Experimental Biotechnology
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Lecture - 23
Affinity Chromatography - Part - 1

Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT, Guwahati. And in this series what we are discussing? We were discussing about the different types of chromatography techniques. So, today we are going to start about discussing about the affinity chromatography.

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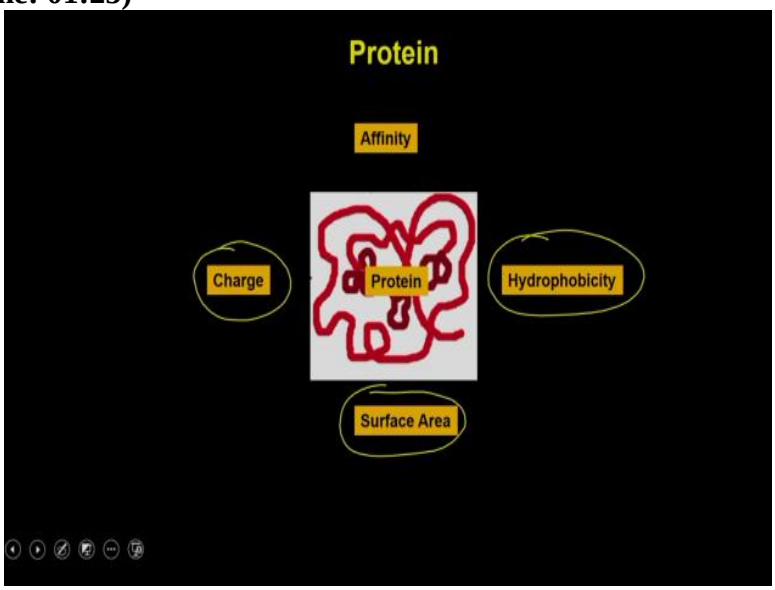

So, as you can recall that the protein is providing the different types of properties, and so, far we have discussed about how to exploit the charge, how to exploit the hydrophobicity and how to exploit the surface area of a protein by discussing about different types of chromatography techniques.

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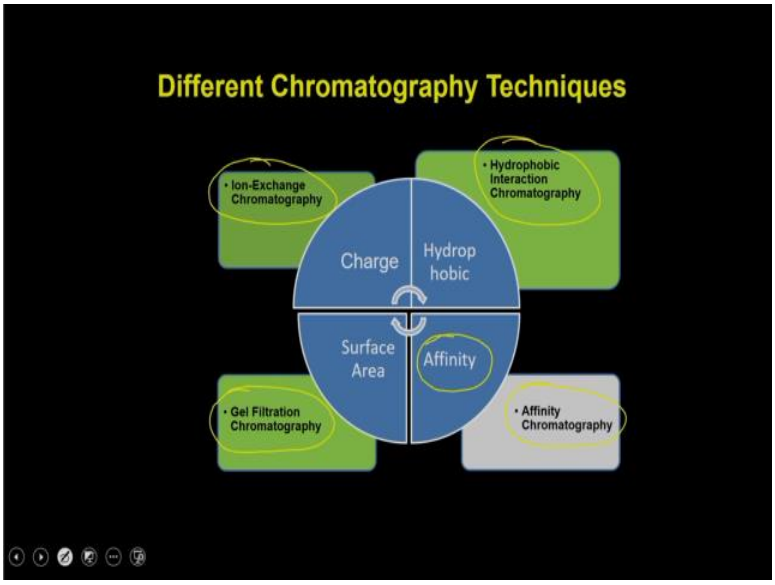

In the case of charge, we have discussed about the ion exchange chromatography, in the case of hydrophobic patches, we have discussed about the hydrophobic interaction chromatography and in the previous lecture, we have discussed about how you can be able to exploit the surface area of a protein in the utilizing the gel filtration chromatography? So, today we are going to start discussing about the affinity as a parameter and the technique what we are going to discuss is the affinity chromatography.

So, as the name suggests the affinity chromatography it actually works on the exclusive recognition principles of a particular receptor for a ligand and that is the basic principle of the affinity chromatography.

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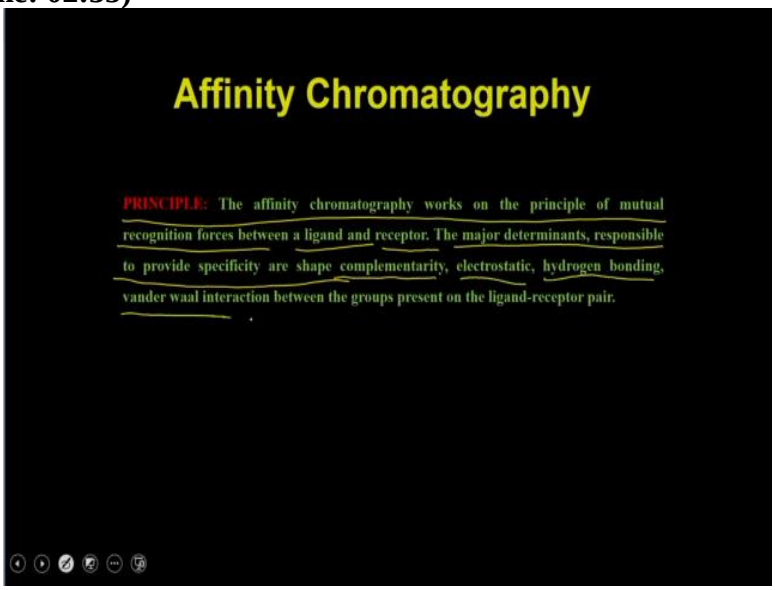

So, the affinity chromatography relies on the basic principle that it works on the principle of the mutual recognition forces between the ligand and the receptor. The major determinants

which are responsible to provide the specificity are the shape complementarity, electrostatic interactions, hydrogen bonding, vander waal interactions between the groups present on the ligand and receptor pair.

