Enzyme Science and Technology
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Module - V
Enzyme Production (Part 3: Purification)
Lecture - 25
Chromatography (Part-I)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing, we were discussing about the different properties of the enzyme in the course, Enzyme Science and Technology. And in the couple of modules, we are discussing about the enzyme productions.

And if you recall, what we have ah; what we have discussed that the you can actually be able to utilize the different approaches to isolate the gene of your interest, which is going to code the enzyme. And you can have the option of using the genomic library or CDNA library to screen out the gene of your interest or you can actually be able to use the site specific primers to amplify the gene of your interest.

And that you can be able to put it into the cloning vectors with the help of the restriction enzyme and the ligase enzymes. So, once you have got a clone, then you can actually be able to deliver that clone into a suitable host, it can be a bacterial host or the eukaryotic host. So, in the bacterial host, we have discussed about the different approaches, what you can use for protein production.

And then we have also discussed about the protein production in the yeast, bacteriophage waste systems, yeast sex alliance and as well as the mammalian system. Once you have generated the product, then the next task is that you are actually going to utilize the different techniques to purify the protein. And in this current module, we are discussing about how you can be able to isolate the protein or the enzyme from the cell and how you can be able to utilize the different techniques to purify the enzyme.

So, if you recall in the previous lecture, we have discussed about the cell disruption methods and when very briefly we have discussed about the basic principle of chromatography. And we have also discussed about the purification system and how you

what are the different components are present in the purification system and how they are actually going to be helpful for the protein purifications.

Now, in today's lecture, we are going to discuss more about the chromatography. So, let us start today's discussion.

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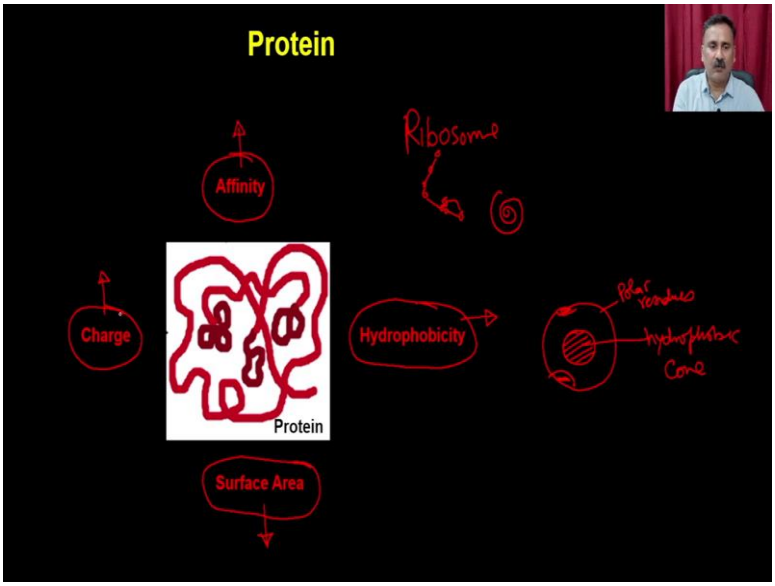

Now, what we want to do is, we want to purify a protein ok. So, remember that when we were discussing about the protein folding. So, protein is going to be produced from the ribosome and it is actually going to be produced as a chain right, a chain of amino acids, right. So, all these chain of amino acid, as soon as they comes out from the ribosome, they start folding because of the intramolecular interactions.

And as a result, they will actually going to fold around a central, ok. And as a as a result of this folding, it is actually going to arrange all the amino acid in such a way that if you are actually going to see the cross section of a protein, what you will see is that it is actually going to have the hydrophobic core at the center.

So, this is actually the hydrophobic core and in the center and on the periphery, it is actually going to have the polar residues. And apart from that, it also going to have the multiple patches, these patches can be recognized by the different system. So, if you talk about the enzyme, they are actually going to give you the different properties that can be exploited in a typical chromatography.

So, the amino acids are going to have the different amounts, different types of amino acids and that is why they can be able to have the positively charged amino acids or the negatively charged amino acid and that can be exploited in a chromatography technique. Similarly, it can also have the hydrophobic residues, so that also can be exploited in a chromatography technique.

Apart from that, proteins are globular in nature. So, they are actually acquiring a surface area and that surface area could vary between the different proteins because the different proteins are going to be of different diameters and that is how they are actually going to be of the different surface area. So, that also is a very very crucial property that can be also be exploited.

Apart from that, it can also have the exclusive region which will have the exclusive affinity for a particular molecule and that also can be exploited in the affinity also. So, based on these four criteria's you can have the different types of chromatography techniques.

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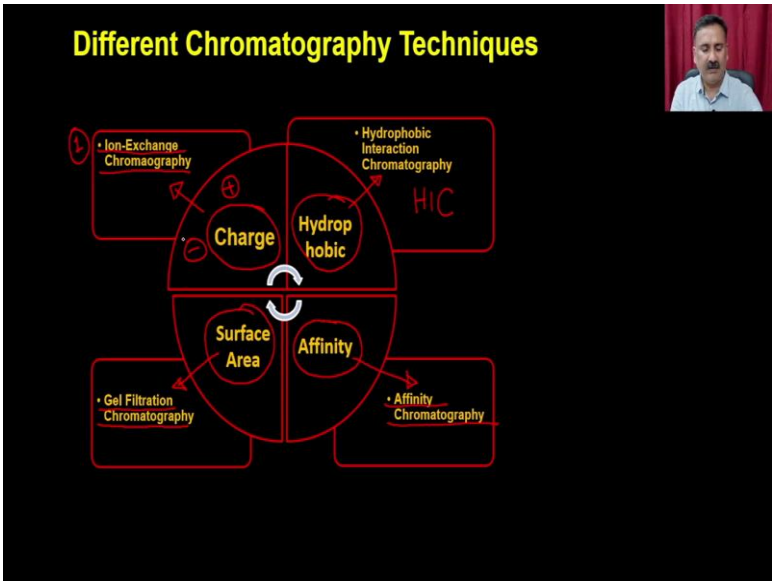

So, if you are going to exploit the charge, whether it is the positive charge or the negative charge, you are actually going to exploit that in a chromatography which is called as ion exchange chromatography. Similarly, if you are going to exploit the presence of the hydrophobic amino acids, then this chromatography is called as the Hydrophobic Interaction Chromatography or the HIC.

Similarly, if you, since the proteins are actually going to have the balls of the different sizes, you can actually have; you can expect that the proteins are going to be of different surface area. That can be also be exploited in a chromatography technique which is called as the gel filtration chromatography.

And as we said, you know proteins are also going to have the region, which are going to be having the exclusive affinity for a particular molecule and that also can be exploited in a technique which is called as the affinity chromatography. So, in this particular module, we are going to discuss about these different chromatography techniques and how you can be able to utilize them to purify the enzyme of your interest.

So, let us start our discussion with the first technique and that is the ion exchange chromatography.

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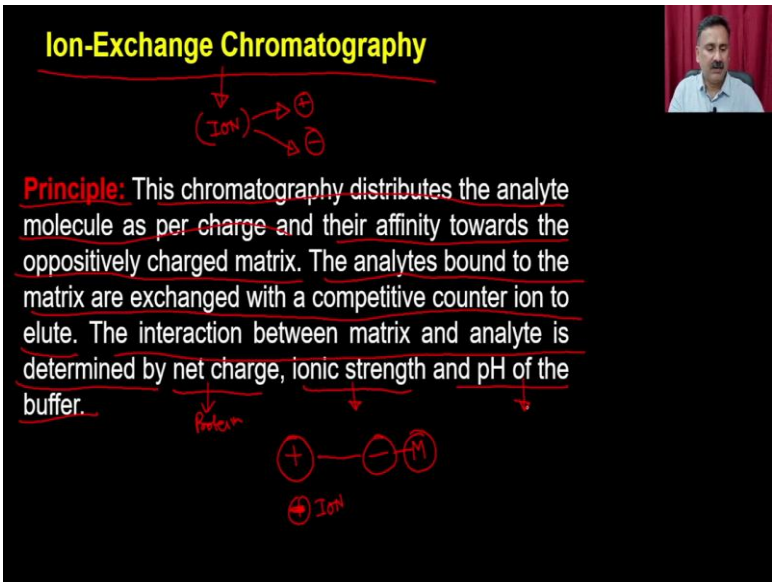

So, as the name suggests, the ion exchange chromatography is the term of chromatography, where you are actually going to play a role of the different types of ions ok. Ions could be positively charged ions or ions could be negatively charged ions. So, what is the principle of this technique? So, this chromatography distributes the analyte molecule as per their charge and their affinity towards the oppositively charged matrix.

The analyte bound to the matrix are exchanged with a competitive counter ion to elute, the interaction between the matrix and the analyte is determined by the net charge, ionic

strength and the pH of the buffer. So, what we are going to say is that, in an ion exchange chromatography, it is actually a chromatography where you are actually going to exploit the different types of ions.

So, if you are going to talk about that the positively charged proteins. So, it is actually going to have an affinity with negatively charged matrix ok. So, in this case, you can actually having a competition with the positively charged ions ok. And that is how sorry negatively charged ions and that is how you are actually going to have the competition.

