Genetic Engineering: Theory & Application
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Module 7 Isolation and Purification of Product Part 1
Lecture 21: Ion-Exchange Chromatography

Hello everyone. This is Dr. Vishal Trivedi from Department of Bioscience and Bioengineering IIT Guwahati and what we were discussing, we were discussing about the purification as well as the isolation of the product from the overexpressing cells in this series. In the previous lecture, we have discussed the basic principle of separation as well as we have discussed the principle of column chromatography.

And then subsequently we have discussed the chromatography system as well as the different components of the chromatography system, their influence on the overall, purification, efficiency as well as purification folds. And, and now in this lecture, we are going to discuss the different types of chromatography techniques. So in this series, before we get into the details of the chromatography techniques, all chromatography approaches what you can use to purify the proteins. Let us see, what are the different properties are present in the protein molecules.

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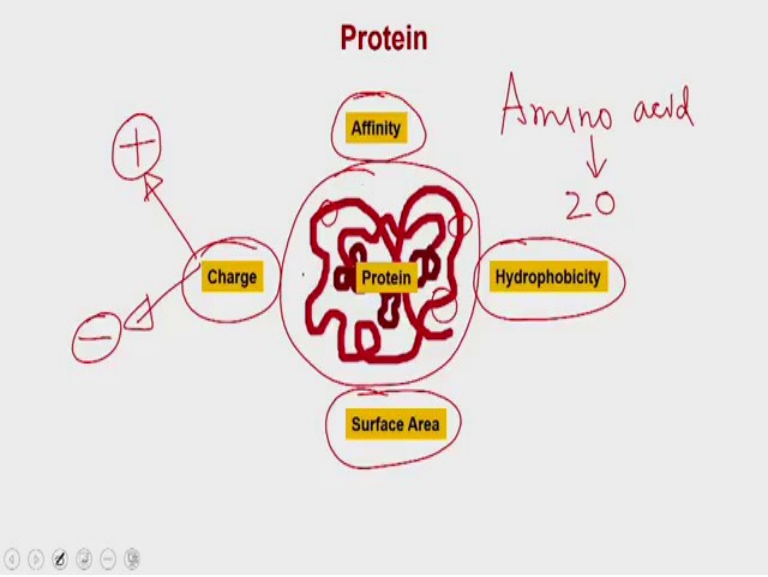

So you can imagine that this is a three-dimensional protein. So protein is made up of with the amino acid and the amino acids are, so 20 different types of amino acids are presenting the protein and these 20 different types of the amino acid are of diversified in nature. Some are polar

amino acids, some are non-polar amino acids, some are hydrophobic amino acids, some are basic amino acids, and some are acidic amino acid so because the protein is having the 20 different types of amino acids and this amino acid is giving different types of features into protein solutions and according to this amino acids, the protein is also getting folded into different three-dimensional conformations.

So as a result, one protein is acquiring entirely different properties from the different proteins, which is completely been controlled by the amino acid sequences for what are the different properties that can be exploited in a chromatography. A protein could have the charge for a protein that could have negative charges. It means if it has the acidic amino acid, it could have negative charges. If it has a basic amino acid, it could be a positive charge for a charge. Could be positive or charge could be negative. Not only the charge, but the distribution of these charges on to the protein is also may vary from the two different proteins, and accordingly they are efficient for the metrics or their distribution for the metrics is also could be different. So the charge is one of the properties which can be exploited for chromatography.

The other parameter is the hydrophobicity so the protein may have the hydrophobic as well as the non-polar amino acids and these are non-polar or hydrophobic amino acids are mostly been present inside the protein, but these hydrophobic amino acids can be exploited for chromatography so that this protein will go and distribute towards the metrics which contains the hydrophobic linkers and as a result, you can be able to purify the proteins. Now the proteins are acquiring those three-dimensional confirmations and based on the amino acid compositions, the protein could have the, a smaller surface area or protein could have the larger surface area, which means the ball, the three-dimensional ball, what you see could be off a smaller diameter or the approach or the three-dimensional ball. What you see is of a larger diameter. So depending on the diameter, the surface area is also going to be different. And that is also the criterion of exploiting during the chromatography.

Now the fourth is the affinity parameter because the protein may have different types of charges, different types of groups that are present. And these combinations of charge hydrophobic patches may acquire an affinity for a certain biomolecule. And that is a criterion that can be used to purify the protein. One of the classical examples is the single standard DNA binding protein or

helicase is the single standard DNA binding protein is binding to the nucleotides when the DNA is going through the process of replications.

So if you remember, if the DNA is preparing themselves for the replications, the DNA is getting unwind and then it is going to be separated into the two strengths. And these two strengths are then being courted by the single standard DNA binding protein so that the DNA template is freely available for the primer to come and sit. And that is how it is going to synthesize the new strength. So the single standard DNA binding protein has the charge distribution and as well as the other kinds of amino acid distribution in such a way that it has a very, very high specificity for the DNA. And that is why if you use the DNA as ligand or DNA as a molecule, you could be able to purify the single standard DNA binding protein from the crude lysate.

This is just one example, there are many other examples where the molecules may have a very specific affinity for some of the ligand. For example, there are so many dehydrogenases which have an affinity for NADH or NADPH. And in those cases, you can use these leggings to purify the protein.

