Genetic Engineering: Theory & Applications
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Module 8 - Isolation and Purification of Product (Part-II)
Lecture 25 - Affinity Chromatography (Part-I)

Hello everybody! This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering. And what we were discussing, we were discussing about the purification of the product from host cells which are over-expressing that particular product and in this particular discussion so far we have discussed about the ion exchange chromatography followed by we have discussed about the hydrophobic interaction chromatography.

And in the previous lecture, we have also discussed about the gel filtration chromatography and now today we are going to start the new chromatography and that is called as the affinitive chromatography and the affinitive chromatography as the name suggest is actually going to have a exclusive affinity for a particular ligand.

So, the first question comes how a particular ligand or the receptor is developing the exclusive affinity for its respective ligand or the receptors. So, the affinity chromatography works on the principles of mutual recognition forces between a ligand and a receptor.

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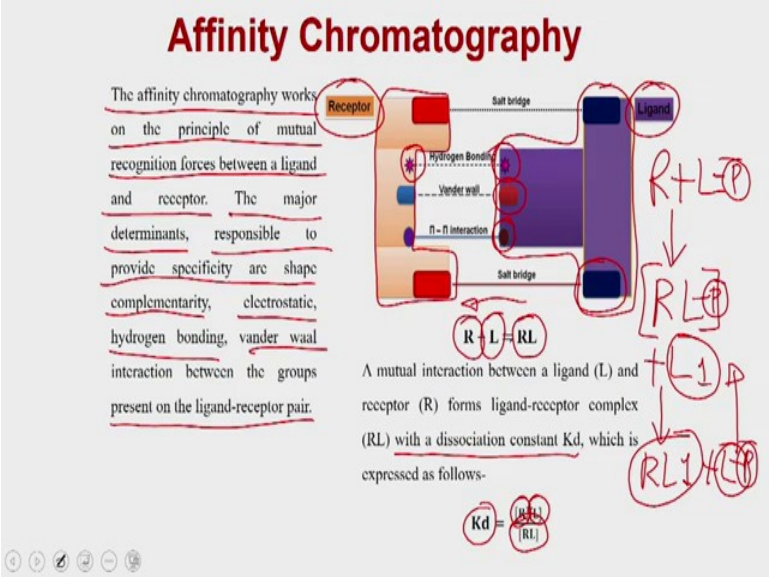

So, you can imagine a situation that there is a receptor. So, you that the cells are containing the receptors and these receptors are having their respective ligands and all these ligands actually go and bind to this receptor and that is how these receptors are either using that

interaction to generate the downstream signal transactions or many of the times the cell is using these receptors to uptake the food particles or uptake the nutrition from the excess cellular media.

So, this particular kind of philosophy that a receptor is going to recognize its ligands simply by having the mutual and exclusive forces, so what are the forces that are going to govern between a receptor and the ligand? The major forces or determinants responsible for providing the spacificity are shape complimentary.

So, the first thing is the shape because the shape complementarity between a receptor and a ligand is actually bringing the receptor and ligand into a close proximity. Once they come into the close proximity then the electrostatic hydrogen bonding, vander waal interactions between the groups which are present on the ligand receptor pairs actually play a role.

So, you can imagine that because of a unique shape of a receptor and the complementary shape of the ligand, these two molecules come together, then they have the respective groups present on the ligand which is actually going to have the corresponding groups present on the receptor.

And that is how these two groups are either going to form the hydrogen bonding then they can also form the vander waals interactions or they can also make the strong pi pi interaction as well as the salt bridge interaction. And all these interactions are going to be exclusive; they are going to provide the specificity in the system. That is why a particular receptor is going to interact with the particular ligand or a set of ligands which are actually going to have these characteristics where they are going to have the complementary shape.

And these complementary shapes are also going to share many of these groups and their specific locations so that when they come together these groups are going to participate in hydrogen bonding, vander waal interactions and very strong pi pi interactions as well as the salt bridge interactions.

So, you can imagine that a receptor is making a interaction with the ligand to make a receptor ligand complex, and this receptor ligand complex can be dissociated to give you the receptor as well as the ligand, that is why this particular approach can be used in chromatography, which means if you form a receptor ligand complex you can add the ligands and that actually is going to break the receptor ligand complex to give you the receptor plus ligand.

That is why the dissociation constant of this interaction is the K d is equivalent to the concentration of R, concentration of L divided by the concentration of RL. So, you can imagine that a receptor is making an interaction with L or the ligand and giving you a receptor-ligand interaction.

