Genetic Engineering: Theory & Applications
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Module VIII - Isolation and Purification of Product (Part-II)
Lecture 27 - Affinity Chromatography (Part –III)

Hello everybody. This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati and what we were discussing? We were discussing about the different chromatography techniques which people are using to purify the protein or the other factors from the cells where they are overexpressing these components. In this context so far but what we have discussed, we have discussed about the ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography and in our previous lecture we have also discussed about the affinity chromatography.

And now today in this lecture we are going to discuss about the different types of affinity chromatography and how to perform this chromatography and at the end of this lecture we are also going to discuss about the application of affinity chromatography. So let us start with the first examples of affinity chromatography. As you know that in our previous lecture we have discussed that the affinity chromatography is of two different types. One is called bio-affinity chromatography where you are using the biological material as or biological pairs like receptor and ligand as a pair to purify or to perform the affinity chromatography.

Then we have the pseudo-affinity chromatography. In the pseudo-affinity chromatography you are using the non-biological receptor ligand pairs and then you are using them for affinity chromatography. Within the pseudo-affinity chromatography you have the dye affinity chromatography as well as the metal affinity chromatography. So let us start with the some of these examples and how to perform this affinity chromatography in your laboratory.

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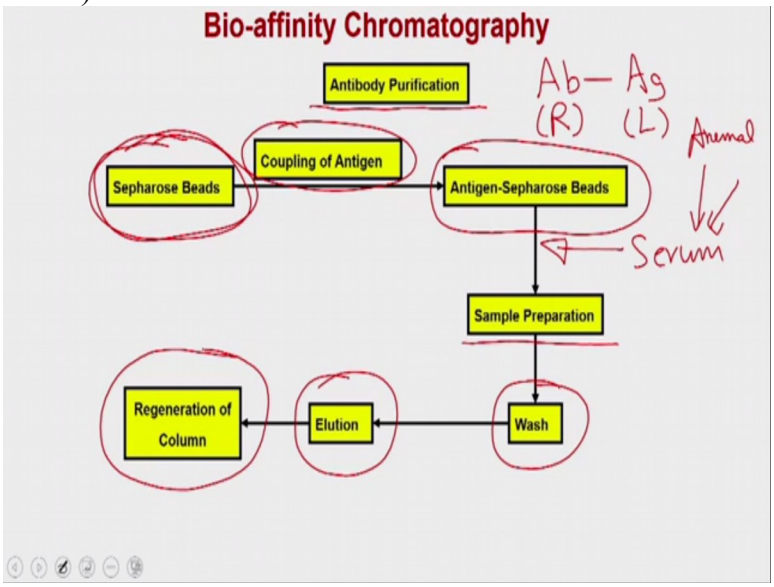

So in the bio-affinity chromatography we have taken two examples. One example is where you can use the bio-affinity chromatography to purify the antibodies and the second example we have taken is to use the GST, GSH as a pair. So we will start with the purification of antibody using the affinity chromatography. So in the purification of antibody you are going to have the sepharose beads and the first step what you have to do is you have to prepare a column where your antigen is going to be coupled.

As you know that every antibody is having its cognate antigen which means the antibody is going to work as a receptor whereas the antigen is going to work as a ligand. So if you would like to purify the antibodies, what you have to do is you have to first prepare a column or affinity column so that you will be able to bind the antibodies which are present in the serum.

If you remember or recall in the previous lecture we have discussed how you can generate the antibodies in rabbit or mice. So now in this we are going to discuss how you can purify the antibodies against the antigen which you are going to use. So the first is you are going to have the sepharose beads, these sepharose beads you have to couple the antigen which is going to complementary to the antibodies or whatever have to purify.

So the coupling of antigen is the first step and once you couple the antigen you are going to have the sepharose beads coupled with the antigens, then what you are going to do is you are going to prepare the samples like you are going to apply the serum which you are going to get from the animals like rabbit or mice or sometime you may get the antibodies from the hybridoma cells.

