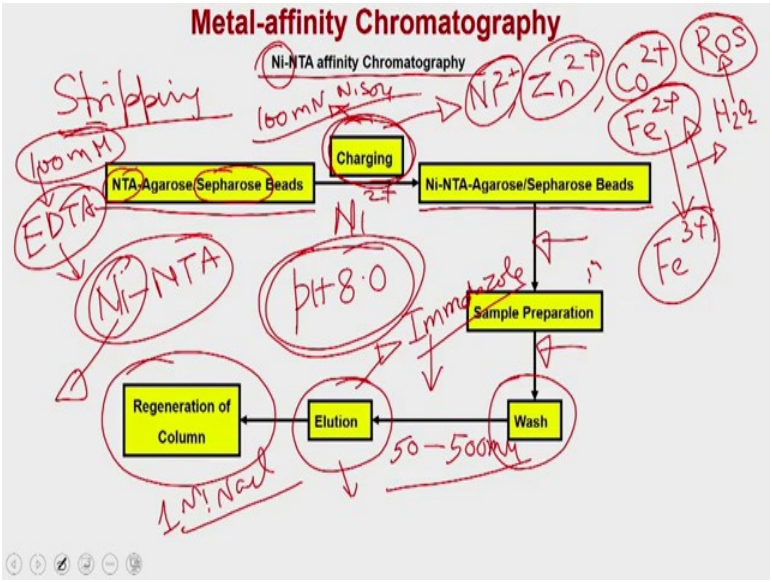
Genetic Engineering: Theory and Applications
Professor Vishal Trivedi
Department of Biosciences and Bioengineering,
Indian Institute of Technology Guwahati Assam, India.
Module VIII
Isolation and Purification of Product (Part II)
Lec 28
Affinity Chromatography (Part 4)

Hello everybody this is doctor vishal Trivedi from department of biosciences and bio engineering IIT Guwahati. And what we were discussing, we were discussing about the different chromatography techniques. Which people are using to purify the fact protein or the other factors from the cells where they are over expressing these components.

(Refer Slide Time: 00:53)



Now, how to perform this affinity chromatography in your laboratory, Let us take so for this I would like to take you to my laboratory. And, where we are going to show you each and every step how to do the charging, how to do the equilibrate the columns and then how to purify the protein using the nickel NTA affinity chromatography.

(Refer Slide Time: 01:17)

So, protein purification first we have to inoculate the culture in to this larger volume of PLB plus then we will induced it. So, first I will show you how to inoculate? This is the single (()) (01:37) grown over night culture. So, we can use for the inoculating into large cultures. So, these classes should be done in aseptic conditions. So, that means we have to use laminar air flow for this purpose. So, and also we have to remember we should include suitable resistant marker like ampicillin or kanamycin in this kind of antibiotics.

Or this is depends upon what vector what resistant vector you are having. So, in this case we are using the ampicillin as a antibiotic so let us start.

(Refer Slide Time: 02:25)

We have lysate cells using sonication, now we have to centrifuge the lysate to get super net end. So, that super net end we wrote on to nickel NTA column and purified protein. So, I will transfer in to 50 ml centrifuge tube then centrifuge. While the centrifugation is going on we have to wash the column using, first this is in 20 percent ethanol.

So, we have to wash first with water then equilibration buffer. So, let it drain completely the 20 percent ethanol. Then we will add water double distill water. So, atleast 5 column volumes also water should be added to remove completely. And, next we will equilibrate with the lysate buffer the buffer which we use it for the lysate of the bacterial cells.

(Refer Slide Time: 03:50)

Before, equilibration of the column we have to charge the column. Nickle NTA there are two types of risk are there one is already readily charged beads which comes from company. And another one is we have to charge they will give only NTR there used beads. So, here what we will do is we will charge the beads with a nickel and then we will incriminate. We already washed the column with water and point 2 normal (())(04:16) again with water, so now we will equilibrate. So, this is a nickel hex chloride solution. So, we will keep in this condition atleast 20 minutes to charge the beads. After that we will remove nickel NTA we will allot the nickel solution and then I equilibrate with the lysate buffer.

