Experimental Biotechnology
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Lecture – 26
Affinity Chromatography - Part-4

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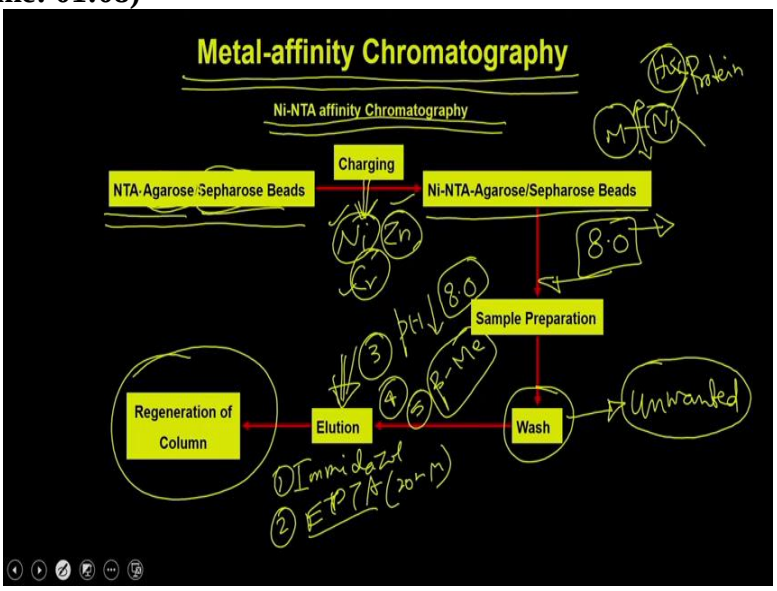

Hello everybody, this is Dr. Vishal Trivedi from Department of bioscieneces and Bioengineering IIT Guwahati. So, now, let us move on to the next affinity chromatography which is called as the metal affinity chromatography, that metal affinity chromatography falls within the category of the pseudo affinity chromatography, where we have the diaffinity chromatography as well as the metal affinity chromatography.

So, we have taken an example of nickel NTA affinity chromatography and a nickel NTA chromatography technique has multiple steps. So, what you have is you have the 2 different columns one is called as the nickel NTA agarose columns and the other one is called as the nickel sepharose beads when you buy this column or when you buy this affinity chromatography. From the vendors you may have the 2 versions one is the agarose beads other one is called as a sepharose beads.

Irrespective of these beads are not going to have the nickel or the condition metals bound to the matrix. So, the first step is you are going to do the charging with the help of the nickel or zinc or the other transition metals you will not going to utilize the iron, because the iron is

very reactive and iron is known to produce the hydrogen peroxide when it is been present in the water.

So, because the iron can go through with the multiple oxidation states for example, Iron can go from Iron 3 to Iron 2 and because of this oxidation reduction states of the Iron, it normally processes the water and then it actually you convert the water into the hydrogen peroxide or the hydroxy radicals and all these hydrogen peroxide or the hydroxy radicals are bad for the protein what you are planning to purify because they will damage the proteins.

So, that is why the iron is not being used in this chromatography technique, but apart from the iron, you are free to use the other affinity other transition metals like nickel, zinc or chromium. So, the charging step you are going to add the metal solutions, either the nickel or zinc or the chromium and that actually will go and bind to the NTA agarose beads. And that is how you are going to develop the affinity column where the matrix is going to have the nickel bond.

And this nickel is going to have the extra coordination bonds available for the protein to bind which means this if all these are transition metals, so they have the multiple you know multiple valences which are available, so, few valences are going to be used by the functional groups which are present on to the NTA agarose beads or NTA Sepharose beads and the few coordination valency or bonds are going to be available for your protein to bind with the help of the protein bond.

Histidine tag which means the histidine is going to have the very high affinity or histidine tag is going to have the very high affinity for this metal bound condition metals whether it is nickel, zinc or chromium. So, now, what you do is you apply the sample which means you apply your cell lysate after that you are going to have a washing step so that you are going to remove the unwanted proteins and once you are done with that, you are going to do the illusion.

So, illusion can be done in multiple ways and illusion can be done with counter ions. So, in this case, the counter ion is the histidine. And but ideally, we do not use the histidine we use something else. So, that anyway we are going to discuss when we will discuss these steps in more in details. After this you have to wash the column and to the regeneration of the

column. So, you remove the unwanted proteins by the washing and then you are going to do the elutions with the help of the counter ions.

So ideally, we should use the counter ions such as the histidine, because the histidine tag is the ligand in this case, which is for the nickel, which is bound to the matrix, but the histidine is a very costly amino acids, and that is why people do not use the histidine instead of histidine. What they do is because the histidine is also contained the immidazol ring so instead of histidine.

What you can do is you can simply use the immidazol and the immidazol. If you use the different concentration of the immidazol you will be able to elute the protein from the column apart from the immidazol you can also use the EDTA because the metal is bound to the matrix by coordinate linkages. So, there is no covalent linkage of nickel to the matrix. So, if you add the small quantity of EDTA for example, if you add the 20 milli molar EDTA you are going to remove the metal from the matrix.

So, if you remove the metal from the matrix, subsequently the protein which is bound to the matrix it is also going to be removed EDTA is a gelating agent, it normally binds to the metals and it has a very high affinity for the metal and that is why it can be able to remove the metal from the matrix. The third is you can use the pH because the nickel NTA Sepharose are the chromatography is always been done above to the 8 pH.