Now once you prepare the samples then you are going to apply this sample onto the column. Because there will be an interaction between the antigen and the antibodies the antibody will go and bind to the antigen and then you are going to have a washing step, that washing step is going to remove the nonspecific material which means you are going to wash with a high salt concentration so that you are going to remove the nonspecific proteins which are present in the serum or the hybridoma culture supernatant and that is how you are going to get rid of most of the protein.

Now what you have left is the antibodies which are bound to the antigen which is coupled to the beads. Now what you are going to do, you are going to do elution, so elution there is a multiple approaches or multiple choices what you have to elute the antibodies from the antigen and once you are done with the purification then you have to regenerate the column. So let us start with the first step that is the coupling of antigen to the sepharose beads to prepare your antigen sepharose beads which are going to be the column for purifying the antibodies.

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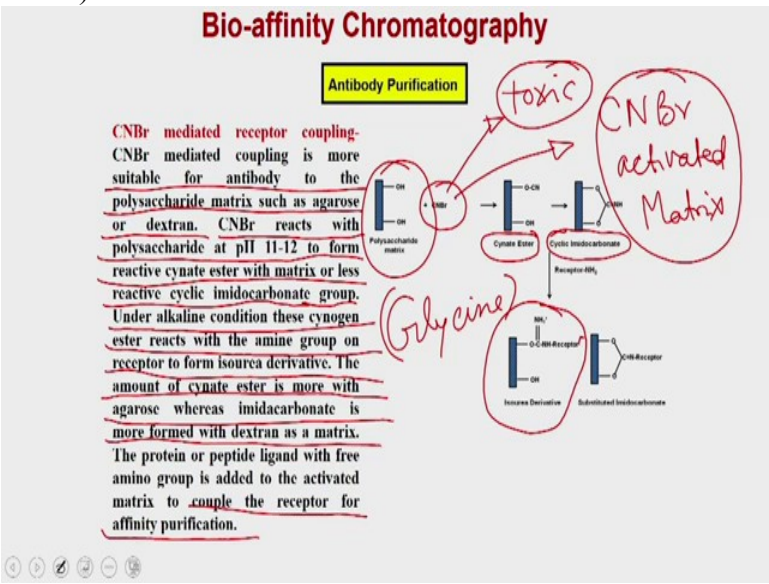

So in a previous lecture we have discussed about some of the coupling methods, those coupling method like also can be used to couple the antigen to the beads but what we are going to discuss today is the CNBr mediated coupling. CNBr mediated coupling is very specific for the proteinaceous material like antibodies, protein and peptide and it is more suitable for the antibodies to the couple to the polysaccharide matrix such as agarose or dextran. CNBr reacts with the polysaccharides at a pH 11 to 12 to form a reactive cynate ester with matrix or less reactive cyclic imidocarbonate group.

Which means if you take the cyanogen Bromide and if you react with the polysaccharides, what will happen is it is going to generate the two material which is called cynate ester and the cyclic imidocarbonate. Once you add the protein which actually may contains the free amino group, so under the alkaline conditions, these cynogen esters are going to react with the amine group which is present on the receptor to form the isourea derivatives.

And that is how your protein is going to be coupled to the beads and the amount of cynate ester is more with the agarose whereas the imidocarbonate is more formed with the dextran as a matrix. The protein or peptide ligand with free amino group is added to the activated matrix to couple the receptor for affinity purifications. As I said, you know the CNBr can be added to the polysaccharide matrix either the agarose or the dextran and then it is going to activate the matrix and then ultimately the matrix is going to bind the antigen through the free amino group.

But there is a problem that cynogen bromide is very toxic in nature, okay? So that is why availability of cynogen bromide is completely banned. And that is why what you are going to get is the CNBr activated matrix. Which means you are going to so instead of doing this whole activation what you are going to get is the CNBr activated matrix. And in that case what you have to do is you are going to buy the CNBr activated matrix from the vendor. And then you can just add simply add the proteins or the antigen or the antibodies. And then antibodies are going to have the free amino group. And then that will be used to couple the antibodies or the antigen to the matrix.

