Genetic Engineering: Theory and Applications
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Module 7
Isolation and Purification of Product (Part- 1)
Lecture-22
Hydrophobic Interaction Chromatography

Hello everyone this is Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. The ion exchange chromatography is being used very extensively to purify the positively charged or the negatively charged proteins and you can have the flexibility of using the cation exchange chromatography or the anion chromatography, so apart from the protein purification the ion exchange chromatography is also being used very extensively in other kind of application.

Let us see in what way the ion exchange chromatography can be exploited to answer few basic question related to science as well as how it can be used in the daily life as well.

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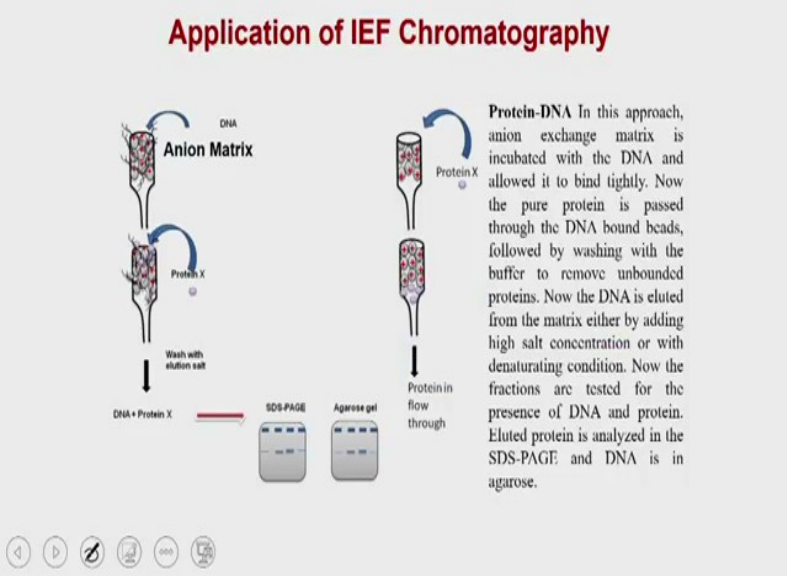

So the first application what we are going to discuss is about the utilization of ion exchange chromatography in developing different types of assays or studying the interaction between the two bio molecules. We have taken an example of the interaction of DNA and protein, but this could be replicated with a little optimization for any two bio molecules in which the criteria is

that one of the bio molecule should have the affinity for a particular type of ion exchange ion exchange matrix whether it would be for cation exchange chromatography or the anion exchange chromatography.

And the other bio molecules should not have the direct affinity for the particular matrix, so if you have this particular type of criteria you could be able to study the interaction between the these two bio molecules. So let us take an example how you can study the interaction between a DNA and a protein.

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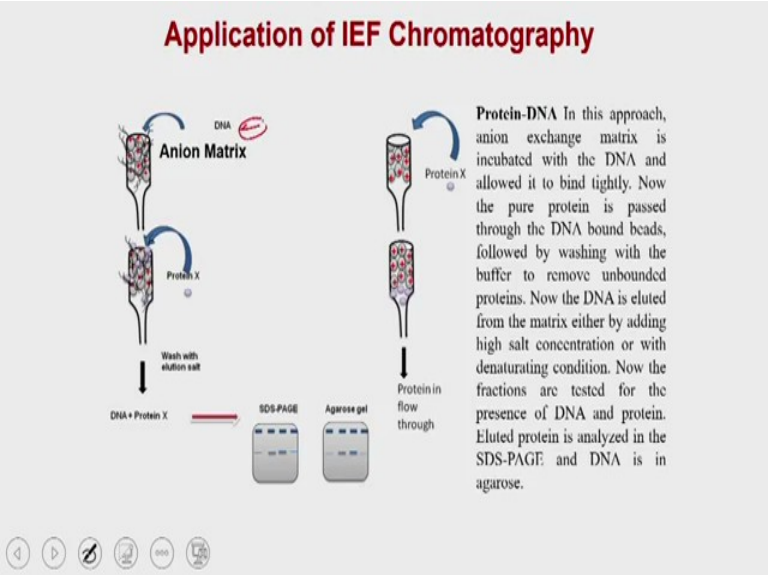

So in this kind of applications you know that the DNA is negatively charged, so for negatively charged DNA you are going to use the anion exchange chromatography which means you are going to use anion matrix and anion matrix are going to be positively charged, so they will very rarely bind the DNA, so in the first step what you are going to do is you are going to load the DNA which you are interested to study the interaction between a protein x.

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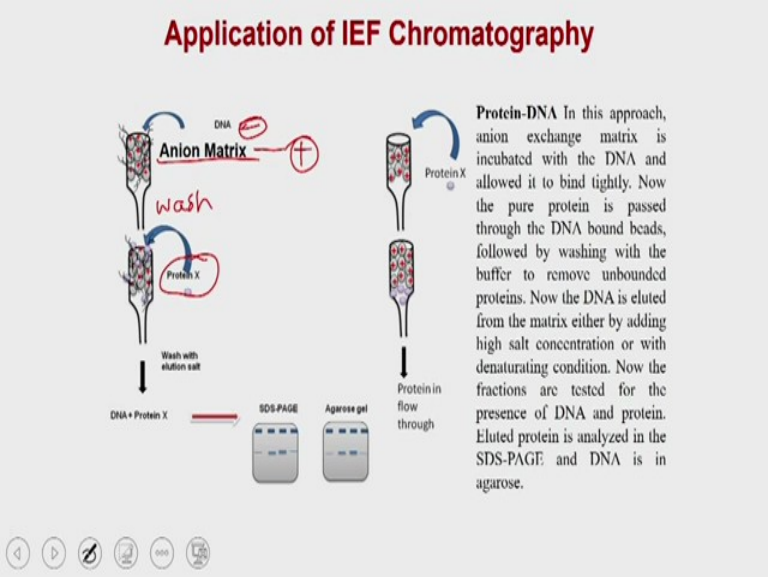

So in the first step you load the DNA and what will happen is that the DNA will go and bind to this anion matrix or anion beads, now the second step and then you wash the beads, so that whatever the DNA is binding to the matrix or whatever the DNA is non specifically binding to the matrix beads should be removed and then what you do is you follow the protein on to this column, so if this protein is going to have the interaction with the DNA, it is going to bind the DNA instead of matrix because this protein should not have the affinity for anion matrix at this PH.

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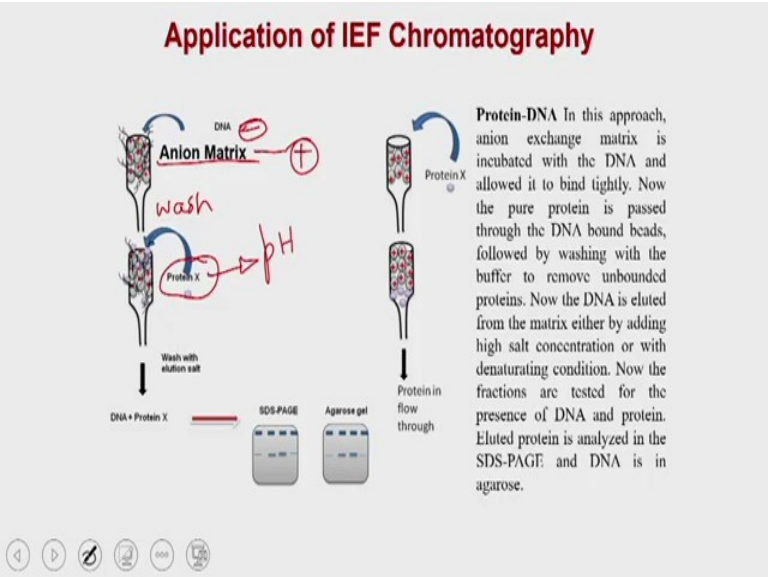

So you can actually optimize the PH in such a way so that the protein is going to have the will not going to bind the anion matrix and now you can elute this whole complex with help of a salt, when you do so the DNA and protein complexes will be removed.

