Experimental Biotechnology
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Module No # 04
Lecture No # 19
Hydrophobic Interaction Chromatography

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(Video Ends: 01:00)

Hello everybody, this is Doctor Vishal Trivrdi from the department of biosciences and bioengineering, IIT Guwahati. And what we were discussing? We were discussing about the chromatography. So in this chromatography series, we were discussing about the different chromatography techniques in the previous lecture. We have discussed about the ion exchange chromatography and now in today's lecture, we are going to discuss about the hydrophobic interaction chromatography.

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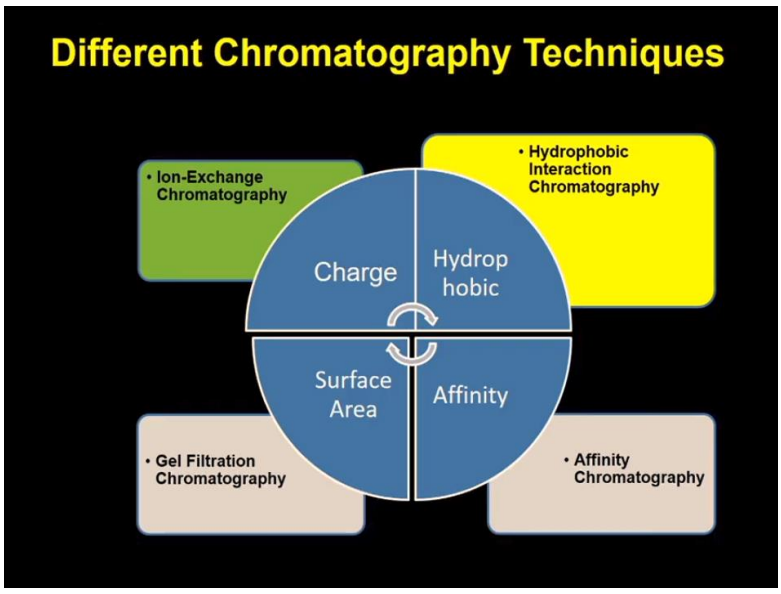

So if you recall what we have said is that the protein is having the different physicochemical properties and all these physicochemical properties can be exploited in different chromatography techniques. What we have discussed so far is that the charge, how the charge present on the protein can be utilized to perform the ion exchange chromatography. And now in today's lecture, we are going to discuss about the hydrophobic interaction chromatography which utilizes the hydrophobic patches present on the proteins surfaces.

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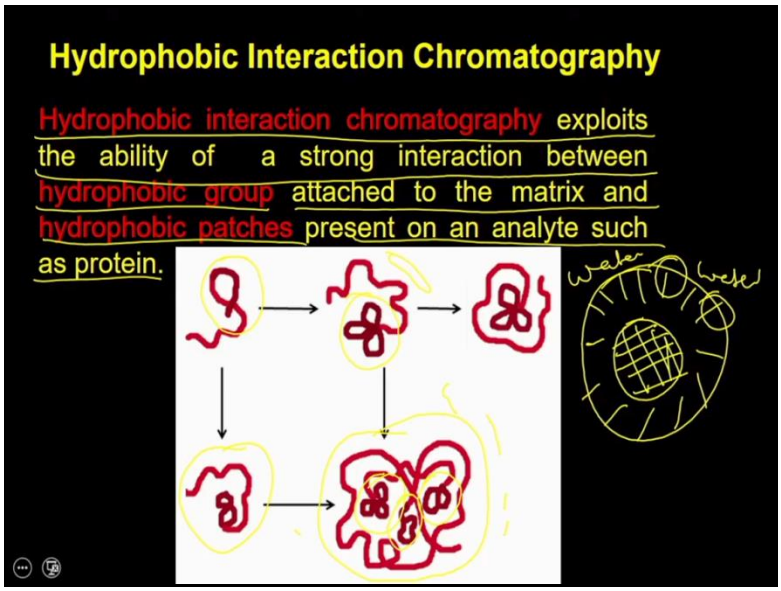

Now what is the basic principle of the hydrophobic chromatography? Hydrophobic interaction chromatography exploits the ability of a strong interaction between the hydrophobic groups attached to the matrix and the hydrophobic patches present on the analyte such as the protein. So what happens, how the hydrophobic patches are being placed in a protein is that when the protein is been synthesized as a single polypeptide chain as soon as the polypeptide is being released from the ribosome, it starts folding because of the intra-molecular interaction between the amino acid side chain..

