Experimental Biochemistry
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Lecture – 24
Chromatographic Techniques – II

We continue our discussion on the Chromatographic Techniques. In the last lecture we learnt about the different methodologies based on chromatography as to how we can get our protein of interest. In this particular lecture we are going to look at the other methods.

As was mentioned to identify or to elute or to get a protein of interest the idea is to exploit a specific property. When we looked at gel filtration chromatography the property the specific property that we exploited was the size or the mass of the protein where we were we looked at a molecular sieve method that just checked out the specific protein of interest based on a polymer bead matrix.

The polymer bead matrix was such that we it would not allow proteins of larger size or mass to enter the matrix network. The medium sized partially entered them and the smaller sized entered them. And they were later in their elution from the chromatographic setup.

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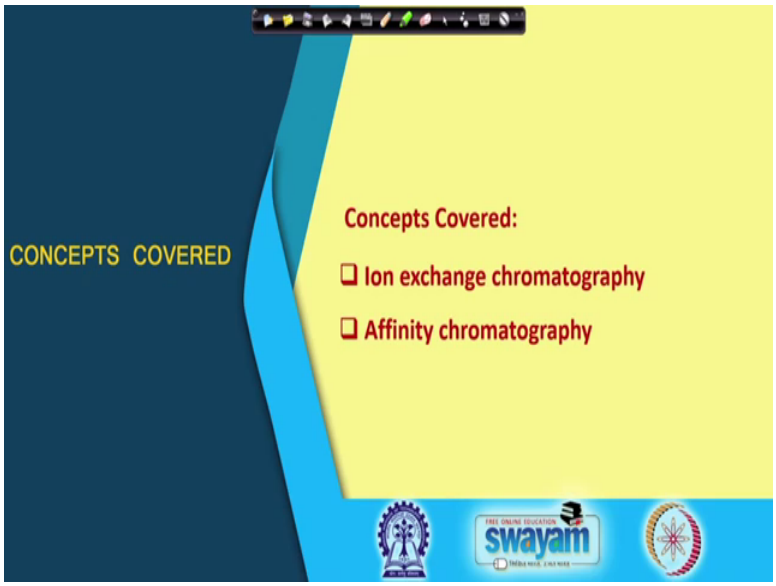

In this case we are going to look at ion exchange chromatography and affinity chromatography. As the name implies or the names imply that we are going to look at an ion exchange which means; in this case our protein has to be charged. In affinity chromatography we are going to look at the specific affinity of our protein of interest for a particular small molecule. This may be also DNA it may be glucose or any other protein that or any other ligand that our protein binds to.

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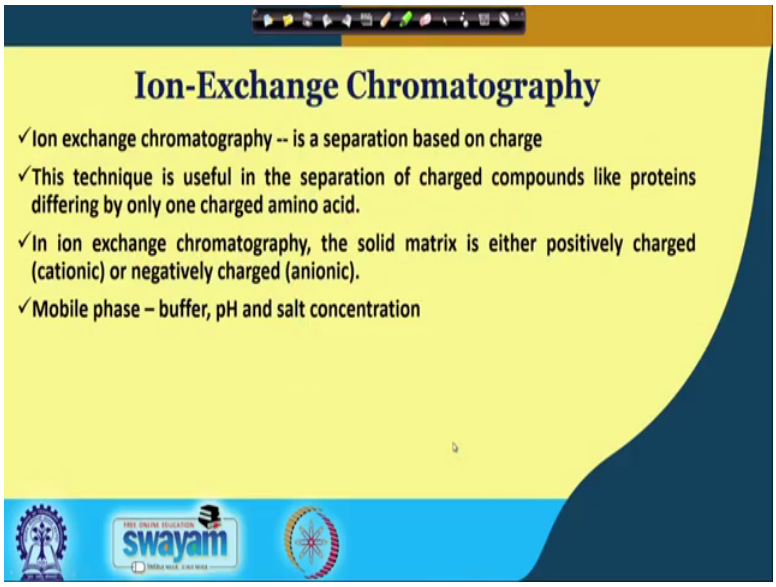

In ion exchange chromatography it is a separation based on the charge of the protein it is useful in the separation of charged compounds. For example, if we have proteins of similar mass. It is difficult to isolate them using gel filtration chromatography. So, we have to exploit the charge on the protein for their separation. In this case the solid matrix our network is either positively charged or negatively charged. And the mobile phase is the buffer; it has a specific pH and a specific concentration for the elution of our particular protein.

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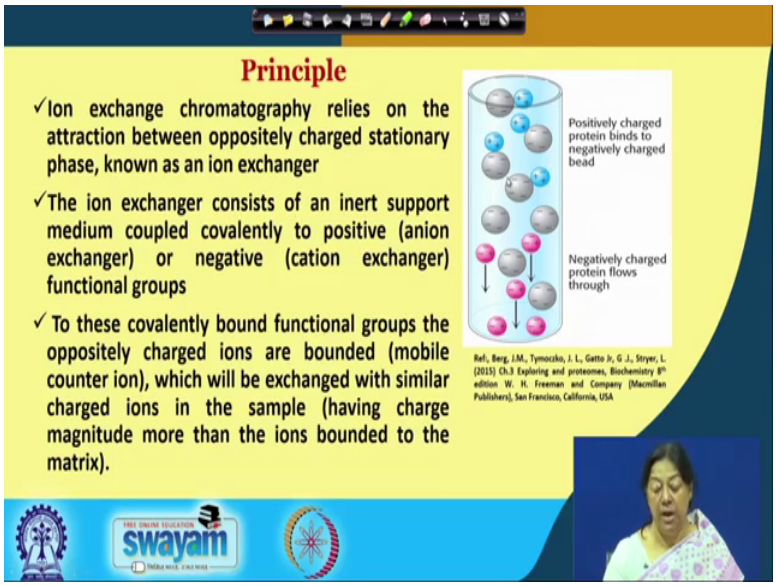

Now, what happens in ion exchange chromatography the property that we are exploiting is the charge. So, it relies on the attraction between the oppositely charged stationary phase and the protein. So, if we have negatively charged beads as we can see here what is going to happen is the positively charged beads the positively charged protein will bind to the beads. And what will happen to the negatively charged proteins? Predominantly negative charged proteins will be repelled by the negatively charged beads.

And that they will come in what is called a flow through. So, the negatively charged protein will flow through, but the positively charged beads will be proteins will be attracted to the polymer matrix that is the anion exchange. In this the cation or the anion exchange beads that we have. So, now, what happens is in this case we have some proteins that are attached to the polymer matrix that is our bead.

