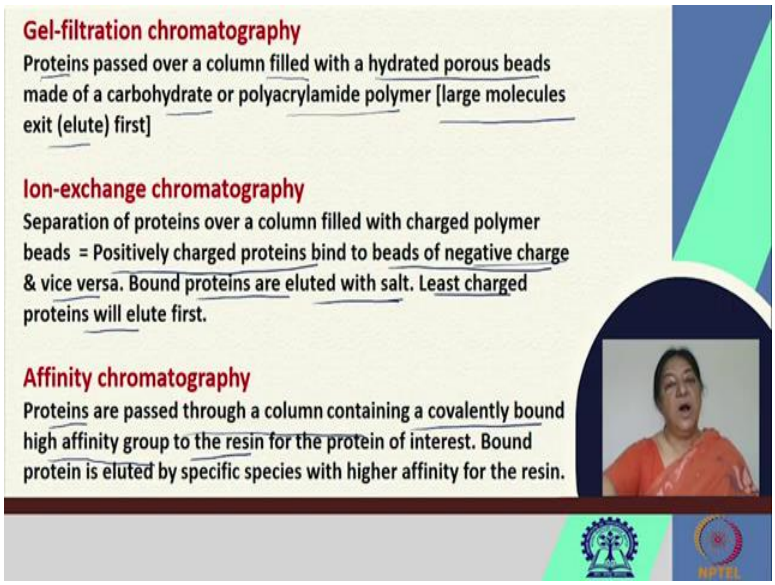

How do we do this procedure? We have three methods that we will follow; gel-filtration chromatography, ion-exchange chromatography and affinity chromatography.

We will see what properties we are exploiting in each of these cases. In gel filtration chromatography, the proteins are passed over a column, that is filled with hydrated porous beads, that are made up of a carbohydrate or polyacrylamide polymer and the larger molecules exit or as in chromatography parlance is called, elute first.

In ion exchange chromatography, we are looking at the separation of proteins over a column filled with charged polymer beads. So what is going to happen? The positively charged proteins will bind to beads of negative charge and vice versa and the brown proteins will be eluted with salt, where the least charged proteins will elute first.

In affinity chromatography what we are doing is, the proteins are passed through a column containing a covalently bound high affinity group to the resin for the specific protein of interest. We are looking at the property of the protein that has a specific affinity for any compound be it glucose, be it DNA.

A protein or the DNA or the glucose is bound to the resin or the bead and our protein of interest will then bind to the specific component that is added to the bead and then can be eluted. We will be briefly visiting each of these types of chromatography to give us an idea of how the procedure is accomplished?

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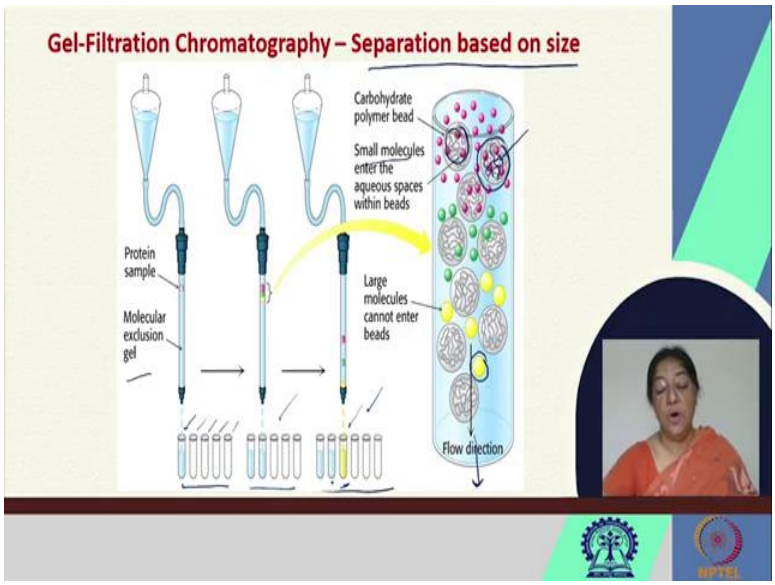

In gel filtration chromatography we have separation based on size. This is also called size exclusion chromatography or molecular sieve chromatography. In this case what happens, we have a carbon polymer bead which has within it a network. In this network the smaller molecules can enter the network; the medium size molecules will partially enter the network, while the larger molecules cannot enter the beads and are removed first.

So we have our protein sample, a molecular exclusion gel and these are the fractions that we collect. Each of these fractions are then checked for absorbance as was shown in a previous slide, where we are looking at the absorbance at 280 nm and these are our specific fraction numbers.

Depending upon the separation of the proteins, we will see that the large molecules that cannot enter the bead will be eluted first. So this particular fraction will give us an absorbance at 280nm, because of the presence of the protein.

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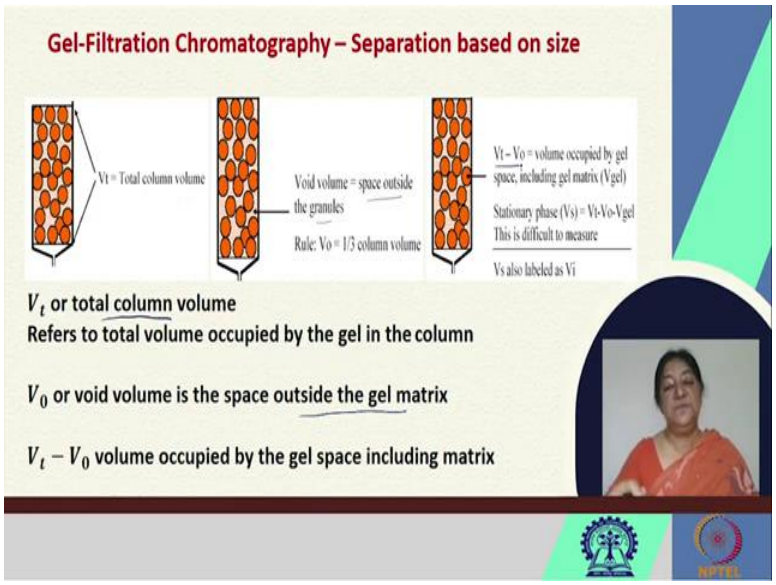