(Refer Slide Time: 05:18)

So, after 20 minutes we equilibrate the nickels solution next we equilibrate with the lysate buffer. We have to wash atleast 2 column volumes to remove any free nickel, which exist in the beads. So, after equilibration next step we will load the lysate and then incubate for binding.

(Refer Slide Time: 06:12)

This is the forum and I am going to close this (())(06:21). So, once column packing is over you have to keep it in ice and you keep in this condition for atleast two hours for binding. So, and that stalk protein we will bind to the nickel NTA and in further steps we will allot proteins. After incubation with beads we have to follow another three steps to get completely purification done. 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 allot the sampling allot the protein his tab the protein using in dissolve containing buffer. All for all this buffers the pH should be adjusted prier ham. Not like you to first you take the buffer lysate buffer. And you have to add into it is not like that it may increase the pH of the buffer. So, after comparing all the lysate buffer with immidazole.

Then we have to adjust pH so that throughout the procedure the pH would not change. So, this is a flow through whatever we are getting is flow through. In next step we will wash with the lysate buffer or equilibration buffer.

(Refer Slide Time: 08:52)

So just this is the lysate buffer, before doing this we have to observe the bead we should not directly load on to this. We just have to pore through corner through the valve of the column. Otherwise it may disturb the beads so protein may also be dead. 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 that time you have to connect the traction. And this can be use it for the running SDS (())(09:45) and testing the purity of the samples. And, also the flow through part and washing part what we have collected you have to keep it safely. After verification of the gel only we have to throw. Say you are getting only 10 percent of the protein in your purified fractions and the 90 percent of the protein denoting in the flow through.

(Refer Slide Time: 10:26)

That time you can reuse the flow through for purification and purifying the protein. So, you have to collect the fractions in a small micro centrifuges tubes and we have to save those fractions name it and save.

(Refer Slide Time: 10:43)

So, we washed with the equilibration buffer and we also collected the flow through. Now, this time we will washed with the 20 mille mole of immidazole. So, this will remove any nonspecific proteins binding to the beads. So will washed with the 20 mille mole and integral containing buffer then we will allotting subsequently allotting 250 mille molar immidazole containing buffering.

(Refer Slide Time: 13:32)


Step 8
Elution with 250mM Immidazole

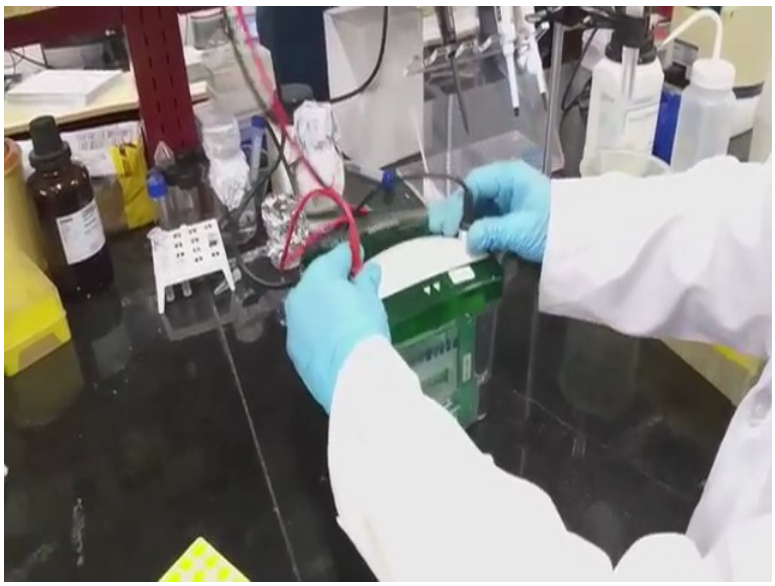

Step 9
Regeneration of Column

In a final step we are going to end with the 250 mille mole immidazole containing buffer. So, what we are going to do is we have to ink bed beads with this buffer at some time and collect the fractions. After allotting the complete fractions we have to wash the column with water with 0.2 normal sodium hydroxide solution. Then again water after final wash with the water then we have to store the beads in 20 percent ethanol. So I will washed it and store it in the ethanol.