Once that is over then what you are going to do is you are going to deactivate all the activated groups which are present on the matrix and that you are going to do simply by putting a glycine. So you know that glycine is the simplest amino acid but the glycine also contains the free amino group.

So once your coupling action is over you guard the antibodies or you guard the antigen coupled onto the beads, then you have to remove or you have to deactivate all other functional groups which are still available to bind the ligand and that you will do simply by adding the large quantity of glycine into the reactions. What will happen is that glycine will go and bind to those beads or those activated groups.

And as a result what will happen is if you use these beads without the deactivation, what will happen is as soon as you will flow the proteins or the serum these activated group which are

present onto the matrix are going to bind the protein. And that will actually going to give you the nonspecific purification. So to avoid that you always add the glycine to deactivate the activated group present on the matrix after your coupling is over. So now we have coupled the antigen onto the matrix or to the beads using the cyanogen bromide activated beads.

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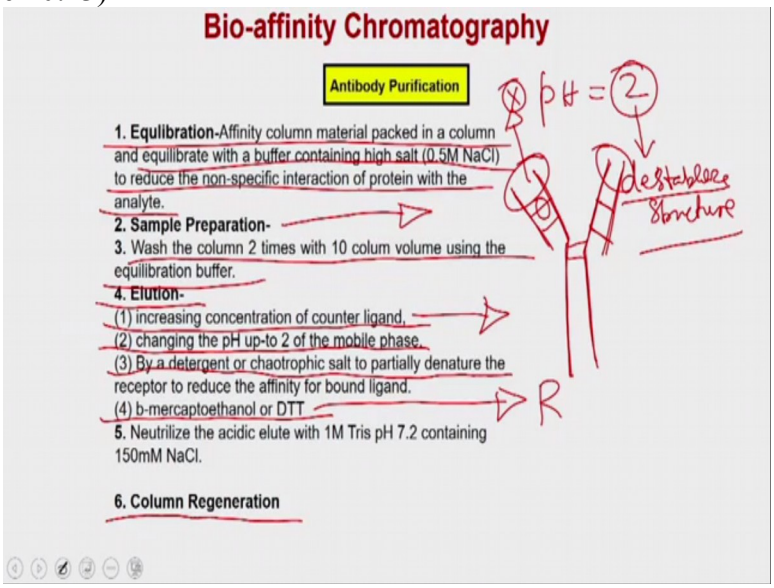

Now let us go to the next step. So in the next step what you are going to do is you are going to equilibrate, which means you are going to first pack the column in affinity column. And then you will equilibrate the buffer with the containing high salt to reduce the nonspecific interaction of the protein with the analyte. Then you are going to prepare the sample, the sample preparation is simple that you should not have any particulate matter so that it should not clog or should not reduce the flow rate or should not affect the flow rate when you are purifying. And then what you have to do is once the sample is being injected into the column, then you wash the column two times with the ten column volume using the equilibration buffer.

After that you have to do, so once the washing is over that actually is going to remove the nonspecific sample or nonspecific proteins which are being bound to the matrix. Now you have to do the elution and since we are talking about the antigen and antibody interactions, we have a couple of choices or couple of options which we can exploit to elute the antibodies which are bound to the antigen present on the matrix.

What you can do is you can increase the concentration of counter ligand. Which means you can add some counter ligand and that actually is going to bind to these antigens instead of and then in that process the antibody is going to be released. Number two, you can change the pH

of mobile phase. So since the antigen-antibody interaction is very very strong, you have to change the pH somewhere around 2 to 3 and that actually is going to release the antibodies which are bound to the antigen. So what will happen when you change the pH to 2? When you change the pH to 2, it is actually going to destabilize the structure of antibody and when it destabilizes the structure of antibody, the antibody is going to have the reduced affinity for the antigen which is bound to the matrix.