There are certain terminologies that we need to know here. There is a total column volume that is the total volume that is occupied by the gel in the column. We have the void volume that is the space outside the gel matrix or outside the granules, which needs to be also known to determine the partition coefficient. This means that, the volume occupied by the gel space including the matrix is V – (V_t - V_0).

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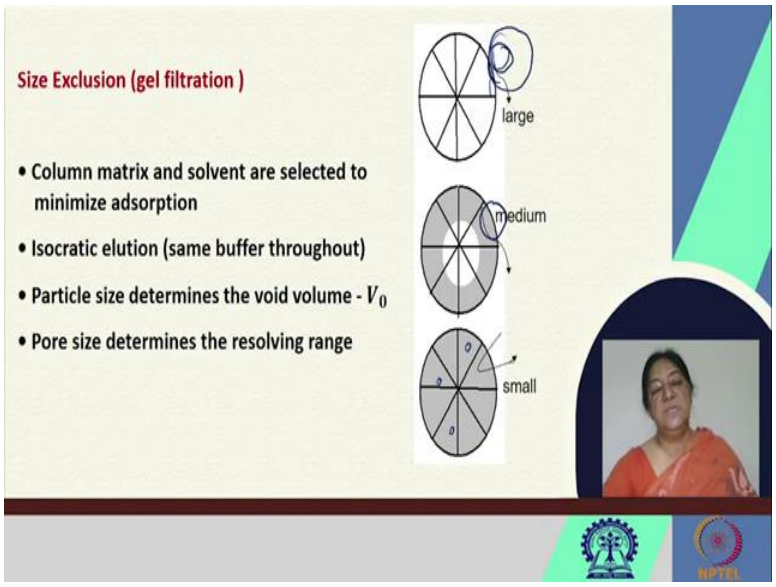

In this size exclusion or gel filtration chromatography, we realize that the larger molecule will not be able to enter the bead, a medium size molecule may partially enter the bead, but a smaller size molecule will definitely enter the bead.

So, what happens is the column matrix and solvent are selected to minimize adsorption. We use the same buffer throughout, usually in an isocratic illusion as is it is called and the particle size determines the void volume, and the pore size determines the resolving range.

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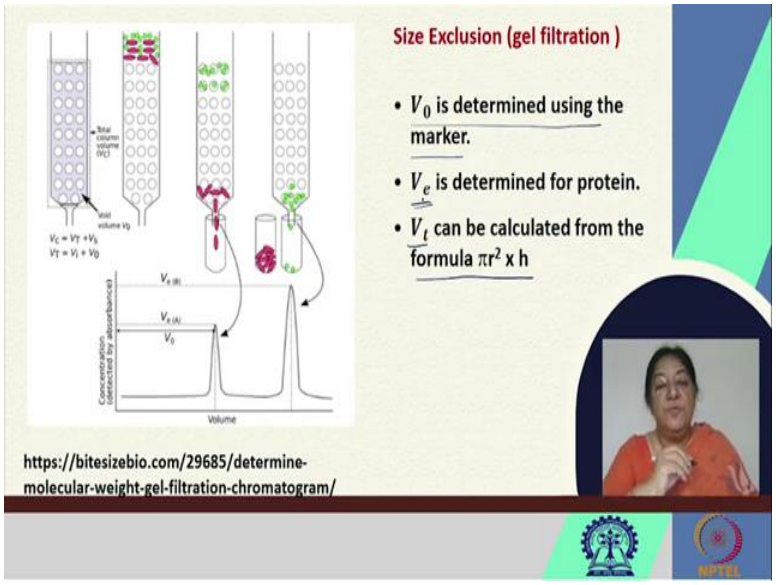

So, when we are looking at a system, we have the V_0 that is determined by using a specific marker. V_t can be calculated from the formula for the volume of a cylinder and the elution volume is determined for the specific protein of interest.

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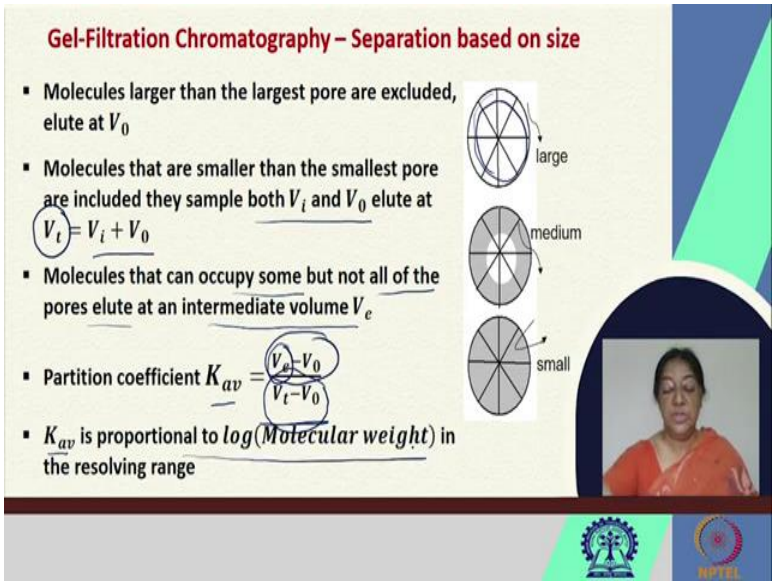

How is this accomplished? We have our specific expressions that we are going to come across.