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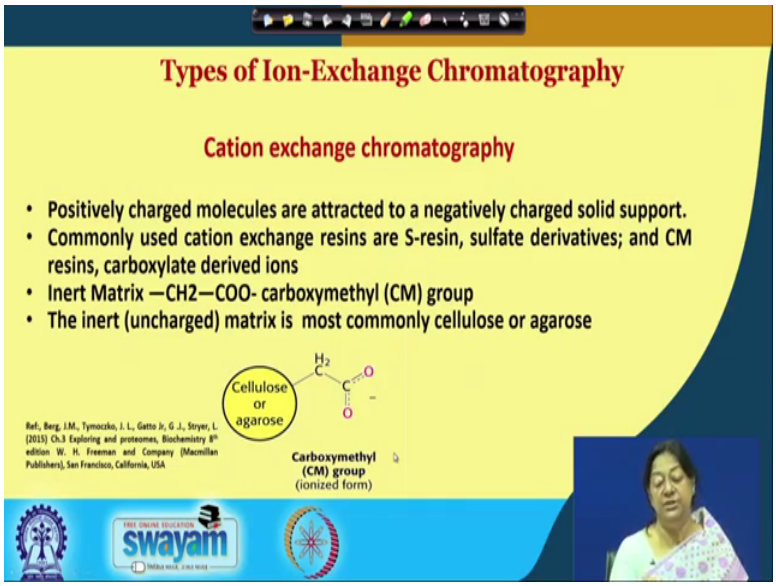

The types of ion exchange chromatography that can occur are cation exchange. What this means is that we will have an ionized form we know now that the positively charged molecules are going to be attracted to a negatively charged solid support.

So, the commonly used cation exchanges are the different ones that are mentioned here and for example, if we look at something like this with a carboxy methyl group with a negatively charged. Now it is easily understandable that this to this negative charge there will be an attraction of a positive protein. So, this would be cation exchange chromatography.

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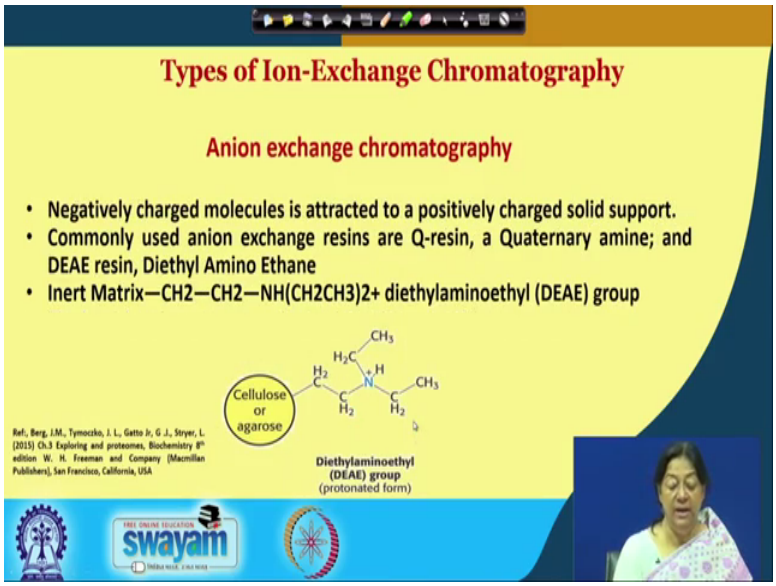

On the other hand if we have a negatively charged molecules, the proteins they are going to be attracted to a positively charged support. So, this DEAE cellulose as it is called has a positively charged group as you can see here the protonated form.

What is going to happen is any protein that if a column is packed with beads that have DAE group to it. Then the negatively charged proteins are going to be attracted to this and we are going to have an anion exchange occur.

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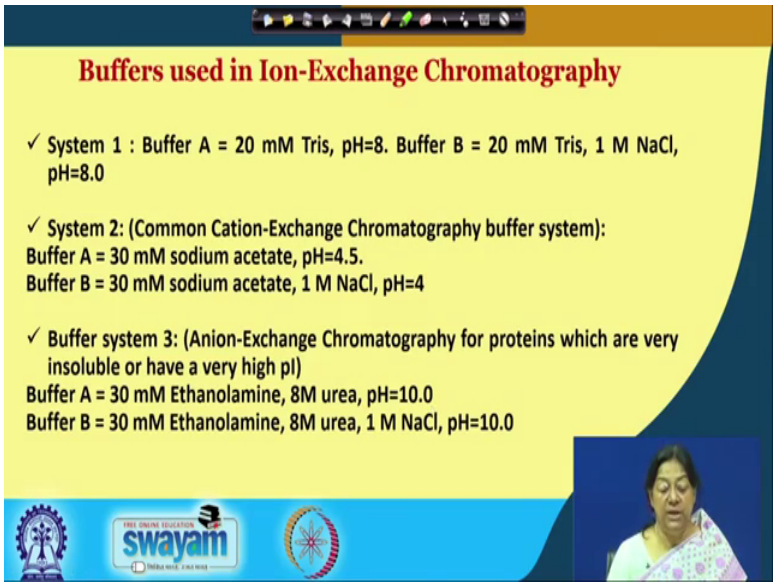

The buffers that are commonly used in ion exchange chromatography are common cation exchange chromatography buffer systems, where we have sodium acetate sodium chloride and so on and so forth. What we need to understand now is when we have the anion or the cation exchange the protein is bound to the column.

So, if we have a cation exchange what we are going to see is in this case the cation exchange means the positively charged protein is attracted to the negatively charged ionized form of the CM group. Similarly a negatively charged protein is going to be attracted to the protonated form of the TAE group. So, once the protein is ionically attracted to the specific bead and the rest of the proteins or the mixture of proteins has flown out, it is now the separation is now going to involve the isolation or the elution of the attracted protein or the one the bound protein of the bead.

So, the question now arises is how can we get the protein now, to come off in the elution procedure from the bead this is where the specific buffers come into the picture. The normal course is to use concentrations of sodium chloride. What happens is the sodium ions which are positively charged will be attracted to the negatively charged beads.

And what will happen is the protein which is loosely bound to the beads will then come off and come into the fraction of interest. Similarly if again we use sodium chloride the chloride ions will take the part of the negatively charged protein and that will also come off the column.

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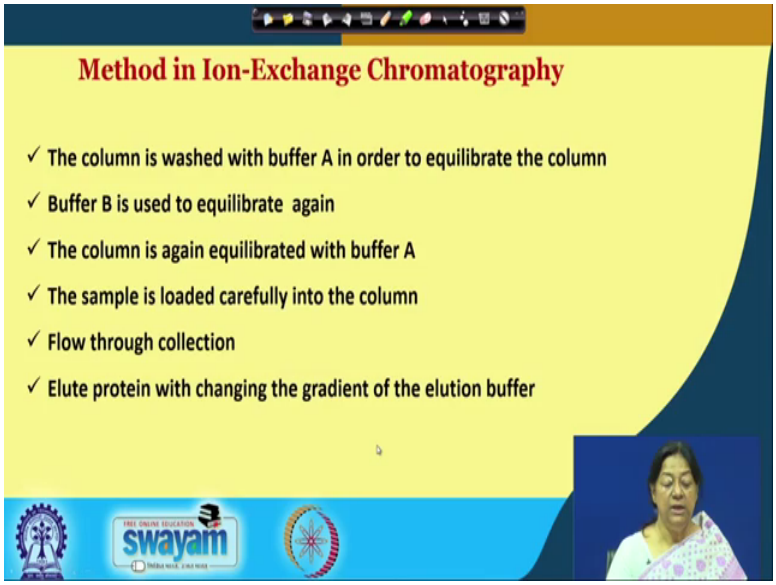

So, we have the column that was washed with specific buffer to equilibrated then we have another buffer to equilibrated again. We have the sample loading the sample loading means we have a mixture of proteins. And say if we have a positively charged protein that is going to be attracted to the negatively charged beads. So, will have a bound protein and we will have then elution through the use of a specific sodium chloride concentration.