And in that process the antibody is going to be eluted from the column. Now the third is you can use the detergent or chaotrophic salt. The when you use the detergent or the chaotrophic salt, chaotrophic salt means you can use the urea or the guanidinium hydrochloride.

All these three options like whether you use the detergent or whether you use the urea or guanidinium hydrochloride, they are going to affect the structure of the antibodies and eventually they are going to compromise the three-dimensional structure of the antibody. And you know the antibody is going to bind the antigen by having a complimentary interactions or complimentary fitting followed by the extensive electrostatic vander waal as well as the salt bridges interaction with the antigen.

So once you supply the urea or GdmCl you are actually going to destroy the three-dimensional structures of antibodies. And in that process the antibody is going to have the reduced affinity for antigen. And that is how the antibody is going to be eluted from the column. Now the fourth is that you can use the beta-mercaptoethanol or the DTT.

You know that the antibody, a general structure of antibody is something like this where this is the antigen binding region. And this is the heavy chain and and this is the light chain. And all these chains are attached to each other by double sulphide linkages. And then these also are bind to each other. So, a complete antibody structure is being formed simply by holding the light and heavy chain together by the sulphide bridges as well as the complete antibody structure is being stabilized by multiple sulphide linkages within the molecule.

And because of this if you add the DTT or whether you if you add the beta-mercaptoethanol, what you are going to do is you are going to break the disulphide linkages. And as a result what will happen is it is actually going to destroy the antibody binding site present on the antibodies. And as a result what will happen is the antibody is going to have the reduced affinity for the antigen. And that is how the antibody is going to be eluted. Once you elute the

antibodies using this, you have to neutralize the DTT or beta-mercaptoethanol present in the solution.

Either you do the dialysis or you put the oxidizing agents so that the sulphide linkages which are being broken down by the beta-mercaptoethanol or the DTT are going to be reformed and once they will be reformed, your antibody is going to be renatured, antibody is going to be reformed and that is how that antibody is going to be available for the other downstream applications.

Once you have done with the purification then you can go ahead with the column regeneration. The column regeneration is very simple that you have to wash the column with the high salt buffer and then followed by you have to wash the buffer with chaotrophic salts so that that will remove the all non-specifically bound proteins and then your this column and then you equilibrate the column with the equilibration buffers which contains the high salt. And now this column is again available for reused to perform the second round of purifications.

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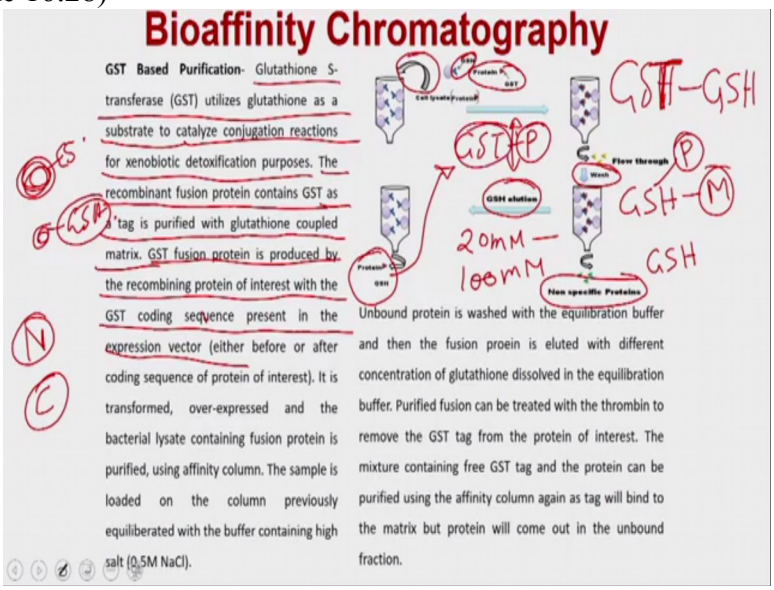

Now let us move onto the next example, the next example is the example where we are using the enzyme. So in our previous example we were using the antigen-antibody interaction as a basis to purify the either antigen or the antibodies. Now we have an example of the enzyme substrate as a partner. So in this case we have taken an enzyme and then we have taken a substrate.