The molecules larger than the largest pore are excluded. So they would elute at V_0 as they do not enter the pores of our gel matrix. Molecules that are smaller than the smallest pore would be included in both the samples V_i and V_0; indicating that this corresponds to the total volume. The volume of the gel matrix that is the internal volume of all the beads that we have, and the V_0, that is the portion that is outside the gel matrix, gives us the total V_t.

Molecules that occupy some but not all of the pores, elute at this intermediate volume V_e. And we have defined a partition coefficient, which is given by K_{av}, the denominator V_t - V_0 depends upon the column of interest. V_0 here is the variable that depends upon our protein and K_{av} is proportional to the log of the molecular weight in the resolving range.

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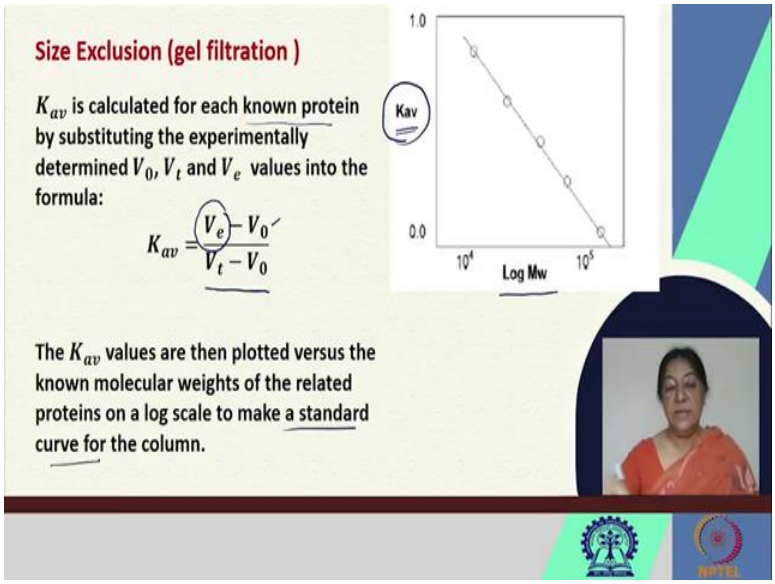

K_{av} is calculated for each known protein, we have a set of known proteins for which the molecular weights are known. Here we have the log molecular weight and the K_{av} value, depending upon the column used and the conditions for the illusion the experiment is conducted, where we have the V_t - V_0 corresponding to the column.

We have the V_0 corresponding to the column and the elution volume for each protein in the known sample is then plotted and the K_{av} is calculated plotted against log M_w, to give us our calibration. So we have a standard calibration curve.

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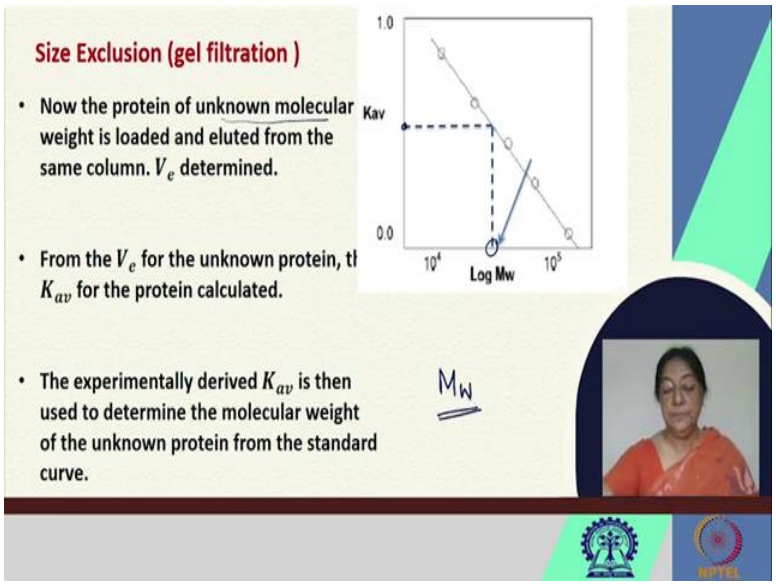

Then the protein of unknown molecular weight is loaded and eluted from the same column under the same conditions of flow rate. From the V_e we can calculate the K_{av} given the formula in the previous slide. So if this corresponds to the K_{av} of the unknown protein, we can from our calibration curve, determine the log molecular weight of the protein and from that we can find out the molecular weight of our unknown protein.

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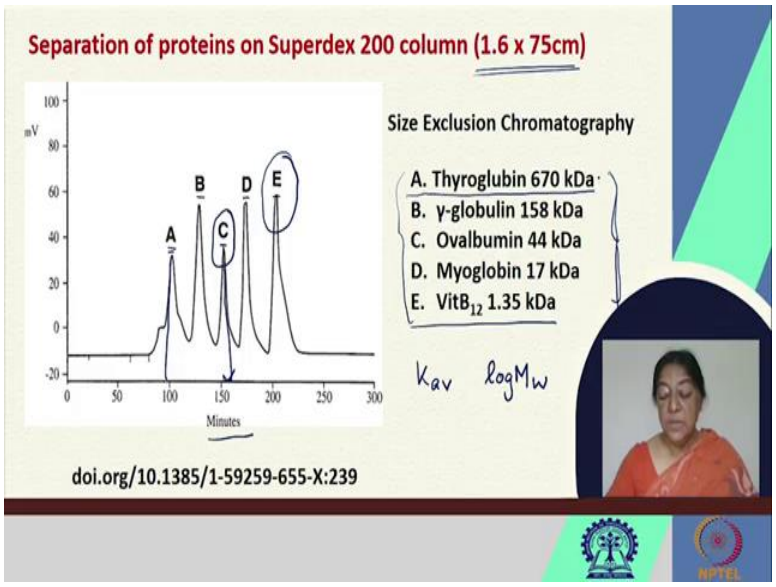