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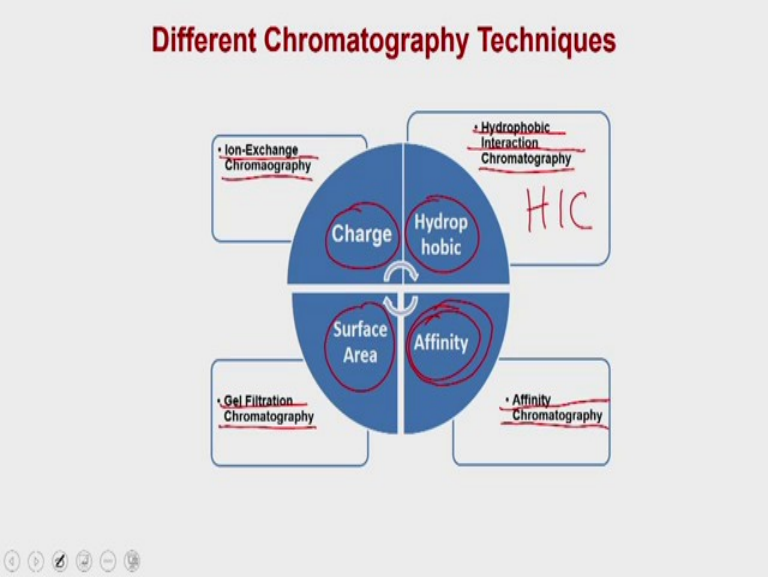

So according to the different properties such as charge hydrophobicity, surface area, and affinity that people have devised different types of chromatography techniques for the charge, people are using the ion exchange chromatography for hydrophobic patches. People are using the hydrophobic interaction chromatography or HIC. Similarly for surface area, people are using the

gel filtration chromatography or gel permeation chromatography and for affinity chromatography depending on the protein and its affinity parameters you can use the affinity chromatography. So in today's lecture, we are going to start our discussion about ion-exchange chromatography.

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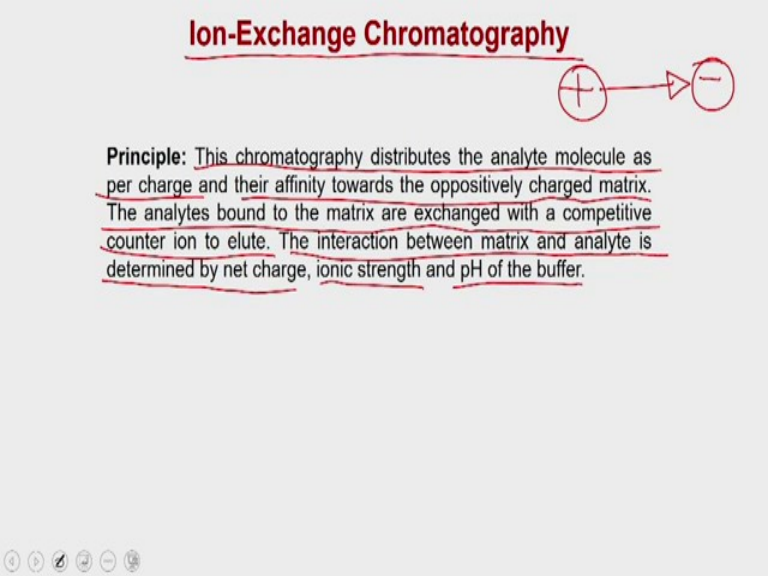

So ion-exchange chromatography, as the name suggests, it is going to work with the positive or negative charges present on the protein. The basic principle of this protein is that this chromatography distribute the analyte molecule as per charge and their affinity towards the appositively charged metrics, which means if the protein is positively charged, it may have the affinity for negatively charged metrics and vice versa. The analyte bounce to the metrics is an exchange with competitive counter ion to elute the interaction between the metrics and analyte is determined by the net charge Ionic strength and the pH of the buffer.

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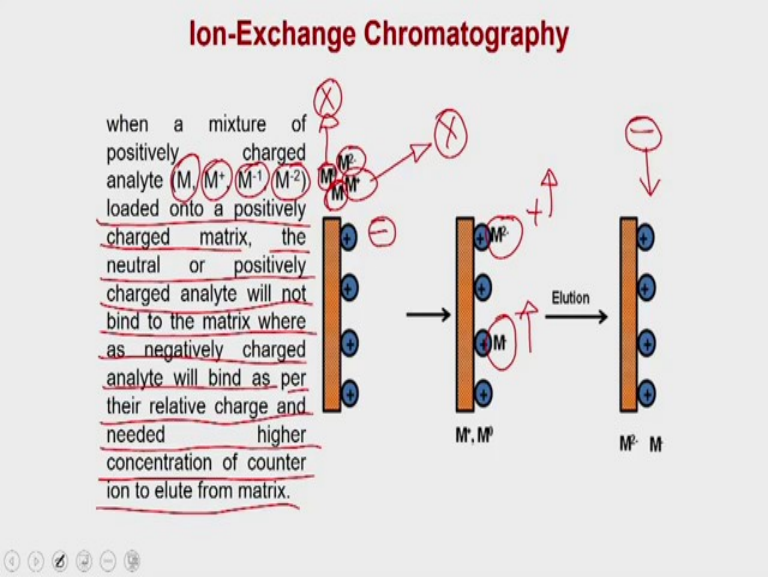

Now let us see how the ion exchange chromatography is separating the two molecules or separating the two proteins of different charges. So in this example, we have taken the three proteins, the protein which is having, (M, M PLUS, M minus 1, M MINUS 2). So you have a complex mixture of the proteins which are M, M PLUS M minus, and M 2 MINUS. And then you have loaded this on to a positive charge metrics. So you have a metric which is positively charged on which you have loaded M, which is neutral molecules and two minutes, which are two negative charges and M minus, which is M a one negative, the negative charge and M PLUS which is one positive charge.