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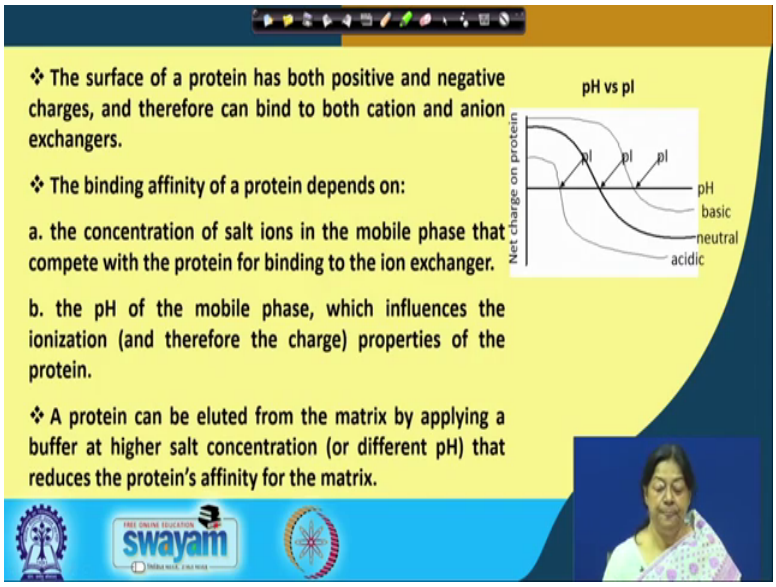

Now if we look at our protein of interest, the pH of any ion exchange chromatography method is extremely important. We know that we have a specific isoelectric point of the protein. Now if we choose a pH that is less than the isoelectric point of the protein the protein is going to be positively charged.

If we have a pH that is greater than the isoelectric point of the protein we have a negatively charged protein. So, the binding of this specific protein will depend upon specific features, one is the concentration of the salt ions in the mobile phase that actually compete with the protein for binding. So, it is not a good idea to have a very high salt concentration at the beginning, but we would want that later. So, that we have a protein of interest come off the column.

The pH of the mobile phase is important because that is going to influence the charge on the protein and in turn it will influence its binding affinity to the specific matrix that is present in the column. So, we can have the protein eluted from the matrix either by

applying a buffer at a higher salt concentration or we can use a different pH where we can change the charge on the protein. And thereby changing the affinity for the specific column beads and the protein will then come off the column and we can isolate a protein of interest.

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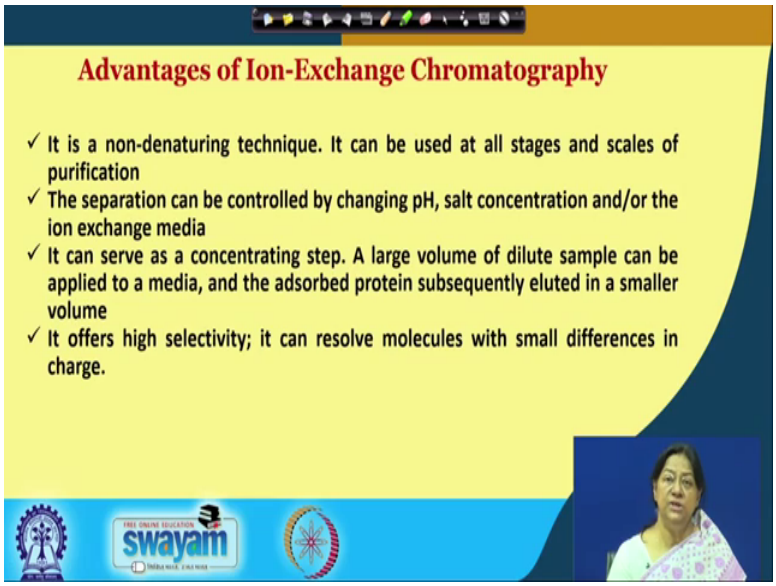

The advantage of using advantages of using ion exchange chromatography is it as a non denaturing technique. And it can be uses used at different stages of protein purification. The separation can be controlled by changing the pH the salt concentration and or the ion exchange media. And it can serve ease even as a concentrating step and it is very selective in it is way of separation which makes it a very convenient method for use.

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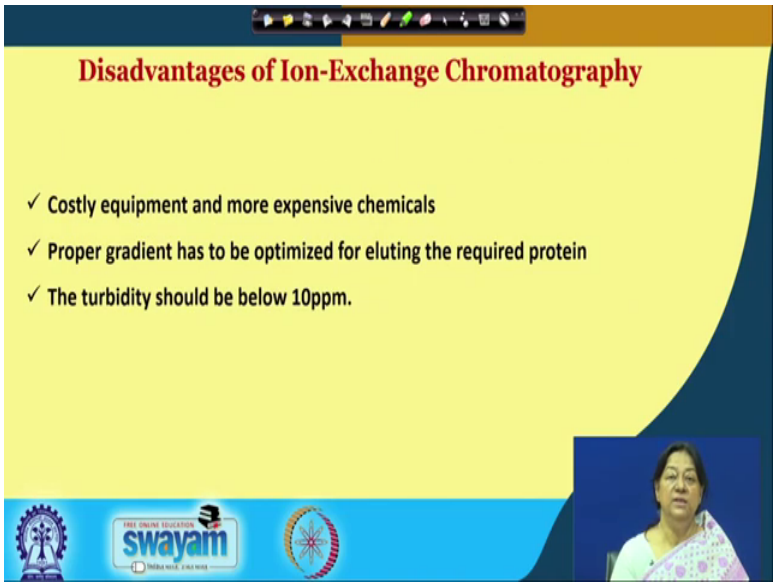

However, the disadvantage is the use of specific columns that are quite costly. And the proper gradient that has to be maintained for proper elution of the protein that is required.