So, it is actually a competitive assay where you are actually first going to bind the protein to the matrix by the; which is going to be oppositively charged and then you are going to elute it with the help of the competition. The interaction between the matrix and the analyte is determined by the net charge onto the protein or the enzyme, ionic strength of the buffer and the pH of the buffer ok. And this all we are going to discuss in detail, then you will able to understand these processes.

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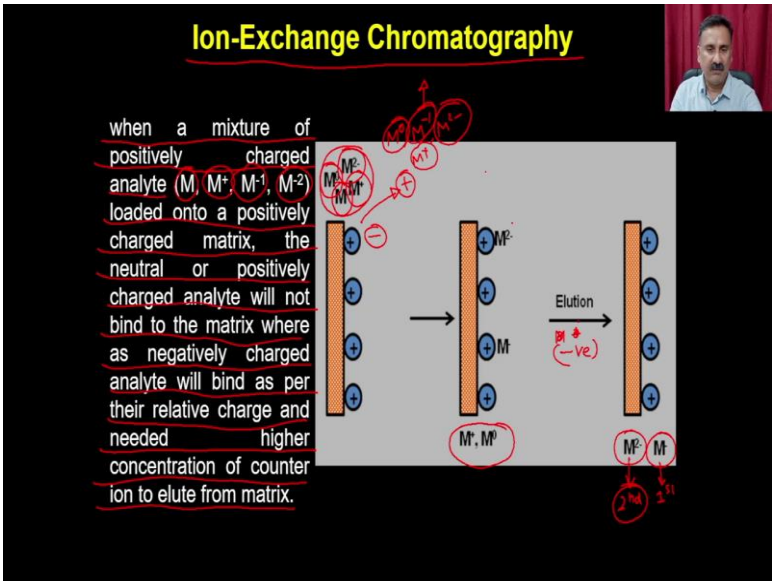

So, imagine that we have analyzed a complex mixture of these protein molecules ok. So, you have a protein molecule which has no charge, you have a protein molecule which has a negatively charged, this is a positively charged and then you also have a protein which has the one negative charge.

So, you have a protein which is one negative charge, you have the protein which is two negative charge, one positive charge and the protein which has no charge. So, when a mixture of positively charged analytes such as M, M plus, M minus 1 and M 2 minus are loaded onto a positively charged matrix, the neutral or the positively charged analyte will not actually going to bind the matrix.

Whereas the negatively charged analyte will bind as per their relative charge and it needed the higher concentration of the counter ions to elute from the matrix. So, what we have is we have this mixture which we have loaded onto a matrix which is positively charged.

So, you know that the positive is actually going to attract negative, but positive is actually going to repel the positive, right. Whereas, the neutral is anyway not going to bind, right. So, this particular column when you are going to load this particular mixture where you have the M 0, M minus 1, M 2 minus 1 and M plus, M plus and M 0 is actually going to not binding the column because the M 0 will not have a charge.

So, it will not going to have any affinity and M positive is actually going to get repel from the column. Similarly, but the M minus 1 and M 2 minus is actually going to have the affinity for this column, but the strength of this affinity is going to be proportional to the amount of charge what is present on this particular molecule.

So, the M minus 1 is actually having the lower strength and M 2 minus is going to have the higher strength. And that is why the M 2 is actually going to bind to the column in the beginning of the column and M 2, M minus 1 is actually going to bind in the lower portion of the column.

And when you are going to elute the small amount of the counter ions, right. So, for example, in this case the negatively charged ions are actually going to elute the M minus 1 first. Because the strength of the m minus to the matrix is lower, right. So, it will come out first right.

So it is going to come out first and M 2 minus is going to have higher strength. So, it is going to come out second. That means you have started with the 4 molecules, you started with M 0, M plus, M minus 1 and M 2 minus 1 and at the end you are going to get all the molecules separately.

This means you have achieved the purification with the help of the ion exchange chromatography. And as I said you know ion exchange chromatography is going to deal with the positively charged ions or the negatively charged ion and that is how the based on these ions the ion exchange chromatography can be of different types.

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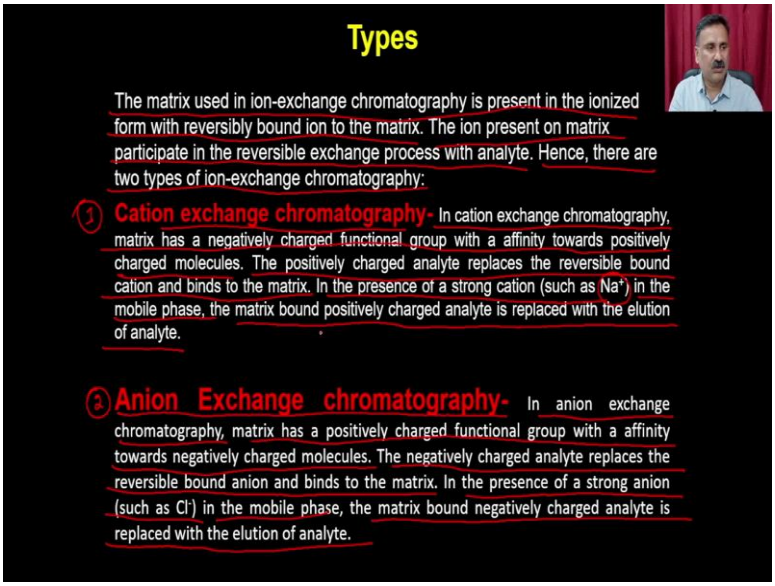

So, the matrix used in the ion exchange chromatography is present in the ionized form the reversibly bound ion onto the matrix. The ion present on the matrix participate in the reversible exchange process with the analyte. Hence there are two types of ion exchange chromatography you can have the cation exchange chromatography or the anion exchange chromatography.

In a cation exchange chromatography, in cation exchange chromatography the matrix has negatively charged functional group with a affinity towards positively charged molecules. The positively charged analyte replaces the reversibly bound cation and binds to the matrix. In the presence of a strong cation such as sodium in the mobile phase, the matrix bound positively charged analyte is replaced with the elution of an analyte.

Similarly, you can have the anion exchange chromatography. In anion exchange chromatography matrix has a positively charged functional group with affinity towards the negative charged molecules. The negatively charged analyte replaces the reversibly bound anion and binds to the matrix.

In the presence of a strong anion such as chloride in the mobile phase, the matrix bound negatively charged analyte is replaced with the elution of the analyte. So, you can have the cation exchange chromatography or the anion exchange chromatography

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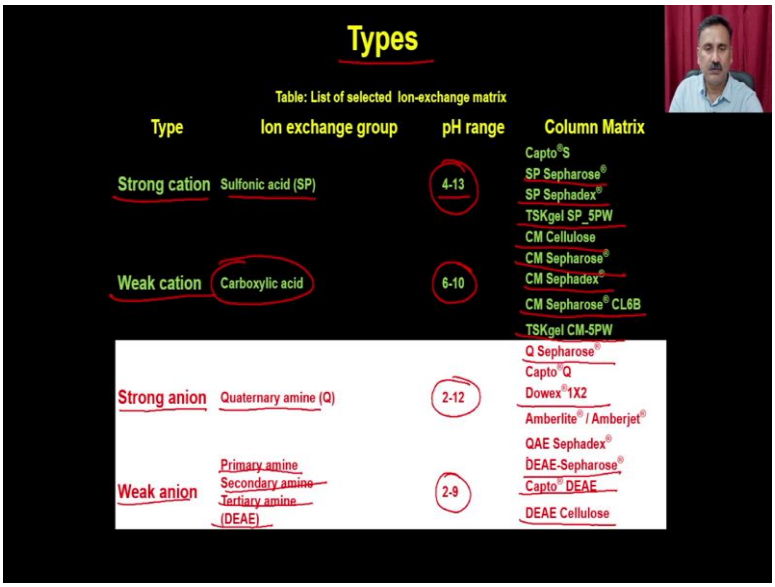

And you can use the different types of matrix. For example, you can have the strong cation exchangers or the weak cation exchangers, strong anions and weak anions. So, these are the different examples of the matrix, what is available for the ion exchange chromatography. So, you can have the strong anions, in which you can have the Sulfonic acid or the SP Sepharose.

So, you can have and the pH range in which you are going to this these matrixes can be work is a 4 to 13. You can have the multiple examples like the SP Sepharose, SP Sephadex, TSKgel, CM Cellulose and CM Sepharose. Similarly, you can have the Weak cation. So, your weak cation you can have the functional group, Carboxylic acid which is going to be attached to the matrix.

And you can use this column in the range of 6 to 10 and the examples are CM Sepharose, CM Sephadex, CM Sepharose, C CL6B and TSKgel CM 5PW ok. In Apart from that you can also have the strong anion or the weak anion. So, strong anion, where you can have the quaternary amine, so quaternary amine can be used in a range of 2 to 12. The examples are Q Sepharose, Dowex and all that. Then we can have the weak anions.

So, in the weak anion you are going to have the secondary amine, and tertiary amine such as, DEAE. And the range in which you are going to use this is 2 to 9 examples are DEAE, Sepharose, Capto and the DEAE Cellulose.