So what will happen while these molecules will go through with the column, they will interact with the charge or the positively charged groups which are present on the matrixes. And what will happen is that because the positive charge is always having, an affinity for the negative charge, the M neutral is not going to bind to the matrixes. M PLUS is having the same charge. So it is going to ripple from the matrixes. So that also is not going to bind the metrics. What it will go to bind is the M MINUS 2 as well as the M^- and the affinity for M MINUS 2 is going to be more compared to the M minus.

Now once they are bind, then you can wash this column and remove the M neutral as well as the M PLUS and now you can put the another negatively charged competitive ion and that is going

to remove the M minus before M minus first and M 2 MINUS on the second occasion. So that is how it is going to separate the complex mixture which contains proteins of different charges.

So the neutral or the positively charged analyte will not be going to bind to the matrixes. Whereas the negative charge analyte will bind. And as per the relative charges and the needed the higher concentration of counterion to elute from the matrix, which means the M two minors is having a higher affinity for the metrics compared to the M minus. And as a result, when you elute with the counter ion, you will have to supply the higher concentration of the counterion to elute the M 2 minus compare to the M minus.

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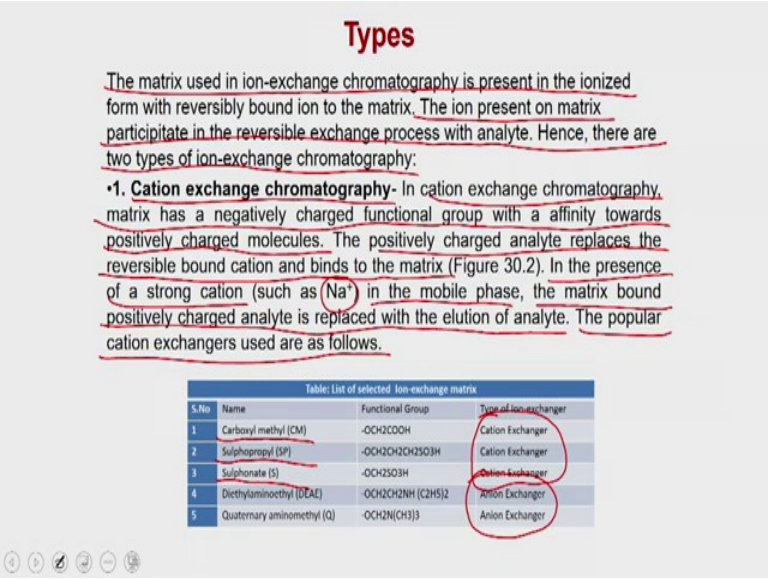

The metrics used in the ion-exchange chromatography is ionized for a reversible bound ion to the metrics, the ion present on the metrics participate in the reversible exchange process. Hence there are two different types of ion-exchange chromatography. One is called Cation exchange chromatography in Cation exchange chromatography metrics has a negatively charged functional group with an affinity towards positively charged molecules.

The positively charged analyte replaces the reversibility bound Cation and binds to the matrixes in the presence of a strong cation such as sodium. In the mobile phase, the metrics bound positively charged analyte is replaced with the elution of analyte. The popular Cation exchanger is as follows, so you can have the Carboxyl methyl, which is a CH2COH. You have the SP Sulphopropyl, you can have the Sulphonate, these, all these three are the Cation exchanger.

Whereas the other possibility is the anion exchanger and in the case of anion exchanger, it is going to be a positively charged functional group, which is having the affinity for the negatively charged groups.

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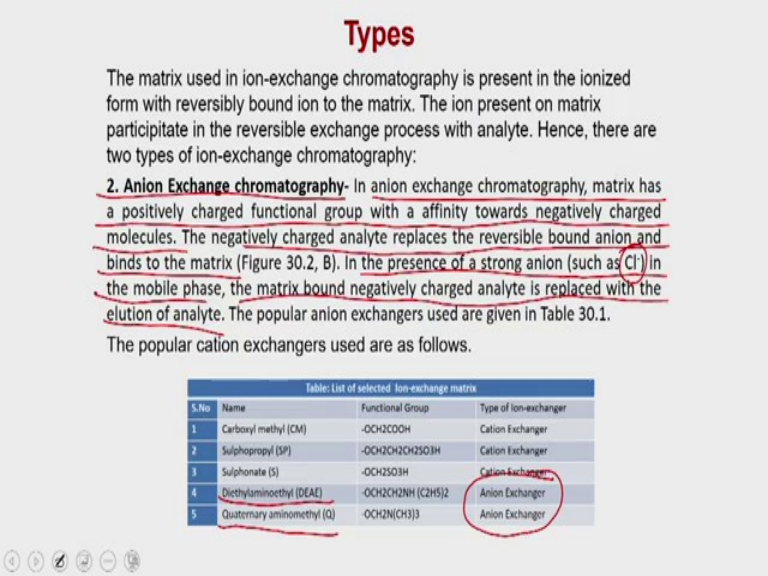