So, you can imagine that we have a receptor and every receptor has its own cognate ligand. So, both of these receptor and the ligands are actually been recognized with the help of the different types of interactions. So, let us take an example and understand this phenomena.

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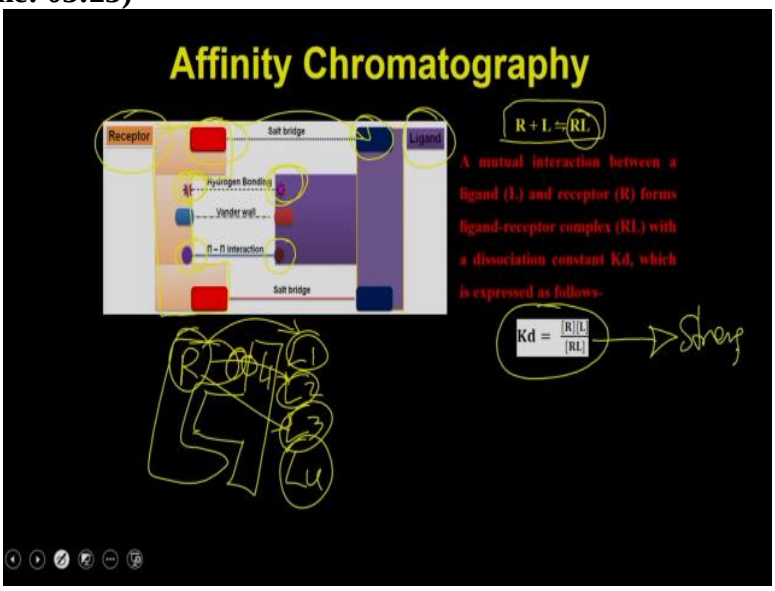

So, you can imagine that we have a receptor and we have a ligand and you can see that the first thing what actually going to bring the receptor to its ligand is the shape complementarity which means, the receptor which is actually maintaining a shape is looking for a ligand which is maintaining the completely you know complimentary shape. For example, if I have to design a ligand, I will go into design a ligand which is going to be a complete complementary to its shape.

But, apart from that, so, you can imagine that receptor is having probably the options between the multiple ligand for example, L 1, L 2 and L 3, which all of these probably could have the similar kind of 3 dimensional confirmations, but, what is actually going to decide whether the receptor is going to make the pair with the L 1 or L 2 or L 3 is actually the additional determinant factor.

For example, you may have some of the hydrogen bonding donor groups or the salt bridge interaction group on the ligand and you might have the complimentary groups on to the

receptor. So, that is what you have a salt bridge interactions on the receptor that actually should have a complimentary group on to the ligand. And that actually is going to increase the affinity or the binding of ligand to the receptor to form the complex in addition to that, it also means going to looking for the hydrogen bonding.

So, if you have a hydrogen bonding donor, you should have the hydrogen bonding acceptor, so that there will be an interaction between these 2 groups. Similarly, if you have a group on to the receptor, which is actually been involved into a pi-pi interaction, then you should also have the complimentary group present onto the ligand and the position as well as the group is going to be identical or going to be complement to each other.

So, that the receptor is going to make a complex with the ligand and once the receptor is going to make pair with the ligand, it is actually going to recognize. So, as you can see, this receptor has the 4 choices, it has the L 1, L 2, L 3 and maybe L 4 and out of these ligands it is actually going to utilize all these additional determinants factor to decide which is actually going to be the actual ligands.

And that is how it is actually going to make the pair with either L 1 or L 4 or L 2 or L 3 and once it makes the pair it is actually going to maintain a equilibrium. So, if the receptor is going to interact with the ligand it is going to make the receptor ligand pairs and if you would like to determine the dissociation constant, you can use this equation where the dissociation constant is the concentration of the receptor ligand divided by the concentration of the receptor ligand complex.

And that can be used to determine the strength of the interaction which means, if you have receptor and ligands are complementary to each other and they are making very tight complex the Kd value is going to be very small because the you are going to have very high concentration of the receptor ligand complex.