The enzyme is coupled or enzyme is coupled to the protein of our interest whereas the substrate is going to be used as a ligand to elute the proteins. So in this process we are taking an example of the GST, GSH as a pair. So the GST is actually making an interaction with the GSH, so GST is making an interaction with the GSH, so GST which is called Glutathione S-transferase utilizes glutathione as a substrate to catalyse the conjugation reaction for xenobiotic detoxification purposes. Now what you have to do is you have to produce a recombinant protein which is going to be having a fusion protein which contains GST as a tag to purify the glutathione coupled matrix.

So in this case what will happen is that your matrix is going to have the GSH. The matrix is going to have the GSH and (the NT) your enzyme is present onto the as a chimeric protein present on the protein. So what will happen is the GST fusion protein is produced by a recombining protein of interest with the GST coding sequence present in the expression vector.

It can be done on the N-terminus of your protein or you can put onto the C-terminus of the protein, irrespective of whether the GST is present on the N-terminus of the protein or whether it is present on the C-terminus of the protein the GST is fully functional. And it is actually can bind to GSH which is present onto the beads. So what will happen is you will transform this fusion protein which contains the GST either on the N-terminus or the C-terminus and then you will overexpress the protein. And then you are going to take the protein lysate and pass through to the column. So once you pass through the lysate to the column what will happen is that you are going to have the GSH which is coupled onto the beads and the protein is going to be having the GST as a tag.

So what will happen is that the protein containing the GST as a tag will go and have an interaction with the GSH which is present onto the beads. And that is how your protein is going to bind. Now in the next step you are going to do a wash, so you are going to do the washing with the high salt concentration so that all other nonspecific proteins should come out from the column and only the protein which contains the GST as a tag will go and bind to the GSH which is present onto the bead.

Now you are going to do the elution, so you can add the large quantity of GSH which means you can add the GSH in the range of 200 (milli) 20 millimolar to 100 millimolar. And that actually is going to compete for the GSH which is present on the beads. So you can imagine

that you have 2 GSH, the GSH which is the matrix bound and then you are adding a GSH from the which is present into the elution buffer.

And this GSH has already bound a protein through GST but what will happen is when you flow a large quantity of GSH which is present in the mobile phase the protein which is binding to the GSH present onto the beads is going to have a distribution between the GSH which is present onto the bead, the GSH which is present in the solution. And since the GSH which is present in the solution is very very high concentration compared to the GSH which is present onto the beads, the protein is going to bind the GSH which is present in the solution.

And as a result it is no longer is going to bind to the beads and that is how it will be present into the supernatant. And that is how the protein will come out from the column as the GST protein complex. Now you can use some of the proteases, so GST and protein is being coupled to each other with a protease cutting side.

And that is how if you want to purify you can add that particular protein and that actually is going to remove the GST from the purified protein. And it will give you the pure protein into the supernatant. So this is about the utilization of bio-affinity chromatography. We have taken an example of antibody, now we have taken an example of enzyme-substrate complexes.

Now let us move onto the next examples where we are going to use the pseudo-affinity column and in the pseudo-affinity chromatography we are going to use either the dye affinity column or to the metal affinity column.