Now in anion exchange chromatography in anion exchange chromatography, the Matrix has a positively charged functional group with the affinity towards the negatively charged molecules. The negatively charged analyte replaces the reversibly about anion and binds to the metrics. In the presence of a strong anion such as the chloride. In the mobile phase, the metrics bound negatively charged analyte is replaced with the elution of analyte. The popular anion exchanger is DAE Afros or quaternary amine or the mono Q. So these are the examples of anion exchanger. So these are just a simple example to explain to you about the ligands which are being present on two the anion exchanger or the Cation exchanger. How these two techniques are performing the chromatography and making separation is being given in this figure.

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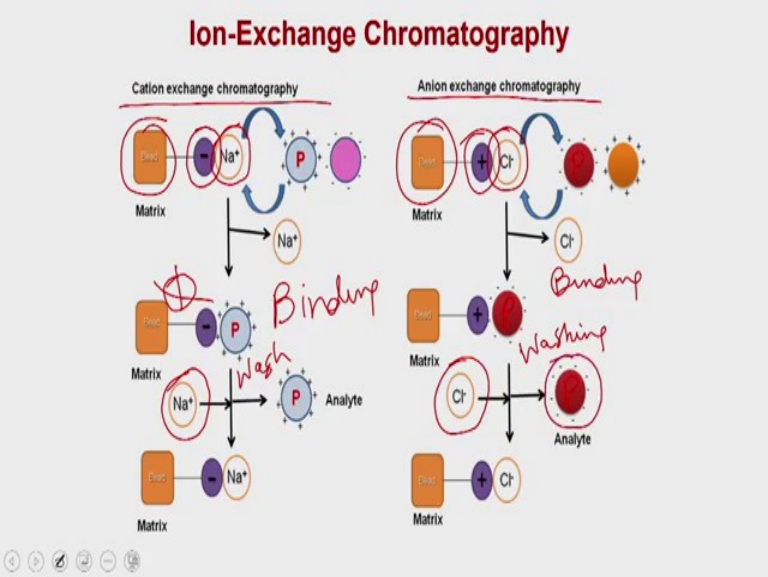

So in the case of Cation exchange chromatography, you have the metrics which are actually having the negatively charged groups. And these negatively charged groups are having the bound sodium. So once you add a protein that is positively charged, the protein is going to replace the sodium and it is going to bind to your weights in the first step. When you add the protein to the bead, it is going to go and bind. So in this, this is your binding step.

Now once the binding is over, then you can wash and by when you wash, what will happen is the proteins which are going to bind the two these beads because of the nonspecific interaction such as the, because the beats, these beads are made up of Agros or Zephyros or some other kind of material. These beads are also having some affinity or nonspecific affinity for the proteins. So once you watch these beads or once these columns with the washing buffer, these protein molecules are going to be removed which are not interacting with the functional group present on the beads. Now you have the beads, the functional group, and the protein is bound to that particular functional group. Now what you are going to do is you are going to do the same the reversal. Now what you do is you will supply the NaCl or sodium.

Now once you add the sodium, it is going to replace the protein and it will bind to your functional group, but the amount of sodium that is required to replace the protein bound to the metrics beads is going to be different for different proteins. For example, if you remember in our previous example, M 2 minus is having the two negative charges so it is going to bind very

strongly to the beach compared to M to M 1 minus, so because of that, it may require a higher concentration of your elution sample or your salt compared to this in anion exchange chromatography exactly the reverse the beads.

Then you have the positively charged functional group which is being protected by the chloride which is present in the buffer. Once you add the protein, the protein is going to replace the chloride and it will go into a bind. Now you at this stage you are going to do so this is your binding stage. Now at this stage, you can do a washing just like as we discussed for the Cation exchange. Once you do the washing it is going to remove the nonspecific molecules. Afterward you will put a competitive and anion that competitive anion is going to replace the protein and this replacement is also going to be in correlation to the amount of negative charge present on that particular protein and its affinity for the matrix.

So by this, the Cation or anion exchanger can be used for a protein to purify with the help of the negative or the positively charged ions on present on the product present on the proteins. But the question comes under what condition you will use anion exchanger or under what conditions you will use the Cation exchanger chromatography.