And now if you add another ligand which is actually L1 and that is going to have more affinity for the receptor then what will happen is this receptor ligand is going to be broken down and that is how it is going to form this and your ligand is going to be free and this is exactly the principle of the affinity chromatography where what are going to do is you are going to have the receptors on one side and then you are going to have the ligands from the other side.

And in most of the affinity chromatographies either you will couple the receptors to the matrix or you will couple the ligands to the matrix and then the cock-net pair you are going to generate or you are going to be present onto the proteins.

So, you can imagine that a receptor is making an interaction with the ligand and this ligand is associated with the protein. So in this case what will happen is, you are going to have a receptor ligand protein complex. Now when you put a competitive ligand, the competitive ligand is going to replace the ligand protein interaction, like it is going to replace the ligand protein and that is how it is going to make a interaction of receptor ligand one and your ligand protein complex is going to be released and that can be put it into the different fractions and that is how you are going to achieve the purifications.

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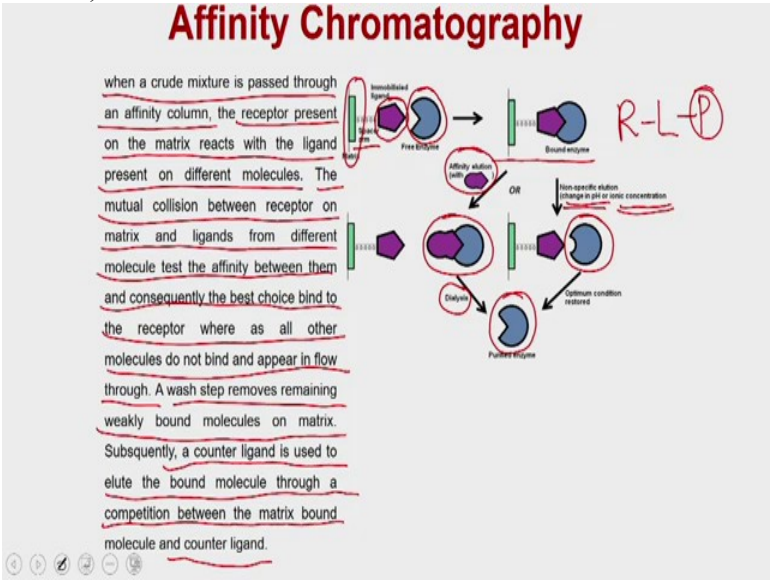

Now, you can imagine when a crude mixture is passed through an affinity column, the receptor present on the matrix react with the ligand which is present on the different molecules, which means in a typical affinity chromatography what you have is the matrix on which the ligands are being immobilized with a special arm. And this is your column. Now, what you are doing is you are adding the enzyme which has an affinity for this particular ligand. Now only this enzyme is going to bind and rest of the bimolecule which are present in the crude mixture are not going to bind.

So, you are going to have the receptor ligand protein complex and ultimately you are going to have, you can add another ligand which is going to be the affinity elusion which means you are going to add a competitive ligand, once you add the competitive ligand that is going to breakdown these receptor ligand protein complexes and this competitive ligand is going to bind to your enzyme.

And that is how this enzyme is going to be dissociated from the matrix and once you dialyze this particular sample, it is going to give you the purified enzyme or you can use the non-specific approaches. For example, you can use a non-specific elusion approach where you can actually change the pH or the ionic interaction.

If you remember in the previous slide we have discussed that if these receptor ligand interactions are mainly depends on the hydrogen bonding, vander waal interactions, pi pi interaction, as well as the salt bridge interactions. So, these interactions also could be sensitive to the change in pH or the ionic strength. So, if you change the pH or the ionic strength you are also could be able to break the interaction between the receptor ligand complexes.

And that actually will give you the free protein or free enzyme and that is how also you can get the purified enzyme. The only difference between a specific elution using a competitive ligand versus a non-specific elution is that when you do a non-specific elution the enzyme may come off along with the impurities whereas when you use the affinity elutions the enzyme will only come out when it is going to have the specific ligand. In those cases what will happen is the level of purity is going to be better.

So, that is what the mutual collision between the receptor on the matrix and the ligand from the different molecule test the affinity between them and consequently the best choice binds to the receptor whereas all other molecules do not bind and appear in a flow through. So, this

is about the binding of your enzyme because it is going to have a very high affinity for the immobilized ligands. All other enzymes are not going to have the affinity, that is why they will go through into the flow through. Then you do a mild wash step so that that will remove the weakly bound molecules on the matrix.

And then what you do is you add a counter ligand which is going to elute the bound molecule through a competition between the matrix and the counter ligand. Now, what is the advantage of affinity chromatography versus the earlier chromatography what we have discussed like ion exchange chromatography, hydrophobic interaction chromatography, or the gel filtration chromatography?