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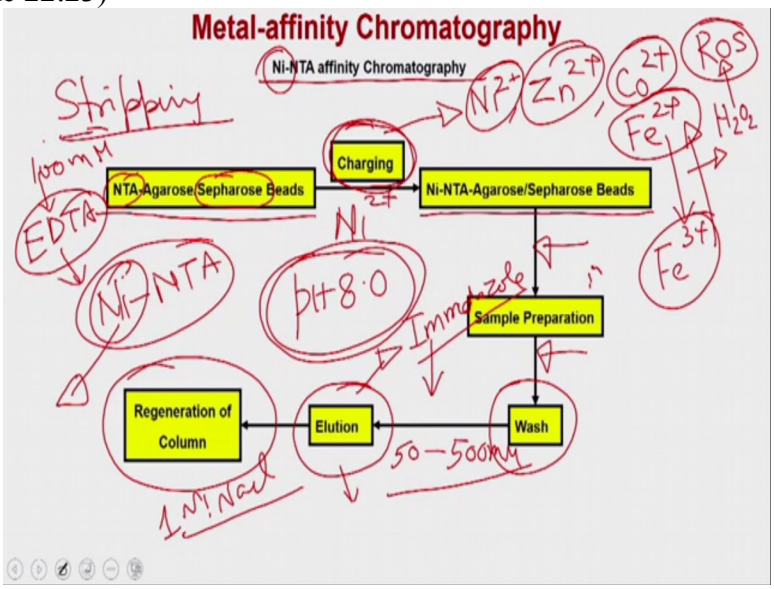

So in the metal affinity column, we have taken an example of nickel NTA affinity chromatography. So the nickel NTA affinity chromatography is the example of metal affinity chromatography where the metal which is bound to the beads is actually having an affinity for a small biophore which is being present onto the protein and that is how it is going to bind those proteins which contains the six histidine either present on the N-terminus or present onto the C-terminus. When you buy the nickel NTA beads or when you buy the nickel NTA column, you are not going to get as nickel NTA. What you are going to get is the column from the vendor as the NTA agarose beads or sometimes you will get the NTA sepharose beads.

So this column material it does not contain the nickel bound to the beads, either you will get the NTA sepharose beads or NTA agarose beads. So the first step what you are going to do is you are going to do the charging, which means you are going to charge the column with the transition metals. You have the option of using the nickel, you have the option of using the zinc or you have the option of using the cobalt and you are having no option of using the iron. Why we do not use the iron as a transition metal?

Because the transition metals which we are talking about like nickel, zinc and cobalt are highly stable compared to the iron as a transition metals, because the iron 2 plus is very readily get converted into iron 3 plus. And in this process what will happen is the iron 2 plus get converted into iron 3 plus and iron 3 plus get reverted back because the protein maybe having the reducing environment and in this process what will happen is if there is be a switching of iron 2 plus to iron 3 plus and 3 plus to 2 plus, it actually generates very large

quantity of hydrogen peroxide or sometimes it also produces very large quantity of free radicals and these free radicals are going to be generated within the column while you are doing the purifications.

And that is why and if these free radicals are being generated, they are going to oxidize the amino acids which are present on your protein and as a result it is going to damage some of this amino acid and ultimately it is going to damage the protein as well. So that is why to avoid this you have the flexibility of using nickel, zinc or cobalt but not to the iron. So the first step is that you bought the column which is present as an NTA agarose or saphrose beads.

Then you are going to do the charging, charging you can use either of this transition metal salt. Suppose we have done with the nickel then what you are going to get? You are going to get the nickel NTA agarose or saphrose beads. Then you are going to apply the sample, so the sample preparation is very simple. You have to apply the samples and you have to filter it so that there will be no particulate matter so that the column should not get clogged or the column's flow rate should not get compromised.

And then it was going to bind to the matrix. Before you apply the samples you have to equilibrate the column with the equilibration buffer and in most of the nickel NTA affinity chromatography you have to keep the pH which is somewhere around 8. So you cannot work into the nickel NTA affinity chromatography if the pH is less than 7 because the histidine tag which is present onto the protein is not going to bind very effectively to the nickel which is present onto the beads.