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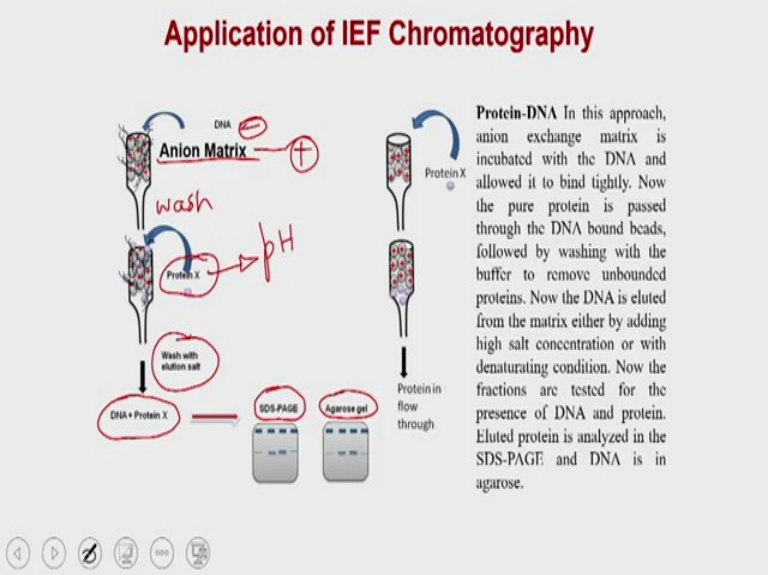

Now once you analyze this so protein can be analyzed on the SDS page, whereas the DNA can be analyzed on the agarose. So once you elute the complexes from this beads you are going to get the different fractions and all this different fraction can be can be analyzed on the SDS page as well as on the agarose gel.

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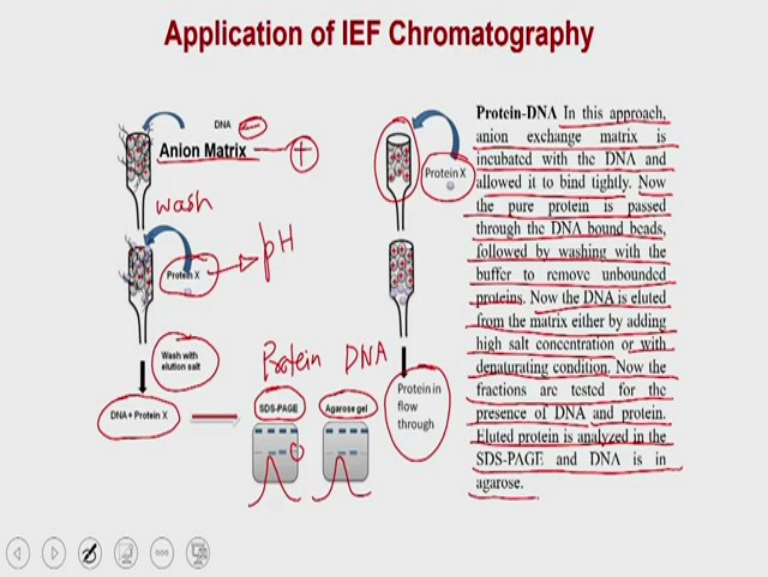

So the protein is going to be analyzed on the SDS page, whereas the DNA is going to analyzed on the agarose gel, when you analyze them you will find that there is descript pattern in which if you see here what you see is that low concentration of protein and then if the protein is making a peak and then it is going down which means if you plot this what you will see is the protein is actually following this pattern, whereas if you follow the DNA on the agarose gel what you will see that the DNA is also following the similar kind of pattern.

To understand or to clarify whether this interaction or this pattern you are getting simply because the protein x is interacting with the DNA you have to run a control reaction where you can load the protein x into the column anion column and in that case the protein should not bind and protein should present in the flow through.

So that is a control reaction you have to do to ensure that the protein which is binding to the column is not directly binding to the column but it is binding to the DNA which is bound to the beads. So that is what is written in this approach. The anion exchange matrix is incubated with the DNA and allowed it to bind tightly. Now the pure protein is passed through the DNA bound beads, followed by washing with the buffer to remove unbounded proteins.

Now the DNA is eluted from the matrix either by adding high salt concentration or with denaturating condition for that you can add the urea or GNCL or something so that actually is going to remove the complexes.

Now you can get the fractions and the fraction can be tested for the presence of DNA on the agarose gel and the protein on the SDS page and the eluted protein is analyzed in the SDS page and the DNA is in the agarose gel and that is what we have said. What you are supposed to do when you do experiment it will say ok protein x is interacting with the DNA but to verify this things you can actually do like control experiment such as that you can add the protein and it's mutant and some things like that or perform the similar experiments and different PH because you know that the DNA and proteins are making an interaction through electrostatic interaction.

So if you change PH's the pattern may vary and so, that is how you actually can study the DNA and protein interaction. But as said this approach can be used for any two bio molecule the condition is that the bio molecule one should have the affinity for the anion exchange chromatography whether it is cation exchange or the anion exchange chromatography and the bio molecule number two should not have the direct affinity for the matrix.

So that if you find the bio molecule two into the purification that will be only possible if it is interacting with the your bio molecule number one, so this is all about studying the interaction between the two bio molecules in this case we have taken an example of DNA and protein.

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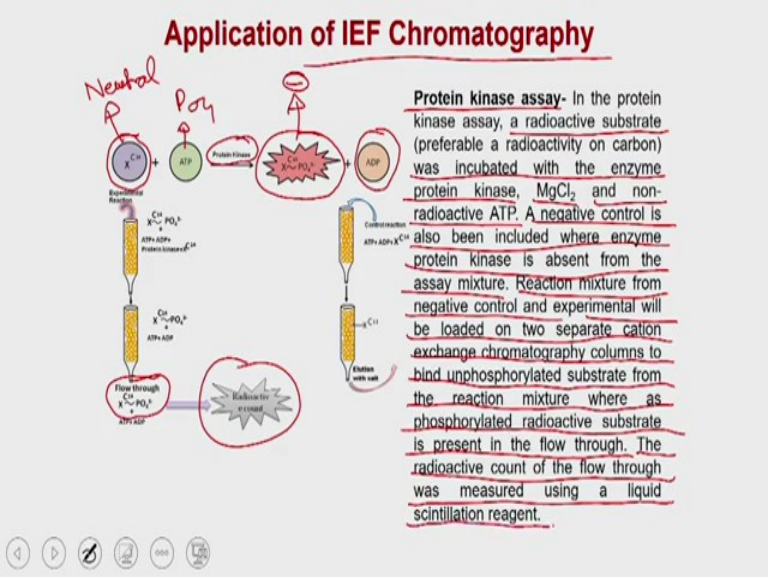

Now you can also use the anion exchange chromatography to study the protein kinase assay. You know the protein kinase assay, so if you use the protein x and you add the ATP the protein kinase what protein kinase will do is it is going to take up the phosphate from the ATP and that

phosphate will be transferred on to the substrate and you will get the x phosphate and the ATP is converted into the ADP so in this kinase assays what you have to do is a radioactive substrate was incubated with the enzyme protein kinase MgCl2 and a non radioactive ATP.

A negative control is also been included where the enzyme protein is absent from the assay mixture, so that will give you the background counts reaction mixture from the negative control and the experimental will be loaded on two separate cation exchange chromatography columns to bind the unphosphorylated substrate from the reaction mixture where as phosphorylated radioactive substrate is present in the flow through. The radioactive count of the flow through was measured using a liquid scintillation reagent.