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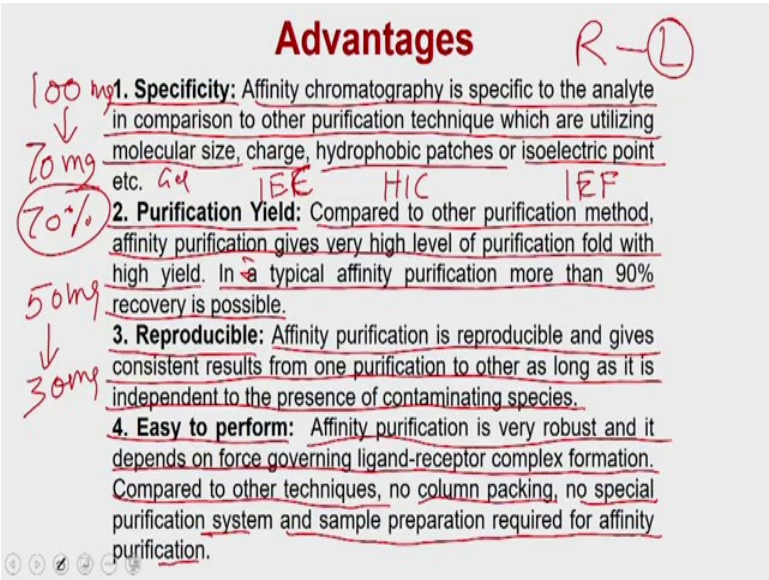

One of the major advantage of affinity chromatography is that it is very-very specific. So, that actually is going to give you the, that will make the purification easier. So, it is very-very specific. As we discussed the receptor is going to interact only with the ligand.

And that is why the affinity chromatography is going to be specific to the ligand or the receptor what you are using. In comparison to other purification techniques which you are utilizing either the molecular sizes which is actually the case in the case of gel filtration or the charge which is actually in the case of ion exchange chromatography or hydrophobic patches which is actually in the case of HIC or hydrophobic interaction chromatography or isoelectric point which is actually in the case of isoelectric focusing, so this is the ion exchange chromatography.

And the second advantage is the purification. So, in the first the specificity is concerned like when you use the ion exchange chromatography, you are actually exploiting the affinity between positively charged matrix versus the negatively charged analytes but what it actually recognizes is the positive and negative charges. It does not recognize whether the negative charge is present on your protein or the negative charge is present on the impurities.

That is why the ion exchange chromatography is not very specific for your analyte whereas in the case of affinity chromatography you are going to have the receptor or the ligand whichever you have immobilized onto the matrix, it is going to have a very exclusive affinity for the counterparts. Which means, if you have immobilized the receptor it is going to have the affinity for ligands or if you have immobilized the ligand it is going to have the affinity for the receptor.

Now, the purification yield, the compared to the other purification method, for example the molecular size, gel filtration, ion exchange or HIC, the affinity purification gives very high level of purification fold with a high yield. What is meant by the yield is, that suppose you have the 100 mg of protein in your crude lysate. And the yield means the out of 100 mg how many milligrams of purified protein you are getting at the end of the purification?

Suppose I am getting 70 mg of purification or 70 mg of protein, that means my yield is 70 percent. So, if you divide the 70 by 100, you are going to get the 70 percent. So, that is why the yield is very high which means if you have even very small amount of protein because the yield is very high you are going to have the very less losses in the purification protocols. For example, if you use the ion exchange chromatography what will happen is, that when you put

the elution, the protein of your interest as well as the impurities are also going to be removed and then you have to choose the fractions where the impurities are less and yours protein is going to be more.

And in this process what will happen is that some of the fractions where your protein is less but the impurities are more, you have to discard or you should not include those into the subsequent purification steps.

For example, if you would like to purify your protein using the conventional chromatography techniques then what you will do is, you will first run the ion exchange chromatography. From the ion exchange chromatography you are going to have few fractions where the protein of your interest is going to be high, impurities are going to be less but you are going to discard all those fractions where your protein of interest is less.

So, now suppose you got the four fractions where your protein of interest is more and there you have the 50 mg of protein in those all four fractions. Rest 50 mg protein is distributed in all other fractions, that you are going to lose in first step itself.

Now this 50 mg of protein you have loaded onto the hydrophobic interaction chromatography, out of this 50 mg suppose again you got the similar kind of pattern, you got the five or six fractions where your protein of interest is more compared to the all other protein. Then you will take those fractions but you will discard all other fractions where your protein is less but the impurities are more.