(Refer Slide Time: 15:47)

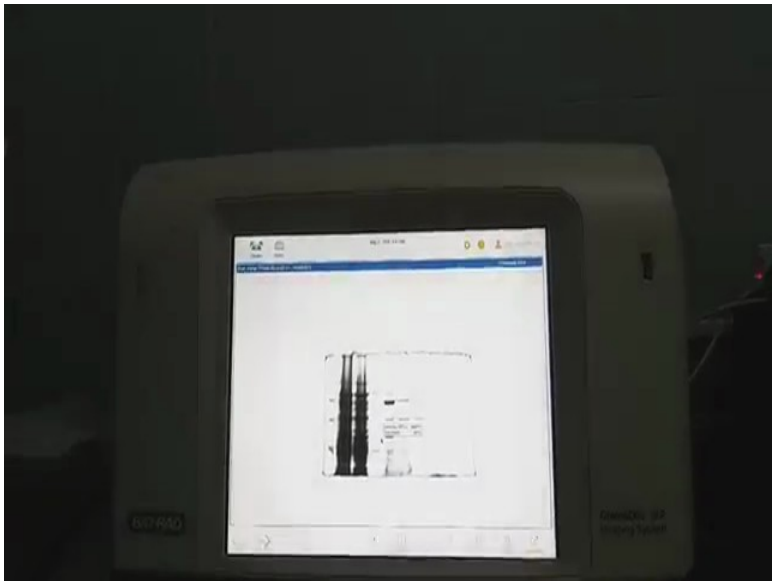
While the washing is going on we have to take 15 micro liter of each fraction and write on SDS plates that will give the purity of the fractions. We have to heat the samples before loading on to SDS plates and also we have to keep this all this all the fractions what we have collected at 4 degree Celsius further confirmation of the purity. Once the purity is confirm we have to dialyze those fractions against the our buffer of interest then use for the further studies.

(Refer Slide Time: 17:04)



So, we purified the protein using nickel NTA column with under gel and state a distant. So, now stand to (())(19:35). So, we have to identify whether we got any single back fraction or not?

(Refer Slide Time: 19:46)



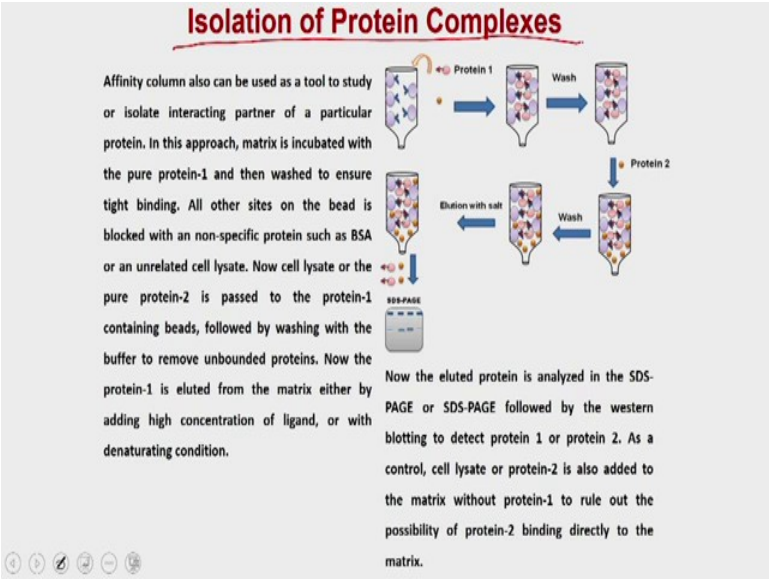
So, this is the gel I keep on white tray now just close it. So, we have loaded marker and this from this side second when one is the load. This is flow through vast 1, vast 2 and this fractions are filtered fractions 1, 2, 3, 4, 5. So, as we can see the allotted fractions showing at the end corresponding to this protein.