So this just an example where are actually allowing the modified substrate to be flow through from the column and you can measure that the modified substrate and that is how you can actually co relate that with the activity of that particular protein kinase. But this is not true only for the protein kinase any x reactions where you will a change in charge could be used.

For example if you are would like to study the acetylation, acetylation is going to bring the negative charge so acetylation is nothing but the addition of carboxyl group on to the protein molecule or sometime you can also use this kind of ion exchange chromatography also to study the other kind of protein modification as well.

The condition is that this modification should give you a modified charge on to the substrate. For example in this case the substrate which is radio enabled when it gets modified by the protein kinase activity it is getting the negatively charged, so that the negatively charged molecule will not going to bind the cation exchange chromatography where this molecule is going to bind the cation exchange chromatography and that is how you could be able to separate the modified substrate from the un modified substrate.

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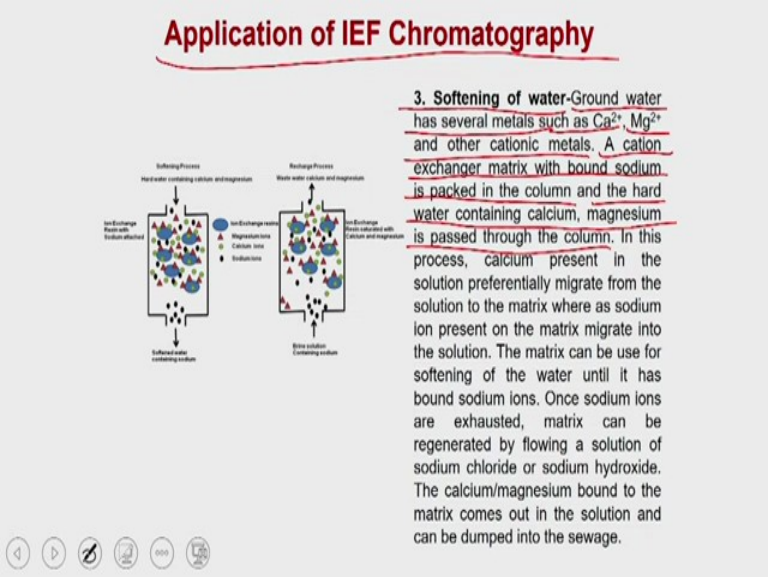

Now this the daily use application of ion exchange chromatography where the ion exchange chromatography can be used for the softening of water, so you that the water what we are getting from the ground water has several metals as a contaminations such as calcium, magnesium and other cationic metals. A cation exchange chromatography matrix with bound sodium is packed in a column and the hard water containing calcium magnesium is passed through the column.

So this cartridges you might have seen in your home as well when you buy a aqua guard or some kind of water purification system what you will see is that it has two chambers in one of the chambers it actually contains these kind of beads and what these beads are these beads are cation exchange beads and this cation exchange beads are masked by the sodium, so the sodium is actually bind to the cation exchange beads.

And when you follow the hard water which actually contains calcium and magnesium and arsenic and all other kind of heavy metals so all this heavy metals are actually having the positive charges and they actually are going to replace this sodium which is bound to the beads and the sodium is going to be removed from the column on a daily bases.

So as long as you are purifying the water the bound metals are binding to the column and the sodium which is coming out from these cartridges are getting mixed to the water. But this going to be exhausted very after sometime because the amount of sodium which is bound is limited so after that you have to do a regeneration steps.

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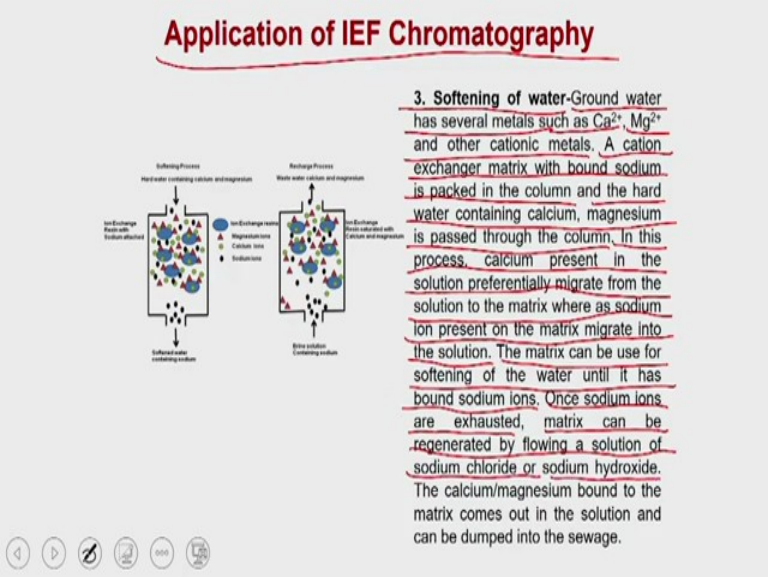

So once this is done, so in this process what happens is that the calcium present in the solution calcium or magnesium or all other heavy metals present in the solution preferentially migrates from the solution to the matrix whereas sodium ion present on the matrix migrates to the solution. So there would be ionic exchange take in which the sodium from the beads moves towards the solution and the magnesium or calcium or other heavy metals moves towards the beads.

The matrix can be used for softening of the water unit until it has bound sodium ions, once the sodium ions are exhausted matrix can be regenerated by flowing a solution of sodium chloride of sodium hydroxide.

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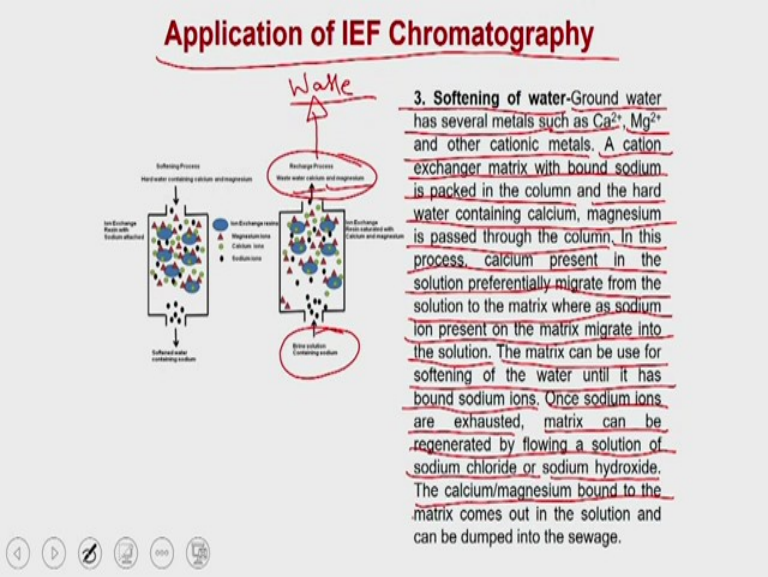

So once the beads are getting is going to lose all the sodium, they are not going to bind anymore these metals so in those case what you do is you follow a solution of sodium either the NACL or the NAOH and that actually is going to remove the bead bounds calcium as well as magnesium and other metals and this heavy metals can be collected in a waste and that waste can be disposed into a sewage.

So a calcium or magnesium bounds to the matrix comes out in the solution and can be dumped into the sewage. So that how you actually removes the heavy metals or the other kind of contaminating metals from the water.

And once the column is getting chocked or once the column is getting saturated and it does not have any more sodium for available for making an exchange then what you do is you flow very high concentration of sodium and that actually is going to regenerate this cartridges and the bead bounds calcium and magnesium will come out from the cartridges and that can be stored and dumped into a sewage.