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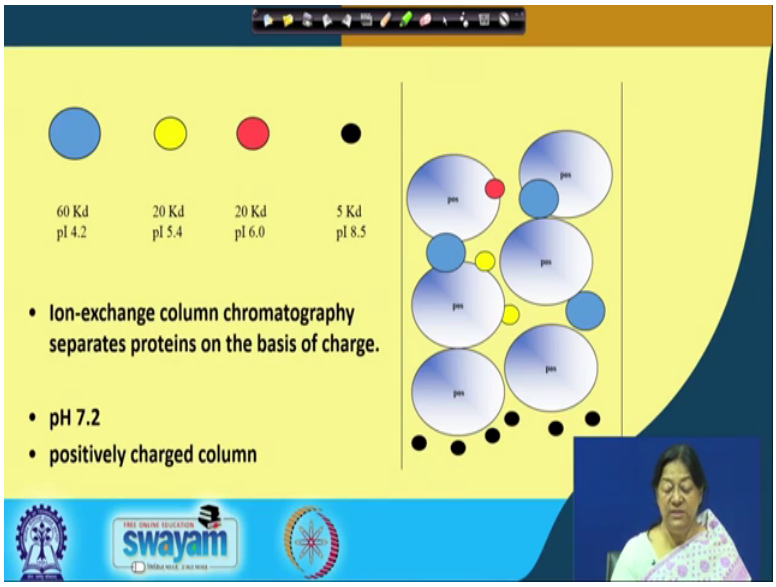

So, if we have a specific system like this and we have four proteins that have been shown here of the different molecular weights, we see that we have the specific molecular weights at 60 kilo Dalton 20 kilo Dalton another 20 kilo Dalton a 5 kilo Dalton. If we

look at these two proteins it is difficult to isolate them by gel filtration chromatography because they have the same molecular weight.

The pI in this case is 4.2, 5.46 and 8.5. So, if we choose a pH of 7.2 these proteins are all going to be positively charged and this protein is going to be negatively charged. So, what is going to happen? If this is our protein of interest the one that is coloured in black and we take a positively charged column with the pH of 7.4 well all of these have the pI less so the pH is higher than the pI. So, these are going to be negatively charged and this is going to be positively charged.

So, what is going to happen is the positively charged raisins will repel this set of proteins because the protein is positively charged, why because the pH is less than the pI when the pH of 7.2 is greater than the pI of these proteins making all of them negatively charged. So, all of them are going to be bound to the positively charged beads. And so we can separate the positively charged protein from this.

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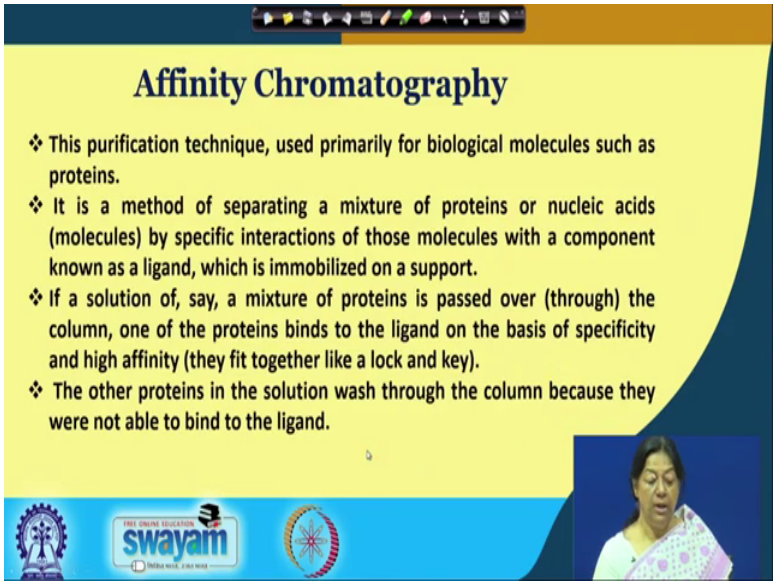

In the affinity chromatography it is used for primarily for biological molecules that show a specific affinity for either say glucose or DNA. And it is a method of separating a mixture of proteins or nucleic acid by specific interactions of those molecules with a specific component that is known as a ligand which is immobilized on a support. Now what do we mean by this?

If we have a mixture of proteins and one of these proteins has an affinity say for glucose or has an affinity for DNA. If we can have the matrix or the column beads have a linker to the beads and glucose attached to this linker. Then of the mixture of proteins if one of them has an affinity for glucose it will bind to the column. Whereas, the others that do not have an affinity for glucose will be washed off the column in the beginning and they will not be able to bind.

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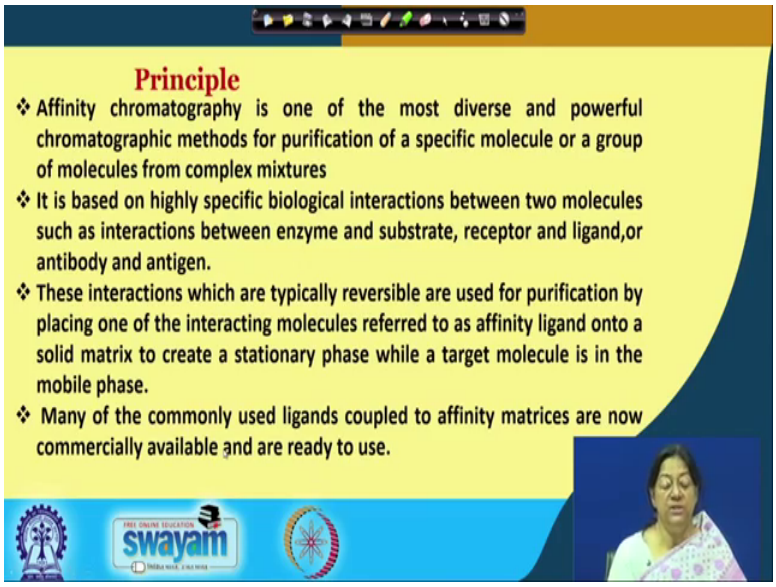

So, this is one of the methodologies and probably the most diverse and powerful methods that is used; where we are looking at a specific biological interaction between two molecules such interactions could be between the enzyme and the substrate any receptor ligand the antibody antigen.

And since they are typically reversible it is easier for them to sometimes wash off the column due to the creation of a specific mobile phase that is going to then be attracted to the column attracted to the matrix present in the column and the protein is going to be washed off.

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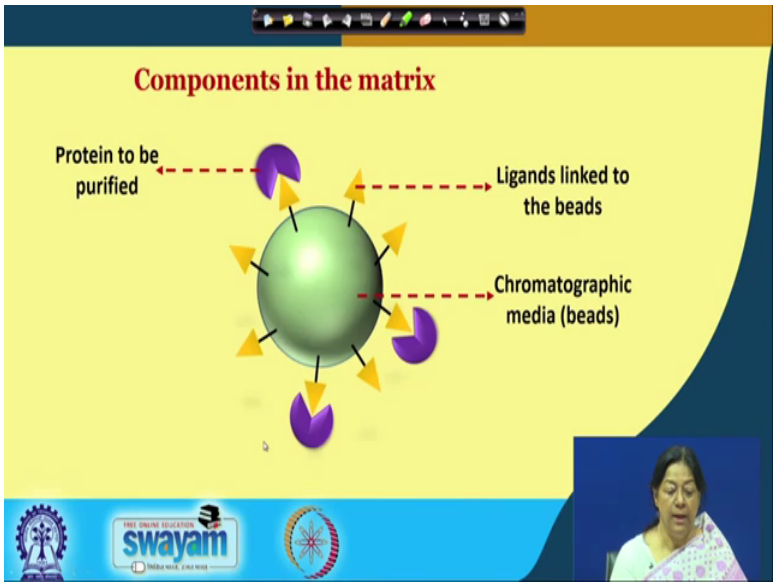

So, we have say a protein to be purified this is a chromatographic bead and these yellow triangles are the ligands that are linked to the beads. So, if we have a mixture of proteins that is poured down the column which has these beads that and the ligands linked to these beads. The protein to be purified will link to the specific ligands present.