But, the molecular weight can be calculated using this software image (())(20:23) software. So, as we can see in the protein corresponding to this purification is third 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 fraction again for purification of the protein. You can incubate this flow through with the same beads. And, you can re purify again so that will increase the productivity getting the protein.

So, this are all other than whatever we are seeing in the protein this allotted banks. Those are because of the contaminates or degraded protein. Contaminates sometimes may come because of a instead in your 3 or 4 instead in having in folded state that will give possibility to buying to nickel NTA column.

And also washing vigorous washing should be done. If you do not wash properly with the high amount of immidazole that will give you this kind of nonspecific binding so with this will compute video. So, I hoped it will help you to help you in your work for during protein purification or help you to understand how protein purification works.

(Refer Slide Time: 22:09)



Let us move on to the application part of the nickel to the affinity chromatography. So, affinity chromatography is a very very robust technique or robust chromatography techniques

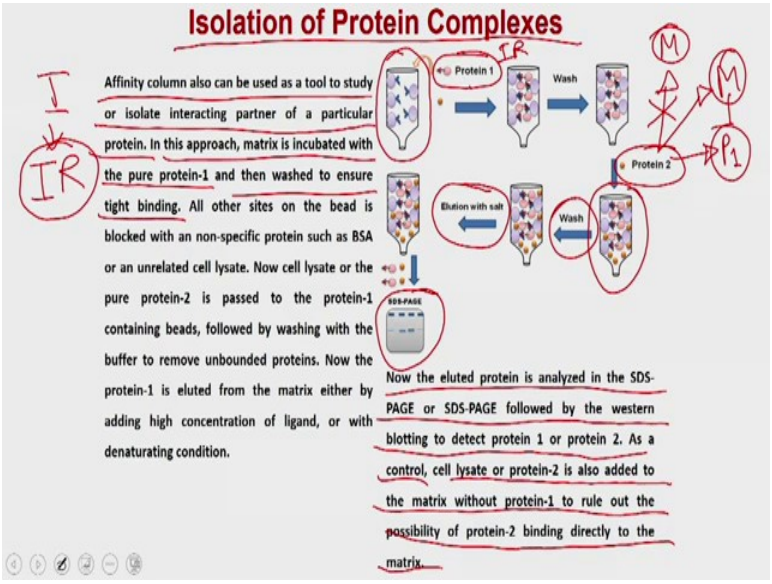
which can be used for multiple applications. One of the application is that you can use the affinity chromatography to isolate the protein complexes. If you remember in our earlier lectures we have discussed that suppose you stimulate a mammalian cell with the insulin what will happen is that insulin is go and buying to the insulin receptor.

And, in this process the insulin receptor is going to get phosphorylated. Once, the insulin receptor is getting phosphorylated, it brings the binding side for many of the adaptor proteins. Once, this adaptor protein goes and binds to the insulin receptor. They actually relay this phosphorylation signal and that is how they actually give this signal to the nucleus. And, that is how the gene expression profiling is going to be changed. And, that actually is going to change overall physiology of this particular protein.

So, suppose you are working in your laboratory and you would, you are exploring the this problem or the similar problem where you are stimulating a cell. And, you are looking for that what are the proteins are binding to this receptor. Or what are the proteins are coming together to make a multimeric complexes. Like some of the signal of formed which are being informed when you are going through when B cell or T cell is under the active immune responses. Like against the cancer cells or against the infectious organism.