So this the very briefly we will discuss about the application of ion exchange chromatography in protein purification as well as in terms of other applications, so that you use the ion exchange chromatography for developing the different types of assays or you can use them for studying the interaction between bio molecule or we have taken once example of daily use where you can use ion exchange chromatography.

To prepare to purify the water and make the water hard water into the clean water and you can remove the contaminating metals because all this metals are toxic for the human being and this is all about the ion exchange chromatography and its application in the bio technology related processes.

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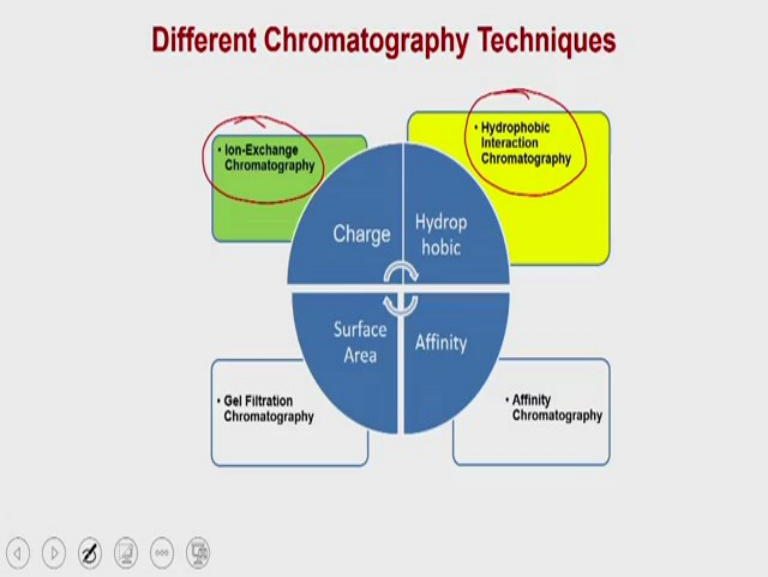

Now let us move on to the next chromatography, so we are already done with the ion exchange chromatography in terms of the mechanism in terms of principal how to operate the ion exchange chromatography and as well as its application and let us move on to the another technique which is called as the hydrophobic interaction chromatography. So as the name suggest the hydrophobic interaction chromatography is going to exploit or going to utilize the hydrophobic groups which are present on the protein.

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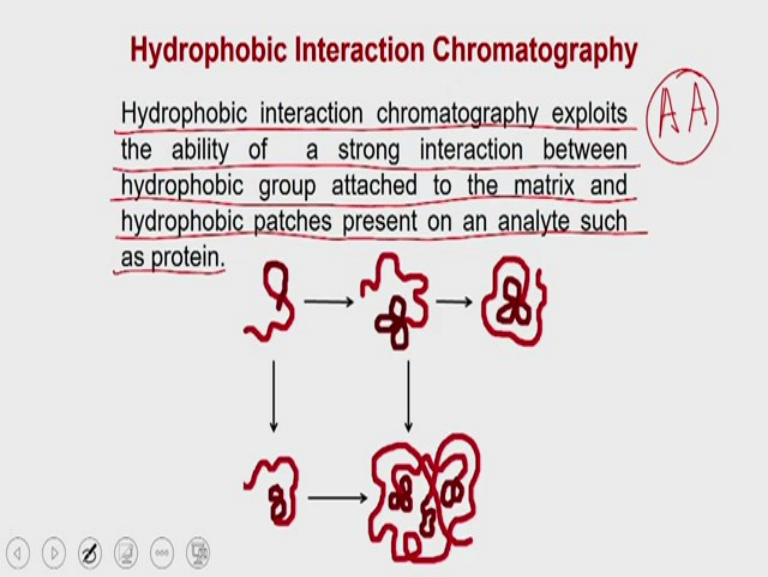

So the hydrophobic interaction chromatography, exploits the ability of a strong interaction between hydrophobic groups attached to the matrix and the hydrophobic patches present on the analyte such as protein. So as we discuss in the past the protein is made up of amino acids and these amino acids are of diversified nature they would be positively charged amino acids negatively charged amino acids, non polar amino acids, polar amino acids and the hydrophobic amino acids.

But the hydrophobic amino acids there is… does not like the water, because they would like to shield from the water so while protein is been synthesized as a chain of amino acid it is start folding and the folding is always been guided by the sequence of amino acid.

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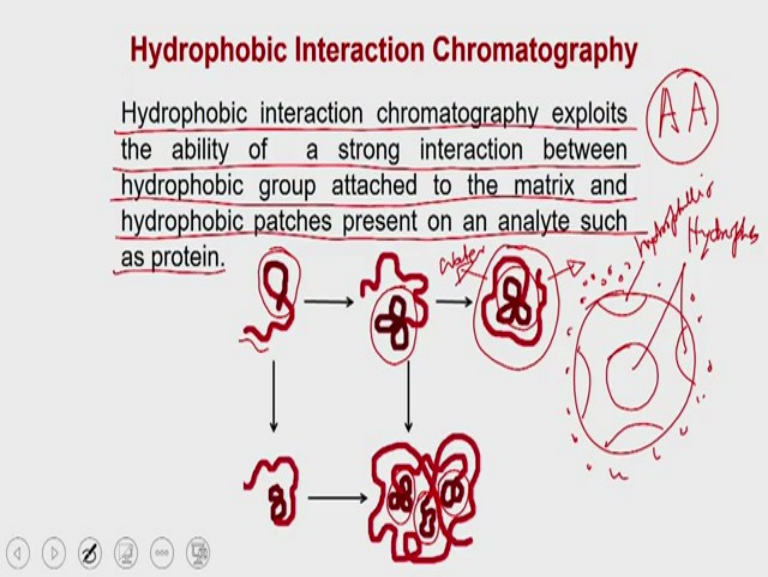

So you can see that as the synthesis of protein starts and you can see this dark region what you see is actually the region which is containing the hydrophobic amino acid such as tryptophan and phenyl aniline and tyrosine and so this so now the protein has started folding, so now this a your hydrophobic group and what you see is the protein is folding in such a way so that this hydrophobic groups should be present inside.

Whereas it would be covered by the hydrophilic as well as the polar groups, so that this polar groups could be able to interact with the water molecule which are present in the buffer or other micro environments and what will happen is that eventually all the hydrophobic groups would be present in the centre of the protein, whereas all the hydrophilic polar groups would be present outside, so if you see a cross section of a protein what you will see is that this is going to be hydrophobic core which is present in the protein molecules.

And you are going to have the hydrophilic outside. Apart from that the protein molecules are also having the different types of the hydrophobic patches and these patches are also been covered by the presence of water outside. This water is actually a part of the hydration shell, so every protein which actually is present in the biological fluid is been covered by the small tiny water molecules and this tiny water molecules are been covering the protein.

So that the protein should have should be always been present in a aqueous environment and that actually protects proteins and conserve its biological activity

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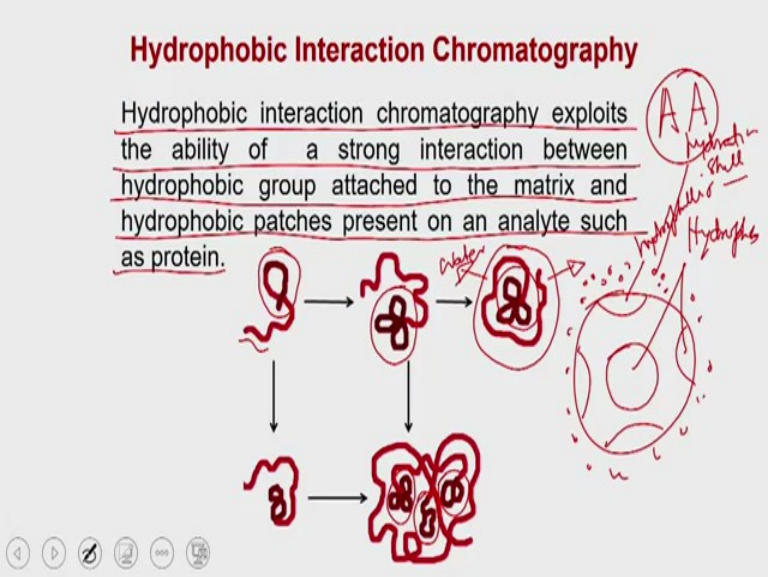

So these water molecules which are present outside are called as the hydration shell. And the amount of hydration shell which is present outside the protein varies from one protein to another protein and it depends on the number of polar molecules the charged molecules the negatively charged the positively charged amino acids which are present on the surface of the protein and this hydration shell is very important in terms of maintaining the three dimensional structure as well as in terms of maintaining the enzymatic activity of this proteins.