In a typical size exclusion chromatography, chromatogram as it is called, if this [refer to slide] is the column dimensions, we have 5 proteins here. We see in the minutes that have been elapsed through the column running, we have molecule A come out first followed by B C D and E. So A comes out first and D comes out last, indicating that A has a large molecular weight and E has the smallest molecular weight.

This is an indication of the molecular weight distribution. Then what we can do, is from the K_{av} value, from the log of the molecular weight values, we can calculate or form the calibration curve and then run the unknown protein and find the molecular weight of the protein of interest, not only finding the molecular weight but also the separation of the proteins.

If we are interested in E or we are interested in the protein C; this [refer to slide] will be the distribution. We would know that the fraction that comes out around this time is exclusively protein C, which means that we have isolated our protein from the mixture of these proteins.

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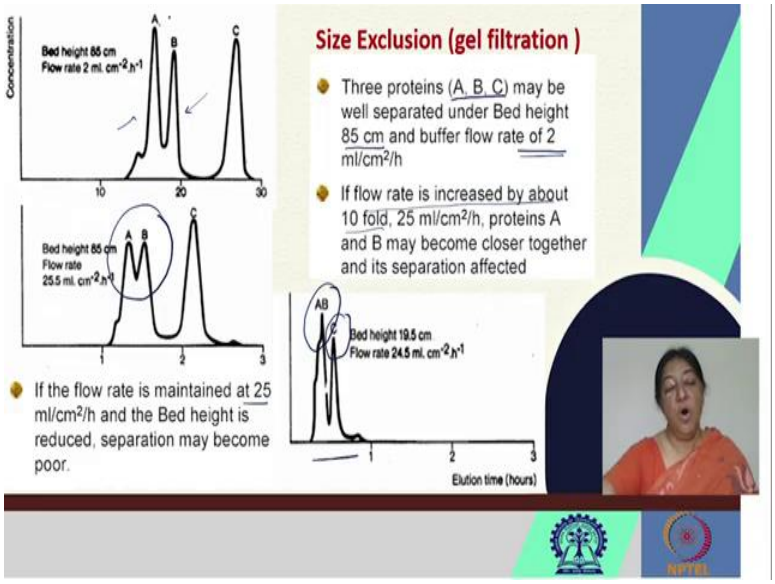

If we look at certain methods or certain important parameters, we have a bed height of our column and we have a flow rate. We want to separate out three proteins based on size exclusion with different molecular weights. Ideally we would want the A, B, and C to form distinctly different peaks.

If we now change the flow rate to about 10 fold. This had 2 ml/cm^2/h, we changed it 10 fold. What may happen is we do not have a good resolution for two proteins A and B, which were distinctly separated in this particular flow rate.

Another condition that we might change is if we maintain the flow rate at this large value, but we reduce the bed height. If we reduce the bed height, we also reduce this resolution of separation. And happens is, there is a combination or an illusion together of the 2 proteins, that does not give us a good separation. So, a combination of a good sized column and a good rate of flow rate is going to give us the proper separation of our protein of interest.

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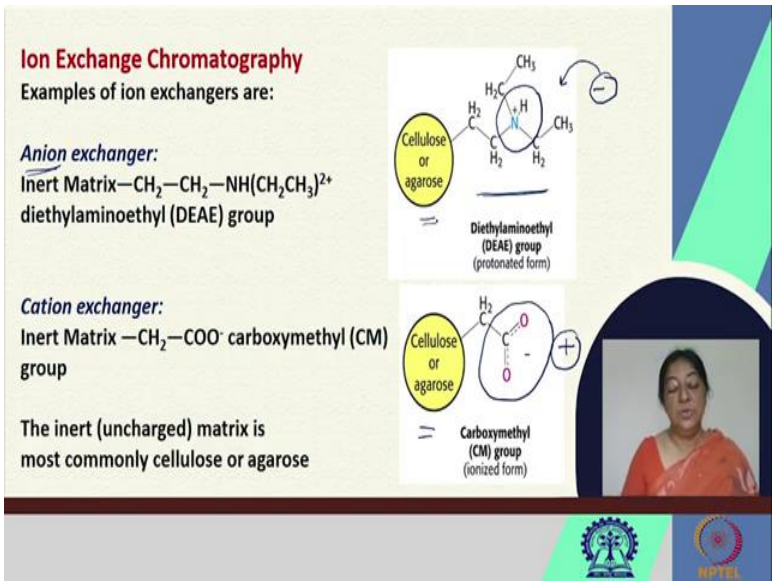

In ion exchange chromatography, we look at anion exchange, where we have the protonated form of the gel matrix. This being positively charged, any negatively charged protein will get attached to it. So this is an anion exchanger. Similarly, we can have a cation exchanger, where our resin is negatively charged and our protein that would bind to this is positively charged.

The inert uncharged matrix is usually cellulose or agarose to which the DAE or the carboxymethyl group is linked. Depending upon the charge of the protein we will have this isolation or we would have the separation.