And there are additional interaction present because of that the initially it folds and then it folds into a multiple ways. The purpose of this folding is to protect the hydrophobic groups from the aqueous environment what is present outside. How to achieve that is that the protein is going to place all the hydrophobic groups within the core of the protein as well as it is going to keep all the hydrophilic amino acids on the outside.

Because the outside is the aqueous environment so it is going to maintain a hydrophobic environment in the center. So what is the achievement by the protein is that it is actually keeping the hydrophobic amino acids in the centre and the hydrophilic amino acids and the periphery, because outside is aqueous which means aqueous means outside is water molecules. So that is why the polar amino acid or the charged amino acids are present outside, where as the hydrophobic amino acids are present inside.

So the question comes if the hydrophobic amino acids are present inside, how this hydrophobic amino acid could be available to make a interaction with the hydrophobic groups attached to the matrix. Because that is what you are supposed to achieve. So what happens is when you add this salt to the proteins, okay the salt is having the higher solubility to the water molecules compared to the protein.

So In this process salt is actually removing the water from the protein molecules. And in that process, it actually exposes the hydrophobic patches, and by adding addition of a low amount of salt you are actually increasing the solubility of a protein and this effect is called as the salting in effect. Whereas when you add the more amount of salt the hydrophobic what is been exposed onto the surface, actually allows the protein to clump together and that is how it is actually decreasing the protein solubility and that effect is known as the salting out.

So with addition of the salt you are doing the 2 things initially, you are removing the water molecules what is present outside the proteins and that is how you are increasing the solubility as well as you are exposing the hydrophobic patches. Once you add the more amount of salt you are actually reducing the solubility simply because the hydrophobic patches are interacting with each other and making a bigger clumps.

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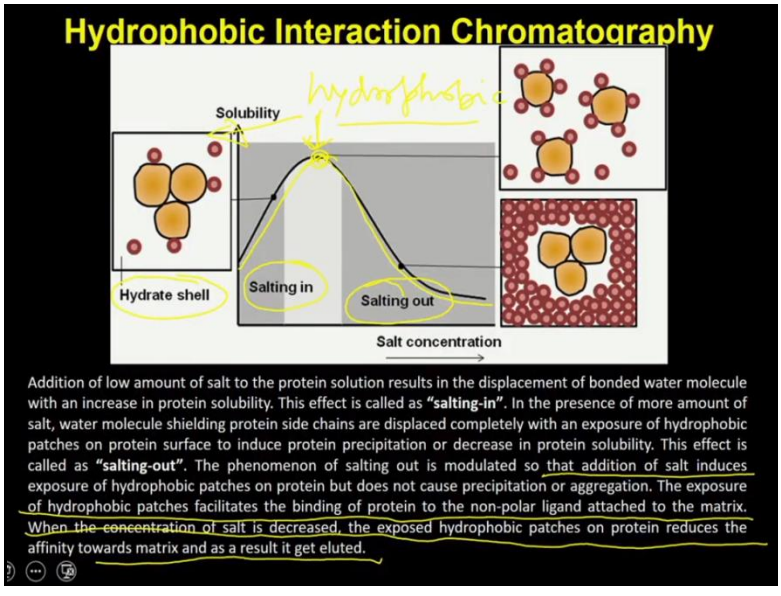

So this is what you have to see this is the when you add the salt is actually taking up the water what is present in the hydration shell and that is how it is actually increasing the solubility of the protein. But it goes up to a limit, okay. After that it actually remove more amount of salt and that is how the hydrophobic patches are been exposed and that is how it reduces the solubility.

This increase in solubility is known as the salting in effect. The decrease in solubility is known as salting out effect. So in the hydrophobic interaction chromatography, what you are supposed to do is you are going to add the salt in such a way so that it is actually reaching to this point and by reaching to this point. It is actually exposing the hydrophobic groups and that is how you can be able to utilize these hydrophobic Groups for the hydrophobic interaction chromatography.

So the addition of the salt induces the hydrophobic patches facilitate the binding of the protein to the non-polar ligands and attached to the matrix. When the concentration of salt is decreased, the exposed hydrophobic patches; on the protein reduces the affinity towards the matrix and as a result it get eluted. So exactly the reverse what you are supposed to do, when you are supposed to elute the protein you can just simply decrease the salt on addition.

And that is how it will actually again go back to this conditions where it will again going to take up the water from the environment and the hydration shell is going to come back and that is how is actually going to disrupt the interaction between the matrix as well as the hydrophobic patches present on the protein.