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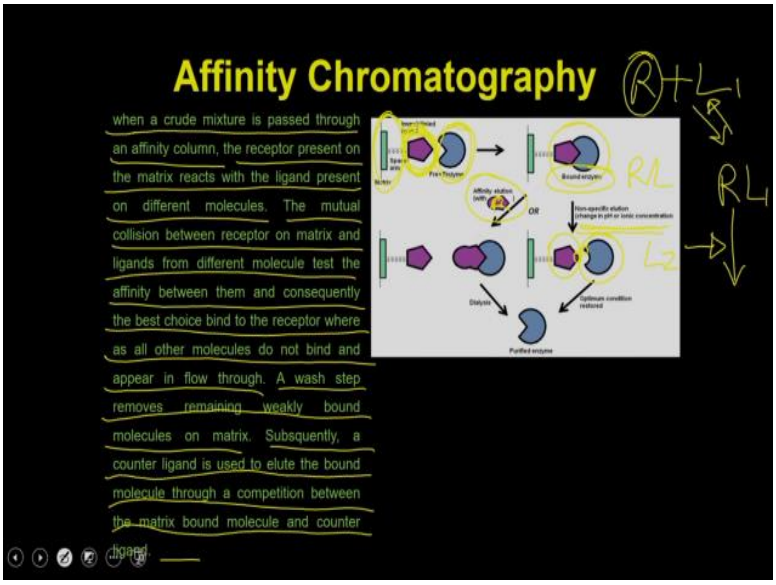

How you can utilize the, this kind of phenomena in affinity chromatography? So, what you can do is you can either tag the receptor or the ligand to the beads and then you can actually be able to purify the complementary partner for example, when a crude mixture. So, in this example, what we have done we have taken a matrix and we have used and we have coupled the ligands and what will happen is if you are going to flow a crude mixture onto an affinity column, where you have actually coupled the ligand onto the matrix.

The receptor is present onto the matrix react with the ligand present on the different molecules, the mutual collisions between the receptor on the matrix and the ligands from the different test molecule will determine the affinity between them and consequently, the best choice binds to the receptor whereas, all other molecules will do not bind and appear in the flow through then what you do a wash step you will going to remove the remaining weakly bound molecules present on the matrix.

And ultimately, you are going to put the counter ligand which is going to be used to elute the bound molecule through a competition between the matrix bound molecule and the counter ligand. So, for example, in this case, what we have done we have taken a matrix we have coupled a ligand. So, what will happen is now, this ligand has an affinity for the enzyme which is actually going to serve as a receptor. So, in this process, it is actually going to form the receptor ligand complex.

Now, this receptor once the receptor ligand complex is bound and you can imagine that you may have the different types of enzyme which actually going probably we have the similar

kind of active site, but they may be having the subtle differences and because of that, this ligand is only going to recognize this enzyme and to form the receptor ligand complex. Now, after this you have the 2 options, either you can actually do the illusion with the help of the nonspecific illusion.

For example, you can change the pH or you can change the ionic strength of the buffer by doing so, what you are actually doing is you are actually changing the interacting residues present onto the ligand or the receptor. So, if you change the pH for example, you may actually have the aspartate in the ionization ionized form. And if you change the pH, you might actually bring the aspartate into the non ionized form or the neutral form.

And because of that, what will happen is the aspartate may not be able to make the interaction with the other residues which is present onto the ligand. And as a result, it is actually going to be having a weaker interaction and as a result ultimately the, it may actually break the complex apart and that is how you can be able to elute the enzyme from the matrix bound the ligand the alternate is that you can actually be able to utilize the affinity illusions with the help of the competitive receptor.

So, for example, in this case, we have put a competitive receptor and if you put the competitive receptor, what will happen is this competitive receptor is going to also compete with the enzyme. So, you can imagine that the receptor is making a complex with the receptor ligand to give you the receptor ligand complex. Now, what you do is you add the L 2 which is also actually going to have the affinity for the receptor and L 2 is probably as good as the L 1.

So, in this case, what will happen is, as you might have seen that this is actually a reversible reaction. So, what will happen is the RL 1 is going to disassociate between the R and plus L 1 and then if you increase the consideration of the RL 2, what will happen is that it will eventually going form the RL 2 complexes and if you keep increasing the concentration of the L 2, this complete RL 1 is going to be broken down and ultimately what you are going to see is we are going to see the formation of the RL 2 complexes.

And ultimately it is actually going to elute the enzyme which is going to be bound to the matrix with the help of the interaction with the ligands. So, this is the basic principle through

which you can be able to exploit the affinity phenomena to purify the different complexes exploiting the receptor ligand counts concept or the affinity concept.

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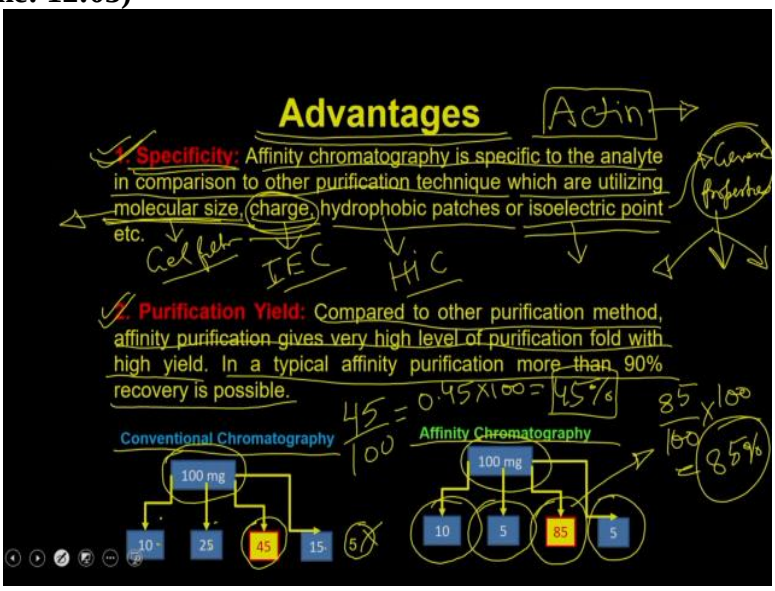

Now, what is the advantage of the affinity chromatography compared to the other conventional or the traditional affinity chromatography techniques? So, the major advantage is that the affinity chromatography is very specific as we discussed the receptor is very specific for the, its particular ligand. So, it actually provide the specificity so, if you are even if you have a very, very crude mixture, you can be able to purify it with the help of the affinity chromatography.