Because at this pH the histidine tag which is present on to the protein has the suitable charges and that is required for it is to bind to the beads. So, if you lower the pH to the less than 8, which means, if you use the pH of 7 or 6 or even 4, you will be able to reduce the affinity of the histidine tag to the nickel which is bound to the matrix and as a result, the histidine bound protein or histidine tag containing protein is going to be removed from the column.

Number 4 way of eluting the protein is that you can add the pure tropic salts like you can use the urea or GDMCL and that also is going to affect the affinity between the histidine tagged versus the nickel NTA and that also is going to remove the protein from the column number 5 you can also use the beta mercapto ethanol or you can use DDT. So, if you use the reducing agents the reducing agent are also going to interfere with the binding of the nickel to the matrix.

And as a result, it is actually also going to allow you to elute the protein from the column. The only thing that you have to remember is that the beta mercapto ethanol or the DDT are very harsh treatment. So, it may sometimes destroy the NTA beads and as a result, it may permanently cause the damage to the column. So, in those cases, you might have to renature the column back very quickly or you have to avoid utilizing the beta mercapto ethanol.

So, with this since, these are the theoretical explanation of how to perform the nickel NTA chromatography, I thought of taking you to my lab, where the students are routinely been utilizing the nickel NTA affinity chromatography and they might be able to show you how do perform the nickel NTA chromatography and how to do all these different steps to do the purifications and how to analyze the purifications after the affinity columns and so, on. And so, in this demo, the student have explained the running of the nickel NTA column with the difference steps.

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For protein purification, first we have to inoculate the culture into this larger volume of conical flask because then we may use it so, first I show you how to inoculate. This is the single colony grown overnight culture. So, we can use for the inoculating into large cultures. So, this process should be done in a septic condition. So that means we have to use laminar air flow for this purpose.

So, and also we have to remember we should include suitable resistor in marker like ampicillin or kanamycin these kind of antibiotics. This is a depends upon what resistance vector you are having. So, in this case we are using ampicillin as a antibiotic. So, in this time we are listing cells using sonication. Now, we are going to centrifuge the lysate to get supernatant.

So, that supernatant we loaded to nickel NTA column and purify the protein. So, I will transfer it to 50 ml centrifuge glue then centrifuge glue. Finally centrifugation is going on we have to wash the colon using first this is in 20% ethanol. So, we have to wash first with water then equilibration buffer. So, let it drain completely the 20 % ethanol and then we will add water double distilled water.

So, it leads to 5 column values of water should be added to remove completely and next we will equilibrate the lysate of the tub of the which we use it for the lysate of bacterial science.

Before the purification of the column we have to charge the column nickel NTA. There are 2 types of lids are there. One is other one is already readily charged bits which comes from company and another one is we have to charge they would give only in India there was abuse.

So, here what we will do is we will charge the beers with chemical and then we will equilibrate. We are already washed the column with water and 0.2 normal NaOH and again with water. So, now, we equilibrate. So, this is a nickel hexachloride solution. So, we will keep in this condition at least 20 minutes to charge the beads. After that, we will remove nickel NTA.

We remove the nickel solution and then we will equilibrate with the lysate buffer. So, after 20 minutes we remove the nickel solution next, we will equilibrate with the lysate buffer. We have to wash at least 2 column volumes to remove any free nickel which exist in the beads. So, after equilibration, next step we have to load the lysate and then we incubate for eluting. It is the paraffin used for packing. So, once column packing is over, we have kept it in ice and keep in this condition for at least 2 hours for binding.

So, that installed protein will bind to the nickel NTA and in further steps and we will remove that. After incubation of beads we have to follow another 3 steps to get complete purification done. The first step is we have to wash with the equilibration buffer. First, after the beads taken out from ice, you have to remove the outlet. So, that all the flow through other than beads will be taken out and the next step is we have to wash with the equilibration buffer.

And the third step is we have to erotic sample erotic protein history protein using in the zone containing buffers. For all these buffers, the pH should be adjusted prior and not like you have to first you take the buffer lysate buffer and you have to add it is not like that. It may increase the pH. So, after compiling all the lysate buffer with methazole, then we have to adjust the pH so that throughout the procedure the pH no change.

So, this is a flow through whatever we are getting this flow through next step we will wash with the lysate buffer in this step we have to wash with the lysate buffer for equilibration. Before doing this, we have to observe the beads we should not directly loaded to this, you just how to pour through column through the wall of the column. Otherwise, it may disrupt the beads. So, protein may also.

So, this we have to keep in mind while doing this washing. While doing purification, we have to remember that every time you are introducing new buffer, you are introducing new buffer and that time you have to correct the fraction and this can be used for the running, testing the purity of the samples and also the flow through part and the washing part what we have collected, we have to keep it safely after verification of the gel only we have to throw.

Say you are getting only 10% of the protein in the purified fractions and the 90% of the protein erotic in the flow through that time you can reuse the flow through for purification or purifying protein. So, we have to collect the fractions in a small micro centrifuge tubes and we have to save those particles name it and save it. So, we washed with the equilibration buffer and we also collected the flow through.