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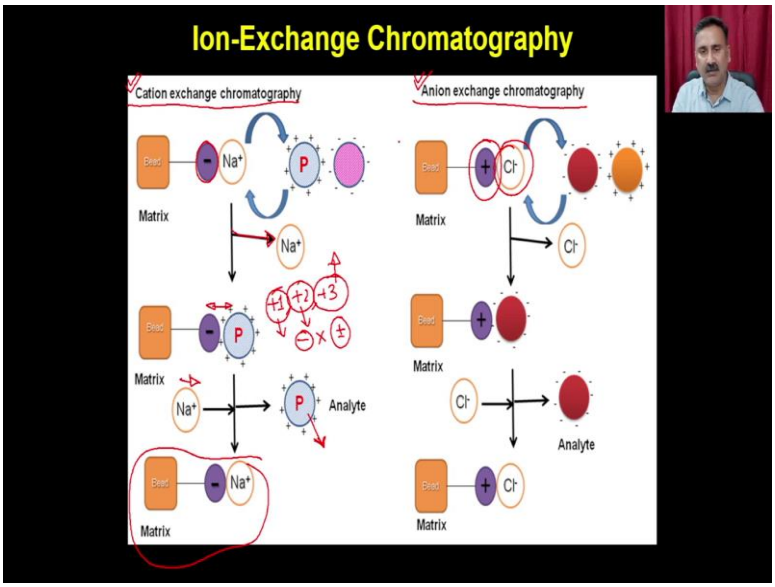

Now, how the ion exchange chromatography is going to use is it is actually going to be dependent on the competition between the counter ions. So, for example, in the cation exchange chromatography you have the beads which is negatively which is attached to a negatively charged groups.

And on this negatively charged group you have the counter ion that is the sodium which is immobilized. So, in the presence of a positively charged protein, what its going to do is it is going to do a competition with the immobilized, so cation onto the matrix. And in this process what will happen is that immobilized cation is actually going to be replaced and the protein will actually going to bind.

And as you remember when we were discussing about the different types of analyte, when you have loaded onto the positively charged matrix. The charge the amount of charge onto this protein is going to be different. So, if suppose you have the plus 1 charge, plus 2 charge, plus 3 charge so this guy is actually going to have the maximum strength, this guy going to have a middle range strength and this guy is going to have the least strength.

Whereas, a negative charge molecule will not bind or the neutral molecule will not bind. So, since the proteins are going to have the different amount of the ionizable groups these charges are going to be different and that is why the strength of the interaction of the protein to the matrix is also going to be different.

And then what you are going to do is you are going to again doing a competition with the counter ion. So, in this case we have supplied the sodium, SP once you supply the sodium sodium is going to replace the protein which is bound to the to the matrix and this protein is going to replace, right.

But the amount of sodium what you are going to use to replace the plus 3 protein or plus 2 protein or plus 1 protein is going to be different. And that is why these proteins are going to be eluted at the different concentration of the sodium. And ultimately you are going to regain the matrix at the end.

Same is true for the anion exchange chromatography, the only difference is that the bead is going to be positively charged and it is going to have the negatively charged anion as a counter ion. And in this case the protein is going to be negatively charged and that is how the negatively charged protein is going to bind the matrix and then when you supply the anions, it is actually going to replace the protein and that is why you are going to get the matrix batch.

So, whether you want to use the cation exchange chromatography or anion exchange chromatography it depends what kind of charge is actually going to be present onto the protein and based on this charge you can be able to use either the cation exchange chromatography or the anion exchange chromatography. Now, how you are going to run the chromatography ion exchange chromatography.

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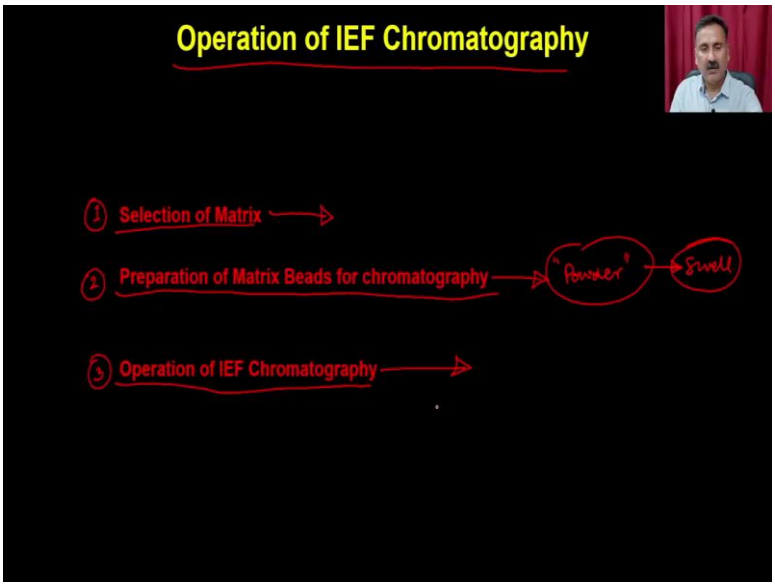

So, when you want to start the running of the ion exchange chromatography, you have to follow the 3 steps. Number 1 you are going to select the matrix, right. So, we have discussed many types of matrix like the weak cations and strong cations and weak anions and all that.

So, you have to choose the matrix. Then we have to prepare the matrix bead for the chromatography. So, these beads are always being supplied as a powder, right. So, this powder has to swell into a buffer where you are actually planning to perform the chromatography and that is how you are going to prepare the beads and then you are actually going to do the ion exchange chromatography.

But ion exchange chromatography operation of the ion exchange chromatography is also a multiple step process because how you are going to select the matrix, how you are going to prepare the matrix and how you are going to operate the ion exchange chromatography is depends on the multiple factors.

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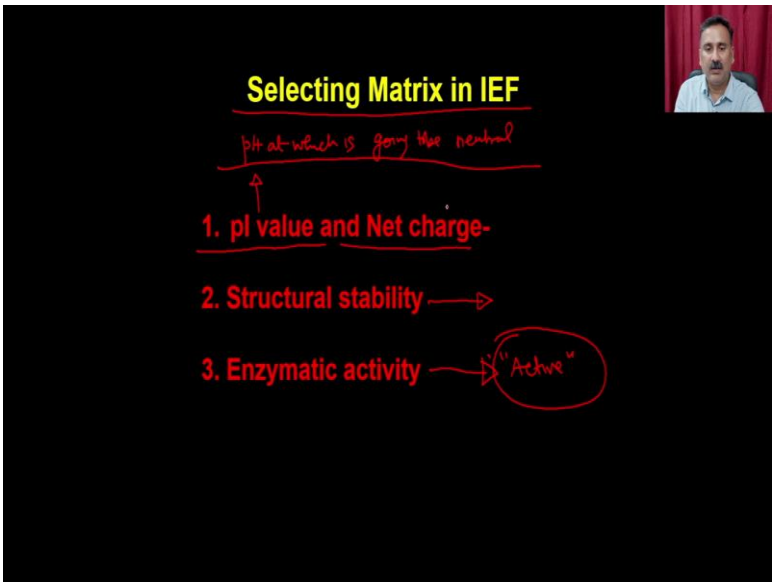

So, let us see, how you can be able to select the matrix, when you are going to select the matrix, what are the different points, what you have to always keep it in mind. First is you are going to be consider the pI values and the net charge onto the protein. So, you know that the pI charge pI value is the pH at which the protein is going to be protein is going to be neutral.

So, keeping this pH and you are actually going to calculate the charge. So, you can actually be able to vary the pH of the buffer and that is how the net charge of the protein is also going to be vary. When you do the pH variation it is actually going to disrupt not only the its also imparting the charge onto the protein molecule, but it also going to disrupt many of the crucial interactions.

So, in those cases you also have to see that particular pH whether the there will be a structural stability or not. And when you are trying to you know purify the enzyme for example, its very important that the enzyme should have been active at that particular pH. It should not be the case that you are purifying an enzyme which is not active at that particular pH.

Because if that happens the enzymes, are you know going to be dead before you actually going to purify. So, there is no point in purifying the enzyme which is dead actually. Now, how you are going to exploit the pI value and you are going to calculate the net charge.

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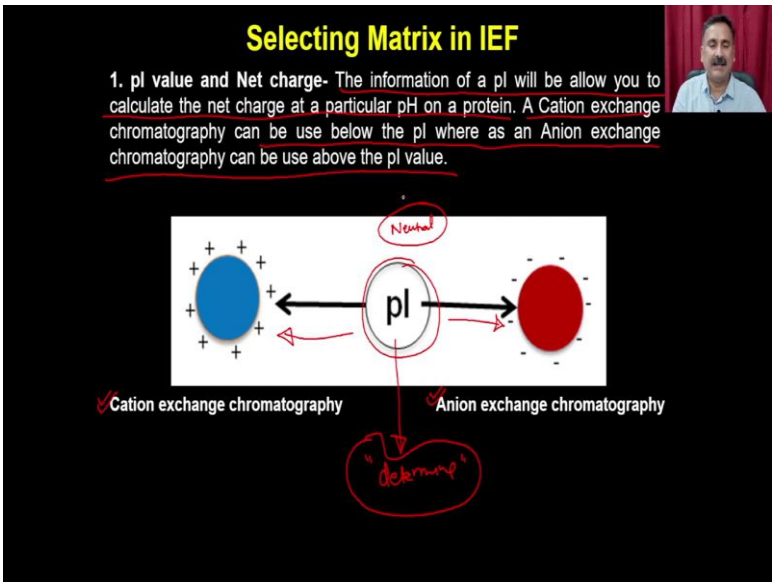

So, pI is the information of the pI will allow you, to calculate the net charge at a particular pH on a protein. A cation exchange chromatography can be used below the pI whereas; the anion exchange chromatography can be used above the pI. So, if you have the pI at this pH it is actually going to be neutral which means the amount of positive and a negative charge are going to be same.