So, affinity chromatography is a specific to the analyte in comparison to the other purification techniques, which are utilizing either the molecular size. So, molecular size is been identified by the gel filtration chromatography, charge is been identified by the ionization chromatography, hydrophobic patches are being identified in the HiC and whereas, some places you also can use the isoelectric point which is actually been utilized in the different chromatography techniques.

But all these parameters with either the molecular size, charge, hydrophobic patches or isoelectric points are actually the generic properties which means, you might have to you know, so, many protein might have these properties and that is how, it actually not going to provide you the specificity to isolate the particular analyte from a crude mixture simply by utilizing these properties because if you go with the molecular size.

For example, the molecular size, every protein is going to have some size some protein might be 10 Kda, some protein could be 50 Kda, some protein could be 80 Kda. So, even if you run a gel filtration chromatography, it is actually going to separate the molecule based on the sizes, but it still it is not going to give you the a specifically this particular analyte it is actually going to give you an analyte which are falling under the within the range of 10 to 50 Kda.

So, if that is the case, you are going to get the multiple analytes. And similarly, if you go with the ion exchange chromatography and you would like to utilize the charge, the same is phenomena the charge the multiple proteins will have the multiple amount of pro charge and you can be able to utilize ion exchange chromatography to purify but you cannot be able to purify the particular analyte, for example, if I have to purify the actin from a crude mixture, I cannot even if the I know the molecular properties of the actin, what is the charge present?

What is the size of the actin? Whether the hydrophobic patches are present on the actin or not? But you cannot be able to purify the actin from the crude extract or crude mixture in a one goal, you might have to utilize multiple these techniques to get a purified actin from the crude mixture; whereas in the case of affinity chromatography, if I would like to utilize the affinity chromatography, I might be able to purify the protein the actin you by utilizing the receptors, which are actually having the affinity for the actin in single goal.

So, that specificity is a very, very important and exclusive advantage of the affinity chromatography, the second is the purification yield so, compared to the other purification methods, the affinity chromatography give very high level of purification fold with a high yield. So, in a typical affinity more than 90% recovery is possible. So, what is mean by the purification yield is that, if suppose you started with the 100 millgrams of crude extract, or if the your molecule is present as like 100 milligrams in the beginning.

And suppose, at the end of the purification, when you got the purified protein, whatever the amount you get is actually been called as a purification yield, for example, you can understand that in a conventional chromatography, for example, the gel filtration chromatography or ion exchange chromatography or hydrophobic interaction chromatography, if you start with the 100 milligrams of protein.

So, what will happen is after the first round of the purifications, you are going to get the different fractions and all these different fractions, suppose, in all these different fractions, you have the different proteins and your protein also is present in the different fractions, but, so, you have 10 mg protein, 25 mg protein, 45 mg protein and 15 mg protein and if you add all these, you are going to see that there will be a loss of 5 mg protein which is not been countable.

And suppose, this is the your purified fraction where your desirable protein is present in the single band and it is 100% pure, then what will happen is the purification yield in this case is 45 / 100, which is actually only the 45% or 0.45. So, if you divide multiplied by this 100, you are going to get the 45% is the yield, which means, you started with the 100 milligrams of protein, and ultimately, you got only 45 milligrams of protein, which means, your yield of the purification in this case is the 45%.

For example, and compared to that, if you go with the affinity chromatography, if you started with 100 milligrams of protein, what will happen is that, it is still going to give you the different fractions where your protein of interest is going to be present, but the major chunk is going to be present in the 1 fraction, and that actually is going to give you the purified protein and imagine that what is going to be purification yield 85 / 100 into 100.

So, that is going to be 85% which means, you are going to recover whatever you have, having in the crude mixture simply by employing the affinity chromatography. So, since the end is going to be more, it is actually going to be economically more viable, because you are not going to waste these your protein in with the other fractions. For example, in these cases, you are actually losing the more than 50% in other fractions where the other proteins are present in a higher quantities.

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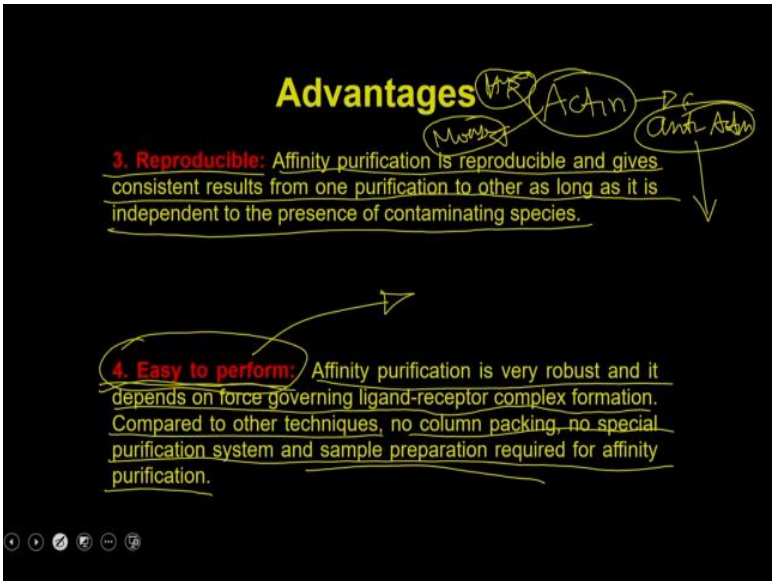