Now it is time we will wash with 20 milli mole of Immidazole. So, this will remove any non-specific proteins binding to the beads. So, we will wash with the 20 milli molar containing buffer then we will eluting, subsequently eluting 250 milli molar into the container. In the final step we are going to eluting with 250 milli molar Immidazole buffer. So, what we are going to do is we have to incubate beads with this buffer at some time and collect the fractions.

Now we have to collect the fractions. After eluting the complete fractions, we have to wash the column with water then 0.2 normal sodium hydroxide solution then again water. After final wash with the water then we have to store the beads in 20% ethanol. So, I wash it and store it in while the washing is going on, we have to take 50 microliters of each fractions and run 10 SDS pages, that will give the purity of the fractions.

We have to heat the samples before loading onto SDS pages and also we have to keep these all these fractions but we have collected at 4 degrees Celsius for further confirmation of the purity. Once the purity is confirmed, we have to dialyse those fractions against the buffer of interest. Then use for the further studies. So, we purify the protein using nickel NTA a column we run the gel and stained at extent. So, now it is time to document detail. So, we have to identify whether we got any single bad fraction or not.

So, this is the gel. I kept on white tray. Now, just close it. So, we have loaded marker and this from this side second one is the load, this is flow through, wash 1, wash 2 and these fractions

are eluted fractions 1, 2, 3, 4 sequentially. So, as we can see the eluted fraction is done corresponding to this protein but the molecular weight can be calculated using this software image lab software.

So, as we can see in the protein corresponding to this purification is tagged one it is going most of the fraction in the flow through. So, we can use as I said in the video earlier, we can use this flow through action again for purification of the protein you can incubate this flow through with the same beads and you can repurify again. So, that will increase the productivity getting the protein.

So, these are all other ones what we are seeing in the protein, this eluted glance those are because of the contaminants or degraded protein contaminants sometimes may come because of histidine 2 or 3 or 4 histidine having in folded state that will give possibility to bind to nickel NTA column and also washing vigorously washing should be done if you do not wash properly with a high amount of imidazole that will give you this kind of nonspecific binding. So, with this will conclude the video.

So, I hope it will help you to help you in your work during protein purification help you to understand how protein purification once.

(Video Ends: 30:47)

So in this demo we have I think the students have explained each and every step in detail and I hope this could have been useful for you to understand the practical aspects of the chromatography techniques. So, with this we would like to move on to address some of the research problems related to the affinity chromatography.

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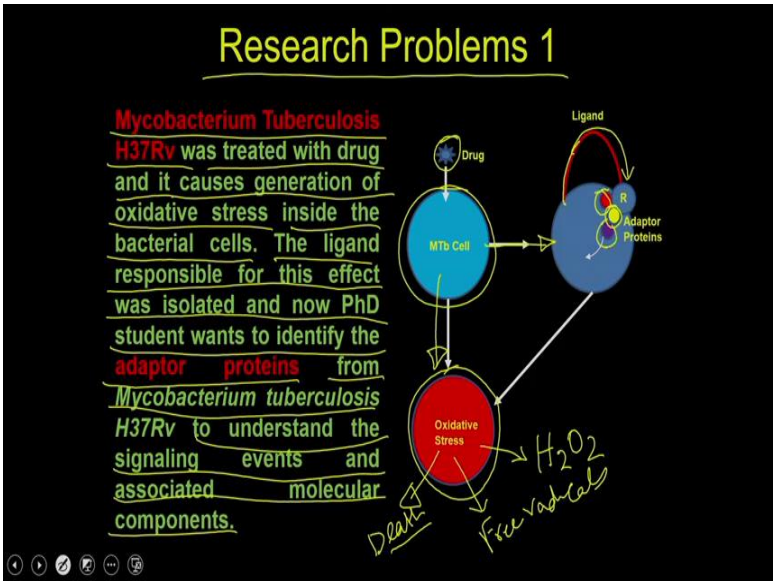

The first problem is where we can use the affinity chromatography is that the mycobacterium tuberculosis H37Rv was treated with the drug, anti TB drug, and it causes the generation of the oxidative stress inside the bacterial cells, the ligand responsible for this effect was isolated and now, the ligand responsible for this effect was isolated and now, PhD student wants to identify the adapter protein from the mycobacterium tuberculosis to understand the signaling events and the associated molecular component.

So, what this question means is that if you are taking a mycobacterium cell to mycobacterium tuberculosis is a bacterial species which causes the TB into the human beings and if you treat it with the drug, so, what will happen is that the mycobacterium tuberculosis is drugs are see mycobacterium cells are developing very high quantity of oxidative stress which means, oxidative stress means the cells are producing very large quantity of hydrogen peroxide, superoxide radicals, hydroxyl radicals and all those kinds of free radicals.

So, it is actually generating very high quantity of free radicals. So, that actually is if you do not control the oxidative stress for a very long time that it is actually going to bring the death of the bacterium. So, how it is happening, it is happening because when you are treating the MTB with the drug, it is actually producing a ligand and that ligand is binding to a receptor and that receptor is associated with the downstream protein molecule which are all technically been called as the adapter protein.

And then these adapter proteins are taking the signal from the receptor and driving the reactions to the bacterium and so, the question is how to identify these adaptor proteins.