So you have the hydrophobic patches but this hydrophobic patches are also been covered by the hydration shell so if you have to perform the hydrophobic interaction chromatography the first question comes how you are going to access the hydrophobic molecule a hydrophobic patches? To so that the hydrophobic groups which are present on these beads are having a accessibility to this groups, so that they will be able to bind.

So for that if you have to achieve this task what is the first thing is that you have to remove the hydration shell in such a way or in milder way so that you will be able to have the access to the hydrophobic patches.

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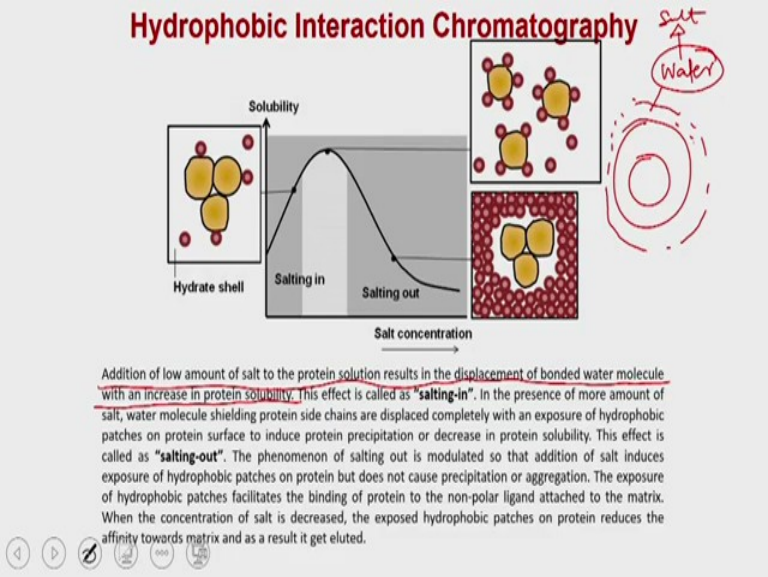

Now let us see how you can do that so you can imagine that the you have a protein and the outside the protein you have the hydration shell, so this hydration shell which actually I said know hydration shell contains the water molecules, so you know that the water molecule is going to have more solubility for salt compare to that it is going to having the association with the protein. So if you want to remove this hydration shell which is present outside the protein you can add the small amount of salt.

So when you add very small amount of salt to the protein solution that actually is going to make the displacement of bonded water molecule which is the part of the hydration shell and that is going to increase the solubility.

Why it is going to increase the solubility because it is going to increase the number of protein molecules which can additionally be used and which can additionally be solubilized because you are removing the you are removing the water and if actually making more space available for more number of protein molecule to be get solubilized.

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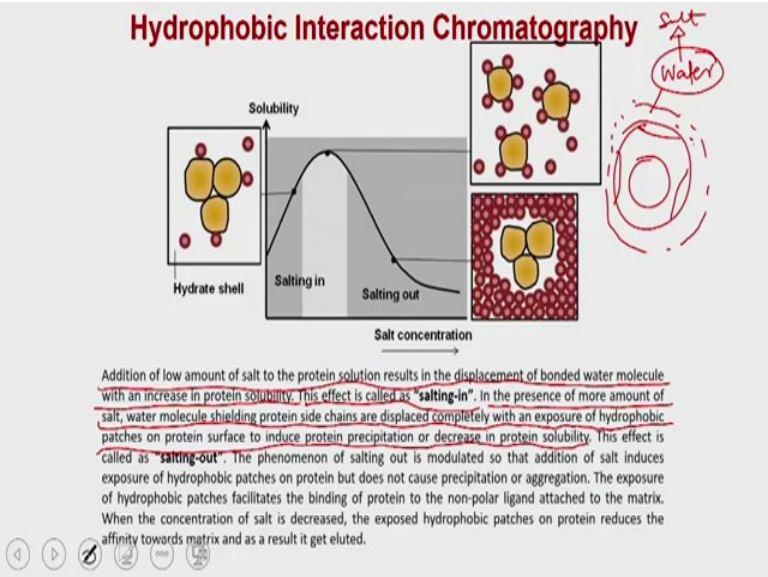

So that actually is going to increase the protein solubility and this effect of increasing the solubility by adding the amount salt is called as the salting in. But if you increase the salt like if you increase amount of the salt the water molecule shielding the protein side chain are also displaced completely with the help of an exposure of the hydrophobic patches. On the protein surface to induce the protein precipitation or decrease insolubility so if this solubility of the protein is going to be increase until you are not removing the hydration shell which are protecting the hydrophobic patches.

Once you remove or remove the water and expose this hydrophobic patches then the hydrophobic patches present on one protein is going to make the interaction with the neighboring and that is how either it will induce the precipitation or it will induce the aggregation of this proteins and this effect of reducing the solubility is called as the salting out.

Which means this is going to remove the protein from the solution. This is actually been always done in a very very traditional protein purification approaches where people actually use the salts and they use the salting out methods to precipitate the protein in a differential manner.

For example if you have a complex protein mixture you can actually use the salting out as an approach to purify the different protein by because every protein is going to have the different amino acid composition and accordingly it is going to have the different amount of the hydration shell present outside, so if you vary the salting out you could be able to separate them and you can precipitate some protein but the other protein will still be remain in the solution because there hydration shell is intact and if you add some more salt those protein also get precipitate, so if you do in a step wise manner you could be able to separate the different proteins.

And that actually is always been a traditional bio chemistry way where you actually precipitates the protein by adding the different amount of salts.

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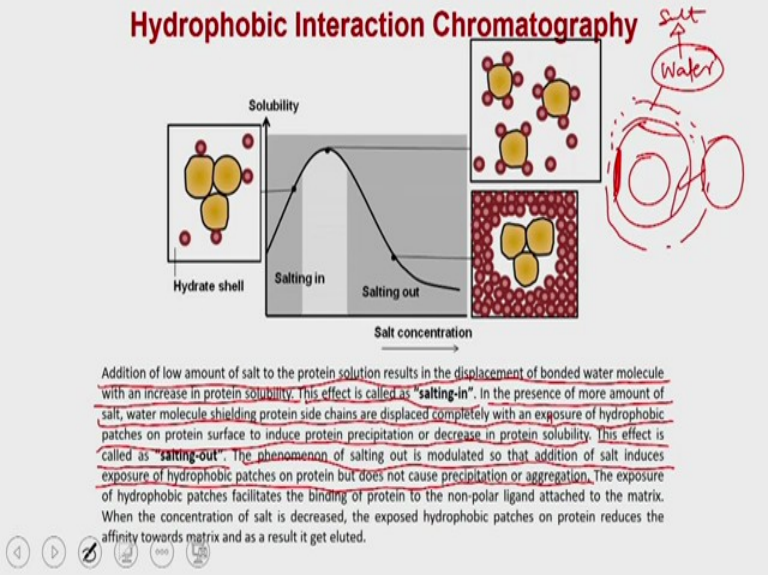

So the phenomena of salting out is modulated in such a way so that you add the salt that will induce the exposure of hydrophobic patches on the surface but does not cause the precipitation or aggregation. So you have to make a very fine control and you have to optimize in such a way that you remove the hydration shell but at the same time you do not allow the protein protein molecules to stick to each other to form the aggregates or the precipitate.