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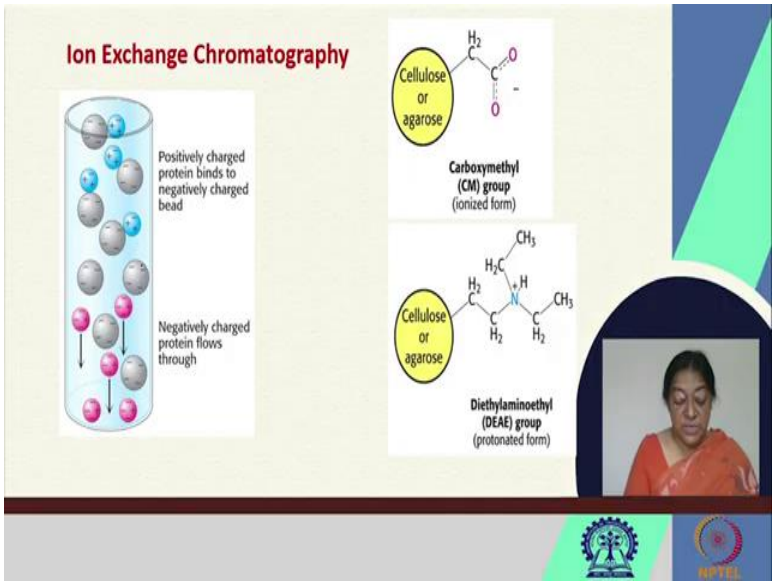

If we have negatively charged beads as is seen in this case, any protein that is negatively charged would not bind to the column but would appear as it is called in the flow through.

But if our resin is negatively charged we would expect the positively charged proteins to bind to the negatively charged beads.

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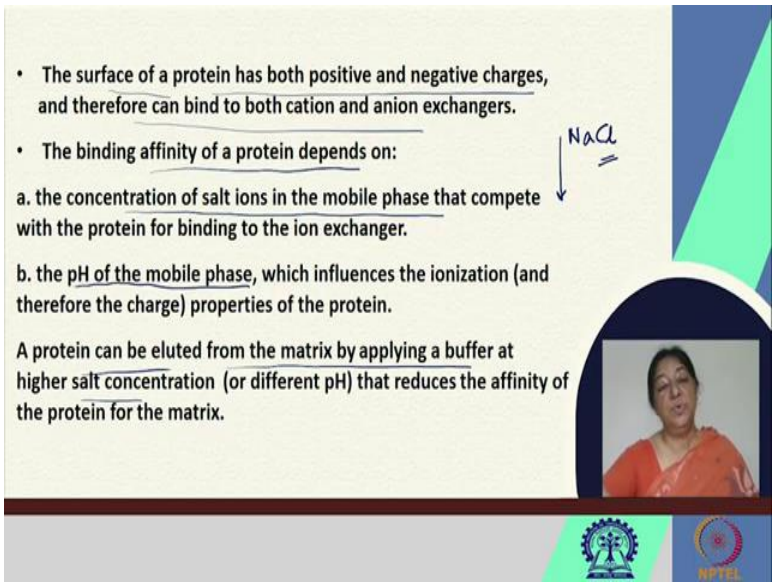

The surface of a protein now has both positive and negative charges. So theoretically it should be able to bind to both cation and anion exchanges.

How do we ensure that we have a proper binding to allow for a proper separation? The binding affinity of a protein will depend upon the concentration of the salt ions in the mobile phase, that are going to compete with the protein for binding to the ion exchanger.

And the pH of the mobile phase, which influences the ionization is extremely important. What we know is, the protein then can be eluted from the matrix by applying a higher salt concentration or a different pH, that would reduce the affinity of the protein for the matrix. So the ion exchange or any affinity chromatography indicates that a protein is bound to the column.

Now we have to elute our protein of interest. In ion exchange chromatography, usually the ionic strength is increased. We can increase the ionic strength of the phase of the buffer that we are flowing through. For example, if we increase the concentration or the ionic strength of NaCl, then if this is an anion exchange, then Cl^- will bind to the beads and we will have the removal of the protein of interest.

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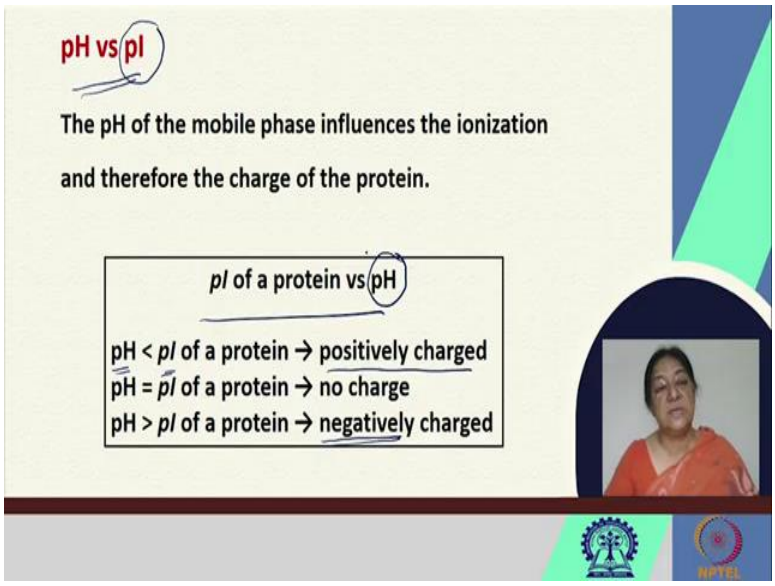

The thing here to understand is that the charge or the pI of the protein is important.