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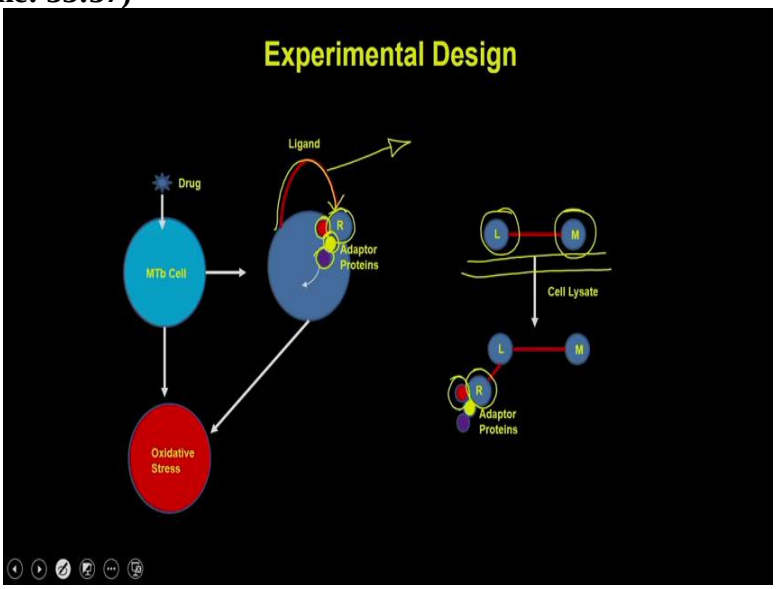

Now, in the experimental design, if you want like to address these questions, what is our issue is that a ligand is being produced, which is bounding to this receptor and then this receptor is additionally binding to the adapter protein. So, in the first step, what you can do is you can generate affinity column which actually contains the ligand which been coupled to the matrix and then what you do is you take out the bacterial lysate and incubate this whole complex we did that.

So, what will happen is this bacterial lysate is going to bind the receptor or to the adapter proteins and then eventually you wash the column for some time and then it is actually going to give you the receptor as well as adapter proteins.

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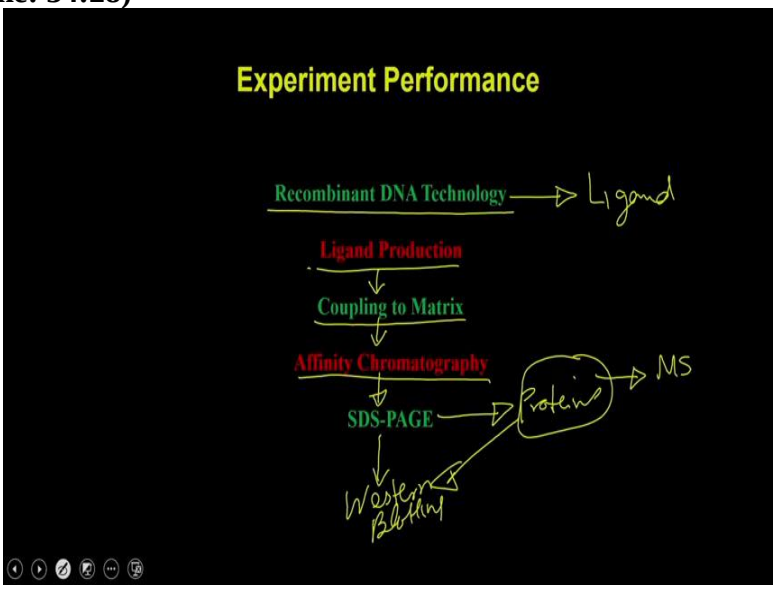

How to perform the reactions to perform this experiments, what you have to do is you have to do the recombinant DNA technology, because you have to produce the ligand then you have to do the ligand production because recombinant DNA technology is going to give you the ligand productions, then you have to couple the ligand to the matrix and once that is ready, then you are going to prepare the lysate and perform the affinity chromatography once the affinity chromatography is over.

Then you have to perform the SDS page and SDS page is only going to give you the proteins what are present. That protein can be identified by 2 methods, either you do the mass spectrometry to identify these proteins or you can do the western blotting and that actually also going to give you the identity of these proteins, because you can use the specific antibodies and that actually is going to give you the proteins.

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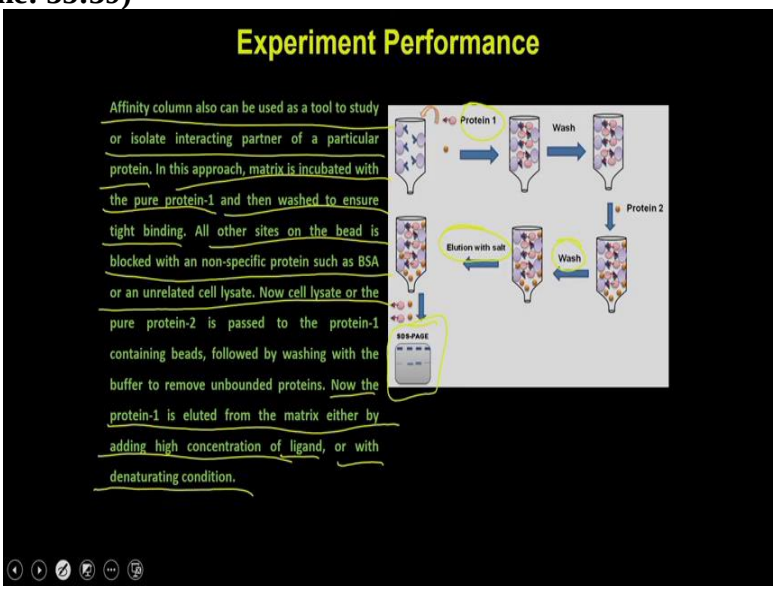

So, how to do that affinity chromatography can be used to study the interacting partners of a particular protein. So, in this approach matrix is incubated with the pure protein which means, in this case, the ligand and then washed to ensure tight binding, all other sites on the bead is blocked with a nonspecific protein such as BSA and then you incubate with the cell lysate which is actually going to bind to the protein or the ligand.