And suppose at the end you are going to get the 30 mg of protein which has your protein and no impurities, which means by using the conventional chromatography techniques such as ion exchange chromatography or the hydrophobic interaction chromatography or the combination of these, you have purified using a 100 mg protein, you have purified approximately 30 mg protein which means your yield is 30 percent compared to that when you are doing the similar kind of purifications in the affinity chromatography, you may get even up to 90 percent yield.

So, in a typical affinity chromatography purifications more than 90 percent recovery is possible because it is going to be very-very exclusive, so it will bind every molecule which is being generated or which is being produced in your host cells compared to that ion exchange chromatography you may have to lose some of those proteins.

Because you would like to not add the impurities, the purpose of purification is that you discard the impurities and you keep the protein of your interest. Now, number three is reproducibility, affinity purification is reproducible and give consistent result from one purification to other as long as it is independent to the presence of contaminating species.

Number four, the affinity purification is easy to perform. Affinity purification is very robust and it depends on the forces governing ligand-receptor complex formation. Compared to the other techniques no column packing, no special purification systems and the sample preparation required for affinity purification.

So, this is actually the point number four that it is very easy because what affinity purification requires that you have the beads, these beads are having the receptor or the ligand immobilized. And then they have the exclusive affinity for their counterparts like ligand or the receptor.

So, in these cases you do not need a very-very sophisticated system so that you will be able to use the purification system or you do not need even to these beads to be packed. What you can do is you just use them as such, you incubate your beads along with the complex mixture and what the beat will going to do is it will going to bind only your specific analyte which are containing the receptor or the ligand.

And then you just do a washing step and that is it. And then you do a elution using the competitive ions and that is how you are going to get a very high yield, reproducible results and it is very easy to use. And at the end because it does not require the column packing, it does not require the purification system, it also is going to keep the things at a very-very low infrastructure, which means even if you do not have the columns you can be able to do the chromatography in simply by in a beaker.

So, that if you keep the beads in a beaker, incubate that beads along with your crude samples then you spin down, remove the flow through, and then you just wash once and then your beads are ready to get eluted by the specific analyte. So that is why affinity chromatography brings lot of advantages compared to the all other chromatography techniques.

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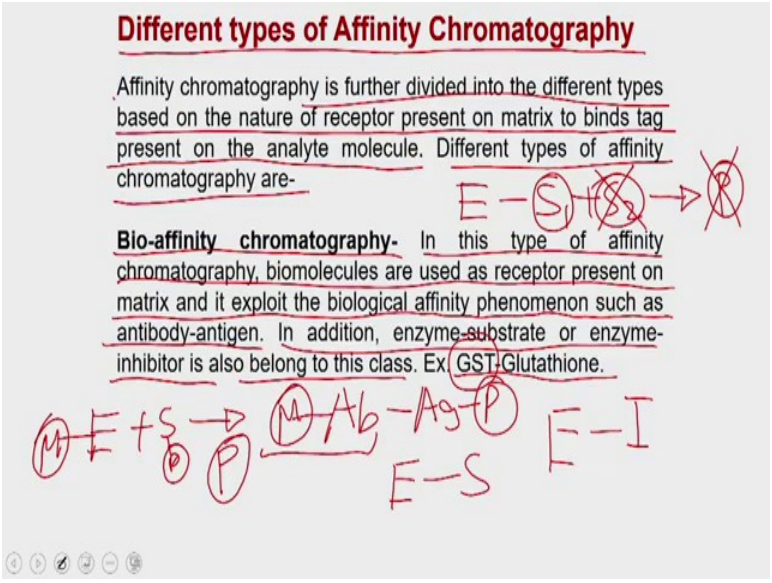

Now we have different types of affinity chromatography; affinity chromatography is further divided into different types based on the nature of the receptor present on the matrix to bind the ligand present on the analyte molecules. Different types of affinity chromatographies are bio-affinity chromatography.

Since the affinity is going to exploit the idea that you can have exclusive affinity between the receptor and ligand, the receptor ligand complexes could be the biological in nature, for example they are already being predetermined where you have a receptor and you have a ligand. So this ligand-receptor pair can be used. Sometimes, you can have the synthetic receptor or synthetic ligand which is going to have a very-very exclusive affinity for a particular type of protein. In those cases it is going to be synthetic. And in sometime you can have the other variants of the affinity chromatography.

And that is why the affinity chromatography exclusively depends on the pair of receptor ligand you have taken for performing the chromatography. So, in the first class is called bio-affinity chromatography, in this type of affinity chromatography the bio-molecules are used as a receptor present on the matrix and it exploits the biological affinity phenomenon such as the antibody-antigen.