We know that each protein has an isoelectric point. The isoelectric point is dependent on the number of positive and negative charges that the protein has and the charge is dependent on the pH. If the pH is less than the pI of the protein, the protein is going to be positively charged. If the pH is equal to the pI of the protein there is going to be no charge. And if the pH is greater than the pI of the protein, the protein will be negatively charged.

So depending upon the pI of our protein of interest, if we are aware of the pI of the protein; say we know it is an acidic protein or we know it is a basic protein, based on that we can choose the pH at which we are going to conduct our ion exchange chromatography experiment.

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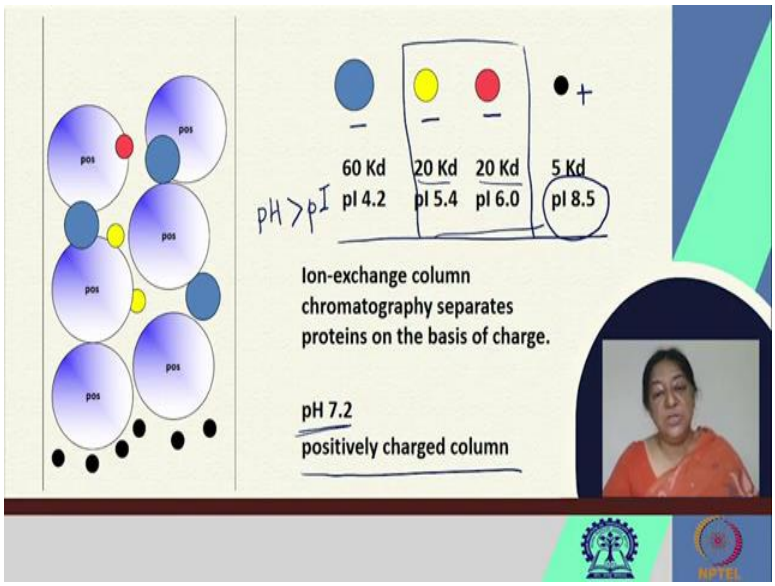

For example, if we have these different proteins we could do a gel filtration chromatography, but as we can see here [refer to slide] these two proteins would elude together because their molecular weights are the same. However, we can isolate them based on charge.

If we look at a pH 7.2 set and we have a positively charged column, then at 7.2, what is going to happen? We have a positively charged column, which means anything that is negatively charged is going to bind to it; the pH is less than the pI of this protein. So this protein is positively charged under these conditions. This is negatively charged, this is negatively charged, and this is negatively charged, because the pH is greater than the pI.

Then what happens is, we have isolation based on their attraction to the column. Anything that has a further negative charge or a larger negative charge is going to be attracted to the column more strongly than the others. And based on the charge of the protein we can bring about a separation.

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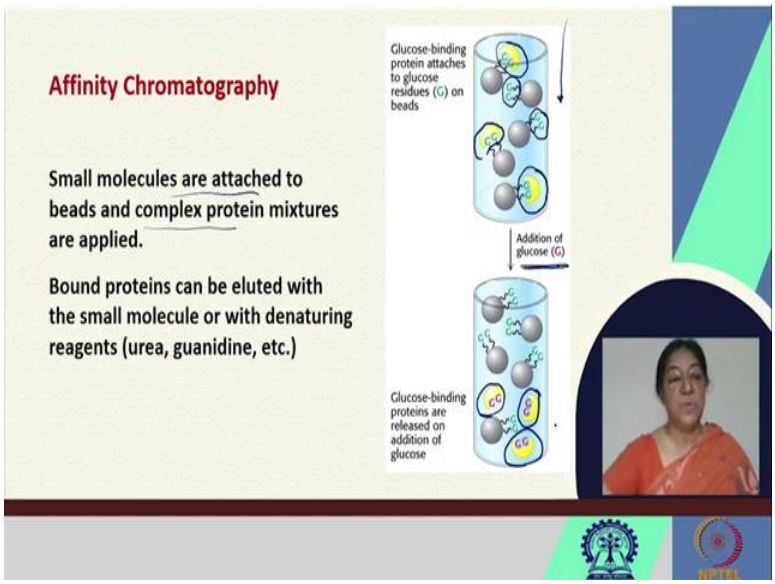

We have affinity chromatography where we have for example, a protein that has an affinity for glucose. In this case the protein binds to the glucose. The yellow protein that we see here [refer to slide] binds to the glucose and the small molecules are attached to the beads and the mixture of proteins is in the column. Now anything that has an affinity for glucose binds to the beads.

We then wash this column. Anything else will be washed out of the column and our protein of interest will be bound to the resin. Then we add additional glucose. The protein then is removed or released from the beads, binds to the additional glucose added and then can be separated out. So we have a separation.

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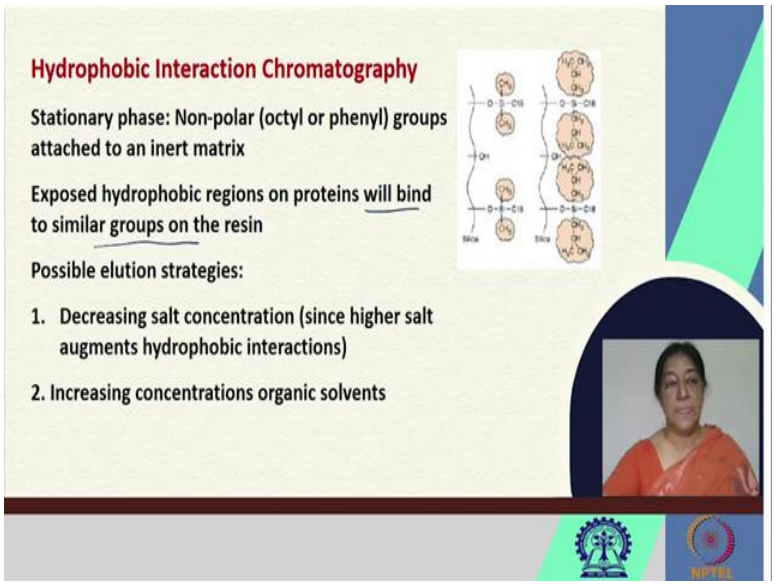

We can also have a separation based upon the hydrophobic capacity of proteins. In this case exposed hydrophobic regions of the proteins will bind to similar groups on the resin. Then how do we remove this?