But if you go lower to this pH it is going to be positively charged, so you can be able to use the cation exchange chromatography. If you go to the above to this particular pH it is actually going to be anion exchange chromatography because the protein is going to be negatively charged.

Now, the question comes, how you can be able to determine the pI of a protein right, because that is very crucial and important information what is required to perform the ion exchange chromatography.

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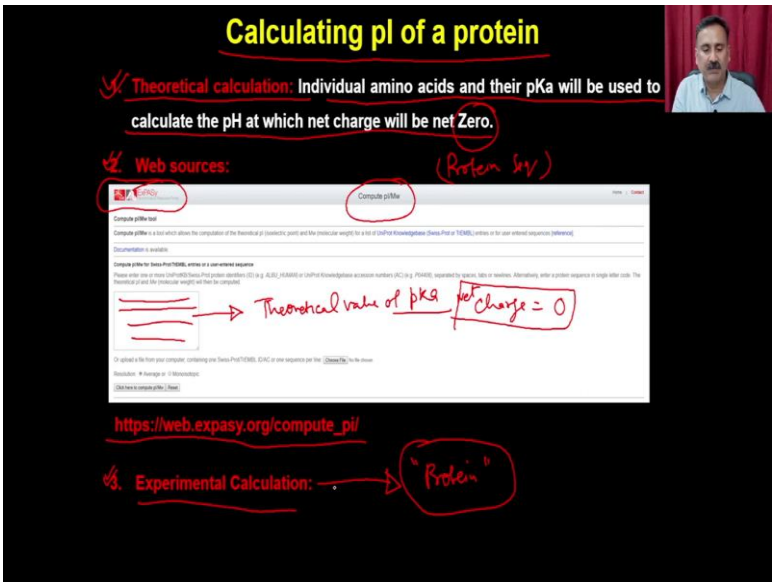

Calculating the pI of a protein, you have multiple options one is critical calculations. So, that you can manually you can will do, right. So, individual amino acids and their pKa will be used to calculate the pH at which the net charge will be 0. So, you can actually have the 4 amino acid, if you take the 4 amino acid and histidine.

So, if you calculate the pKa values of the number of amino acids like number of lysines, number of arginine, number of glutamic acid, number of aspartic acid and number of histidine. So, if you calculate all these and if you use the pKa value of these amino acids, it the average molecule its actually going to tell you what will be the charge and at what pH its actually going to have the 0 charge or net charge as 0 charge.

The second option is the web sources. So, what you can do is? You can actually be able to go to the ExPASy website and you can actually given you the link also. So, if you go to that ExPASy website and you say the compute the pI values. So, what you have to do is you have to just put the amino acid sequence in this particular column and it is actually going to do exactly the same way.

It is actually going to put the theoretical values of the pI of the pKa values and of the individual amino acids and that is how its actually going to tell you the calculation at which ph the pI is actually going to be at which the charge is going to be 0, net charge is going to be 0. And that is going to be your pI values.

The third is the experimental methods. So, experimental methods where you are actually going to use in those cases, so the first two options only possible when you are actually going to know the protein sequence ok. If you do not know the protein sequence, you only have a protein, but you do not know the protein sequence, then you actually going to have no option, but to do experimental calculations. How you are going to do the experimental calculations?

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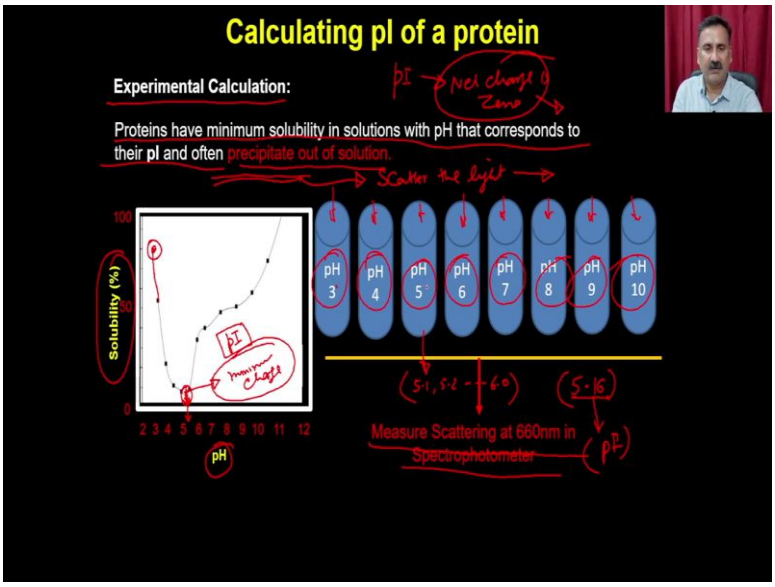

So, experimental calculation is based on the basic principle that the proteins have the minimum solubility in a solution with the pH corresponding to their pN and often precipitated out of the solution which means at pI, the net charge is actually going to be 0 right. So, net charge is 0 and because the net charge is 0, it is not going to interact with the solvent molecule and as a result, it is actually going to have the minimum solubility.

And how you are going to monitor the solubility because if it is having the lower solubility, it is actually going to form the precipitate and these precipitates actually are going to scatter a light and that can be measured with the help of the spectrophotometer ok. So, what you see here is I have giving you a curve between the solubility versus the pH.

So, what I have done is, I have taken the protein, I have incubated that into the different reactions of pH 2, pH 3, 4, 5, 6, 7, 8, 9 and 10. So, all the pH I have prepared, right. So, all these pH are actually going to change the total charge on the protein. So, it possible

that in some charges, some places the protein is going to have acquire or reach to a pI values and in that pi that value, the solubility is going to be very minimum.

So, what I have done is, I have taken the protein, same amount of protein incubated in pH 3, 4, 5, 6, 7, 8, 9 and 10 and then I have incubated it for some time and then I have measured the scattering at 660 nanometer in a spectrophotometer. So, scattering is going to tell me that, how much the protein is actually going to form the particulate matter. So, and that I can use to calculate the solubility.

So, initially it is actually going to have the high solubility because it is actually going to have the charges. So, if it having a charge, its going to interact with the solvent system and that is how it is actually going to this remains solution. As the protein will reach to its pI value, its solubility is actually going to go down and that is how you see here is this is the point at which the pI is the protein is actually having the minimum solubility.

This means at this particular point, I mean protein has the minimum charge, right? It could be neutral or it could be minimum charge. So, at this point this is the point at which you can say that it is actually a pI. If you want you can make it more precise, you can what you can do is for example, in this case this is a value which is closer which is in between the 5 and 6. So, what I can do is, I can just prepare another pH ok of 5.1, 5.2 and dash dash dash dash 6 ok.

So, if I prepare some more pH, I will actually going to get very precisely I will know the number that ok the pI of this particular protein is 1.16, 5.16. So, that is a way you have to do right, initially you have to start with the broader range when you see that ok the precipitate is maximum between 5 and 6, then you can actually be able to prepare a buffer of 5.1 to 6 and you will know that at 5.16, the precipitate is maximum.

This means this is actually going to be the pI value of this particular protein. Now, once you know the pI values, you can be able to use that for selecting the matrix.

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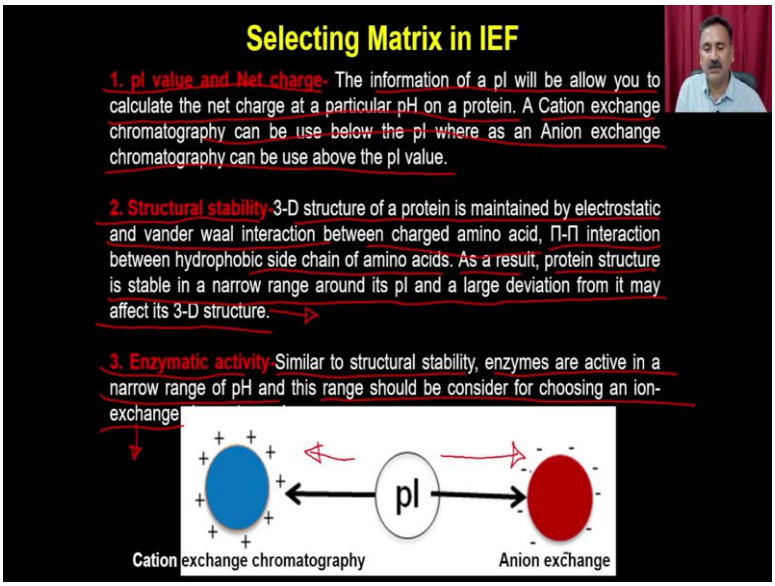

So, the pI value and net charge, so the information of the pI value will allow you to calculate the net charge at a particular pH, a cation exchange chromatography can be used to below the pI value and anion what it can be used above. Once you know this, you can also be talk about the structure stability.