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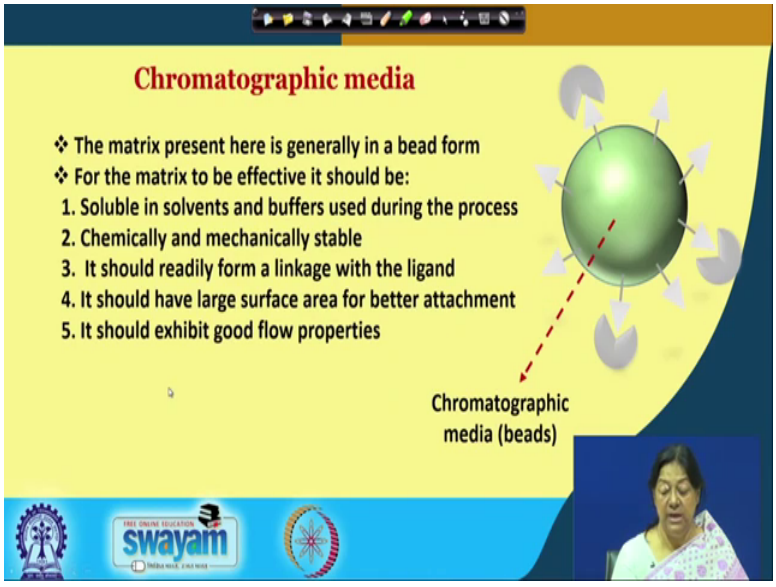

And then what is going to happen is there is going to be a bead and it is going to be for the matrix to be effective it should be soluble in solvents and buffers used during the

process. It has to be chemical and mechanically stable and there should be a linkage with the ligand and a large surface area for better attachment.

Because if we do not have this linker you realize is that this ligand were attract were attached to the bead itself the protein molecule because of steric hindrances would not be able to go directly to the chromatographic bead.

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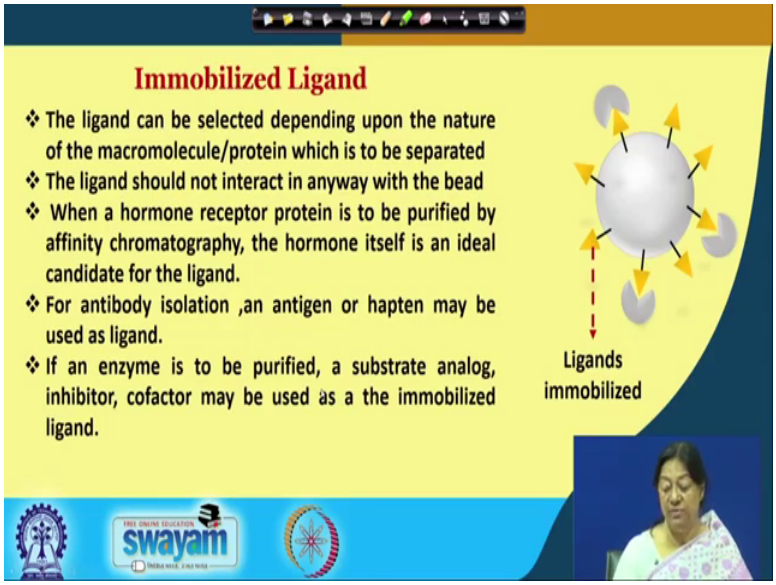

So, we have the immobilized ligands on our bead and the ligands are selected depending upon the nature of the macromolecule or the protein that is to be separated. And the ligand itself should not interact with the bead. And if the enzyme is we can have a substrate analog or the substrate be attracted here and our protein or enzyme of interest is going to be attracted to the specific ligand.

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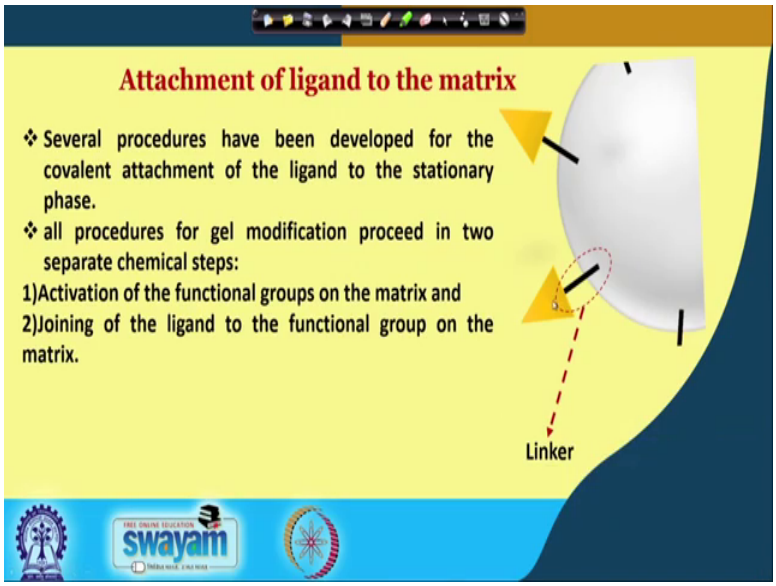

So, here is our linker and specific linkers are going to have a long R and the ligand is going to be attached to the ligand and we are going to have our protein attached to this.

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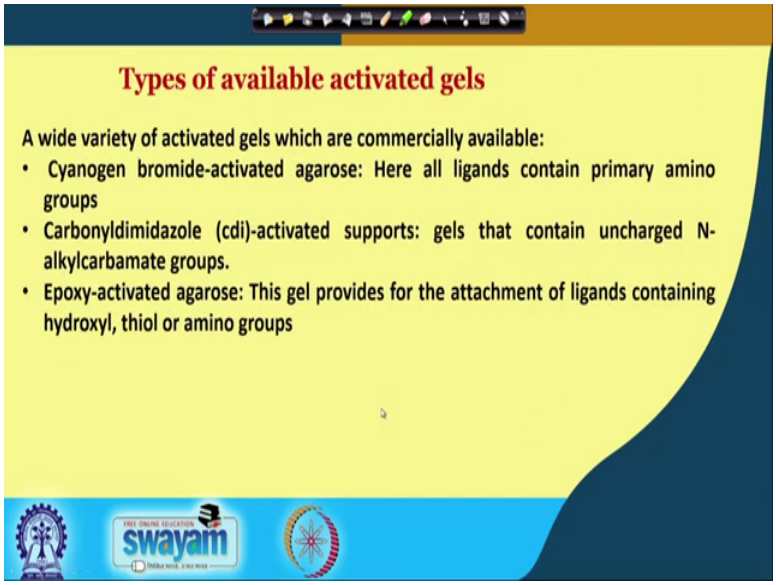

There are specific types of different types of activated ligands that we can use.