In addition, the enzyme-substrate or enzyme-inhibitor is also belong to this class. So, in this particular bio-affinity chromatography what you have is you can have the antibody to anti-gen. So, you know that the all antigens are when they were being injected into the human

body, they are actually generating the antibodies and these antibodies are very-very-very specific for that particular antigen.

So, if you can, you can use the antibody and antigen complexes where you can put the antigens onto the proteins and you can have the antibodies coupled to the matrix. And that you can use this combination, you can use to purify the particular type of protein. So, this antigen you can couple to the protein and the antibodies you can couple to the matrix and that is how you can actually use this particular type of column.

So you can have antibody matrix and antigen containing proteins, you can flow. Then subsequently you can add the free antigen and that will compete with the antigen which is bound to the antibodies along with the protein. That is how it is going to elute the antigen containing proteins from the column, and that is how you are going to get the purified protein.

The other example is where you can have the enzyme either making interaction with the substrate or you can have the enzyme which is making an interaction with the inhibiter. Now what is the important when you are using the enzyme substrate complexes? Now in the case of enzyme-substrate complexes all though the enzyme substrate interaction is also very-very specific but majority of enzyme what people use in the bio-affinity chromatography is the combinations where the enzyme does not process these substrate.

Let me tell you the example, so suppose I have an enzyme and I have added the substrate, what will happen? The first step itself that the enzyme is going to produce the product because it is going to process this particular substrate and ultimately I am going to get the product which means this enzyme is processing this particular substrate and giving me the product.

If I use this particular type of combination where the enzyme has the ability to use this particular substrate then ultimately what will happen? So, while I was doing the chromatography I have coupled this particular substrate to the protein which I would like to purify.

Now what will happen? As soon as the enzyme which is coupled to the matrix, as soon as the enzyme will see the substrate what will happen? It is going to, it will not going to bind, it will go into bind this substrate along with the protein. And while I am washing the enzyme will

catalyze the reaction and ultimately what will happen? It is going to generate a product and as soon as it will generate the product it is going to release the product from the active side.

And that is what the enzyme kinetics is, that it actually processes the substrate and then it generate the product. If you use this particular kind of combinations, what will happen is the substrate will go and bind the enzyme, enzyme will process these substrates and generate the product and ultimately it is not going to bind your protein.

So, in all these combinations where you are using the enzyme substrate or the enzyme inhibitor combinations, you have to ensure this particular enzyme requires additional substrate for catalyzing the reactions. Which means, what I am talking about is that you should have this particular kind of combination where the enzyme requires substrate 1, substrate 2, and then only it will give you the product.

So, what will happen is even if you use the substrate 1 as the ligand and you will not provide the substrate 2, then there will be no product formation. And the interaction between the enzyme and substrate is going to be very-very stable, it is not going to dissociate because the substrate is not going to be processed.

So, that is why this kind of bio-affinity chromatography requires a certain type of conditions and that is why we have to be little careful when we choose these pairs for performing the bio-affinity chromatography. So, in the case of enzyme substrate bio-affinity chromatography, the classical example is the GST-glutathione. So, GST is enzyme which is called Glutathione S-transferase and glutathione is the ligand which is going to bind. But the GST requires additional substrates where, so what is the function of the GST? The function of GST is that it actually takes up the glutathione and then attach those glutathione to the molecules which need to be detoxification reactions. Which means the purpose of GST is to make the toxic products more water soluble so that they could be detoxified, they could be excreted from the body.

So, for this particular type of reactions, Glutathione S-transferase requires glutathione as well as the other substrates where it is going to transfer the glutathione. But in this particular reaction or in these particular combinations we are not providing the second substrate and that is why the glutathione and the GST interaction is going to be stable enough to give you the purifications.