So, the 3D structure of a protein is maintained by the electrostatic and Vander Waal interaction between the charge amino acid, pi pi interactions. And so as a result, the protein structure is stable in a narrow range around its pI and a large deviation from it may affect its 3 dimensional structure. So, that is very, very important.

Then its enzymatic activity similar to the structural stability, enzymes are active in a narrow range of pH and this range should be considered for choosing a ion exchange chromatography because you always want to purify the enzyme which is very very active.

So, as I said, you know if you go; if you want to use the cation exchange chromatography, you have to go below the pI values and if you want to use the ion exchange chromatography, you have to use the above to the anion exchange chromatography.

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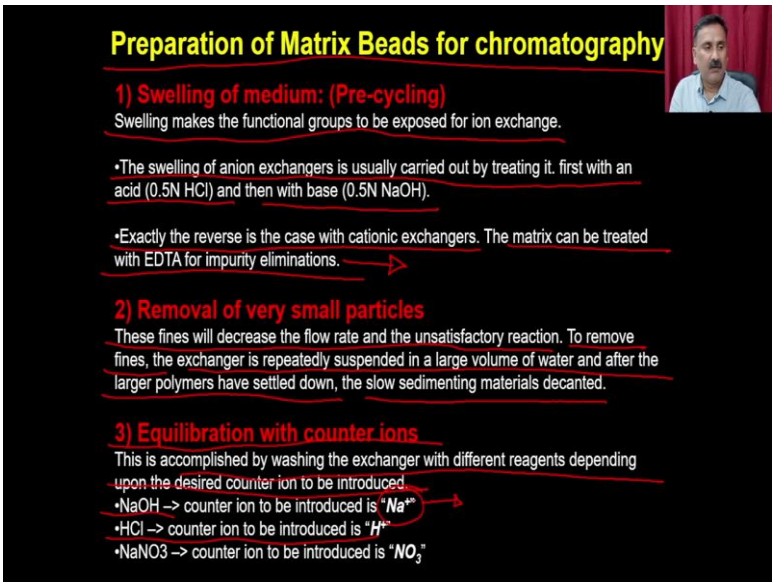

Now, the second step is preparation of the matrix for the chromatography. So, the first step is swelling of the matrix or swelling of the media which is called pre cycling. So, swelling makes the functional group to be exposed for ion exchange chromatography. The swelling of the anion exchange chromatography is usually been creating by treating it first with an acid and then with a base.

So, all this is been done. So, that the matrix functional groups are going to be you know denatured and therefore, they are actually going to be exposed. Exactly the reverse is the case with the cation exchange the matrix can be treated with EDTA for the impurities eliminations. Then you also have to remove these small particles.

So, these fines will decrease the flow rate and unsatisfactory reactions. To remove the fines, the exchanger is repeatedly suspended in a large volume of water and after the large polymer has settled down the slowly sedimentation decanted, which means you are going to wash the matrix multiple times.

So, that the fine particles can be removed, because they are actually going to interfere in terms of reducing the flow rate and the fine particle can also interact with the proteins. Then you are going to equilibrate with the counter ions. So, for example, this is accomplished by washing the exchanger with the different reagents depending upon the desired counter ion to be introduced.

For example, you can wash it with the NaOH, if you want the NaOH, sodium plus to bind. So, that will be you going to do for the cation exchange chromatography, you can use the HCl if you want to introduce the H plus and so on.

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So, once you are going to prepare the matrix, it is actually going to be like this. You are going to have the positively charged ions and it is actually going to have the counter ion in terms of the negative ions. Now, when you at this stage, you can be able to have the different choices of the buffers what you are going to use for performing the ion exchange chromatography.

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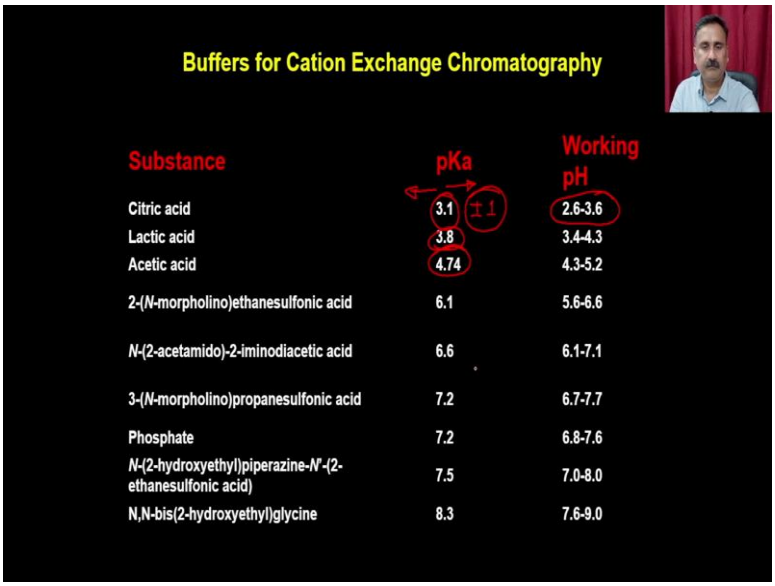

So, these are the some of the buffers what you can use and you can choose the buffers based on the working concentration. So, what is mean by the working concentration is, that it is always going to be plus minus 1 from the pKa value. For example, in this case it is 3.1.

So, it is actually going to be from 2.6 to 3.6 which means 0.5 on this side and 0.5 on this side ok. Similarly, this side also this one also. So, depending on and what buffer range you are working, you can be able to choose these buffers and you can actually be able to work.

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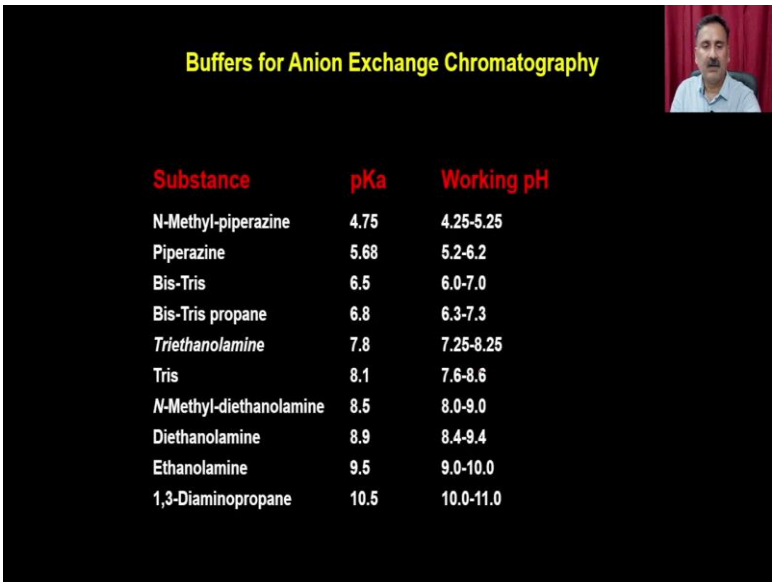

So, these are the buffers for the anion exchange chromatography.

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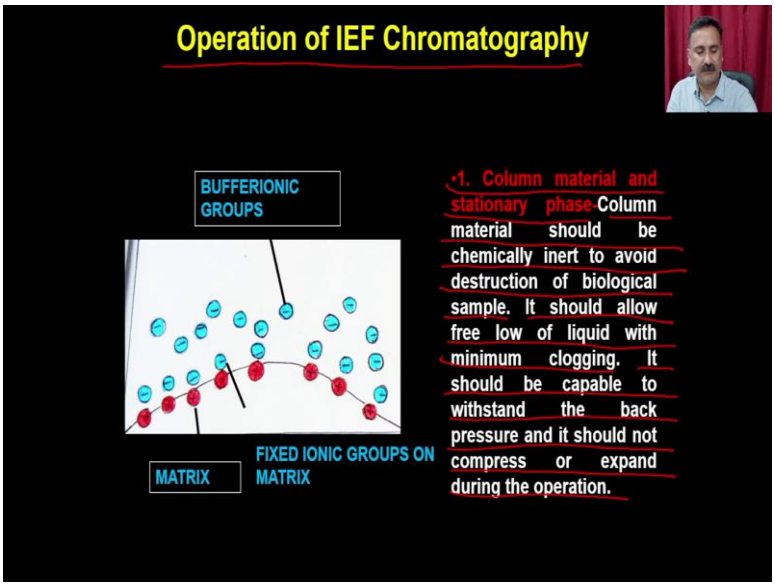

Now, the second point is that how you are going to perform the ion exchange chromatography. So, ion exchange performance of the operation of the ion exchange chromatography is a 3 step process. In the step 1, you are going to prepare the column matrix and the stationary phase. So, column material should be chemically inert to avoid the destruction of the biological sample.

It should allow the free flow of liquid with the minimum clogging. It should be capable to withstand the back pressure and it should not compress or expand during the operation. So, this is going to be the matrix when you are going to prepare. Its going to have the positively charged groups and that are going to have the counter ions.