We can remove this by decreasing the salt concentration, because higher salt will augment hydrophobic interactions or we can increase the concentration of organic solvents to release our protein of interest.

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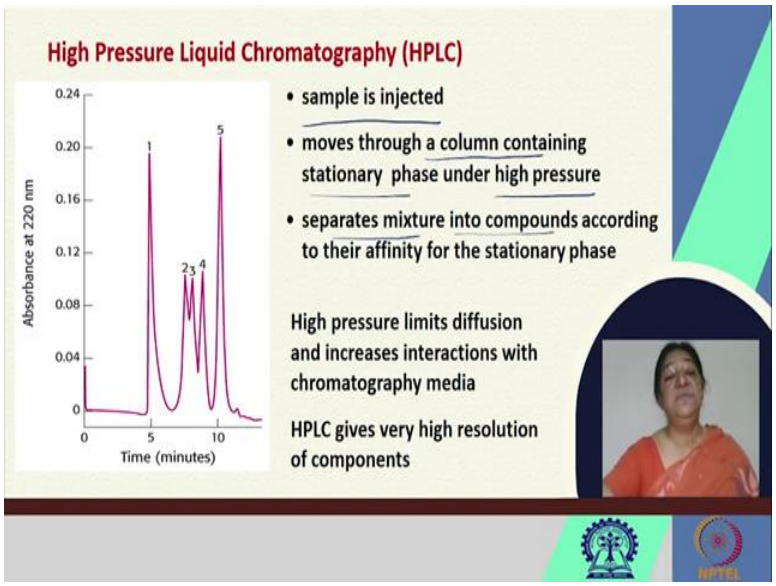

As we do so, we can look at a method called high pressure liquid chromatography. Here we have the movement through the phase under high pressure. This separates the mixture into the compounds according to their affinity for the stationary phase. This is a method that limits any

diffusion possibilities because of the high pressure being applied and it gives very high resolution of our components.

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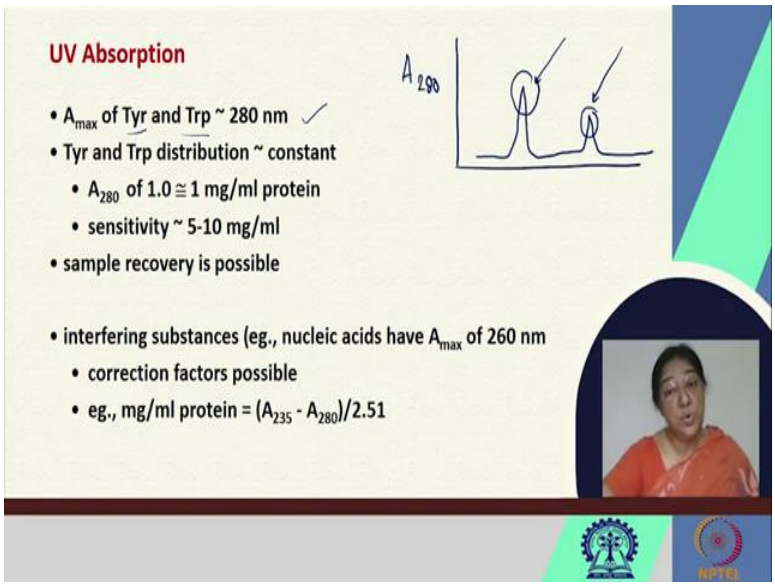

We now look at the methodologies to see whether we have in the fractions any protein present, we use methodologies such as UV absorption. If we look at the absorption at 280 nm and we have tyrosine and tryptophan present in our proteins, then we will have a typical absorption that we will see at A 280 nm. And if we plot this absorbance at 280 nm with our fraction number, we get separate peaks, say for a gel filtration chromatography. We will know that we have a distinct separation of 2 proteins, where we have the larger protein come out first, followed by the smaller one.

With UV absorption sample recovery is possible. We have interfering substances such as nucleic acids but nevertheless correction factors are possible, that would tell us or give us an idea of whether we have our protein present in the specific set.

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Another method is using a dye, where the absorption maximum shifts from 465 to 595, when this coomassie-blue G-250 is bound to the protein because it reacts primarily with arginine and we have a good sensitivity. So we can take a drop of our eluted sample and mix it with the Bradford reagent to see whether we have our protein of interest.

It is a single step, with less interfering substances, but we do cannot recover the protein. We can then find the protein concentration from the standard curve but we cannot recover the protein. So this is usually checked by a drop to see whether the proteins are being eluted from the column.