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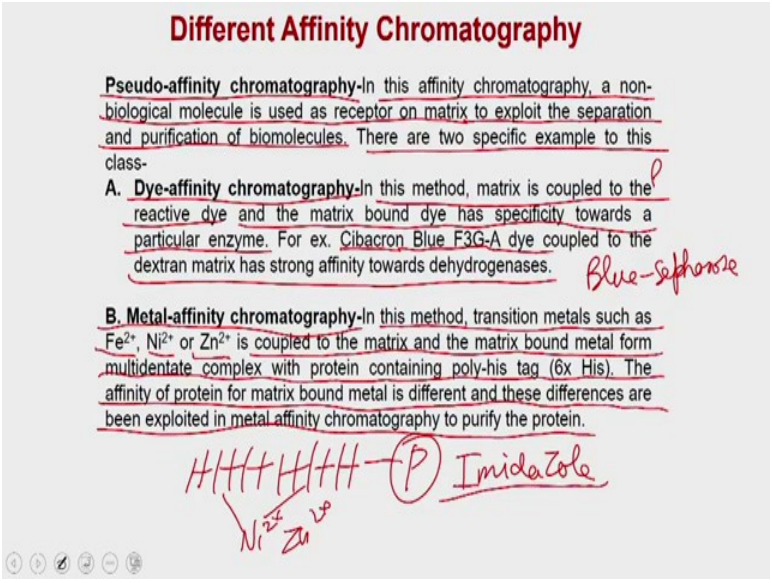

And the second is the Pseudo-affinity chromatography, so Pseudo-affinity chromatography is in this affinity chromatography a non-biological molecule is used as a receptor on the matrix to exploit the separation and purification of biomolecules.

There are two specific examples. Dye-affinity chromatography, so in the Dye-affinity chromatography the matrix is coupled to a reactive dye and the matrix bound dye has a very specific specificity towards a particular enzyme class. One of the classical example is Cibacron Blue F3G-A dye, so this is called blue Sepharose and this particular dye when it is coupled to a Sepharose beads, is actually making affinity chromatography column which is called as the Blue Sepharose.

And the blue-sepharose is having a very-very strong affinity from the dehydrogenases. The enzyme which contains NADH. And it is actually having affinity for the dehydrogenase which contains NADH, but as I said this is not the affinity which is exclusive for a particular type of dehydrogenase.

It is going to bind pyruvate dehydrogenase, it is going to bind lactate dehydrogenase, it can bind any dehydrogenase which actually utilizes the NADH as a cofactor or the substrate. Now then second is the metal-affinity chromatography, in this particular method the transition metals such as iron, nickel, or zinc is coupled to the matrix and the matrix bound metal form multidentate complex with protein containing poly-his tag. The affinity of the protein for matrix bound metal is different and these differences are being exploited in metal-affinity chromatography to purify the protein.

So, in metal affinity chromatography what you have is, you have the matrix on which the transition metals such as the nickel or zinc is being coordinately bound and what will happen is the nickel or the zinc is having the two coordinate bonds which are empty and these two coordinates bonds are actually having a very high affinity for a stretch of peptide which contains a series of histidines.

So this series of histidine molecules mostly the six histidine molecules are making a small biofore, and this small biofore has a very strong affinity for the matrix bound nickel or matrix bound zinc. And because of that it will go and bind. So, all other proteins does not contain a stretch of histidine or even if they contain the histidine, they may not contain a stretch of histidine at the one corner of the protein.

So, what will happen is your protein is over expressed like having a histidine tag on the one corner of the protein. Either it could be on the n terminals or on the c terminals, so in that case what will happen is this particular type of hitsidine tag will go and bind to the nickel metals which are being immobilized onto the matrix and then what will happen is you will do a washing step, so that will remove all other protein because all other proteins are not going to have the histidine tag.

But they will nonspecifically adsorb onto the matrix and then you will elute using the competitive ligand. In this case we do not use the histidine but you can use the imidazole as a competitive ligand and that actually will go into elute these proteins from the matrix.

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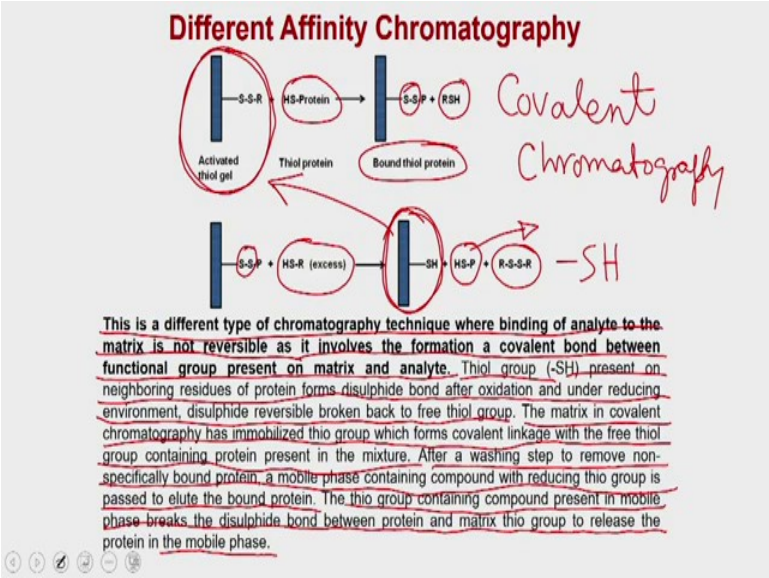

Now the third is covalent affinity chromatography, covalent chromatography. So this is a different type of chromatography techniques where binding of analyte to the matrix is not reversible as it involves the formation of a covalent bond between the functional group present on the matrix and the analyte. So, this is actually a very-very different types of affinity chromatography, that is why it is called as covalent affinity chromatography.