And that is how you could be able to expose the hydrophobic patches present on the protein molecules.

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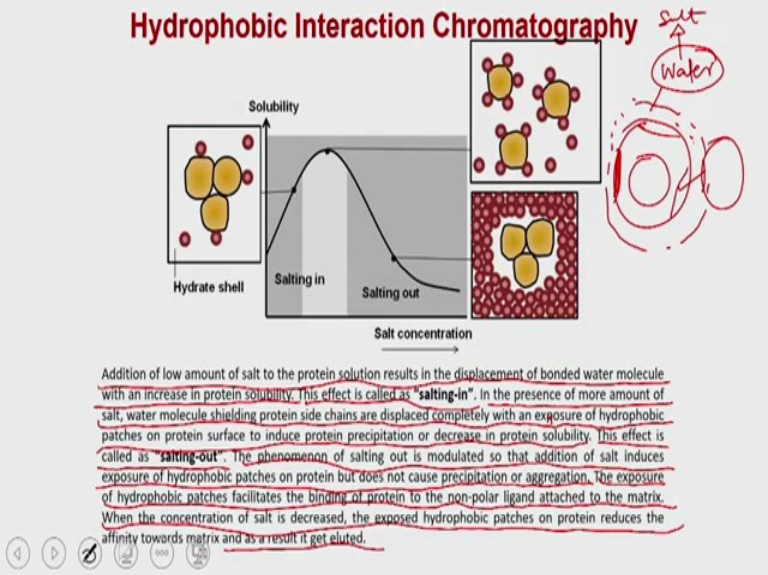

The exposure of hydrophobic patches facilitate to the binding of the protein to the non polar ligands attached to the matrix when the concentration of salt is decreased the expose hydrophobic patches on the protein reduces the affinity towards and as a result it get eluted. So once you increase the salt the hydrophobic patches are going to be exposed and that actually will facilitate the binding of the protein to the matrix and once you do the washing and then after washing you can actually reduce the salt.

Once you reduce the salt actually you will bring the hydration shell back and that actually going to protect the patches and again the interaction between the hydrophobic patch and the ligand which is present on the matrix is going to be broken down that is how your protein will going to be elute from the beads.

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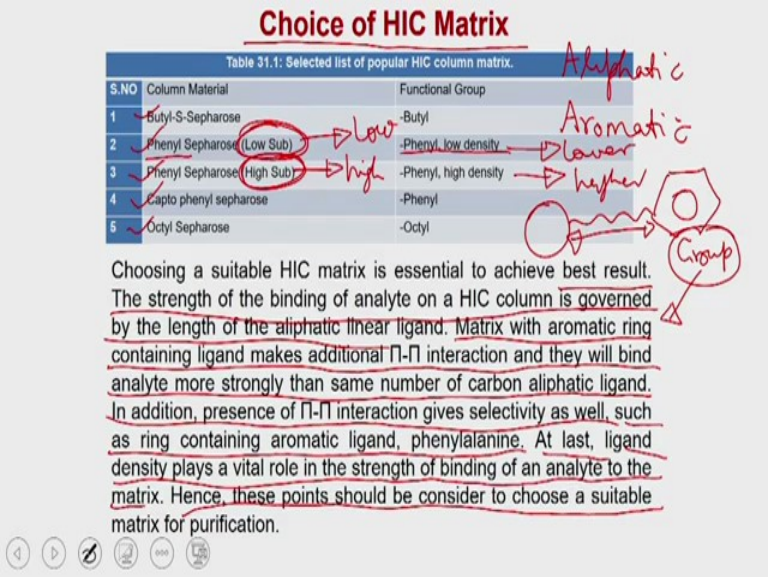

So the choice of HIC the choice of HIC matrix I have given a few examples like butyl sepharose, phenyl sepharose, phenyl sepharose high substitute and low substituted, then we have the capto phenyl sepharose octyl sepharose. You can have the many more molecules, so you have the actually the flexibility of either having the aliphatic groups or to the aromatic groups attached to the beads and accordingly this molecules are going to have the differential affinity.

In some cases you can have the benzene based aromatic ligands and what you see is the that we the two variants low substituted and high substituted which means this is going to have the low amount of ligands attached to the beads, whereas this going to have the high amount of phenyl sepharose phenyl groups attached to the sepharose beads that is why this column the the phenyl sepharose low substituted is going to have the lower affinity for hydrophobic interaction.

Whereas this is going to have the higher affinity, so you can choose the suitable matrix which is essential to achieve the best result. The strength of the binding of analyte on a is governed by the two factor one what ligand you are using for the interaction and the chain length what you are using, because if you increase the chain length you are actually giving the more flexibility for the ligand to interact with hydrophobic groups of the inner core hydrophobic groups present on the proteins.

So the chain length the suppose this is beads and so this is your HIC matrix so the length of this linker which you are going to use to attach the group is very very important and the group if this

group could be aliphatic groups or these could be aromatic groups and depending on whether you use the aliphatic groups or the aromatic groups the affinity of this ligands would be different.

Because the matrix with aromatic rings containing ligands make additional pi pi interaction they will bind analyte more more strongly then the same number of carbon containing aliphatic ligands so in addition, the presence of pi pi interaction gives selectivity as well, such as ring containing aromatic ligand phenylalanine. At last the ligands and density play a vital role in the strength of binding of an analyte to the matrix.

Hence, these points should be considered to choose a suitable matrix for purification. In one of the major issue with the hydrophobic interaction chromatography is that you have optimally see that you should have an interaction but it should not be so strong that while you reduce the salt and you should not get the protein being eluted from the column. Because if this interaction is going to be so strong that the protein got stuck to the beads and should not getting removed.

Then in those cases the protein is going to be denatured or it will not be there will be no recovery of this protein from the beads and then in you will not going have the no option you have no option but to discard this beads.

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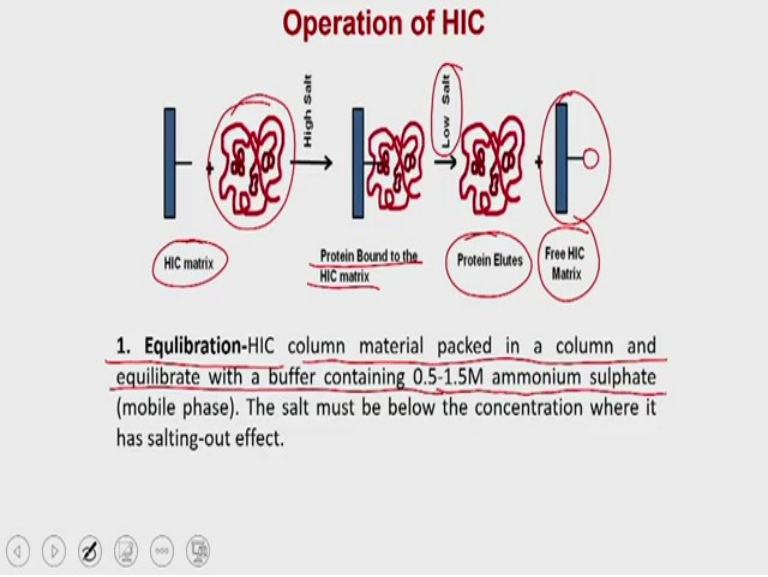

So this what you have HIC matrix you add your protein which is already been incubated with the high salt, so once you put the under the high salt the protein will bind to the HIC matrix and the

you do a washing step and then you are going to reduce the salt concentration as soon as you reduce the salt concentration the protein is going to be eluted and that actually is going to leave your HIC matrix and that can be used for next round of chromatography.