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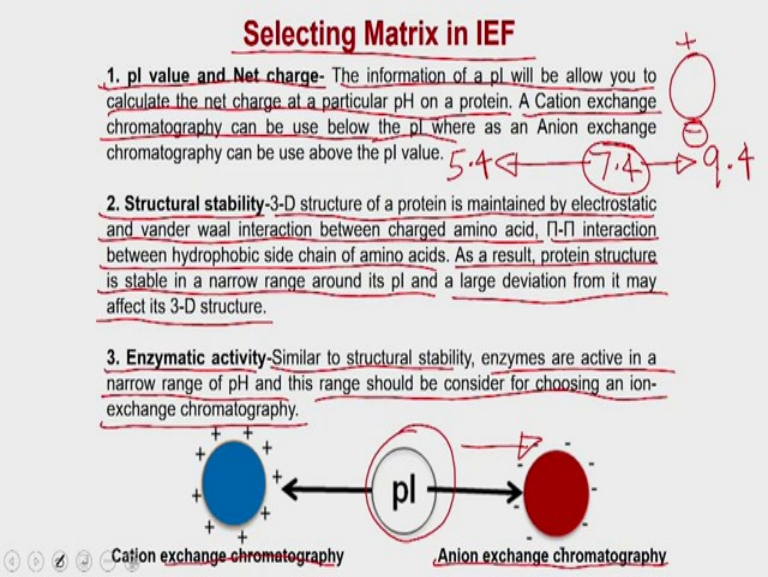

So how you will select the metrics in the ion exchange chromatography one of the crucial parameters is the PI values and the net charge. You know that every protein has the different types of amino acids and these amino acids are collectively providing negative or positive

charges. So the protein is having the positive charges as well as the negative charges, but if you titrate these proteins at different pH, what will happen is that these negative charges or the positive charges are going to be neutralized by the ions which are present in the buffers at different pH and as a result, you will achieve a pH at which the net negative charge or net charge onto your protein is going to be zero and this pH at which your P protein is going to show you a net zero charge.

That pH is called the isoelectric point or the PI values and that PI is going to allow you to calculate the net charge off present on the protein at any pH. The information of a PI will allow you to calculate the net charge at a particular pH on a protein, a Cation exchanger chromatography can be used with the below of the PI so the thumb rule is that if you go below to the Pi value, you can be able to use the Cation exchange chromatography.

If you go above to the Pi values, you will be able to use the anion exchange chromatography for practical purposes, how much we should go down, and how much we should go up. There is no such rule or such formula, but what people have realized by their experiences that you, you have to use at least two units in pH to go down or two units of pH to go up. Which means if you have a protein whose PI value is 7.4 this means if you would like to use the Cation exchange chromatography, you have to perform the Cation exchange chromatography at 5.4 but if you would like to use the anion exchange chromatography, you will have to use the pH, which is 9.4 which means you have to bring the two-unit differences from the Pi values. Why the two units of PI difference why the 2 unit of pH is required. Because what people have realized from their experiences that this much difference is good enough to impart the sufficient charge onto the protein and that actually will allow them to bind to the ligand or the positive or the negatively charged ligand present on to the matrix.

If you go lower to that, it will still bind, but then the affinity would be so low that as soon as you will put the washing buffer, the protein will come out so that is why this is the thumb rule that you use the two pH differences either onto the side or to the upper side. Now the second criteria are structural stability. You know that the protein is three-dimensionally 3 dimensional structures and the three-dimensional structure of a protein is maintained by the electrostatic and Vander Waal interaction between the charged amino acid. For example, the arginine is making a group or making your interaction with the glutamic acid or, some kind of salt bridge interactions, or

sometimes the arginine is making the Vander Waal or hydrogen bonding interaction. And most of these interactions are ionic. So you have the Vander Waal interaction between the charged amino acid. You have the pipe interaction between the hydrophobic side chain of amino acid.

So as a result, the protein structure is a stable in a very narrow range around its PI and a large deviation from it may affect its three-dimensional structure and you know that the three-dimensional structure or the integrity of the three-dimensional structure of a protein is very important for its enzymatic activity or its functional activity. So that is why you have to ensure that the, whatever the changes you do in terms of the BH or buffer to run the Cation or the anion exchange chromatography, you could be able to, you should not destroy the 3-dimensional structures. Now the third is the enzymatic activity in case you are purifying the enzyme.

Then the similar to structural stability, the enzymes are active in a very narrow range of pH. And this range should we consider for choosing anion exchange chromatography. So as it is given, if you go lower to the PI values, you will be able to use the Cation exchange chromatography. If you go above the PI value, you will be able to use the anion exchange chromatography.

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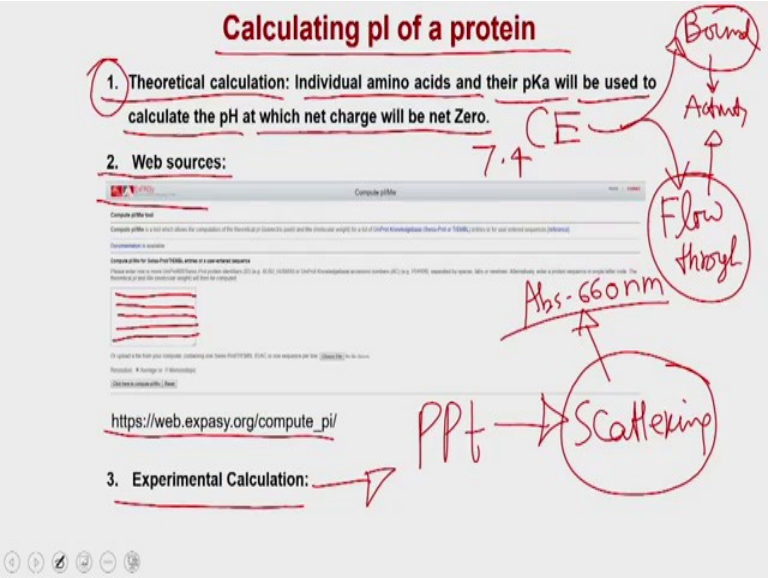

Now the question comes how you could be able to calculate the PI of a protein because that is very important if you would like to start using an anion or the cation exchange chromatography before the genomic era started or before people were not having the sequence information of the

particular protein both in terms of the amino acid sequence as well as the gene sequences. People were going with the trial error methods when they were trying to use the anion exchange chromatography. What they are going to do is suppose they use the Cation exchange chromatography so they will use the Cation exchange chromatography.