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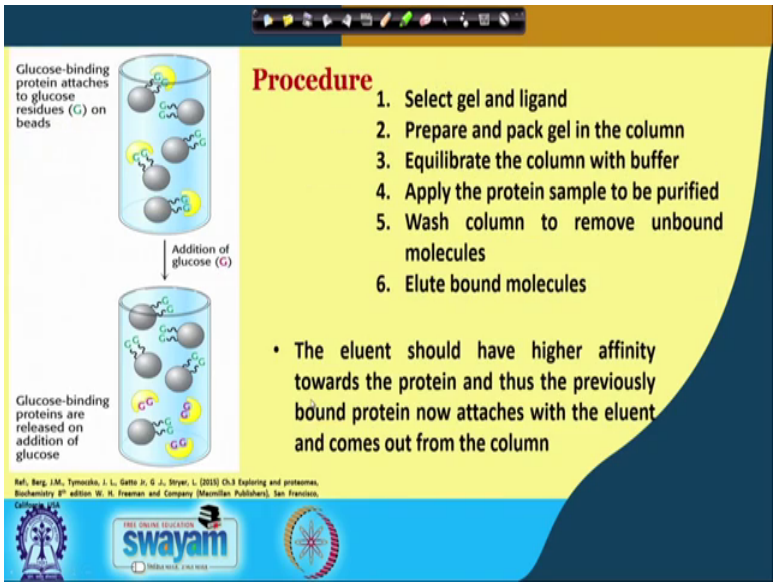

For example, if we have a specific linker here we can see there is a glucose binding protein. So, we select a specific gel and a specific ligand the specific beads in this case are going to have glucose attached to it. And the protein of interest has an affinity for glucose. So, what is going to happen is the glucose bound protein is going to bind to the glucose present in the beads. Now, the next question again comes is how are we going to separate the protein out.

So, if we have a mixture of proteins that is poured down the column that is going to be the specific protein attached to the linker that has or the beads that have glucose on it. And how do we wash it off? We add more glucose. So, what is going to happen the protein molecules will bind to the extra glucose that is obtained and come out in the bound form of the protein and the ligand and then it can be isolated.

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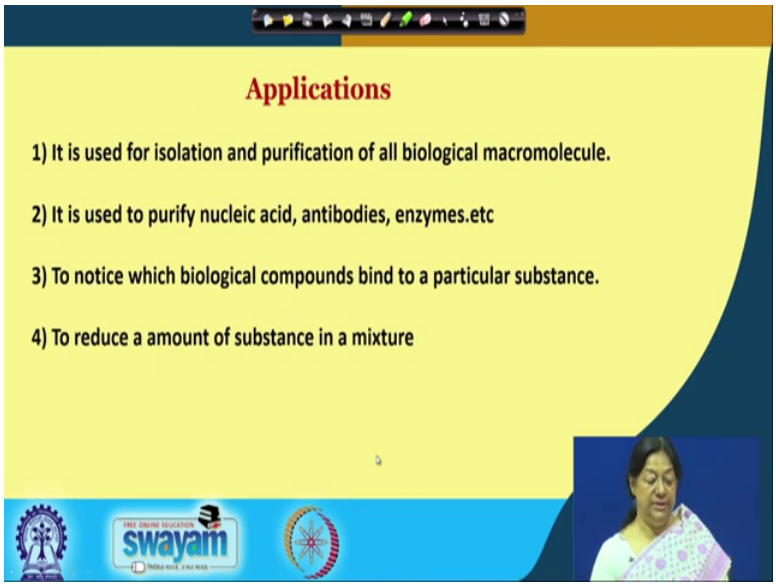

So, the applications of all these chromatographic techniques for isolation and purification of the biological macromolecules and the particular ligand attraction is used in affinity chromatography and exploited for the specific isolation.

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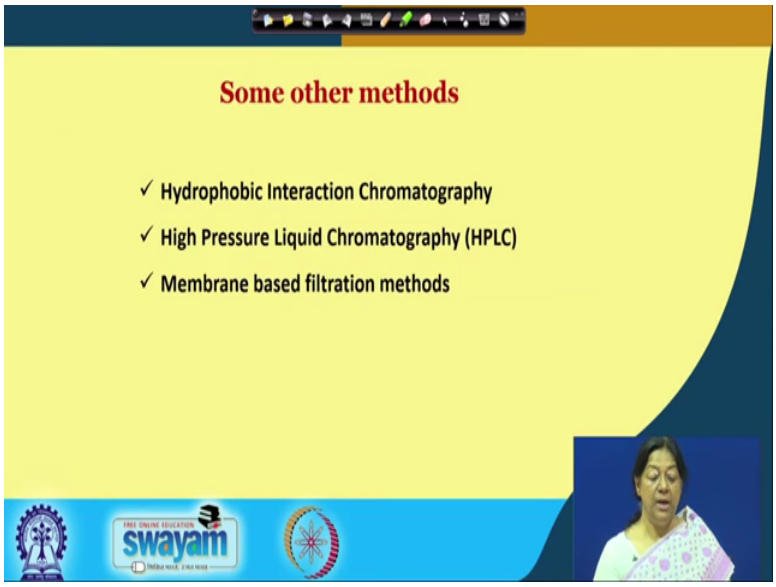

There are other methods that are used hydrophobic interaction chromatography, high pressure liquid chromatography, and also membrane based filtration methods.

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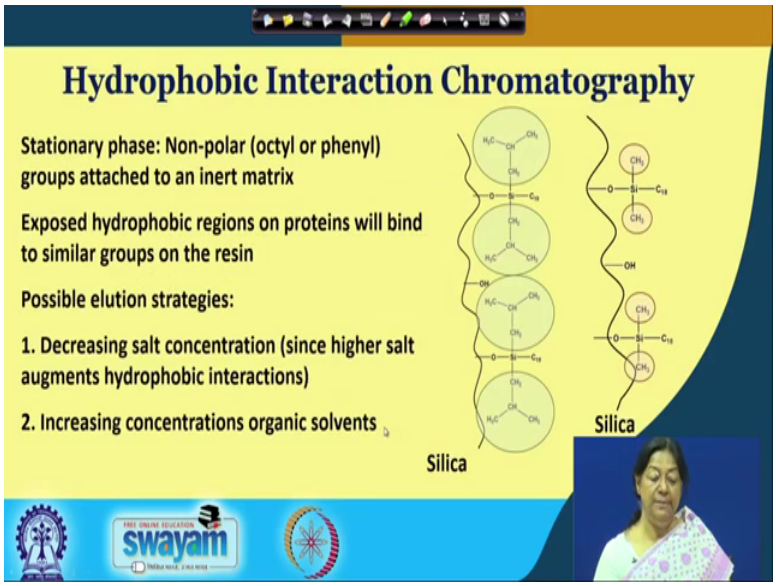

As can be understood from the name itself hydrophobic interaction chromatography takes into account the hydrophobicity of the molecule to be separated. So, in this case what happens in the stationary phase we have a non polar set. So, we have a non polar stationary phase and what happens is the exposed hydrophobic regions in this case now are going to be attracted to the non polar regions.