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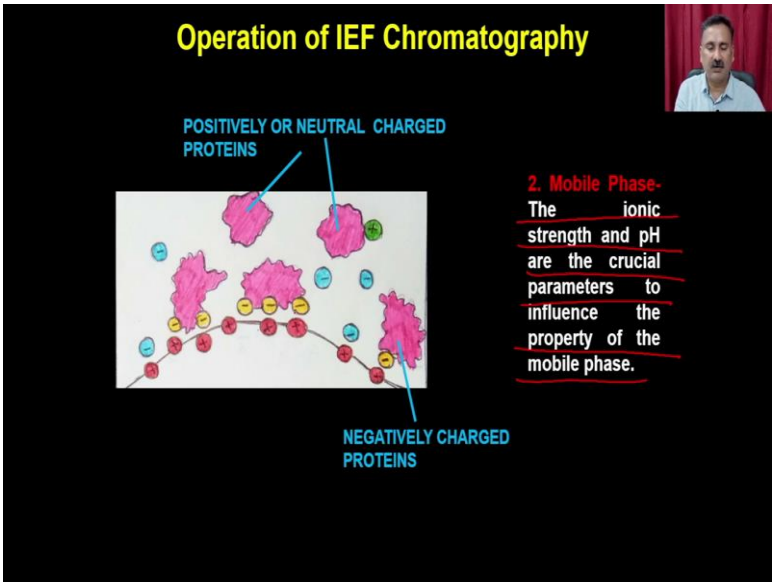

Then the step 2, you are actually going to equilibrate the column with the mobile phase, this ionic strength and the pH are the crucial parameter to influence the property of the mobile phase. And what will happen is that once you are going to supply the mobile phase, it is actually going to have the counter ions what are going to be present in the buffer. And that is how this bead or this matrix is now ready to bind the columns or bind the proteins.

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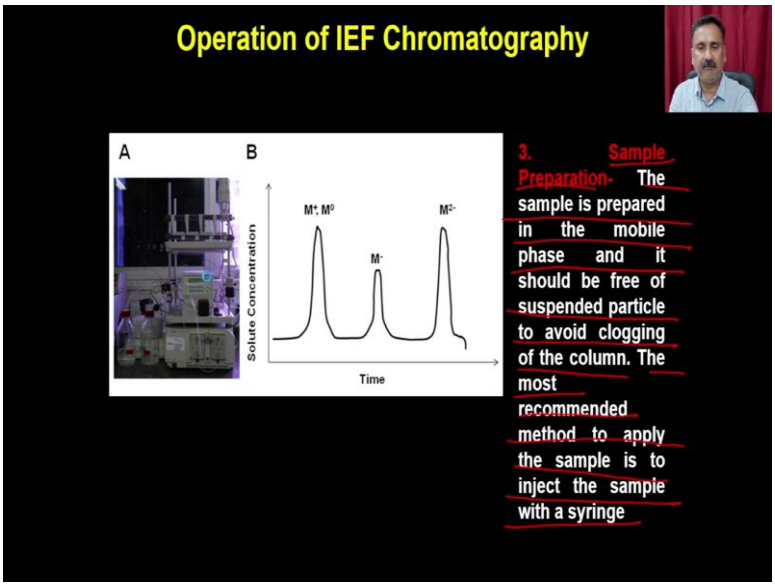

In the 3rd step, you are going to prepare the sample. So, the sample is prepared in the mobile phase and it should be the free of suspended particle to avoid the clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe. So, this is the protein purification system and you can actually be able to use the syringe to inject the sample onto the column.

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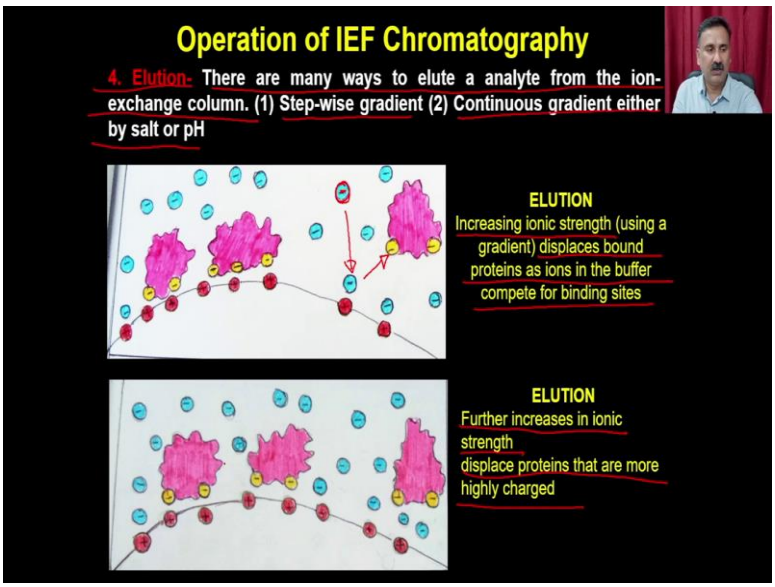

Then in the step 4, you are going to do the elution. So, there are many ways to elute the analyte from the ion exchange problem. You can do the step wise gradient or you can

actually be able to do the continuous gradient by the salt or the pH. So, elution can be done by increasing the ionic strength using a gradient, displacing the bound protein as ion in the buffer compete with the binding sites.

So, you can imagine that if I add supply more amount of negatively charged ions, it is actually going to compete for the protein which has negative charge and that is how it is binding to the matrix and as a result, it is actually going to have the competition. So, these negative ions are actually come and bind to the matrix and the protein which is bound is actually going to be replaced. And that is how it is actually going to come out into the solution.

If you further increase in the ionic strength, the displaced protein that are more highly charged, so its actually going to be in proportion to the amount of protein. So, initially you see when I when I supplied the low amount of ions, only the two charged protein are going to be eluted. But when I am supplying the more amount of charge, the 3 and 4 charge containing proteins are also going to be eluted.

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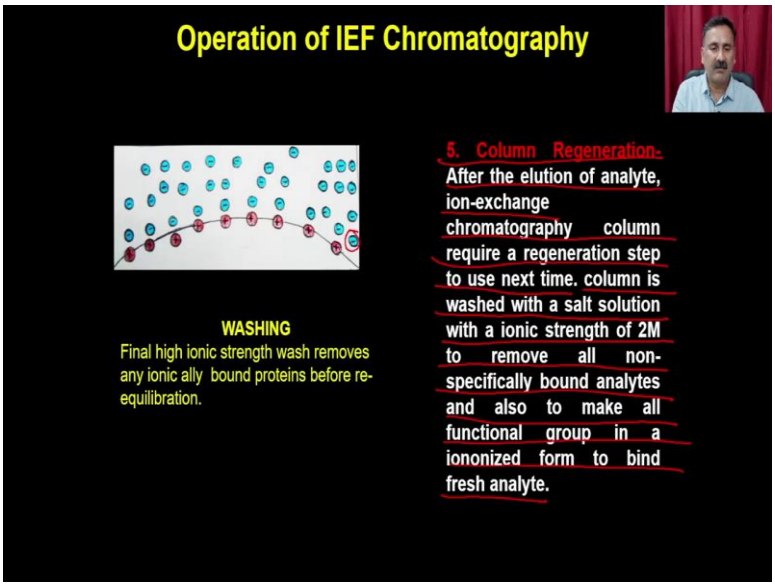

Once you are done with the chromatography, the last step is actually the column regenerations. So, after the elution of the analyte ion exchange chromatography columns requires a regeneration step to use for the next time. Column is washed with a salt solution with a ionic strength of two molar to remove the all non-specifically bound

analyte and also to make all the functional group in a ionized form to bind the fresh analyte.

So, this is what going to happen when you regenerate, you wash it with the high ionic salt, it is actually going to remove all the proteins and it is also going to bring the counter ion back because this counter ion is actually going to replaced, when you are going to load the protein with the negatively charges. So, this is all about the ion exchange chromatography. Now, let us move on to the next technique.

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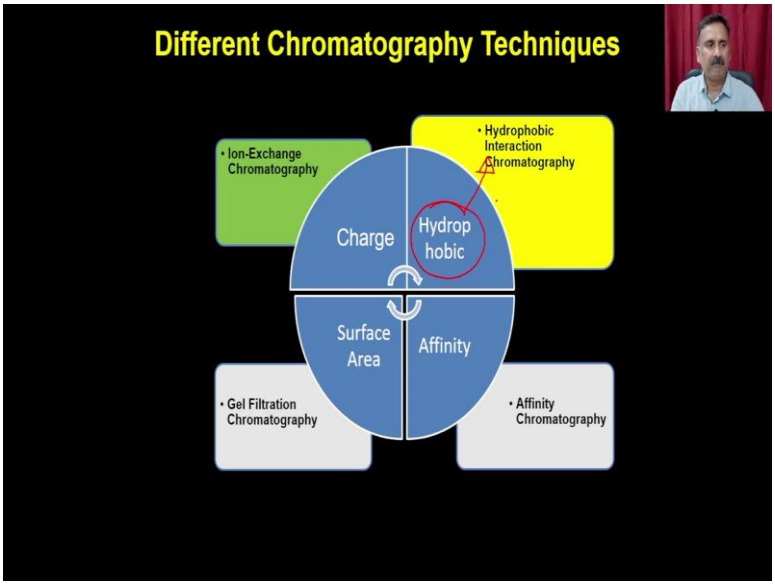

And next technique is go is going to utilize the hydrophobic groups, what is present on to the protein structures and the technique is called as the hydrophobic interaction chromatography.