Once you do the Cation exchange chromatography and you will load your lysate, you are going to get two fractions, one you are going to get the beat bound fraction or you are going to get the flow through. Now since the genomic era was not there, what they will do is and supposes you are purifying an enzymatic activity. What they were doing is they were doing the activity in both of the fractions now and they have, they have already done the Cation exchange chromatography. Okay, so and suppose they have done the cation exchange chromatography at pH 7.4 that is a standard thing because 7.4 is a physiological pH so once they do the cation exchange chromatography at 7.4 either the protein will be present is when the metrics bound phase or it will be present in the flow-through phase which means it is not going to bind to the metrics.

Now they will analyze these two fractions for activity. Suppose they Guard the protein in this fraction, then they will realize that the protein is having PI values which are closer to eight or nine because if you remember you have four cation exchange chromatography. You have to go lower to the PI values, but suppose they got the protein into the flow-through which means at 7.4 the protein is not binding into the cation exchange chromatography. Then what they do is at 7.4 itself, they will go to try the and anion exchange chromatography and in that process, the protein will go and bind to the anion exchange chromatography. But this kind of trial error method was, people were doing when they were not having any information about the PI values, they were not having any information about the amino acid sequences and they were not having any information about the genomic sequences.

But nowadays you have all different types of approaches so that you should not go by the trial error methods you can very precisely be able to use Cation exchange chromatography or anion exchange chromatography by simply calculating the PI values and there are three approaches by which you could be able to calculate the PI values. Approach number one is it Theoretical calculations. So you know that the effective charge of a protein is being decided by the charge provided by the individual amino acids.

So what you can do is you can take the individual amino acids and their PK values and that information can be used to calculate the pH as well as the charge onto the protein at a particular pH. And by doing so you could be able to calculate at which pH the protein is going to have the net charge, net charge zero and that would be the PI values. So the Theoretical calculation is also going to give you a rough idea of the PI values. It may not be very precise, but it will good enough to run the Cation exchange chromatography or the anion exchange chromatography.

The second source is the web source. So there are multiple web forces people are using. One of the examples which I have shown is expressive websites. So the link is given here, you can use this to calculate the PI values as well as the molecular weight of the protein. What you are supposed to do is you in this block you will provide the amino acid sequence of the protein and, and then you ask the computer or the When you ask the web servers to calculate the PI as well as the molecular weight. As soon as you click this button, it will be going to give you the PI values and it is a web source.

The expressive web source is doing the same thing. It is taking the PK values of the individual amino acid, the sequence, what you have put and it is just calculating and putting those values, making the average and calculating the PI values, now the third method is the experimental method or experimental way off calculating the PI values. You know that the protein is soluble in a buffer simply because it has some positive charges and some negative charges and in those positive and negative charges are aline the protein to interact with the molecules of the buffer. And because of that, the protein will remain in the solutions.

But at the PI value when the net positive or negative charge or net charge is going to be zero, the protein is going to be the least soluble and because of that, the protein will start going to precipitate. So when the protein will precipitate or it will make the precipitate, it is going to give you a very high scattering. Now scattering is something that can be calculated by taking absorbance at 660 nanometers. If you remember when we were discussing the counting of bacteria, we were also we have discussed this method of counting the bacteria simply by taking its absorbance at 660 nanometers. That is measuring the scattering events instead of the absorbance events.

So that will give you the scattering and if you see, so what you can do is you take the protein solutions, incubated the protein solution with different pH, and then you take the scattering of these, then you record the scattering. As soon as the protein will start coming out from the solution, it will go into the form of the particles, which means it is not going to be solvable. And that is how it is going to give you more and more scattering. And the pH at which the scattering is going to be highest that is the PI values and that is how you can be able to calculate very precisely the PI values alternate method of calculating the PI is that if you use the isoelectric focusing strips and you load your protein, that also actually can give you the PI value because at the, at the PI value it is not going to migrate. So this is all about calculating the PI. Once you have calculated the PI, you could be able to choose the pH at which you can, you would like to or operate or you would like to run the Cation or the anion exchange chromatography.