The third advantage is that the affinity chromatography is very, very reproducible. So, affinity chromatography is reproducible and it gives the consistent result from one purification to another, as long as it is independent to the presence of the contaminating species, which means, whether, so, suppose I want to purify the actin and I am using the anti actin antibodies as the receptor to purify the actin.

So, what will happen is what is mean by reproducible is that even if I am verifying the actin from the mouse or if I am purifying the actin from the human the process is going to be the remain same whereas, you know that the contaminating protein which are present in the mouse or the contaminating protein which are present in the human are going to be different. So, affinity chromatography does not depends on the other molecules or other proteins which are present in the cell lysate.

Whereas, all other chromatography techniques suppose I have use I will use the ion exchange chromatography, then what will happen, I have to devise a statergy for the purification of actin from the mouse, I have to devise the strategies to purify the actin from the human, because what we have to consider is the contaminating proteins, what we have to consider is how many proteins are still matching with the other proteins which is actually closer to the actin.

So, if you have 10 different proteins, which are matching very close to actin in the mouse, you might have to devise a strategy so, that you get rid of those proteins otherwise, whatever the property you would like to exploit to purify the actin, all those proteins are also going to

show the similar properties. So, that is why the purification with the conventional chromatographic technique.

Whether it is the ion exchange chromatography, hydrophobic interaction chromatography or gel filtration chromatography will depends on contaminating species or contaminating proteins. Whereas, in this case, it is hardly matters as long as your anti actin antibodies anti actin is good enough to bind the actin it hardly matters whether you are purifying the actin from the mouse or the human.

Then the fourth which is actually the most desirable phenomena of the chromatographic technique is that it is easy to perform. So, compared to all other conventional chromatography, the affinity chromatography is very robust and it depends on the force governing the ligand receptor complex formations compared to the other techniques, no column packing, no special purification systems and sample preparation required for the affinity chromatography which means, if it is easy to perform.

It has additional advantage that you do not need to pack the columns because if you remember in the case of gel filtration chromatography, you need to pack you need to know how to pack the column then only you will be able to exploit the gel filtration chromatography column to fractionate the proteins based on the molecular sizes because if you do not pack the column properly.

If you have defects in that column, it is actually going to affect the overall purification yield as well as it is also going to overall affect the resolution of the column. Whereas, in this case, there is no packing required you can actually do the affinity chromatography in a beaker or any other thing apart from that, it actually does not require the sample preparation and all other kinds of things.

And because it is easy to perform, you do not need to train the people which means the affinity chromatography is actually going to save your time to train the manpower or train the people to learn the technique. Whereas, in all other chromatography technique, if it is ion exchange chromatography, hydrophobic interaction chromatography or gel filtration chromatography, the person has to learn the or person has to know that what are the molecular properties and how to utilize and exploit those molecular properties?

How to set up the gradient how to you know? Whether I should use the step gradient or whether I should use the linear gradient and also on? So, these are the these exploiting the gradients and all those requires very, very experienced person to perform those chromatography technique to purify the proteins whereas, in this case, you do not need to have that you just need matrix coupled to the beads and then you just pass through the your sample, it will actually go into bind the sample your ligand.

And then ultimately you just flow through the competitive ligands and that actually is going to remove the proteins or your analyte from the column.

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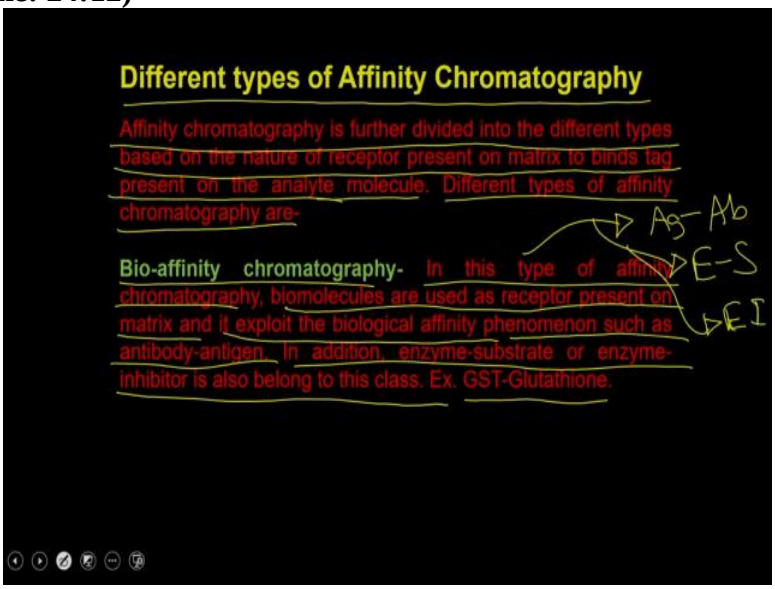

So, affinity chromatography is different types. So, there are different types of affinity chromatography; affinity chromatography is further divided into the different type based on the nature of the receptor present on to the matrix to bind the analyte molecules, different types of affinity chromatography is are bio affinity chromatography. So, in this type of affinity chromatography, the bio molecules are used as a receptor present on the matrix and it exploits the biological affinity phenomena such as the antibody and antigen.