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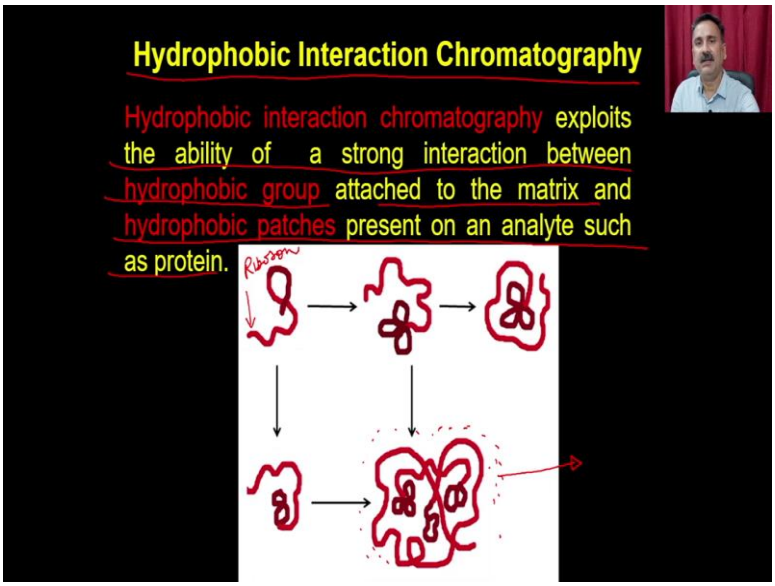

So, hydrophobic interaction chromatography as the name suggest is going to exploit the ability of a strong interaction between the hydrophobic groups, attached to the matrix and the hydrophobic patches present on the analytes such as protein. So, what happen is that when the protein is going to be produced from the ribosome, it is going to be produced as a string of the amino acids, which are attached to the peptide bond.

But when it rotates, it actually try to hide the hydrophobic groups from the polar residues or from the polar environment, right. And that is why it actually keeps these hydrophobic groups in the center and all the protein actually rolls over this. So, that these hydrophobic groups should not be able to see the polar environment because you are outside it is polar because you have the water molecule outside, right.

And that is how it is actually going to give you the functionally developed; fully developed or folded protein, where all the hydrophobic groups are going to be present in the center and all the polar groups are present outside because outside its you are going to have the water molecules.

Now, the question is because you want to utilize this for the hydrophobic interaction chromatography, these hydrophobic patches should be available for the protein to interact with the hydrophobic groups, what are present onto the matrix. And that you are going to achieve by a process which is called as the salting in.

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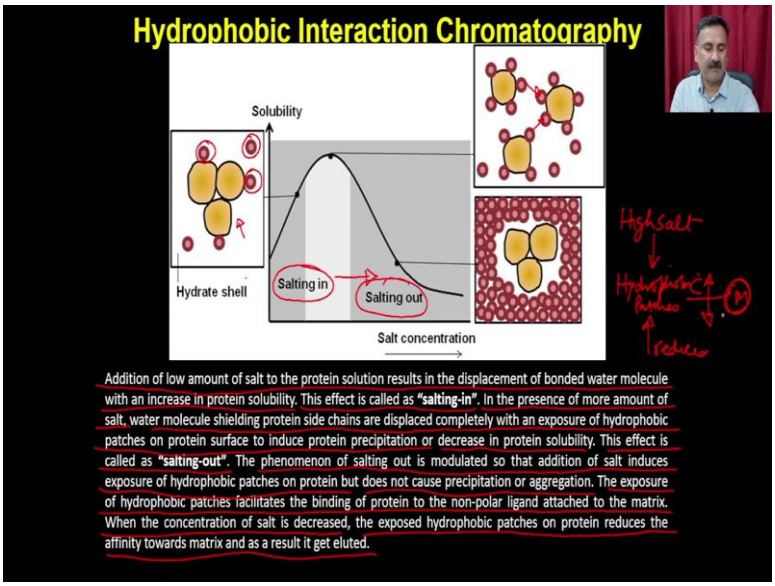

So, what is happened is that if you are actually going to add the small amount of salt, what will happen is that, it is actually going to remove the bound protein molecules. So, it is actually going to remove the some of the water molecules. And as a result, it is actually going to bring more and more space into the solution and that is why it is actually going to increase the solubility of the protein molecule.

And this effect is called as the salting in. So, addition of a low amount of salt to the protein solution results in the displacement of the bound water molecule with the increase in protein solubility and this effect is called as salting in. In the presence of more amount of salt, water molecule shielding, water protein side chains are displaced completely with an exposure of hydrophobic patches on the protein surface to induce the protein precipitate or decrease in solubility. This effect is called as salting out.

So, what happen is if you allow if you add the small amount of salt, it is actually going to remove the you know the water molecules and that is how it is actually going to bring more space and that is how it is actually going to increase the solubility. But you increase more amount of salt, right. Then it is actually going to remove the even the it is going to expose the hydrophobic patches.

And as a result, the protein protein molecules are actually going to interact with each other and they are actually going to form the precipitate. And this effect is called as the salting out. The phenomenon of this salting out is modulated, so that the addition of a

salt induces, exposure of the hydrophobic patches on the protein, but does not cause the precipitation or aggregation.

The exposure of hydrophobic patches facilitate the binding of protein to the non-polar ligand present onto the matrix, when the concentration of the salt is decreased the exposure, exposed hydrophobic patches onto the protein reduces the affinity towards the matrix. And as a result, it is actually going to elute.

So, what we are doing is? We are actually adding the high salt. So, that it is actually going to expose the hydrophobic patches, right. And hydrophobic groups on the protein, right. And as a result, it is going to bind the matrix. Now, what you going to do is you are going to reduce the salt.

So, if you reduce the salt, if you reduce the salt; the hydrophobic patches are going to be covered with the water molecules. And as a result, it is actually going to destroy the interaction between the hydrophobic patch and the functional group what is present onto the matrix.

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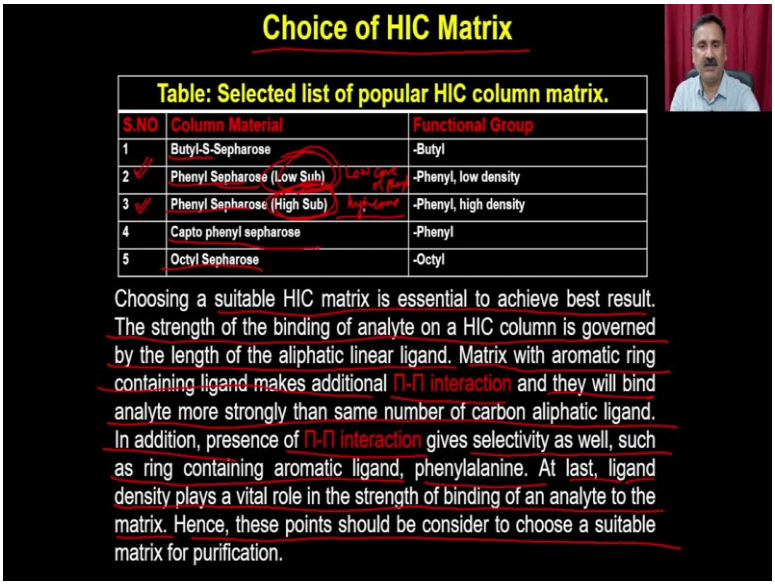

There are different types of choice, the choice of the matrix, what you can use? You can use the Butyl-Sepharose, you can use Phenyl Sepharose, Phenyl Sepharose, a high sub and low sub. You can use the Capto phenyl sepharose and you can also use the Octyl Sepharose. So, choosing a suitable matrix is essential to achieve the best results.

The strength of the binding of analyte on the HIC, column is governed by the length of the aliphatic linear ligands, right. Matrix with the aromatic rings containing ligand makes, additional pi pi interaction and they will bind, analyte more strongly than the same number of carbon aliphatic ligands.

In addition, the presence of pi pi interaction gives the selectivity as well as ring containing aromatic ligands, phenylalanine. At least at last, the ligand density play a vital role in the strength of binding of an analyte to the matrix, hence these points should be consider to choose a suitable matrix for the for the purifications.

For example, you have the two choices, you can use the phenyl sepharose, low substituted and phenyl sepharose is high substituted. So, low substituted means low concentration of the phenyl group, what is present onto the matrix, whereas, here you are going to have the high concentration.

So, depending on your affinity of the, the depending on the affinity of the protein for a matrix, you can actually be able to choose whether you want the low substituted or the high substituted. Now, how you are going to perform the hydrophobic interaction chromatography.