And, if you are exploring any such problems then you could be able to use the affinity chromatography to isolate this bigger complexes or multimeric complexes. And, you could be able to identify each and every protein. And, you could be able to answer many of the biological questions. What you are supposed to do is?

(Refer Slide Time: 24:25)



So affinity chromatography is can used to study or isolate interacting partner of a particular protein. Which means in this particular problem you are going to have 1 protein which is going to have the affinity for the matrix. In this case suppose we are this protein is insulin receptor. Because, we are stimulating with the insulin so our protein number 1 is the insulin receptor. Now, insulin receptor you are going to put some kind of affinity tag for example I have put the histidine tag or I have put the GST tag.

So, it should have the exclusive affinity for the beads. Now, what we have in this approach the matrix is incubated with the protein number 1, which means the insulin receptor. And, then you washed to insure the tight binding. Which means you insure that the beads are having the insulin receptor present on its surface, so you what you have done you have taken a column which has the affinity for the protein number 1. In this case this is the insulin receptor so what will happen if the protein will go and bind to the beads which are present in the column.

Then you washed to ensure that is non-specific protein present. So, now your protein number 1 is bound. Now, you take the protein number 2 or you can take the cell lysate. Whichever, you would like to if you are would like study the interaction between protein 1 and 2. Then you can add the pure protein 2 and you can ask this question. Or if you would like to flow the floor lysate you can flow the floor lysate. So, what will happen is, if you will flow the pure lysate? The lysate protein will interact with the beads.

So, the protein 2 has the option of interacting with the matrix or interacting with the matrix bound protein 1. Means it is interacting with the protein number 1 which is bound to the

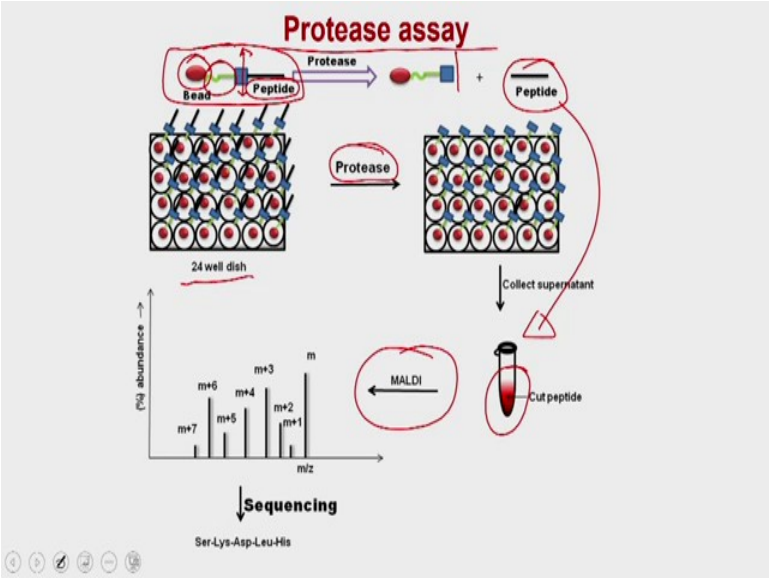
matrix or it has an option that it interacts with the matrix directly. If this is the case this protein is not going to give you the answer. So, if the protein 2 does not have any affinity for the matrix. Suppose you change the condition in such a way that the protein 2 does not have direct affinity to the matrix.

But, it will bind only if it is having the affinity for protein number 1. Then in that case the protein 2 will be bound to the column only in a one condition that the protein 2 has an affinity for protein 1, which is bound to the matrix. Now, you wash so that the nonspecific protein which are binding directly to the matrix are going to be removed. And, then you do the elution of this with the salt. So, what will happen is the both the factors protein a protein1 protein 2 are going to be eluted.