And then you do the washing and then you do illusion with the salt and once you do the illusion with the salt, it is actually going to come out from the column and then you can be able to see the pattern on to the SDS page. Once you see the pattern on the SDS page, you can be able to purify or identify these proteins. Now, the protein bond is eluted from the

matrix either by adding high sites condition of ligand or with a denaturating conditions, once you do that, you are going to see the pattern of the protein on to the SDS page.

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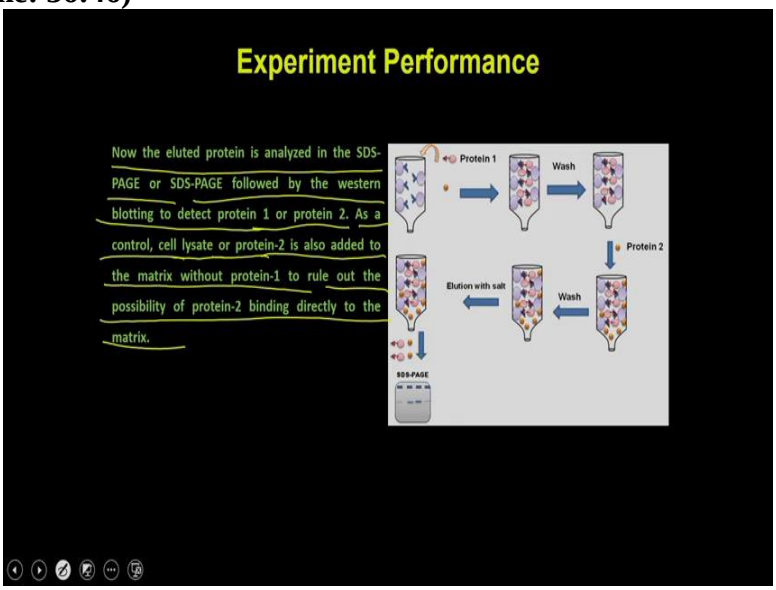

That eluted protein can be analyzed on to the SDS page and SDS page can be followed by the western blotting to detect the protein 1 and protein 2 as a control, because you also need to understand that the analysis speak experiments on experiment could have some nonspecific reaction as well. So, you can be able to lysate you can just simply load the cell lysate without the ligand.

Or you can add the protein 2 with added without a protein 1 to rule out the possibility of protein 2 binding directly to the matrix, which means you can run the 2 control, you can have the 1 control where the cell lysate is the bacterial cells or not been treated with the drug or number 2, where you do not have the you have the matrix, but you do not have the ligand present onto the matrix.

Either of these will actually going to give you the clear idea whether the protein but I am identifying or the adapter protein, what I am identifying at the end are authentic or not. So, this is the result what you are going to get and then you will be able to verify these results simply by running the control the actions.

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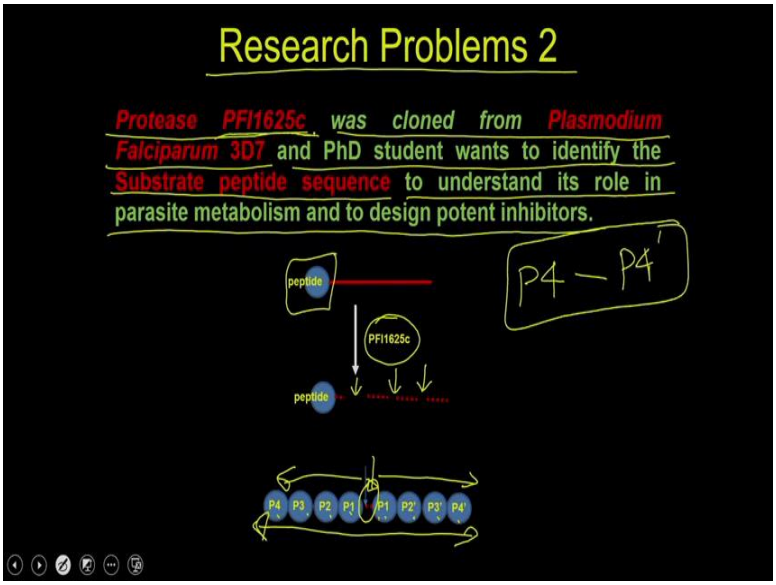

Now, the research problem 2, for research problem 2 is there is a protease called PFI1625c was cloned from plasmodium falciparum 3D7, and the PhD student wants to identify the substrate peptide sequence to understand its role in parasite metabolism and to design the potent inhibitor. So in this case, what is the problem is that you have a peptide sequence, if you incubate this peptide sequence with the protease like PFI1625c, the peptide is going to be broken down into the multiple pieces.