So if the operation of HIC has multiple steps in the first you are going to a equilibration, so HIC column matrix packed in a column and equilibrate with a buffer containing 0.5 to 1.5 molar ammonium sulphate. You can use any salt but the people always preferring to use the ammonium sulphate because ionic strength of the ammonium sulphate is very high compared to the NaCL or the KCL which people use for other application.

So if you use the NaCL or the KCL you might have to add the more concentration of salts ammonium compare to the sulphate.

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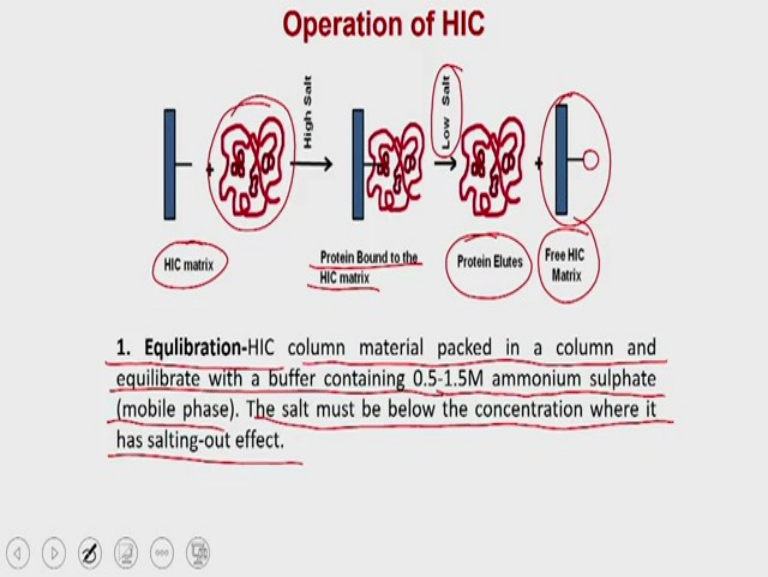

As a mobile phase the salt must below the concentration where it has salting out of it which means it should not precipitate or aggregate the protein. So you have to use the high concentration of ammonium sulphate, but it should not be so high that it is not going precipitate the protein, because otherwise the protein is going to be precipitated and it will not be available for making an interaction with the ligand which are present on the beads.

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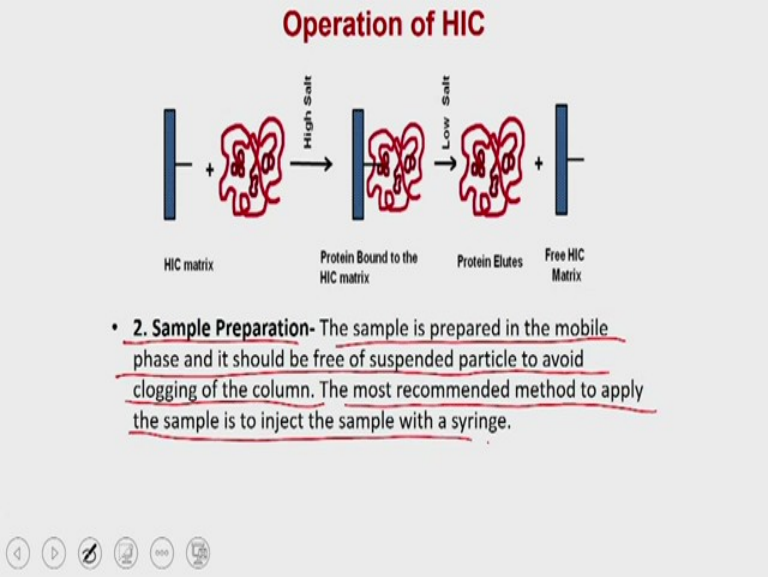

Now in the second step you are going to prepare the samples, the sample is prepared in the mobile phase and if should be free of suspended particle to avoid the clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

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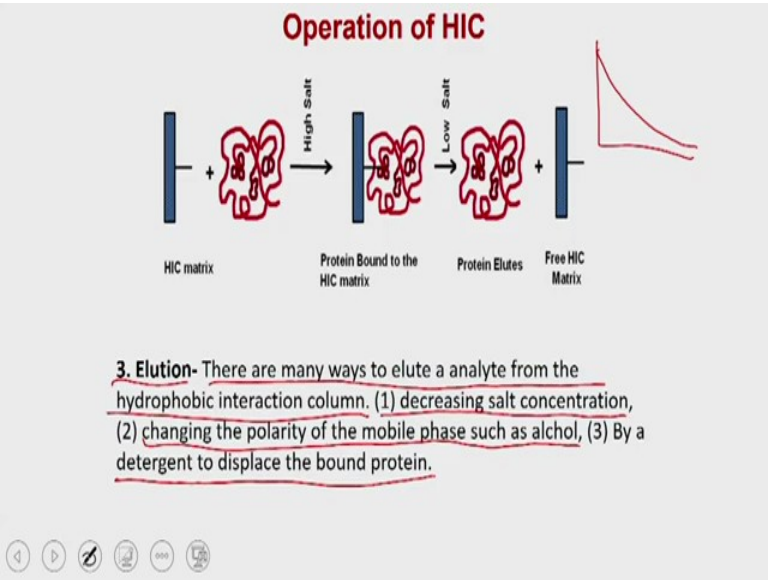

Now the third step you have to do the elution. There are many ways to elute a analyte from the hydrophobic interaction chromatography, number one decreasing the salt concentration, so you can actually decrease salt concentration and that actually will bring bring the hydration shell

back and that actually is going to destroy the interaction between the ligand as well as the hydrophobic patches present on the protein.

Then you can change the polarity of the mobile phase such as you can have the alcohol, so you can also have the competitive molecule because the is going to be more hydrophobic compared to the water molecules, so you can use if you add the alcohol that alcohol is going to make the interaction with the ligands present on the beads compare to the protein and that is going to displace the protein.

Or by a detergent to displace the bound protein, you can use the detergent also because detergent is also hydrophobic in nature, so if you supply the detergent, detergent is going bind to the ligand and it is going to displace the protein.

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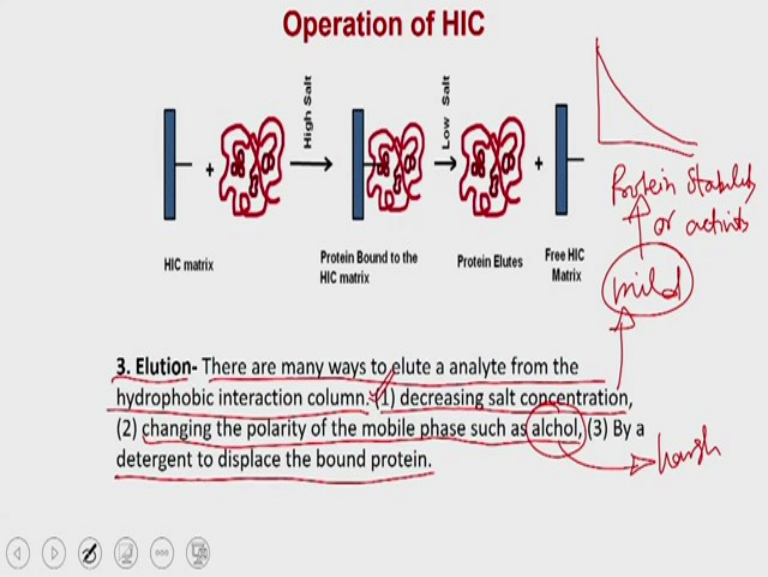

But the method number one which is decreasing the salt concentration is actually the mild method because that actually will not going to affect the protein stability or proteins activity. But apart from that if you use the alcohol, the alcohol method is going to be very harsh and it may actually destroy the activity as well as the three dimensional structure of protein and the same is true for the detergent as well.