So it is preferred or it is recommended that you use the buffer which is above to pH 8 or at least above to the pH 7.4. Now you prepare the sample in this particular pH buffer, you can use the pH 8 or you can use the phosphate buffers. Most of the time people do not prefer the phosphate buffer because it contains a large amount of salts but so you can use the Tris which is actually the more pure salts because the phosphate buffer also contains the many types of salt and other kind of things. So people do not prefer the PBS compared to the or phosphate buffer compared to the Tris, so you can use the Tris pH 8.8 or pH 8, whatever the Tris you want to use.

And then you prepare the sample in suppose 100 millimolar Tris pH 8 and then you apply the sample. The sample will going to bind the beads and the protein which contains the histidine

tag, so just like the GST tag you are going to have the histidine tag present onto the protein simply by recombinant technology.

Either the histidine tag will be present onto the n-terminus or to the c-terminus, then the histidine tag which actually is a stretch of histidine the six histidines and these six histidine actually forms a small biophore for which actually has a very high strong affinity to the nickel which is bound to the beads or to the this particular active group present onto the matrix.

So the sample will bind, then you will wash the sample with the washing buffer which actually is going to be of high salt buffer and then once your washing is over then you are going to do the elution. In this case the elution is going to do with the competitive buffer, so ideally we should apply histidine because histidine is the competitive amino acid which you can use. But because the histidine is very very costly and histidine actually contains the imidazole ring, so you can actually use any compound which contains a imidazole ring actually. So in that case people use the imidazole instead of the histidine. So using the imidazole is the simplest way, you can use the imidazole somewhere around 5 to 500 millimolar concentration and that should give you the protein, that should elute the protein from the column.

The alternate way of eluting the protein from the column is that you can also change the pH as I said, the optimal pH is that you use above the pH 8 because the binding of the histidine tag to the nickel present onto the matrix is very very high or very very good. If you have the pH above to 8 or at least to pH 8 but if you change the pH and bring it towards the acidic side, the affinity of the nickel which is bound to the matrix is going to be compromised to the histidine tag and that also is one of the option which you can use to elute the protein from the column. The third is that you can use the urea or chaotrophic salts and you can actually elute the proteins. The fourth is that the nickel is actually binding to the matrix to nickel NTA.

And if you apply any type of the chelating agent like EDTA, for example if you supply the EDTA to the column, the EDTA is going to remove the nickel from the column. So if you want to use then EDTA, you can also use the EDTA and that can be remove the nickel from the column and that can be used as a method to elute the protein. Once you are done with the elution then you have to do the regeneration of the column.

And the regeneration of the column is that you wash the column with 1 molar NaCl, that actually is going to remove the nonspecific proteins which are binding to the matrix not to the functional group and that actually is going to clear the column. Since we are doing the charging step and in the charging step you are actually loading the nickel to the beads, you are also need to perform the stripping step because what happen is the even the nickel is present onto the matrix, sometimes its binding efficiency get compromised or the amount of nickel which is bound to the protein is going to be reduced with every purification cycle.

So if you would like to have the consistent purification, what you are supposed to do or what is recommended this that if you do 5 to 6 time purification, after that you should strip the column. You should remove the nickel from the column and do the recharging again which means you do the charging step again so that you ensure that the every bead has a sufficient quantity of nickel so that it should bind the protein.

So for that you have to do the stripping step. So how to do the stripping is that you flow at least 100 millimolar EDTA and once you flow the 100 millimolar EDTA, what will happen is that is going to remove the large quantity of nickel from the column and then you can do, you can wash this column with the water so that the EDTA will be removed from the column and then you can do the next round of charging step. So in the charging step you are going to incubate the column with the 100 millimolar nickel sulphate solution. So once you supply the nickel sulphate column the nickel which is present into the nickel sulphate solution will go and bind to the NTA groups. So NTA is a functional group which is present onto the agarose or sapharose beads depending on from which vendor you buy these beads.

And then the nickel will go and bind to the beads. So this is all about the metal affinity chromatography. So with this we would like to conclude our lecture here. Thank you.