And what you could understand is that it is not nonspecific it is starting at a very specific point, what are the specific point so in a typical protease, when you have a cutting side, what you see is that the 4 amino acid onto the right side as well as the 4 amino acid onto the left side are actually be responsible for this protease to recognize the cutting side and what the person wants to identify is he wants to identify this particular sequence, which has been recognized or which has been identified by the protease and then it is cutting.

So, he wants to know this sequence. So, the sequence onto the right side is known as the p 1 p 2 p 3 and P 4 sites, whereas the sequence present onto the left side is known as the p 1 prime p 2 prime, p 3 prime and p 4 prime. So, you want to know the p 4 to p 4 prime sequence for the protease PFI1625c.

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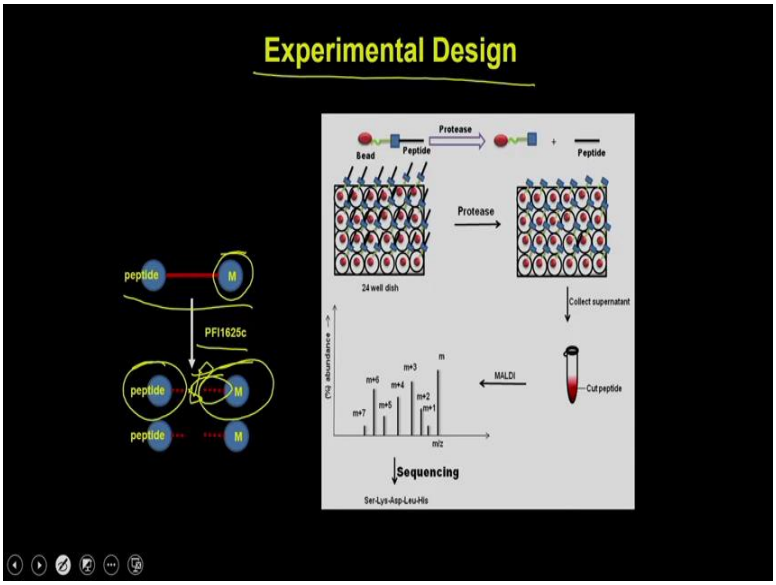

Now, what is the experimental design? In the experimental design, you have to first produce the peptides and you have to couple it to the matrix. If you do so, and incubate these with the PFI1625c, it is going to break this peptide into 2 fragments. So, one fragment will go along with the peptide fragment, the other fragment, which is also going to present along with the matrix sites.

So, if you have a complete library of different peptide sequences, where the one end is bound to the matrix, the other end is free. So, the free end is actually going to be washed away into the buffer whereas the bound form you can be able to recover and if you can be able to sequence this particular bound peptide, you will be able to deduce the protease cutting sides.

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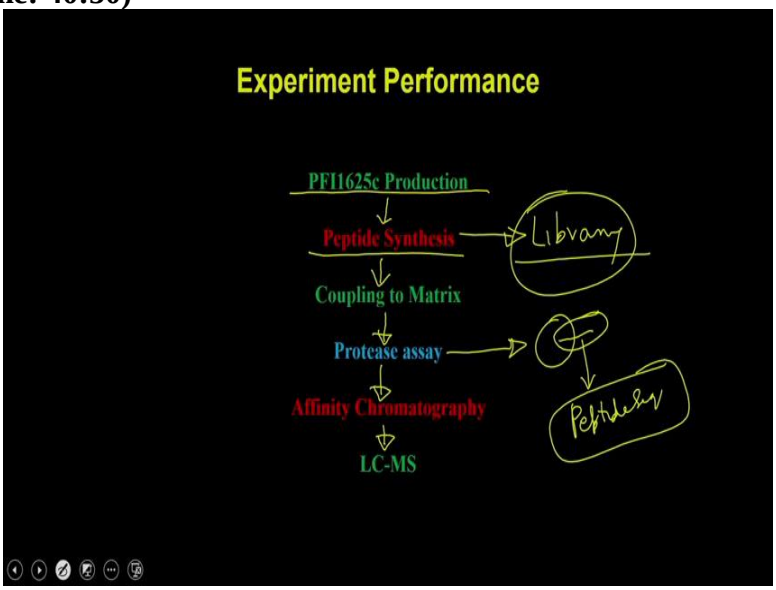

So, for experimental performance, what you have to do first you have to produce the proteins. You also have to synthesize the peptide, different peptide sequences, so you have to actually

generate a library, this library you can generate either in a de-novo made which means you can just start with the scrambled sequence and then you refine the sequences based on your finding or you can take the pre-existing library, then you couple these peptides to the matrix.

Once you have done that, then you incubate these with their protease assay reactions and then you are going to do the affinity chromatography. So, that the matrix bound peptide is going to be remain with you and then you are going to do the LCMS of this peptide to identify the peptide sequence. And that actually, if you analyze all the peptide sequences from this library, you will be able to deduce the peptide cutting sites for this particular protease.