Where you are going to have the irreversible bond being formed between the functional group which are present on the protein and the functional group which are present on the matrix. If you remember most of the chromatography techniques what we have discussed so far whether it is ion exchange chromatography, hydrophobic interaction chromatography or gel filtration chromatography or even other classes of affinity chromatography, you are making a receptor like an interaction, but these interactions are non-covalent. These interactions only involves either the hydrogen bonding, vander waal forces, pi pi interactions, salt bridge interactions and all other kind of interactions but they are not making a covalent interactions.

So, because of that you can actually break these interactions and you can be able to reuse these columns again and again. Whereas in the case of covalent chromatography you are going to have a specific group which is present on your protein, so as soon as it will react with the column it is going to make a specific ligand or a specific bond. That bond will be very specific, it will not going to form with other proteins and that is why the covalent chromatography will give a specificity and that is why it is fall under the category of affinity chromatography.

But at the end what will going into happen is, the protein will go and tag permanently to your beads and that is why when you remove this particular protein from the beads, these beads are not going to be functional for reusing it again. If you want to reuse them again, you have to use or you have to prepare fresh batch of the beads. So, what will happen is the Thiol groups the minus SH or groups present on the neighboring residues of protein forms disulphide bond after oxidation and under reducing environment. So, disulphide reversible broken back to the free Thiol group.

The matrix in covalent chromatography has immobilized thiol group which forms covalent linkage with the free thiol groups containing protein present in the mixture. So, in the covalent chromatography what you have is, you have the activated thiol group which is present onto the beads.

Then what you do is, you add your protein which also contains the free thiol groups and under the reducing and oxidizing environment the protein will go and bind covalently to the matrix with the help of a disulphide linkage and the group which is attached to the matrix is being released.

Now once you have this formed then what you have to do is, you do a washing step to remove the non-specifically bound protein and then you add a mobile phase which contains the reducing thiol groups and that you pass to elute the protein. Then what you add is you add the elution buffer which contains the reducing thiol groups and what will happen is that actually is going to reduce the disulphide linkages between the two sulphurs and that actually is going to generate the matrix which is containing the free thiol groups.

And your protein is going to be eluted and ultimately the both functional groups are going to form. But you see this particular type of matrix and the matrix what you have is very-very different which means you have to generate or regenerate from this particular matrix to this matrix to perform the another round of purifications.

The thiol groups containing compound present in the mobile phase break the disulphide bond between the protein and matrix thiol group to release the protein in the mobile phase and that is why the covalent chromatography is a destructive chromatography where it actually provides the specicity because not all the proteins are going to go and couple to this particular functional group which is present on the matrix.

But once it binds you have to add the free thiol groups and that free thiol groups are not only going to elute the protein from the matrix but it also going to destroy the functional group which is present on the matrix and as a result, this matrix need to be processed again. You need to activate them again so that they can be able to reuse, or they can able to be use for another round of protein purifications.

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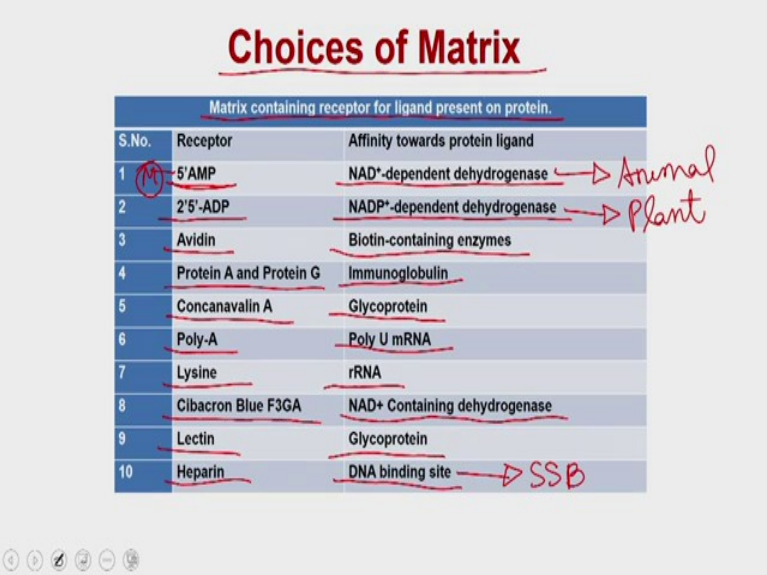

So, now what are the matrix which are available for the affinity chromatography? So, I am giving you a simple example of different matrix. We have a very extensive list of the matrix that people can use. So, in this you have the receptor I have given a receptor. And then I have given you the ligand on which towards what they have the affinity, so all these receptors are coupled to the beads or the matrix.