And in addition, the enzyme substrate or the enzyme inhibitor is also belong to this class for example, the GST and glutathione and so, bio affinity chromatography is a chromatography where you are actually utilizing the pre existing natural ligand receptor complexes, which means you are actually knowing that this ligand actually binds to a receptor and you can actually be able to utilize this pair.

So, in this case, you are either tagging the receptor to the beads or to the ligand to the beads and then you can use the cognate pair for the purification substances. So, in this case you have the multiple classes for example, you can use the antigen antibody complexes you can use the enzyme or the substrate complexes or you can use the enzyme as well as the inhibitor as a complexes and either of these combinations since they are naturally occurring into the system, they all are falling under the category of the bio affinity chromatography.

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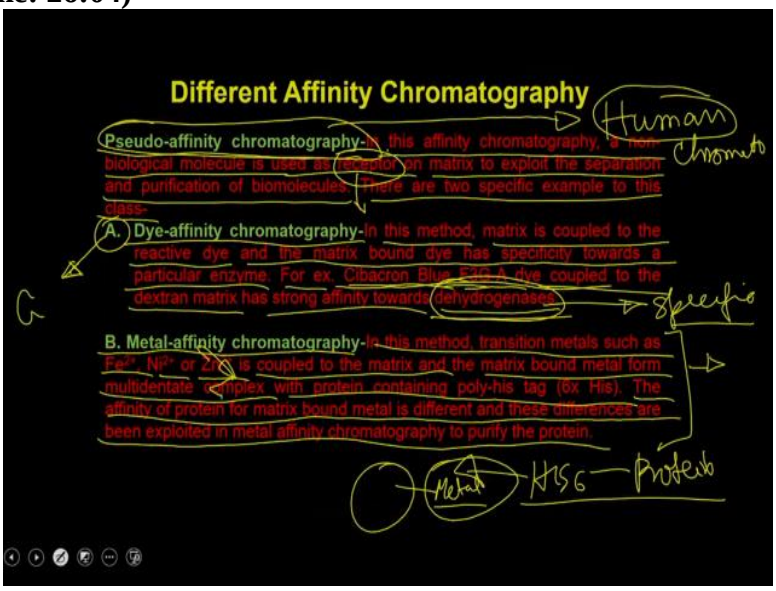

Now, the second class is called as a pseudo affinity chromatography in this affinity chromatography a non biological molecule is used as a receptor on the matrix to exploit the separation and purification of the molecules. These are the 2 specific examples to this class. So, the pseudo affinity chromatography is different from the bio affinity chromatography if I say pseudo affinity chromatography is the manmade chromatography manmade affinity chromatography because, all these receptors are not existing into the nature.

But people have found is that instead of utilizing the actual receptor or the ligand, you can be able to use a chemical molecule which is also going to do the same job. So, in this category, you have the 2 different examples, that for example, that dye affinity chromatography in this method, the matrix is coupled to a reactive dye and the matrix bound dye has the specificity towards a particular enzyme.

For example, you can use the cibacron blue F3GA dye which is and then if you couple it to the dextran matrix, it has a strong affinity for the dehydrogenases. So, the dye affinity

chromatography is having the chromatography where you have the dye molecules or the chemical molecules and you couple it to the matrix and then it has the affinity for a particular class of molecules.

So, what you can actually see is that this affinity is actually generic in nature, which means, it is actually working against the dehydrogenases. So, it does not work against a specific dehydrogenase which means, it does not work against the pyruvate dehydrogenase or some other dehydrogenases it actually works simply against the dehydrogenases which means it will bind all the dehydrogenases with the varying special affinity, but it is actually a generic affinity chromatography.

Now, what you have is you have the metal affinity chromatography in this method the transition metals such as the iron, nickel or zinc is coupled to the matrix and the matrix bound metal form multidentate complex with protein containing polyhistidine tag, this affinity of the protein for the matrix bound metal is different from these differences are being exploited in the metal affinity chromatography to purify the protein.

So, in the metal affinity chromatography, the metal is been coupled to the matrix and then this metal actually when it couples to the matrix, it actually acquires a very, very exclusive affinity for protein which has the histidine tags. And that is how you can be able to utilize this affinity chromatography is where you can be able to put the his tag on to the your protein of your interest which means you can actually produce the protein which has the his tag.

And then that actually is going to bind the beads which are actually containing the metal as the functional group. And as a result this is actually going to form a complex because most of the other proteins does not contain the histidine tag. So, they all other proteins are not going to bind these beads whereas, the metal the your recombinant protein which actually contains the histidine tag is going to bind and ultimate you can just do a competitive ligand, you can supply the competency ligand and that is how you can be able to elute the bound protein from the column.