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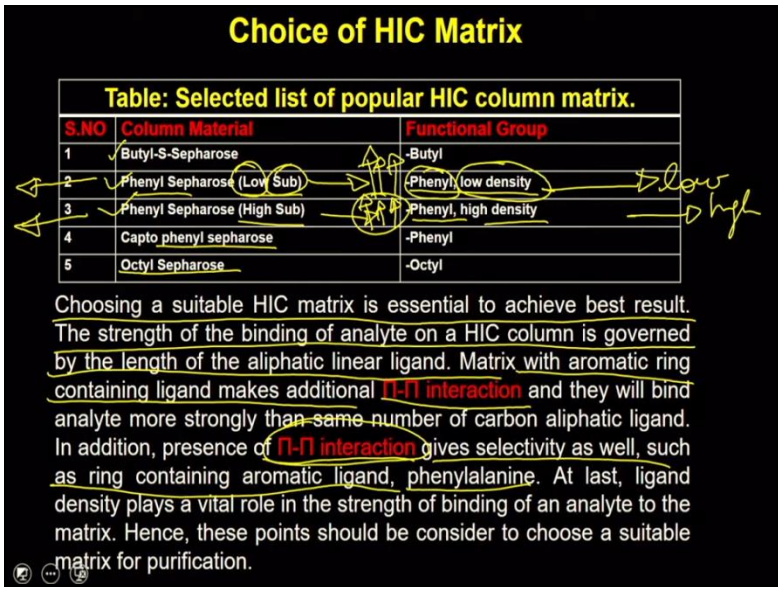

There are different types of matrix, what you are going to use, you have the Butyl- Sepharose, you have the phenyl Sepharose, low substituted, you have the phenyl Sepharose, high substituted, you have the phenyl Sepharose and then you have the Octyl Sepharose. What is mean by the low substituted or high substituted is that here you have the high amount of the phenyl groups attached to the Sepharose beads whereas here you have the high substitutions of the phenyl group much more than the low substitutes.

Which means the affinity for the low substitutes is going to be lesser compared to the high substituted. This is what is written. So the functional group is phenyl and here the density is low that is why is called as the low sub. And in this case, it is the phenyl group, but it has the high density, which means this is going to have the low affinity and this is going to have the high affinity.

Now choosing a suitable HIC matrix is essential to achieve the best results. The strength of the binding of a analyte on a HIC column is governed by the length of the aliphatic linear chain. Matrix with the aromatic ring containing ligand makes additional Pi Pi interactions. So if you have the aliphatic side chains which mean if you have the aliphatic groups attached to the matrix, you are going to have the lower affinity if you have the aromatic then it is going to show the additional interactions with the hydrophobic patches present on the protein and that it is going to have the higher affinity.

Depending on how much your protein can withstand the salt concentration and how much you can be able to affinity you require to bind your protein of interest, you can be able to choose the matrix which are containing the aliphatic side chains or side chains or the aromatic side chains. Because the pi pi interaction always gives the selectivity such as the ring containing aromatic ligand, phenylalanine, ok.

So because the additional advantage of using the aromatic or the ring containing groups are that it also give you the selectivity because the phenylalanine like, for example has more affinity or the selectivity to the bind to the ring containing groups present on to the matrix compared to the aliphatic amino acids. At last the ligand density plays a vital role in a strength of the binding of an analyte to the matrix.

Hence, these points should be considered to choose a suitable matrix for the purification. One thing what you have to remember is that HIC always utilizes the hydrophobic interaction between the groups present on to the matrix as well as the hydrophobic patches present on the proteins. And these interactions are very strong so you cannot choose a matrix, which is also very densely packed with the group.

Otherwise what will happen is it is actually going to bind the protein and then it is this binding is going to be irreversible or it will not get eluted, even if you reduce the salt concentrations and in those conditions you will not be able to recover the protein from the column.

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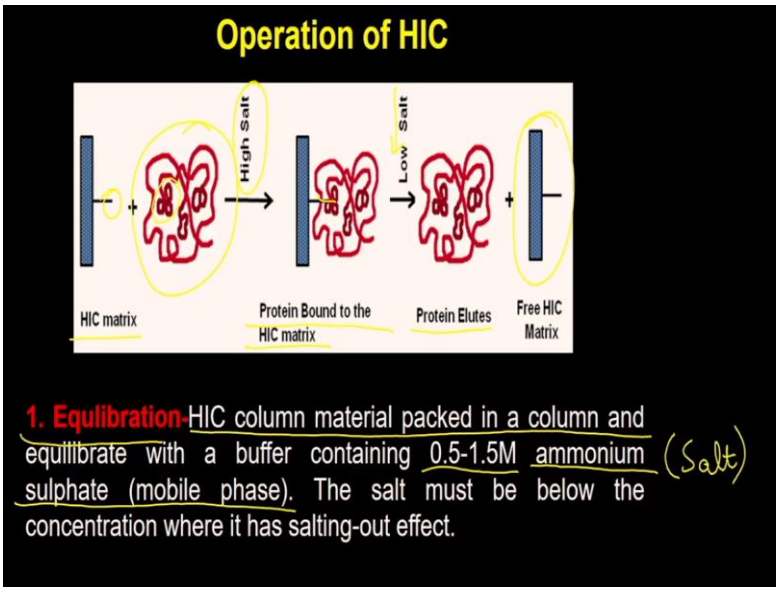