So, in the case of 5 prime AMP, 5 prime AMP is having a very high affinity for NAD plus dependent dehydrogenase. Then you have 2 prime 5 prime ADP that is having the affinity towards the NAD plus dependent dehydrogenase. So remember that NAD plus dependent dehydrogenases are mostly being present in the animal, whereas the NADP plus dependent dehydrogenases are mostly being present in the plant.

Then you have the avidin, so avidin has affinity for the biotin. Then we have a protein A and protein G which has the affinity for the IGG. Then we have the concavalin A that has an affinity for the glycoprotein. Then we have a poly-A, poly-A has the affinity for poly U. Then we have a lysine that has the affinity for ribosomal RNA. Then we have the cibacron blue F3GA and that has an affinity for NAD plus containing dehydrogenase. Then we have the lectin and lectin have affinity for glycoprotein. Then we have the heparin and heparin has an affinity for DNA binding protein such as the SSB and all other proteins which are binding directly to the DNA and are important for performing the replication, transcription and all that.

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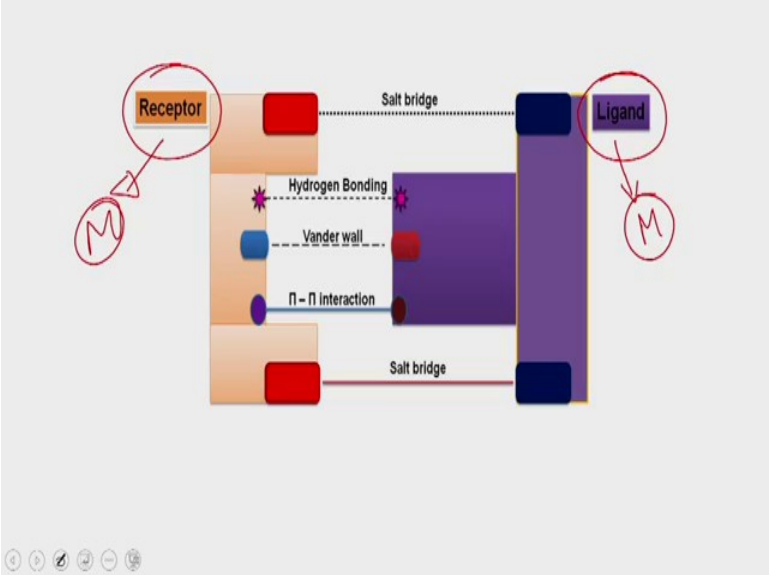

So, at the end now what we have discussed? We have discussed about the receptors as well as the ligand. And if you would like to perform the affinity chromatography you have to generate the receptors or you have to generate the ligands and then depending on the easiness of the coupling you have either couple the receptor to the matrix or you have to couple the ligands to the matrix.

And then in the second step suppose you have coupled the receptor to the ligand, then you are going to produce the protein which are going to contain the ligand through recombinant DNA technology, you are going to generate the ligands onto the protein and vice-versa.

And then you are going to perform the chromatography techniques, so if you would like to perform affinity chromatography, the first step is that you have to generate the receptors or the ligands. Then the second step is you have to couple this receptor to the matrix. And the third step is you have to produce a protein which contains the ligands attached to these. And the fourth is then you have to perform the chromatography and you have to purify the protein.

So, with this we would like to conclude our lecture here. In our subsequent lecture we are going to study or we are going to discuss how to generate the receptors, how to couple these receptors to the matrix. And at the end, we are also going to discuss about the purification, what are the different steps, what you have to use for purifying the protein.

Then we will take couple of examples of the different chromatography techniques or different affinity chromatography techniques what we have discussed, which means we are going to take an example of bio-affinity chromatography or we are going to take few examples of

pseudo-affinity chromatography like metal affinity chromatography or the dye based affinity chromatography technique.

So, all these examples also we are going to discuss in our subsequent lecture. So, with this I would to conclude our lecture here and in the next lecture we are going to discuss about how to generate the receptors and how to couple them, thank you.