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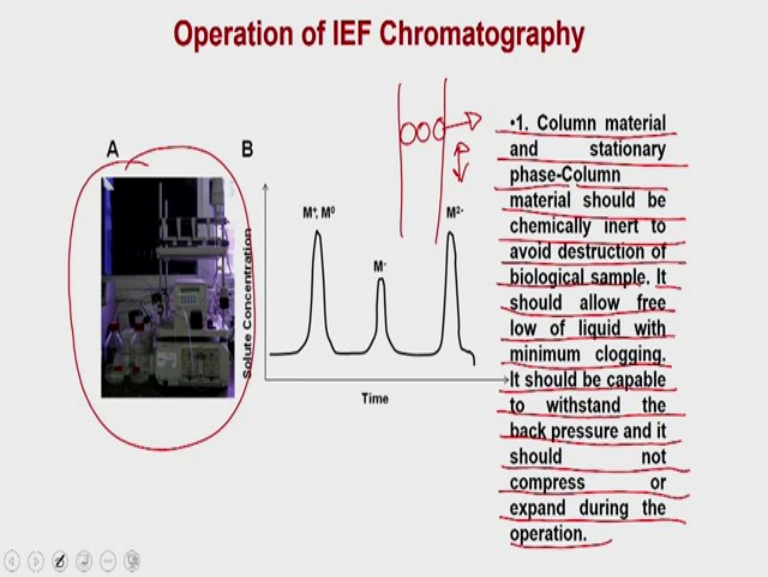

Now let us discuss how to perform this particular chromatography for a crude mixture lysate or a crude mixture of the proteins. So this is a chromatography system and this is the pattern you will get when you will perform the chromatography, and exchange chromatography in the first, first step. What you are going to do is first you add the column material as well as the stationary

phase. So the first is that you prepare your column. The column material should be chemically inert to avoid the destruction or biological samples.

That is very important that whatever the material or whatever the metrics you take that should not react with the proteins, it should allow the free, flow of the liquid with the minimum clogging, which means the column what you prepare should not allow the protein should allow the running of the buffer and it should be able to capable of to stand deep back pressure and you should not compress or expand during the operation, which means if you are taking a column and you are putting the beats, these beats should withstand some pressure.

So that it should not shrink or it should not expand because if they shrink or if they expand, they are going to change the overall arrangement of the beats within the column and that actually is going to destroy the packing and that may eventually affect the purification. The other thing is that the chemical or the molecule, material, whatever you use, it should be chemically inert so that it should not react with the protein. If it reacts with the protein, the protein will get modified and the protein will go and bind to these beats and then they will not come out even you put the reversible competitive and anion or cation.

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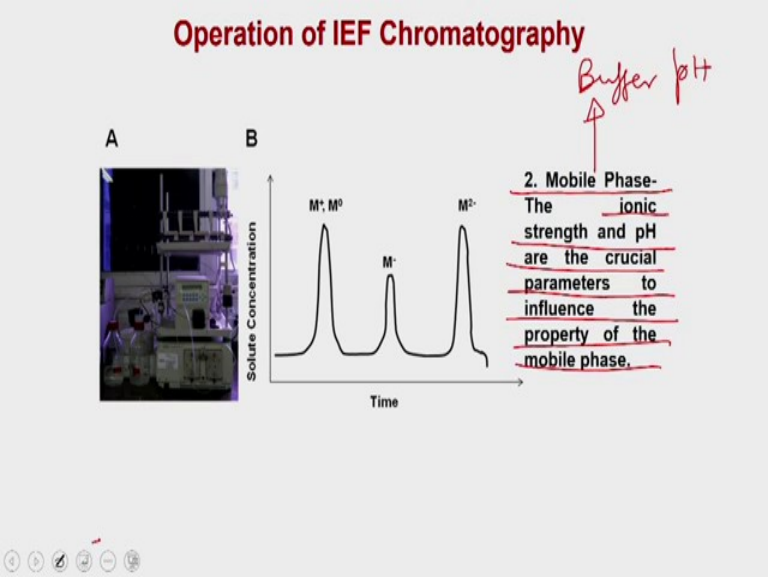

Now the first, you are going to prepare the column. Once a column is ready, then you load your sample so then you equilibrate the column with the mobile phase. The mobile phase is nothing but the buffer. So you have to choose the buffer as well as the pH at which you would like to perform. The Cation or the anion exchange chromatography so I need strength and the pH are the crucial parameter to influence the property of the mobile phase, which means if you use the high ionic strength of the buffer, it may withstand the minute changes of the pH.

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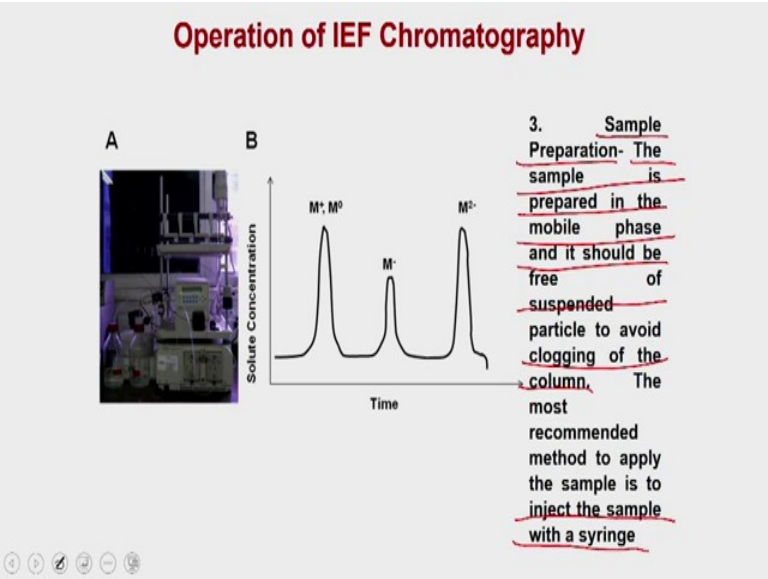

Now the third step is you prepare the sample, you prepare the sample and the sample is prepared in the mobile phase and should be free of these suspended particular to avoid clogging of the column. So when you prepare the sample, you prepare the sample into the mobile phase so that there should be no change in pH and the mobile phase should not have any particulate matter so that it should not clog the column. So otherwise it will not allow the run of the buffer and then you can use the injection wall and, inject the samples.