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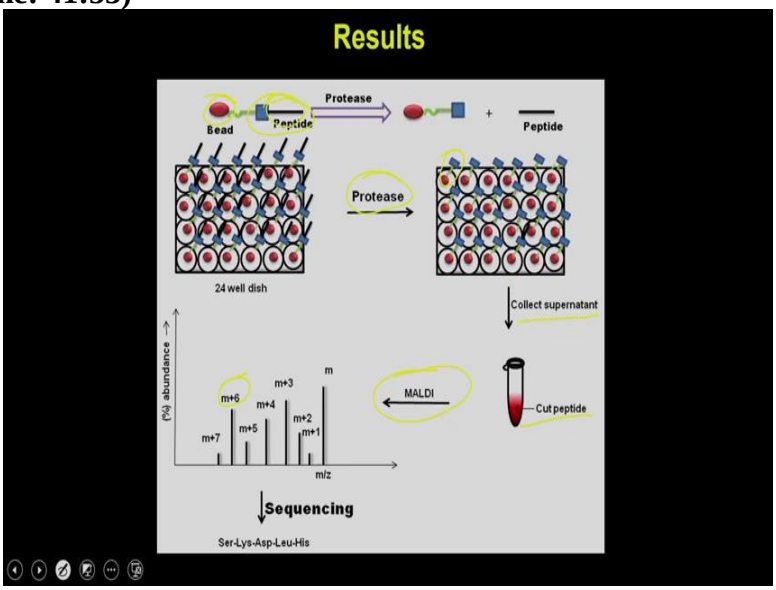

So, in a typical thing, what you have is you have a peptide sequence then you have matrix and then you have the different such peptides which are present in a 24 well dish, then you treat it with protease what will happen is the this protease sequence is going to be chewed off by the protease and you are what is left over is the remaining peptide sequence, you can collect all these bead bound peptide you can collect them.

Then you cut the peptides and then you do the MALDI and what to MALDI is going to do is it is going to give you the masses of different peptides from individual reactions. And if you can interpret these masses, you will be able to know the sequences present in the each well because you know the peptide what is present. So, what is remaining, that is actually you can be able to deduce from the masses and then you can reconstruct and that actually is going to give you the peptide protease cutting sites.

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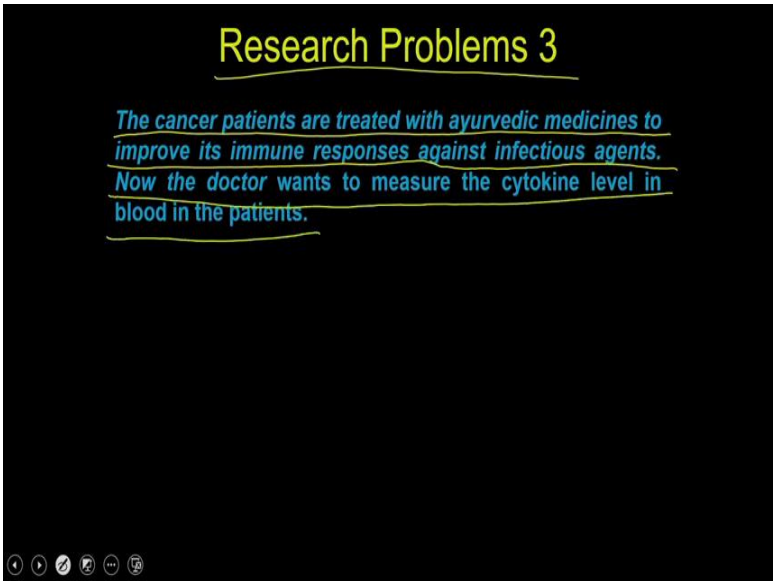

Now, next move on to the research problem number 3. So, in the research problem number 3, the cancer patients are treated with the ayurvedic medicines to improve its immune responses against infectious agent. Now, the doctor wants to measure the cytokine level in the blood of the particular patient to understand whether diabetic medicine is working or not.

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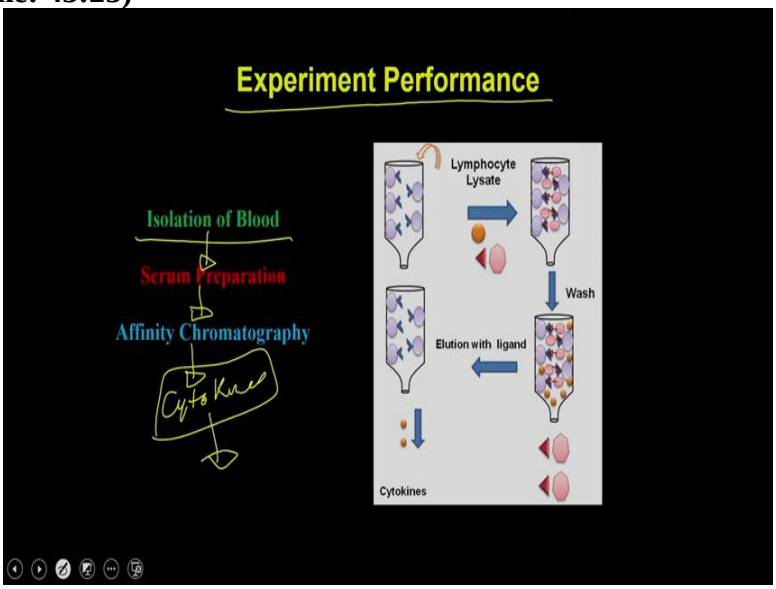

So, what is the experimental design the experimental design is that you are actually going to generate the affinity column where you are actually going to have the antibodies coupled to the matrix against the particular cytokine or a series of cytokines, and then what you do is you take the cell lysate or the lymphocyte lysate from the patient's incubate it into the column and then you wash it and subsequently you can elute.