So this is what you have to do you have HIC Matrix where you have a functional group, then you have a protein, what do you do is? First maintain the high salt concentration, once you maintain the high salt concentration, the protein hydrophobic groups are going to be exposed and as a result it will go and bind to matrix. So it will bind to the matrix in the presence of the high salt concentration.

And then slowly you can reduce the salt concentration either in a step gradient or a linear gradient and that actually is going to reduce the affinity of the bound protein to the salt and that the reduction in the affinity is going to be differential for the different proteins. And that is how the protein is going to be eluted and the column is going to be free to perform the chromatography again.

You have the multiple steps to perform the HIC columns. The first step is the equilibration, in the equilibration, you take the HIC column and equilibrate with the buffer containing 0.5 to 1.5 molar ammonium sulfate. So Ammonium sulfate is being used as a salt so that it is going to do salting in effect to the proteins and on the other hand it also going to provide the suitable environment. So that the protein going to show the hydrophobic patches or going to expose the hydrophobic patches and that is how it is going to bind to the matrix.

This salt concentration whatever you are using 0.5 to 1.5 molar should be the concentration; where it should not have the salting out effect. Otherwise once you add the Ammonium Sulfate it is going to precipitate the protein from the solutions.

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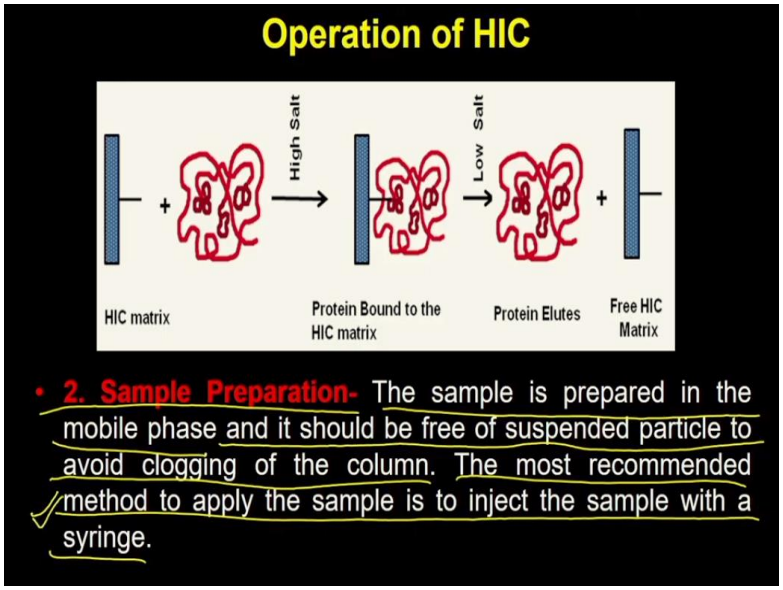

Now once the equation is over you can do the sample preparation. For the sample preparation the precaution remains the same that you have to prepare the sample in the mobile phase. Which means in this case the buffer containing 0.5 to 1.5 molar ammonium sulfate and you have to avoid clogging of the column which means you are sample should be free of suspended particle, free of dust particles and free of the precipitated proteins.

Otherwise, it is going to clog the column and the flow rate of the column is going to be affected. If you do not have the samples free of the debris. The most recommended method to apply the sample is to inject the sample with a syringe. So this is applicable only if you are using a protein purification system and you are would like to utilize the loops and other things then you have to use a syringe. Otherwise you can manually load the sample.