And then you are going to test this protein 1 and protein 2 on to the SDS page. And the pattern of the illusion is going to tell you that both the proteins are interacting or not. Or, whether if suppose you using the cell lysate and then the multiple proteins are going to come out. In that case you have to do the control experiments where you are going to incubate the lysate to the beads without containing the protein 1. And that actually is going to work as a negative control.

So, that you will know that these are the ten factors which are binding to the matrix directly. Now, the eluted protein is analyzed in the SDS page or the SDS page followed by the western blotting to detect protein 1 or protein 2. As a control the cell lysate or the protein 2 is also added to the matrix without protein 1 to rule out the possibility of protein 2 binding directly to the matrix. So, the control is very much important at you should be able to know that this are the lysate protein which are directly binding to the beads not the protein 1.

(Refer Slide time: 28:47)

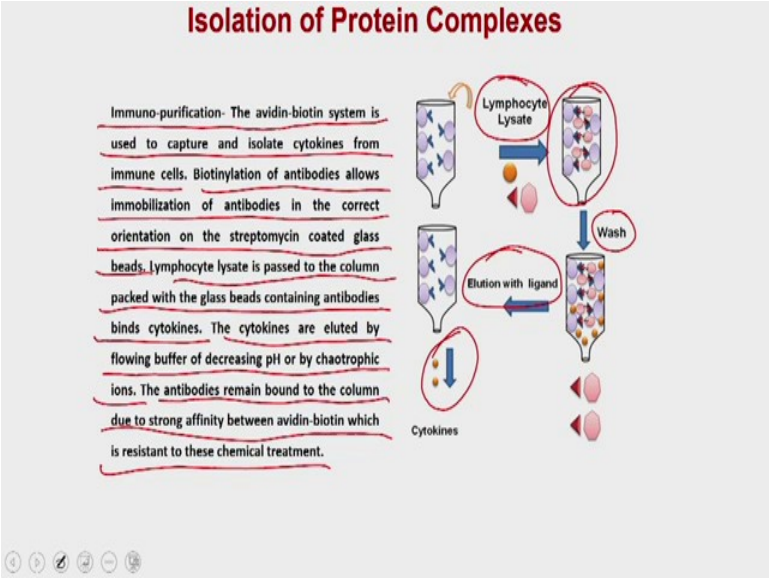


In an alternate experiment you can use the affinity chromatography to generate or to design or protease assay. So, in a typically protease assay what you are going to do is? You are going to have a peptide. Then you put a affinity tag on to this peptide. And then use the affinity beads so what will happen is this is going to be your sample. So, you can change the peptide you can have the complete peptide library. You put it into the 24 well dish so if this 24 well dish is going to have the different different peptide sequences which are bound to the beads in separate wells.

Now, what you are going to do is you treat them with protease. And what will happen? The protease is going to cut the peptide at this point, which means it is going to keep the tag bind to the beads. And the remaining peptide portion is going to be eluted this peptide you can collect in to the super net end form all the beads all the wells. And, then you can perform the maldi analysis which means you can collect these peptides and do a mass analysis and depending on this mass profile.

You could be able to deduce the sequences or deduce the places where the proteases is cutting each well. And, depending on this analysis you will be able to determine the protease cutting side of that particular protease.

(Refer Slide Time: 30:24)