But in some cases, sometime what happens is that you have chosen a wrong matrix. For example if you are instead of using the phenyl sepharose low substituted, suppose you have use the

phenyl sepharose high substituted and high substituted is going to have the higher affinity for the same protein. In those cases what will happen is even if you flowing the salt buffer without salt which means you have decrease the salt even then you are not bringing the hydration shell you are not brining the you are bringing the hydration shell back but still it is not good enough to destroy the interaction between the ligand as well as the patches or the hydrophobic patches present on the protein.

And in those cases you might have to supply the non polar solvent, so people start with alcohol and then they can go up acetone and all those kind of benzene and hexane and all that. But as long as they go upto the alcohol there is a possibility that the protein what they could elute probably will be active. But beyond that if they supply the acetone or hexane, you would still be able elute the protein from the beads, but that eluted protein may not be active.

Because most the protein structure are not stable enough to sustain such kind of harsh hydrophobic solvents, because once you put the solvents you are actually going to destroy the structure of the protein because the hydrophobic core which is present in the centre of the protein will try to come out from the protein. Because in that process what will happen is the three dimensional arrangement of the protein is going to be completely altered.

So that is why it is advisable before you start an HIC application or HIC operation, you should very briefly use test the affinity of a protein in a smaller volume, so that you will know that protein is going to be eluted from the beads.

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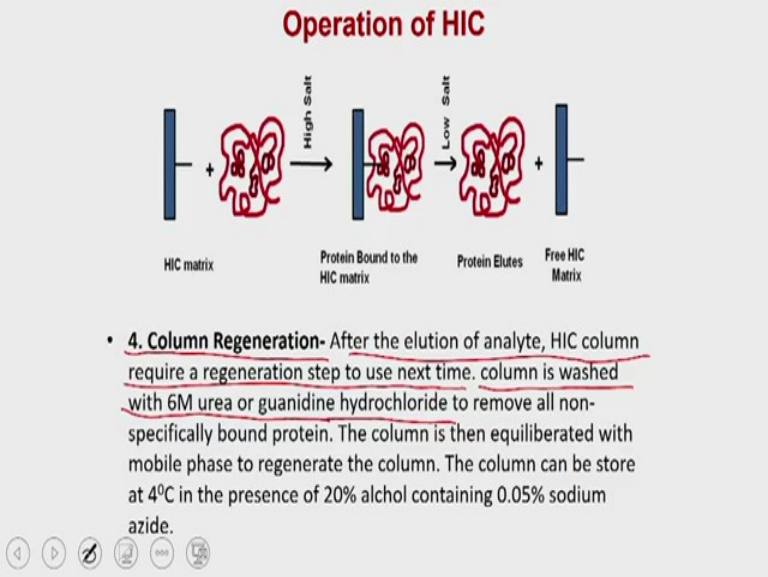

Now once you elution is over you have to do a column regeneration because you have to reuse this column, so after the elution of the analyte the HIC column require a regeneration step to use it for the next time. The column is washed with 6 molar urea or guanidine hydrochloride, this 6 molar urea or gyanidine hydrochloride is going to denature all the proteins and it is going to remove the non specifically protein bound to the beads as well as it going to remove all the protein which is bound to the ligand as well.

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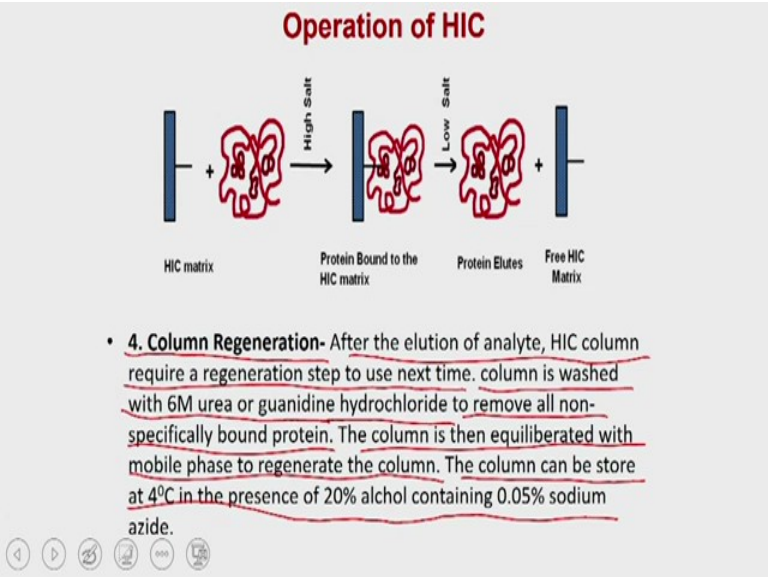

So that it will remove all specifically bound protein. The column is then equilibrated with the mobile phase to regenerate the column. The column can be stored at 4 degree in the presence of 20 percent alcohol containing the sodium azide, so while you are not using any of the column even it is a ion exchange column or HIC column or gel filtration column or any other column. You should ensure that you should store this column in a 20 percent alcohol containing the 0.2 percent azide, so alcohol and azide combination is going to protect this column material from getting the bacterial infection or getting the degradation.

Because most these columns are made of the sepharose or the agarose beads, agarose is nothing but the sugar molecules, so this sugar molecules so if have a sugar sub solution the bacteria is going to love to eat this sugar molecule, so that is why you have to have the preserved this beads in alcohol, so 20 percent alcohol is going to be bacterial sidel and in addition you can also add the small amount of azide, so the combination alcohol and azide is going to protect your column from the bacterial contamination or bacterial growth.

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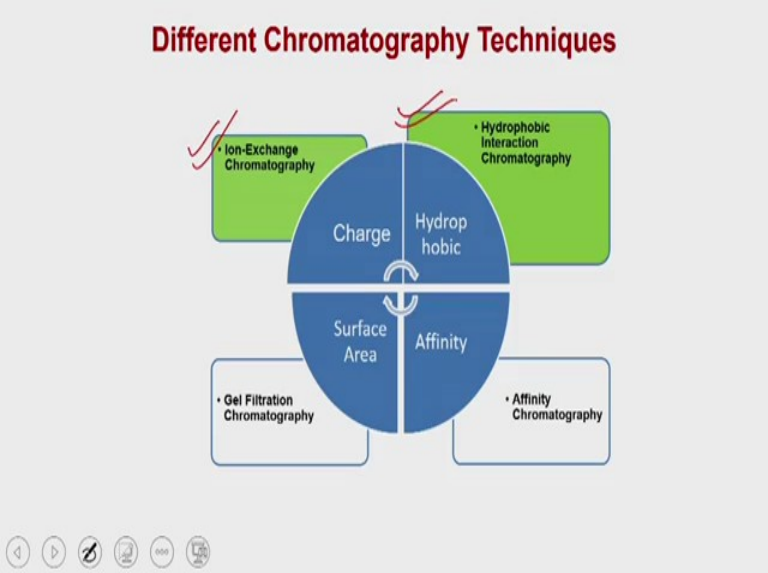

So this all about the two chromatography techniques, the ion exchange chromatography technique and the hydrophobic interaction chromatography which we have discussed both are these chromatography techniques are utilizing the charged group or the hydrophobic patches present on the protein surface. And now in our subsequent lecture we are going to talk about the

chromatography which is going to work on the principal of the surface area or the molecular size of the protein.

So with this I would like to conclude our lecture here and in the next lecture we are going to discuss about the gel filtration chromatography. Thank you.