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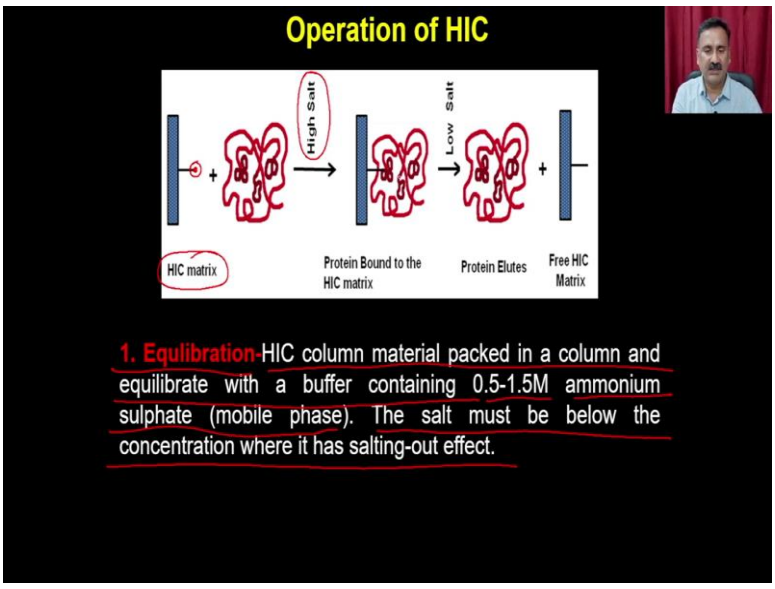

So, what you are going to do is 1st you are going to do the equilibration. So, in the step 1, you are going to do the equilibration. So, HIC column matrix packed in a column and

equilibrate with a buffer containing 0.5 to 1.5 molar ammonium sulphate, right. So, when you are actually going to put the high salt into the equilibration buffer, the salt must be below the concentration where it has a salting out effect.

So, when you put the high concentration, it is actually going to remove the hydration shell from the protein and it is actually going to expose the hydrophobic patches. And as a result, the functional group, what is present onto the protein on the matrix is actually going to bind these hydrophobic patches.

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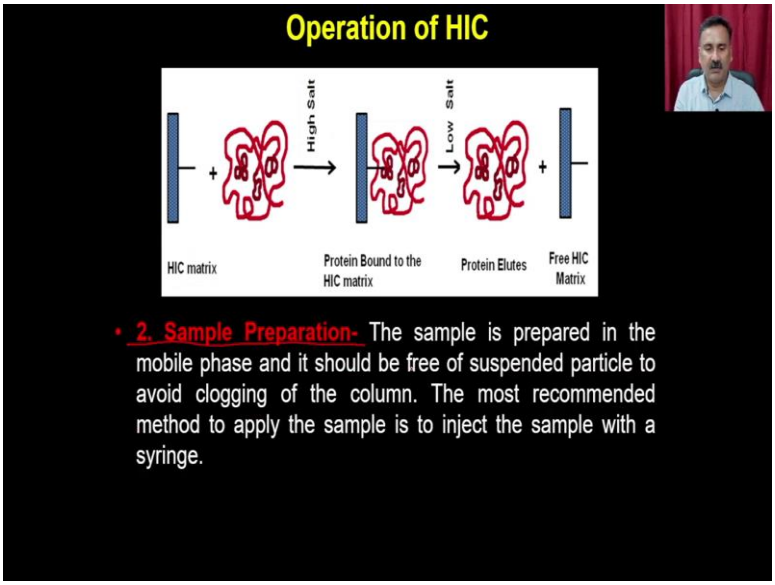

Now, in the 2nd step, you are going to do the, you are going to do the washing, right.

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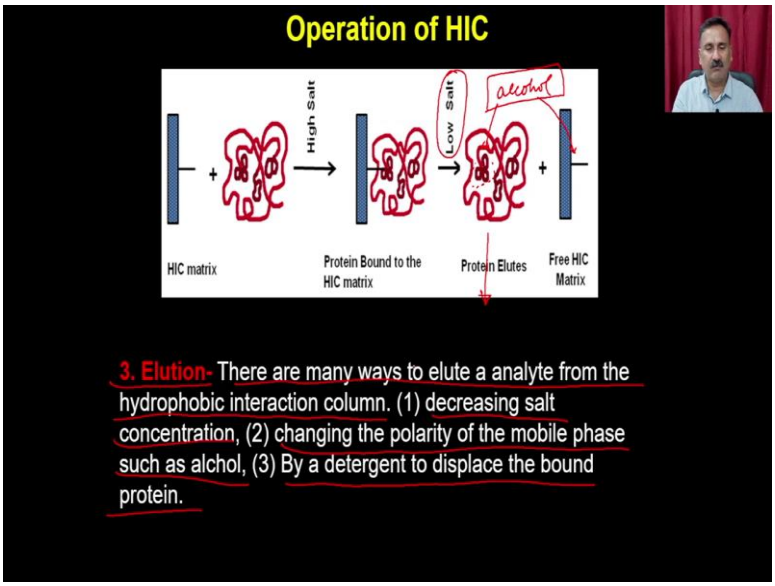

So, you are, and then in the 3rd step, you are going to do the elution. So, there are many ways to elute and analyte from the hydrophobic interaction chromatography. You can decrease the salt concentration. So, if you decrease the salt concentration, what will happen is that it is actually going to bring the hydration shell back. And once it actually brings the hydration shell back, all these hydrophobic patches are actually going to be covered by the water molecule.

And as a result, it will not be able to make an interaction with the HIC matrix, and as all, it actually going to come out into the solution. Then you can also change the polarity of the mobile phase such as alcohol, right. So, if you actually change the polarity of the mobile phase, then also you are actually going to do the same, because then there will be a competition of the molecules what is present in the mobile phase.

So, for example, if you are going to use the alcohol, right. Alcohol molecules then are going to compete for the hydrophobic patches with the matrix. And as a result, the alcohol is actually going to bind these hydrophobic patches and it is actually going to come out.

By a detergent to displace the bound protein, so you can also use a detergent to dislodge or destroy this interaction between the HIC matrix and the protein. And that is why you can be able to use that for elutions.

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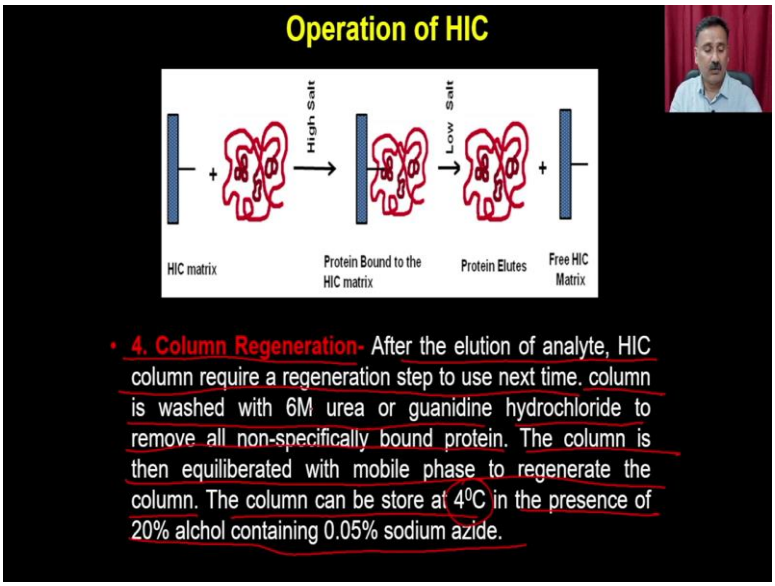

Now, in the step 4, you have to do a column regeneration. So, after the elution of the analyte, HIC column requires a regeneration step, we use it for the next time. Column is washed with a 6 molar urea or guanidine hydrochloride to remove all non-specifically bound protein.

The column is then equilibrated with a mobile phase to regenerate the column. The column can be stored at four degree Celsius in the presence of the 20 percent alcohol containing 0.05 percent sodium azide.

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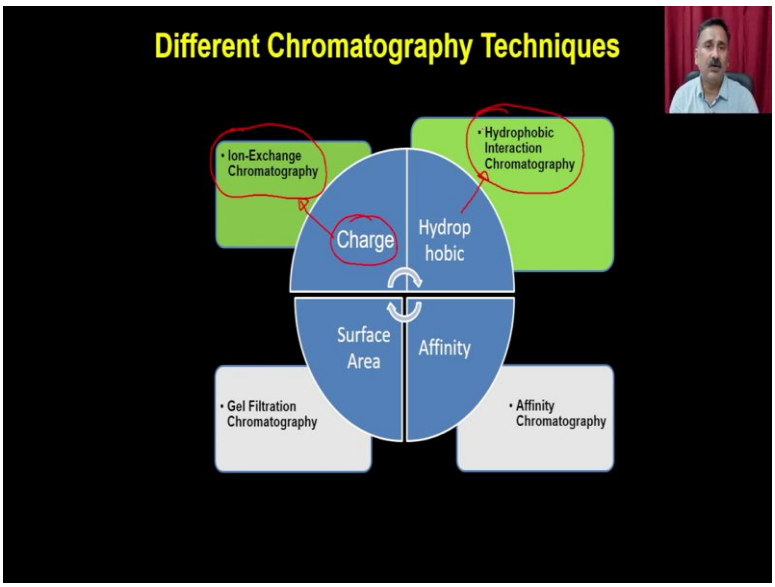

So, this is all about the chromatography techniques, where we have actually discussed about the exploitation of the charge. So, we have discussed about the different steps what we have to do for the ion exchange chromatography. And then we also discuss about the hydrophobic interaction chromatography.

So, we have discussed about the what are the different steps, how you are going to perform the hydrophobic interaction chromatography. So, with this, I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss more about the gel filtration chromatography and as well as the affinity chromatography.

Thank you.