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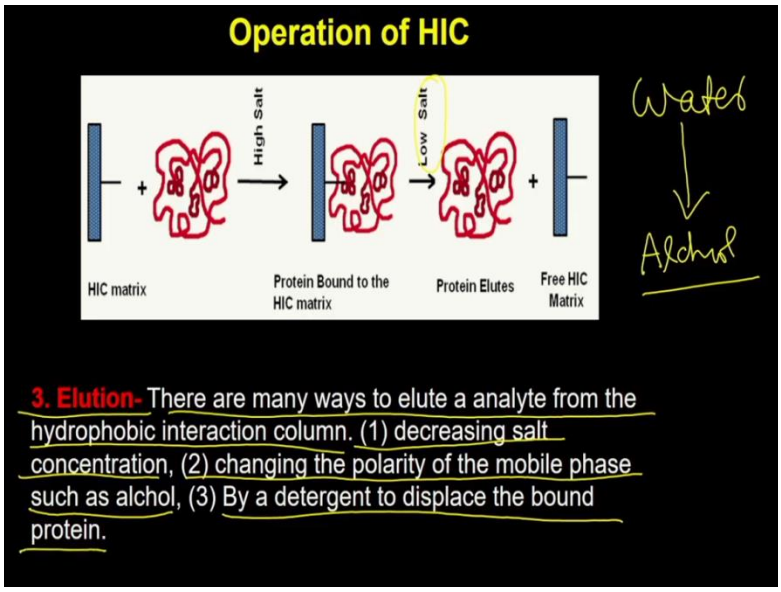

Then you have to do the elution's. So there are many ways to elute analyte from the hydrophobic interaction column. The most popular is to decrease the salt concentration so once you decrease the salt concentration, it is actually going to bring the hydration shell and that is how of hydrophobic patches are going to be hidden and they will not be available for making an interaction with the groups present on to the HIC matrix and that is how you can able to elute the proteins.

The second is that changing the polarity of the mobile phase such as you can bring the alcohol. So normally what we are using we are using the buffer which is prepared in water. But if you want you can also use the alcohol for elution's as well. In fact some time people are using the more different types of solvent such as Acetone and other things because as I said you know, if you do not choose the appropriate matrix the protein may bind to the matrix and then it may not be able to elute even by bringing the salt concentration 0.

In those cases only you are going to use the other polar solvents such as alcohol and acetone. But the only disadvantages are once you bring the elution into the alcohol or acetone, the protein may be come out from the column, but it may get denatured. So the people are using the changing the solvent as well means bringing the more polar solvent, but that is always giving the denatured protein. Sometimes people are always also using the detergent to denature the protein and that is how they are also can be eluted from the column.

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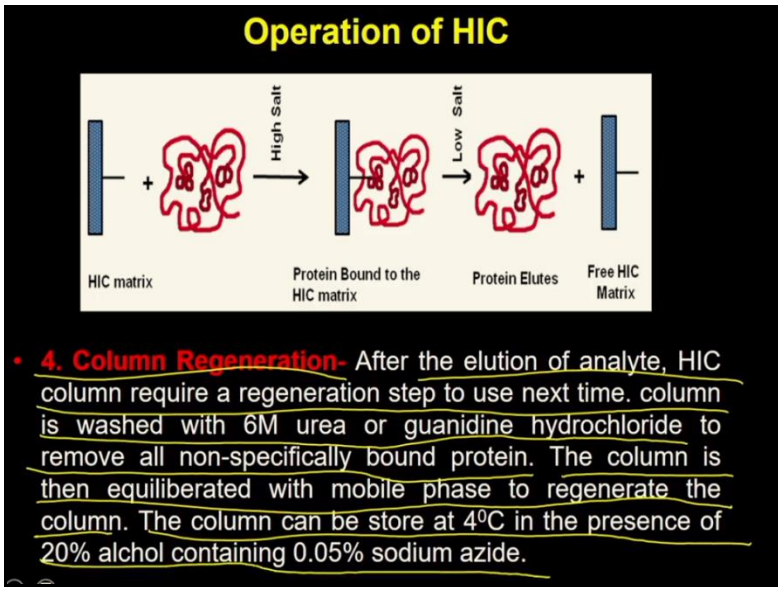

Now once the column running is over you have to regenerate the column. So after the elution of analyte, the HIC column requires a regeneration step to use for the next time. Column is washed with the 6 polar urea or guanidine hydrochloride to remove all non specifically bound proteins. The column is then equilibrated with the mobile phase to regenerate the column, and ultimately the column can be stored in 4 degree in the presence of 20% alcohol containing 0.05% sodium azide.

Because you do not want this column to be, you know to any kind of bacteria or other micro organism to grow because most of these columns are made up of sugar. So you just add to have the 20% alcohol and containing the antimicrobial substance such as the sodium azide. So this is all about the hydrophobic interaction chromatography. So far what we have discussed? We have discussed about the ion exchange chromatography, we have discussed about the hydrophobic interaction chromatography.

And in our subsequent lecture, we are also going to discuss about gel filtration chromatography as well as the affinity chromatography. So with this I would like to conclude our lecture here. Thank you.