Or the nonpolar part of the beads because we have now a stationary phase that is nonpolar. In the other case what happens is we have if we have decreasing salt concentration because higher salt concentrations are going to augment hydrophobic interactions if we increase the concentrations of the specific interactions. So, increase the concentration of organic solvents or we decrease the salt concentration what is going to happen? Is initially when we have the hydrophobic content of the protein high or the hydrophobic content of one of in the mixture.

So, we have a mixture of proteins those with higher hydrophobic content or higher hydrophobic capacity are going to get bound to our column. Once they bind to the column they have to be released how are they released by reducing the hydrophobic interaction. So, that they come off the column.

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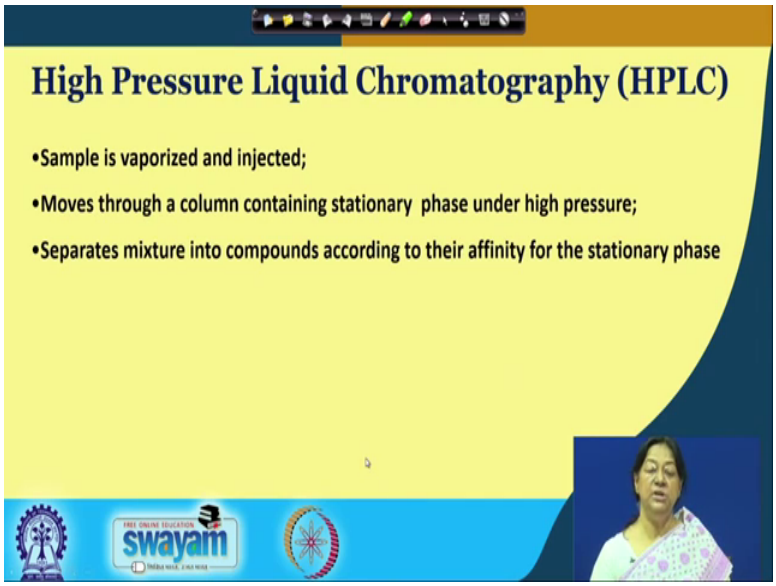

In high pressure liquid chromatography which you will study later on in a more specific setup is here also we have the movement through a column that contains a stationary phase under very high pressure and the elution. Again the affinity for the stationary phase is based on a hydrophobic setup if we are using a specific reverse phase as it called. And the pressure is going to push out the specific isolation is going to occur.

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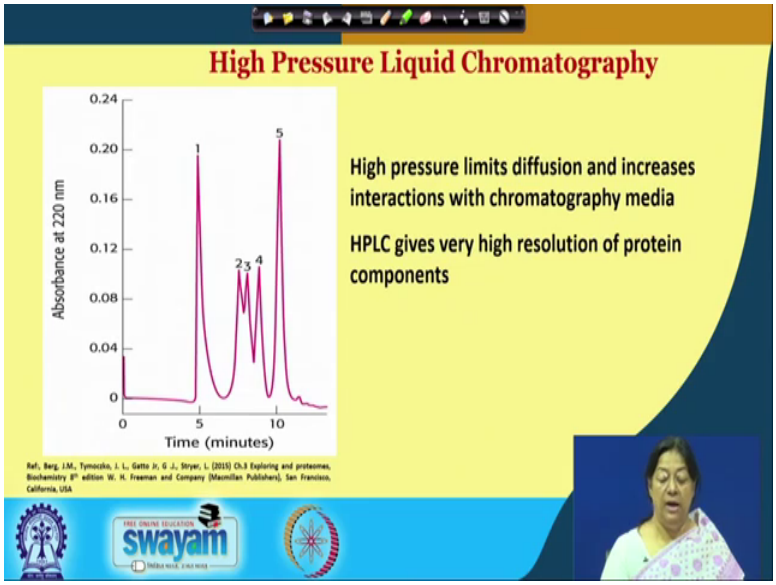

So, the high pressure limits diffusion and increases interactions with the chromatographic media and it gives very high resolution of protein components. And we have again a

specific elution or what is called a specific retention time telling you how strongly your specific protein component is attached to the column.

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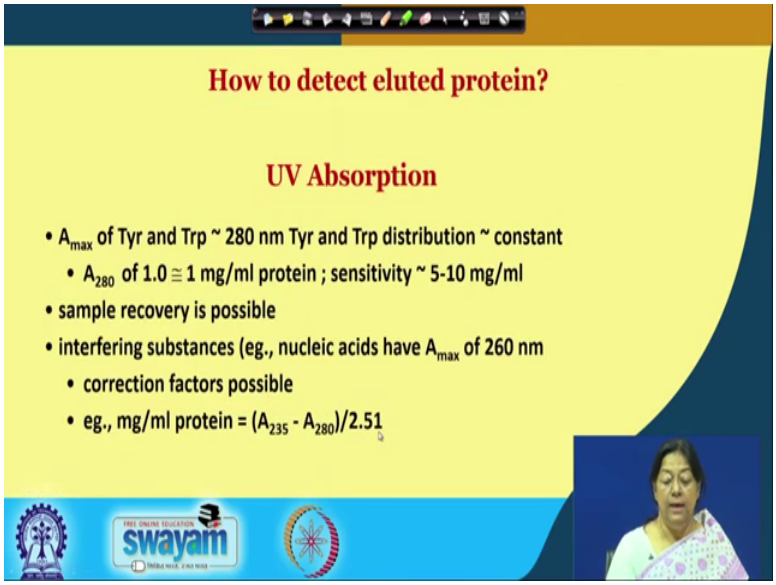

Now how do we detect an eluted protein this was mentioned a briefly in the last lecture. Knowing that the aromatic amino acids residues have a specific UV absorbance associated with them. We can calculate t or we can determine the absorbance at 280 nanometer of each of the fractions that are a eluted from the column. And based on this decide whether our fraction fractions contain the protein of interest.

It may so happen that there will be a set of fractions that contain our protein of interest what is done then is these set of fractions are then pulled together. And a dialysis technique is used to concentrate the protein for interest or a lyophilization technique to remove the solvent and obtain the protein of interest. So, we get the advantage of using UV absorption is that we can the sample recovery is possible.

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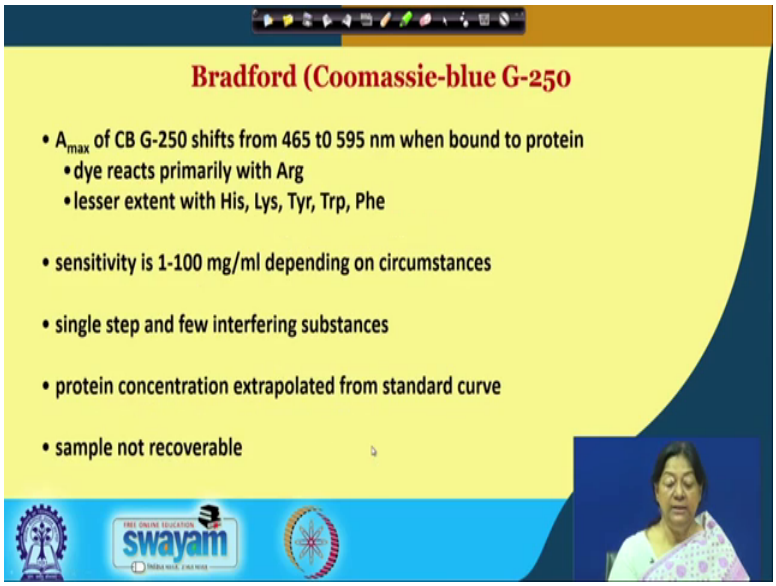

Another way is to use a dye where we you can use a Bradford dye. And the dye interacts specifically with specific amino acid residue. And it also gives us an idea of whether our solution has a protein present in it or not. And the protein concentrations can then be determined from a standard curve.