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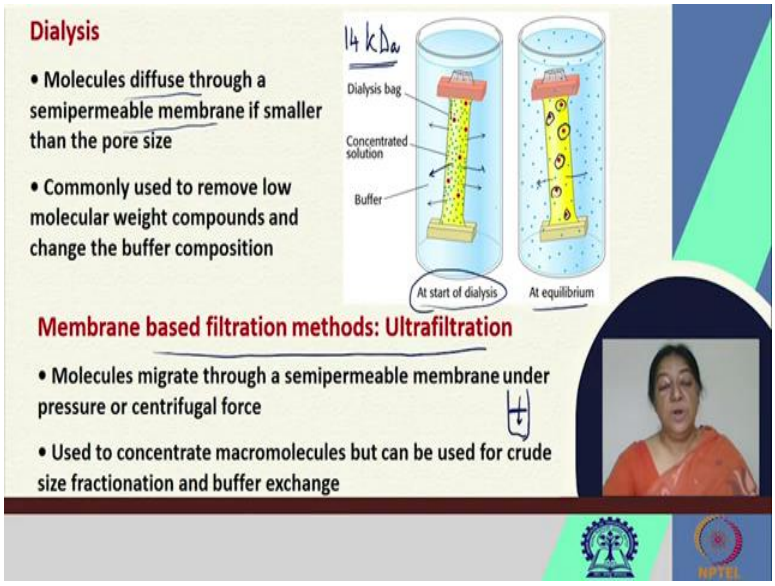

Now that we have our protein present in our fraction, we want to get the protein from the concentrated solution. So we have the molecules diffused through a semi permeable membrane in a process called dialysis, where it is commonly used to remove low molecular weight

compounds. We have extracted our protein of interest using a high ionic strength. This dialysis bag that we see has a specific molecular weight cut off.

For example, if we have a molecular weight cut off of 14 kDa. This means that any molecular weight less than 14 kDa is going to easily pass through the column. We now have a buffer solution kept outside, a dialysis bag dipped in the buffer. At the start of dialysis this is what we observe, where we have a concentrated solution with several ions present.

As the dialysis continues at equilibrium, we see that the protein molecules which were larger inside did not pass through the semi-permeable membrane or the dialysis membrane with the specific molecular weight cut off. It was the smaller molecules that were lost and we get a dilute solution of a protein.

The membrane based filtrations are also there with ultrafiltration. In this case the molecules migrate through a semi-permeable membrane under the pressure of the centrifugal force. Just like we saw in the previous lecture; if we apply a sedimentation in our tube and within the tube we keep a specific membrane, a semi-permeable membrane allowing only specific molecules to pass through, we can concentrate our macromolecules for a fractionating procedure.

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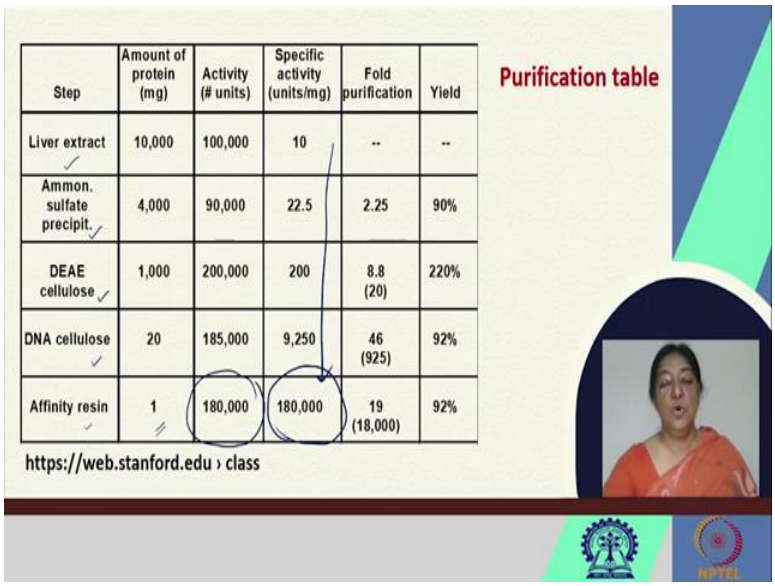

A typical purification table will look like this [refer to slide], where if we start off with liver extract, go for ammonium sulfate precipitation, then a DEAE cellulose, then a DNA cellulose followed by an affinity resin; we will get the amount of protein to be lessed. But the activity will gradually increase and the specific activity increases many many folds depending upon our method of purification. So we learnt how we can purify proteins using chromatographic techniques.

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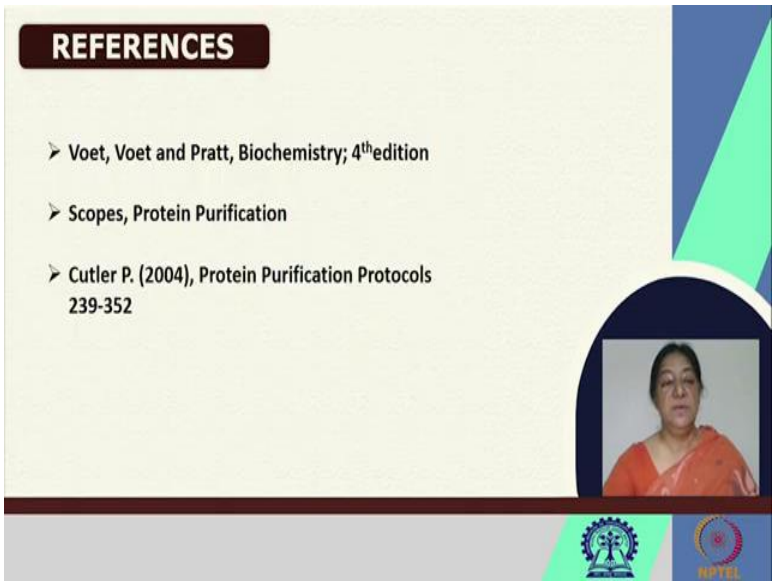

These [refer to slide] are the references.