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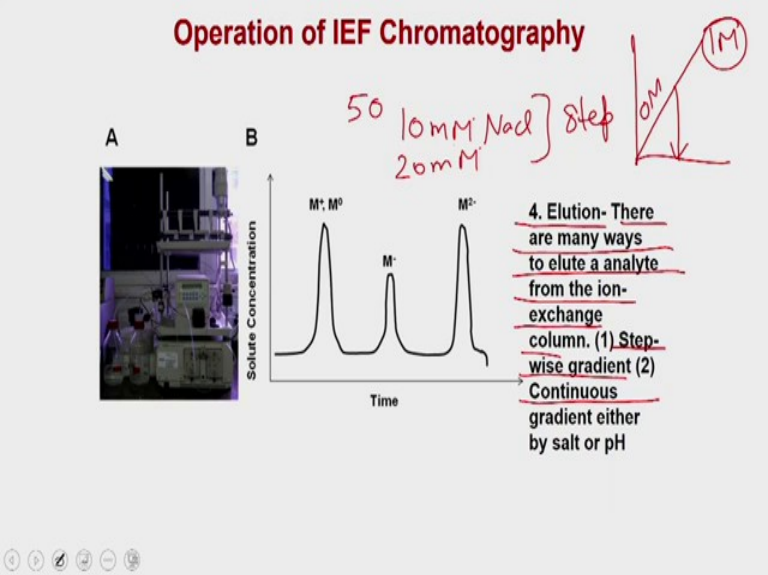

Then the fourth step is once the column will go and once the protein will go and bind to the column, then you can use the Elution. There are many ways to elute an analyte from the ion, from the ion exchange column. One is the stepwise gradient; the second is the continuous gradient so stepwise gradient means that you will elute the column at very very discreet steps, for example, 10 million molar NaCl, 20 million molars NaCl. So those are called a step gradient whereas in the linear gradient you are going to make a gradient of the NaCl starting from zero molars to one molar. So that is a linear gradient and that will allow, that actually will allow you to optimize at what a P a NaCl concentration. You can get the protein of your in charge.

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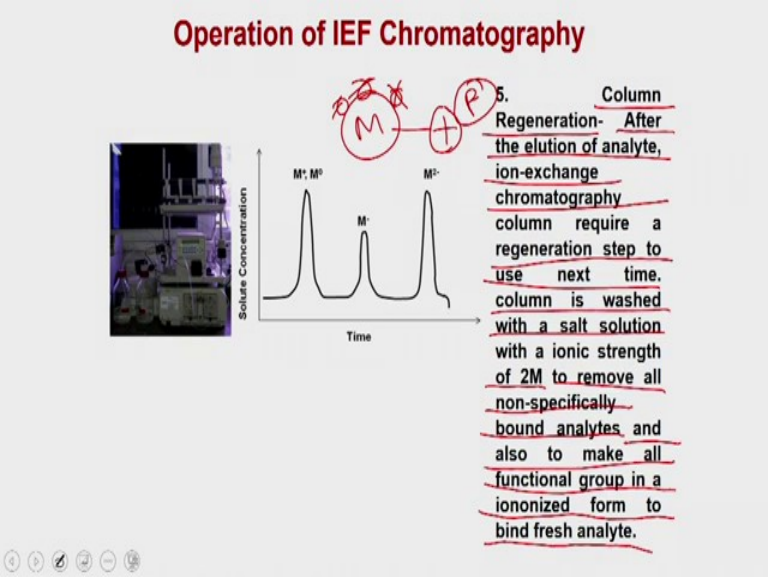

Once the elution is over, then you are going to do the column regeneration because after the purification is over, you have to ensure that there is no material which is bound to the column and that is the column regeneration steps, in the column regeneration step, you are going to wash the column with a very, very high ionic strength buffer so that whatever the material is bound to the column should come out.

So after the elution of the analyte, the ion exchange chromatography column requires a regeneration step to use the column next time. The column is washed with this salt solution with the ionic strength of two molars to remove all nonspecifically bound analytes. If you remember, I said, right, if you have a bead, the protein may bind to these beats, whereas this is a functional group, either the positive or negative. So your protein is binding to this functional group, whereas the other protein may be binding to the matrix.

So this, if you wash it with the two molars NaCl, you are going to remove all these nonspecifically bound protein. Because if you do not do that, the nonsensically bound protein is going to reduce the flow rate and eventually it is going to clog the columns or it will going to reduce the surface area of the beats, which is available for the protein of your interest to go and bind, so that will remove the nonspecifically bound analytes and also make all functional groups in an ionized form to bind the fresh analytes. So with this, we would like to conclude our lecture

here and the subsequent lecture, we are going to talk about the hydrophobic interaction chromatography. Thank you.