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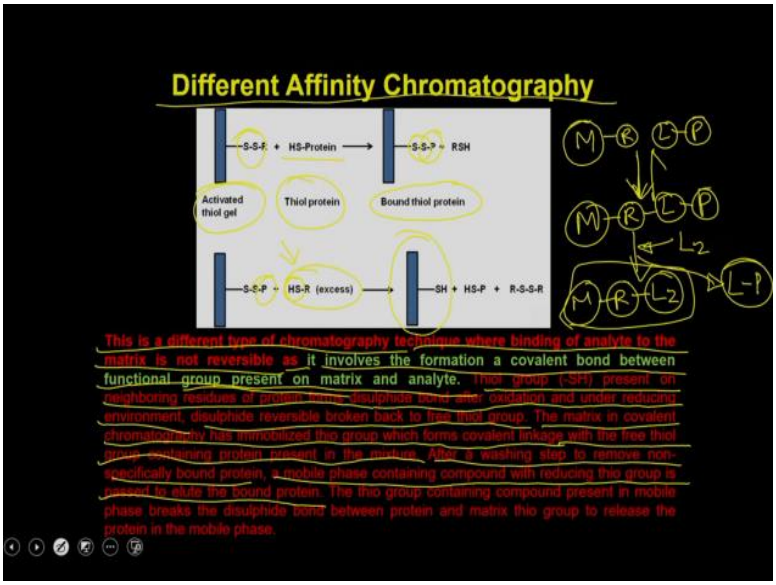

Now, we have a, very different types of affinity chromatography. So, this is a, very different types of affinity chromatography, where the binding of the analyte to the matrix is not reversible as it involves the formation of a covalent bond between the functional group present on the matrix and the analyte. So, what you have discussed in conventional affinity chromatography is that you have a matrix you have a receptor what is being coupled and then what you have is you have a ligand which is coupled with your protein.

So, when you pass through what will happen is the matrix bound receptor is making a complex with the ligand which is present onto the protein and then the third step you are actually going to flow the ligand number 2. So, what will happen is that the matrix is going to where the receptor is bound is actually going to bind the L 2 and then your L bound protein is going to be eluted which means, this process is reversible so, and because it is reversible you can be able to utilize and these beads for multiple rounds.

Whereas, in this case, which is actually called as covalent affinity chromatography, the matrix is actually the functional group which are present onto the matrix is actually going to bind the analyte and there will be a covalent bond between them and because of that, this chromatographic cannot be able to reuse because this is a irreversible bound. So, what happened is that you have the thiol groups present on the neighboring residue of a protein form that disulphide bond after the oxidation.

And under the reducing environment, disulphide reversibly broken back to the free thiol group. The matrix in colon chromatography has immobilized thiol group, which forms the

covalent linkage with the free thiol group containing protein present in the mixture for example, is what you have is do you have the activated thiol gel which actually contains the disulfide bridge.

So, the protein has the sulfhydryl groups and when you incubate this thiol containing proteins, what will happen is that the bound thiol is actually going to bind the protein with the help of the disulfide linkages. And after a washing step you will remove the nonspecifically bound protein a mobile phase containing the compound with reducing thiol group is passed to elute the bound protein.

So, what will happen is to first you do the washing step and then you actually going to add the excess amount of the thiol group. So, what will happen is this thiol group containing chemical agent is going to replace the proteins from this and it is actually going to generate the matrix which is actually containing the activated thiol and the protein is going to be released from the matrix and this is the complex which is going to be formed.

So, the thiol group containing compound present in the mobile phase break the disulfide bond between the protein and the matrix thiol group to release the protein into the mobile phase and that is how you can be able to elute but, what will happen at the end is that you are actually going to lose the matrix because this matrix is not the same as it was present in the beginning.

So, it is you are actually not regenerating the thiol group or the matrix what you have started with, so, that is how, this is actually irreversible reactions and that is all it is called as the covalent chromatography and where you can be able to use these matrix, but only for 1 time.

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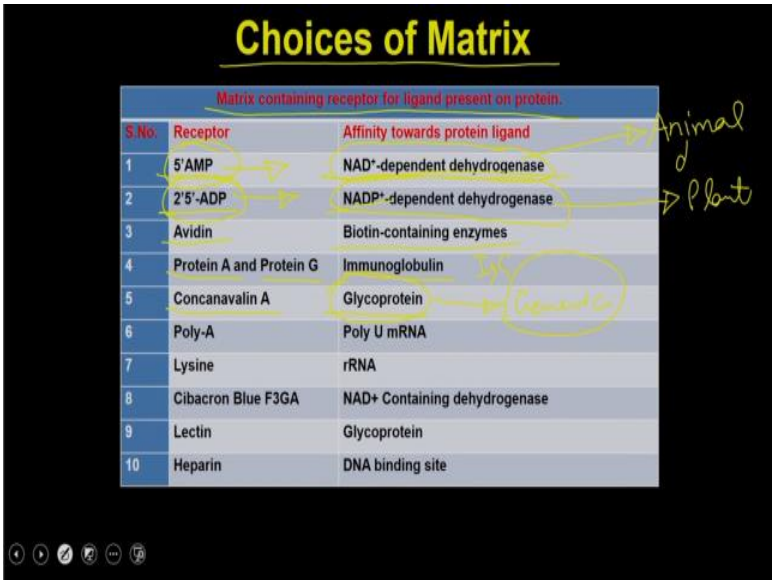

Apart from that, the covalent chromatography cannot be used for multiple proteins, it can only be used for the proteins which are actually containing the free thiol groups, which means with the proteins which are actually containing the cysteine residues and these cysteine residues are free as well as they are present onto the surface of the protein so, that they will be able to interact with the sulfhydral group present on to the matrix.