So, when you wash the all other proteins are going to be removed except the cytokines which are actually binding to the antibodies present on to the matrix. So, what happened is that in

the matrix you have the antibodies, so, this antibody is a specific for a particular cytokine. So, what happened is that does that particular cytokine will go and bind to this matrix or you can have the combination of the antibodies, then you what you do is you do the illusion with the ligands ultimately.

You are going to get the cytokines into you can get the cytokines from this particular patient, and then what you can do is you can just measure the level of these cytokines with the help of the ELIZA. So, the experimental which you want to perform the experiment. The first thing what you have to do is you have to isolate the blood of that particular patient, then from there, you have to first prepare the serum.

I think we have already discussed how to prepare the serum from the blood and then you have to do affinity chromatography to identify the cytokines. So, that actually is going to give you the cytokines and once you have these cytokines purified from the serum, you can be able to identify or measure the individual cytokines with the help of the ELIZA.

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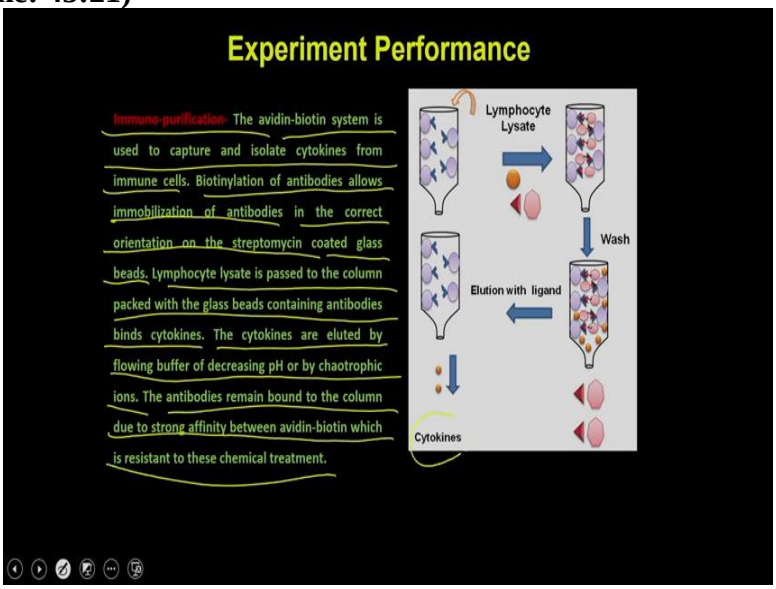

So, in this what you can do is you can do a evident biotin kind of system to capture and isolate the cytokine from the immune cells. So, biotinylated antibodies allowed the mobilization of the antibodies in the correct orientation onto the streptomycin. Streptomycin coated glass beads lymphocyte lysate is passed to the column packed with the glass bead containing antibodies bound bind passed to the glass bead containing antibodies bind cytokines.

The cytokines are eluted by flowing the buffer of decreasing pH or by the pure tropic ions, the antibody remain bound to the column due to the stronger affinity which is resistant to these chemical treatment and ultimately, you are going to get the cytokines.

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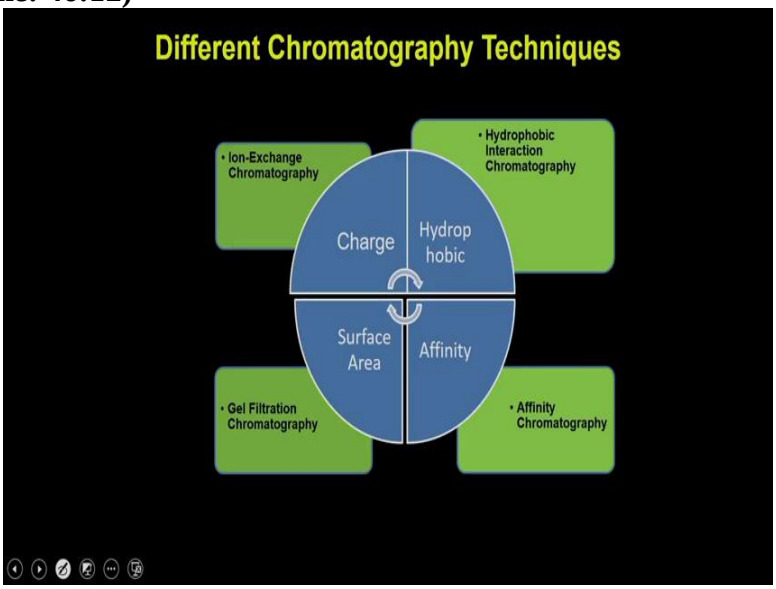

So, with this, we have discussed different chromatography techniques, we started with the basics of the chromatography techniques, then we discuss about the ion exchange chromatography followed by the hydrophobic interaction chromatography gel filtration chromatography and the lastly we have discussed about the affinity chromatography for the each chromatography techniques we have we I tried to discuss the different types of research problems.

So, that you will be able to realize the potential of this affinity chromatography and you will be able to utilize this affinity chromatography for designing the experiments to solving the different types of experimental problems or by solving different types of the research problems related to your work. So, with this, I would like to conclude our lecture here thank you.