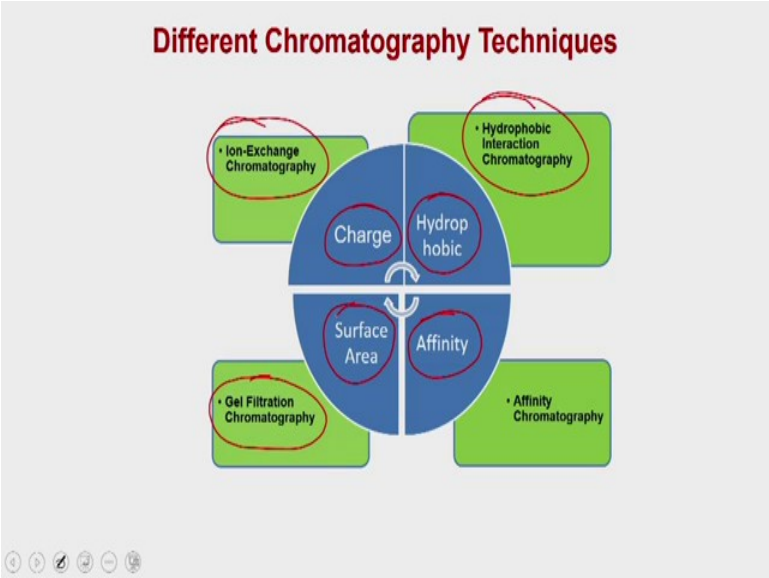
The fourth is you can actually be able to do the immune purification which means could be able to purify the cell of your interest. This is important in terms of the places where you could be able to suppose you would like to purify the bone marrow cells or suppose you want to purify the stems cells and so on. So, those cases what you do is you use the some affinity molecules which will going to have the exclusive affinity for that particular immune cells.

So, the avvidin biotin system is used to capture and isolate cytokines from immune cells. Biotinylation of antibodies allow immobilization of antibodies is in the correct orientation on the streptomycin coated glass beads. Lymphocyte lysate is passed to the column packed with the glass beads containing antibodies binds cytokines. The cytokines are then eluted by the flowing buffer of decreasing pH or by chaotrophic ions. And, the antibodies remains bound to the column due to strong affinity between avvidin biotin which is resistant to these chemical treatments.

So, in this apart from isolating the cells you can also study the cytokine profiling from these cells. So, every cytokine is going to have the its anti body so you can use the that anti body coupled to the avvidin and biotin system. So, avvidin is having a very strong affinity for the biotin and what will happen is that you can take this lymphocyte lysate. And, pass through the column which actually contains the particular kind of anti body.

Then you wash and elute with the ligand. So, what will happen is when you do the illusion only those cytokines will come which are being having the affinity for the anti bodies which are present into these beads. And that is how you could able to identify the cytokines which are being eluted or which are being secreted form this lymphocytes.

(Refer Slide Time: 32:39)

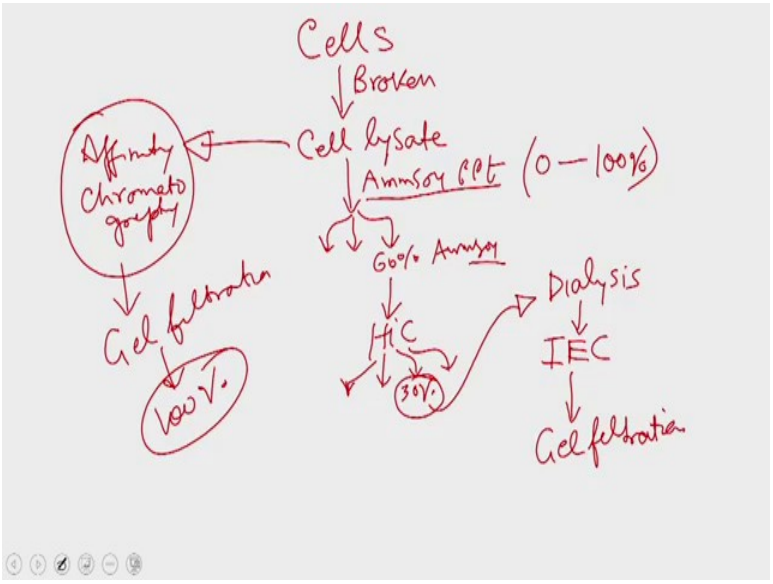


So, this is all about the different chromatography techniques which you can use to purify the factors from the cells over expressing the your protein or other metabolites. Now, let us recap and summaries and also should understand, how you can exploit these different chromatography techniques to perform the protein purification or perform the purification of factors. So, what we have discussed we have discussed about the ions exchange chromatography, which is going to exploit the charge.