So, this is the some of the classical examples of the different types of column material what has been used or what is been available from the affinity chromatography. This is not the very exhaustive list what is the, to all the type of affinity chromatography matrix available, but, these are just simple examples for example, you have the 5 prime AMP. So, if you couple the 5 prime AMP as a receptor that actually is going to have the affinity for NAD plus dependent dehydrogenases.

So, as I said you know these are actually going to have the generic affinity chromatography. Similarly, you have the 2 prime 5 prime ADP if you use that as the receptor, then it is actually going to have the affinity for the NAD plus dehydrogenases which means, this is actually going to allow you to purify the animal dehydrogenases; whereas, this is actually going to allow you to purify the plant dehydrogenases.

If you recall, the plants actually contains the NAD plus based dehydrogenases which are participating into the photosynthesis and all other reactions, which are happening within the chloroplasts animals are mostly containing the dehydrogenases which are actually NADH

dependent dehydrogenases or NAD plus dependent dehydrogenases then we have the avidin which is actually having the affinity for the biotin containing enzymes.

Then you have the protein A or the protein G and that has a very high affinity for the immunoglobulin or the IgG then you have the concanavalin A, concanavalin A is having the affinity for the glycoprotein and this is also a generic affinity chromatography. So, it does not depends on the particular type of glycoprotein it actually works against the glucose or the glycose portion present on most of the proteins if they are glycoproteins, then you have the poly A sepharose which is actually having the affinity against the poly U messenger RNA.

Then you have the lysine that has an affinity for the ribosomal RNA. So, that is also again the same generic it is actually going to let you to purify all the ribosomal RNA present into the cell then this is the example of the dye affinity chromatography where the cibacron F3GA blue and that is called as the blue sepharose is actually having the affinity for the NAD plus containing dehydrogenases then you have the lectin having affinity for the glycoprotein and then you have the heparin sepharose which has the affinity for the DNA binding proteins.

So, these are the some of the classical examples of the affinity chromatography matrix where people are using we have many more column or the matrix available for performing the affinity chromatography. So, if you are interested you can be able to go through with the you know different catalogues of the different companies and that actually will give you the idea or the different types of chromatography columns which you can use for the your specific need.

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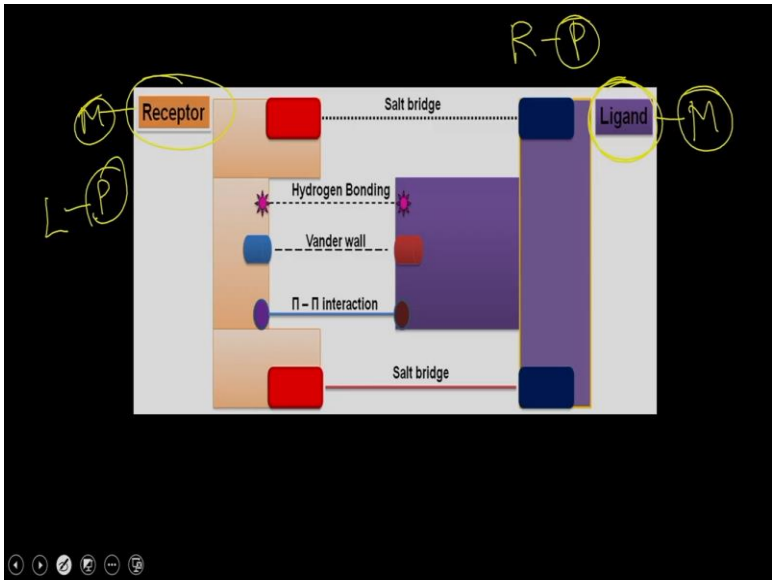

So, for performing affinity chromatography, what you are supposed to do is you have to work with the receptor ligand complexes. So, you have the choice of either coupling the receptor to the matrix or you have the choice to couple the ligand to the matrix depending on which one is easy to perform. For example, if I am working with a ligand which is non proteinaceous in nature.

Then I cannot use that ligand to couple it to the protein because, if I coupled the ligand to the matrix, I have to couple the receptor to the protein if I coupled the receptor to the matrix, then I have to couple the ligand to the protein. So, depending on which one is easy to perform, because the coupling the receptor or to the ligand to the protein is always been done simply by performing the recombinant DNA technology, which means you have to generate the recombinant protein.

If you would like to utilize the affinity chromatography exploiting the receptor ligand complex. So, this is what we are going to discuss in the, our subsequent lectures. So, in the subsequent lecture, we are going to discuss about how to generate the receptor or how to generate the ligands and then how to couple them to the matrix? And then how we can be able to utilize that to purify the analytes utilizing the or exploiting the affinity chromatography?

So, with this I would like to conclude our lecture here. And in the subsequent lecture, we are going to discuss about the preparation of the ligand as well as the receptor and these

generation of the affinity column and how to perform the affinity chromatography. With this I would like to conclude our lecture here. Thank you.