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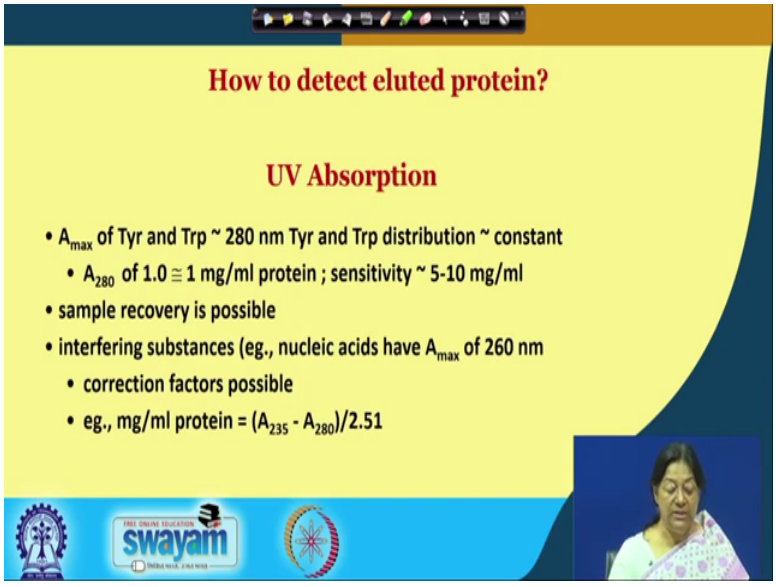

If we go back once in looking at specific milligram per ml of the protein we can actually find out this is a thumb rule that if we have an a 280 that is the absorbance at 280 nanometer about 1 it approximately means we have around 1 milligram per ml of

protein. So, the Bradford assay also tells us whether the protein of interest is present in our specific fraction.

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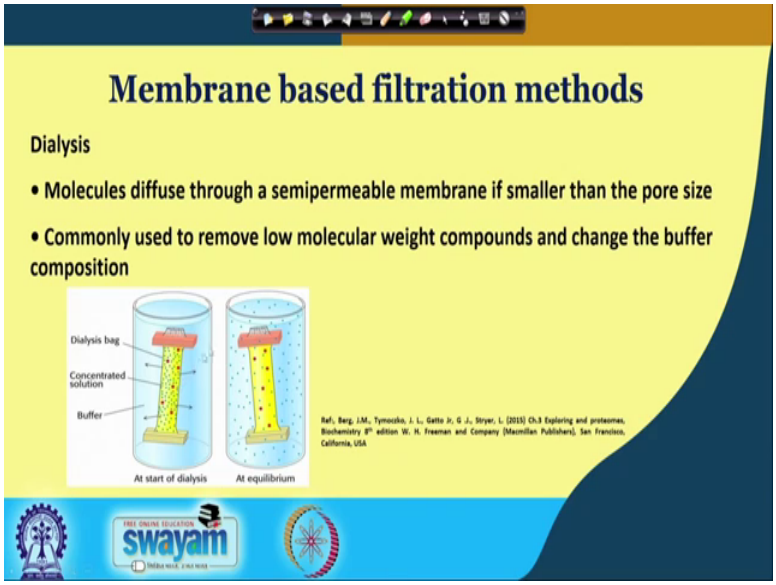

In membrane based filtration methods what we are trying to do is if we have a particular salt concentration that we have used for the specific separation then sometimes the concentrated solution with the salt and we have the buffer. And what we want to do is we want to remove any extra salt that is present in the system. In this case we have a specific dialysis bag that has a molecular weight cut off say if your protein has say a molecular weight of 50 kilo Dalton.

Then you can use a cut of anything that is less than 50 kilo Dalton. So, what is going to happen is the salt is going to move out and the protein of your interest is going to remain in the dialysis bag. So, eventually at the start of dialysis if you have a concentrated solution and you dip your dialysis bag in a buffer solution what happens is you can remove the low molecular weight compounds and if you keep on changing the buffer outside the dialysis tubing.

Then you can actually get your protein of interest in the buffer solution. And to you can also then lyophilization is the protein to get a powder of your protein that you can use depending or you can concentrate your protein even by lyophilization. And then use it depending upon when you have a specific experiment to work out.

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The references that we have used here are the references that are listed in the specific slides themselves. In addition there are specific books on isolation and separation and characterization of proteins.

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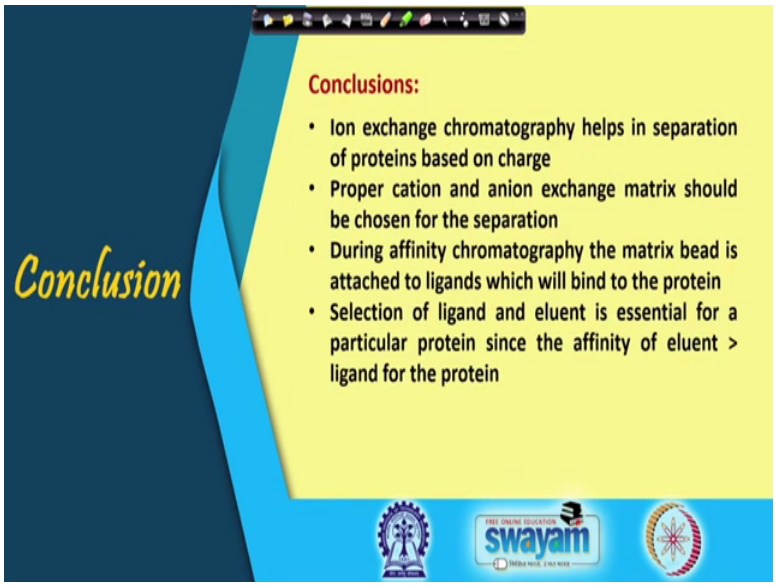

So, what we learned from this lecture is the ion exchange chromatography helps in the separation of proteins based on charge. We have to remember that any of the chromatographic methods that we are using have a specific property that we are exploiting. So, whether it is the size or the mass of the protein and gel filtration, whether

it is the charge and ion exchange chromatography, whether it is the affinity for a specific molecule specific ligand and affinity chromatography. Or even the hydrophobic interaction property that we are looking at it is the properties that we are exploiting to isolate our protein of interest. And it is to our judgment that we utilize the chromatographic technique that suits us the best.

Thank you.