Which is present on to the protein then we have talk about hydrophobic interaction chromatography. Which is going to be exploit the hydrophobic patches which are present on the protein, Then we have discussed about the affinity chromatography which is going to work on the exclusive affinity between the receptor and ligand. And, then we have discussed about the gel filtration chromatography which is going to work on the surface area of particular protein.

And, surface is directly proportional to the molecular weight of that particular protein. As long as we are considering that all this proteins are of globular in nature. Now, let see how you are going to plan out the protein purification? Or, how you are going to plan out the purification of this factor from the cells which are over expressing?

(Refer Slide Time: 34:09)



So, what you have is you have started with the cells. So, first is your going to broken this cells or you are going to destroy the cellular integrity that actually is going to give you the cell lysate. Now, depending on the type of purification strategies you are going to use. So, suppose the factor what I am over expressing is containing a tag then in that case directly I will use the affinity chromatography. But, imagine that I do not have any such thing and would like to use the conventional chromatography.

In that case what I will do is first I will do is what I will do an ammonium sulphate precipitation. What I will do is I will use the different concertation of ammonium sulphate which means from 0 to 100 percent. And, if you nor recount you can actually go through with the google or some other sources and that will actually will tell you that what amount of ammonium sulphate you have to add in to a cell lysate to get the 30 percent saturation. So, the 100 saturation is the maximum what you are going to get.

So, and that is actually theotically you may not get the 100 percent saturation in many of the cases. But, what you can do is the first step you can do is the ammonium sulphate precipitation. And that is actually is going to give you the different fractions all this different fractions can be collected and can be tested for the presence of your protein. Either it would be by western blotting or it would be by the axiomatic acid. Or sometime it may be simply by looking at the SDS page and looking at the molecular weight of your protein as well.

Because, we are talking about the protein cells which are be over expressing your protein. Now, you got the different fractions of the ammonium sulphate. Now, suppose you got 60

percent is the fraction where your protein is present. Now, I have the 60 percent ammonium sulphate fraction what I will do is directly I will start running the HIC column. If you remember we have discussed in the past also that. Why the HIC is a preferred column before the exchange chromatography because, now you are already have the ammonium sulphate in to this solution.

What you can do is you can directly add some more salt which is required to have the exposure of the hydrophobic patches present on these proteins. And, then you can directly run the HIC once you are done with HIC you are going to get different fractions. Now, imagine that I got a fraction where the 30 percent ammonium sulphate is giving me the fraction which contains my factors. Then what I will do id I will take this factors and I will do a dialysis step and that actually is going to reduce the level of the salt present in this particular fraction.

And then after that I will do ion exchange chromatography followed by I will do the gel filtration and that actually is going to give me the purified proteins. So, if I will be keep using the combination of hydrophobic interaction chromatography, ion exchange chromatography, and gel filtration chromatography. And, within the ions chromatography I have the option of choosing the an ion exchange chromatography or cation exchange chromatography.

And, using all this combinations probably you will be able to get the purified protein if you follow this particular type of scheme the definitely there will be more losses of your proteins from the cell. Which means the recovery as well as yield is going to be low compare to that if you have the affinity tag then you can directly use the affinity chromatography and that is it in the single step you may get the more 90 percent purified protein.

Once, you are done with this then you can just simply run the gel filtration chromatography and that actually will give you almost hundred percent pure protein, because the gel filtration chromatography may remove some of the aggregated material from your protein. And that is going to give you the close to 100 percent purified protein. So, with this we would like to conclude over lecture here and in our subsequent lecture, now we have produced the protein in the subsequent lecture.

we are going to study how you can validate or how you can actually be able to characterized this isolated factor and how you can be able to show that the factor what you are producing into this host cells is of good quality. So, that you cloud able to use them under the down streamed bio technology applications. So with this we would like to conclude our lecture here thank you.