Experimental Biochemistry
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Lecture – 26
Protein Purification by Affinity Chromatography

Hello, I am Snigdha. Today, I am going to talk about FPLC system and here am going to talk about the Affinity Chromatography and how you can separate your protein of interest based on the affinity chromatography. So, here am going to use a nickel column which going to which is going to bind the his tags which is present in your protein. And the basic of this chromatography is that your protein of interest will bind to the column and again you will run a buffer system which will have another component like imidazole which will displace your protein of interest and remove your protein from the column and you can collect your protein from the eluted.

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So, this is a FPLC system. Here, what we have is a peristaltic pump which will increase the flow rate of the buffer and also display system, where you can manipulate the floor rate or the channels or the buffers which you going to use. This are the wearing or tubing of the whole system which am going to discuss at a later stage. This is a machine or a laptop, where you can see your result or the flow of the buffers and along with this there

is a fraction collector where you can collect the fractions which is passed through your column so that you can collect your protein of interest in this.

Here we have 2 channels buffer a and b. So, simultaneously you can run 2 different types of buffer and these 2 tubes take buffers from two different container and we just after taking the buffer, it will be mix the buffer here in that small tubes. So, it will start rotating and mix your buffer at a proper concentration which you have decided for yourself. Then, this buffer will migrate through this tube and up till this pump. So, this pump will actually determine the flow rate of your buffer and from this pump, the buffer will go into this valve. So, in this system here you load your sample with the help of an injection tube.

So, here you load your sample and after loading a sample, the sample will go in inside a tubing. So, this is a big tube being, but it contains only 10 ml volume of your protein. So, here I have attached a 10 ml volume tube, but you can also attach 2 ml or more than that. So, based on your need, you have to attach the sample tube after you load you inject your sample tube. Then, you going to flow the buffer through this and this buffer will direct your sample into this column. So, this column is a mini column and it contains the NINTA beads. So, basically the (Refer Time: 03:17) bit is attached to the nickel molecules and your protein of interest which has 6 x or more than that his tags will attach itself to the nickel.

So, his tag has an affinity towards the nickel beads. So, your protein of interest will attach itself to the nickel beads, but other proteins which does not have any his tag or more than or less than 2 to 3 his tags, it will flow out from the column and from this column that tube will insert it the sample will insert itself into the this box and this box have a UV lamp. So, from this box you will get a signal how much protein are or how much sample you have in this particular channel. So, after this UV box, the sample goes through the conductivity box where you can measure the conductance of your sample or how many how much salt you have in your sample.

And then, it can it goes to this valve where you can select either you want to collect the sample or the solution. If you want to collect your solution, then it goes directly to the sample column collector, where you attach this collector to the faction collector system. Here, what will happen is the solution will come here and you will collect your sample

inside a fraction collected tube. If you do not want to collect your solution, suppose it is just the flow through where other proteins are present, but your proteins is not present; you can bypass the whole column collector and bypass this whole solution towards the waste collector.

So, basically it will go towards the west. So, now, I am going to talk about some practical aspects of FPLC run so that you can run it properly. So, before remember one thing that before you start a run that all the tubes and your column is kept at 20 percent alcohol which will stop any kind of bacterial or fungal growth and increase the lifespan of your column also of the machines. So, because the column is at 20 percent alcohol you start your run with water. So, use an deionized water and run the water through all the channels and also through your column. So, that the tubings and the columns are clean.

Now, after that you run the wash buffer or the buffer a or any type of buffer which is supposed to make your protein happy and also it will not detach your protein from the column. After you have run the water through all the channels and your column, now you have to run the wash buffer which contains any kind of trace or (Refer Time: 06:24) which will maintain the pH and the salt solution and also if you are collecting your samples through NINTA column, some amount of imidazole. So, that it can inhibit nonspecific binding of other proteins to the column. So, usually we have trace NACL and 10 millimolar to 20 millimolar of imidazole. So, you should equilibrate your column with the wash buffer and that buffer will stabilize the column before you load your protein of interest.

So, after the column is being equilibrated with a buffer wash buffer; then you inject your protein in that injection valve. Now after you inject your protein again you should run the wash buffer and the wash buffer will direct your sample or the solutions which have your protein of interest as well as other proteins which we you do not want and this it will the wash buffer will direct those solutions towards the column. And your protein of interest will bind to the NINTA column, because it has his tag and the other proteins will come out from the column and go into the west and you have to monitor the whole thing in by the UV lamp.

So, the UV lamp is at 280 nanometer it will show that the value because the it is attach the UV will attach. So, at 280 nanometer it will start recording the samples absorbance

because we are running proteins through this whole system and the proteins have tryptophan tyrosine and it will absorb at 280 nanometer. So, you can monitor how much solution how much protein, you have in your solution or after you start elution at where your protein of interest is coming out. So, now, that you have run the wash buffer after your protein injection.

So, basically your protein of interest will get attached to the column and other proteins will come out. Now you have to run another buffer which will have another components in case of nickel NINTA column it is imidazole. So, what imidazole will do is imidazole has higher affinity towards the nickel beads and it will attach itself towards the nickel beads and displays your protein of interest which has his tag. And now the his tag protein will come out as an elutant and you have to collect that those fractions and from the UV absorbance, you will see at which fractions collect higher amount of protein.

It is if you have success his tags it takes around 200 to 300 millimolar of imidazole and you will select those fractions which has your protein of interest, you run a SDC page gel to see if at all your protein of interest is there or at all if your protein of interest is purified or it has other contaminant protein. So, if you got pure protein at those fractions you concentrate it down because the volume will be much higher you concentrate your protein or then, you go for another procedures like buffer exchange and other steps for your own experiment.

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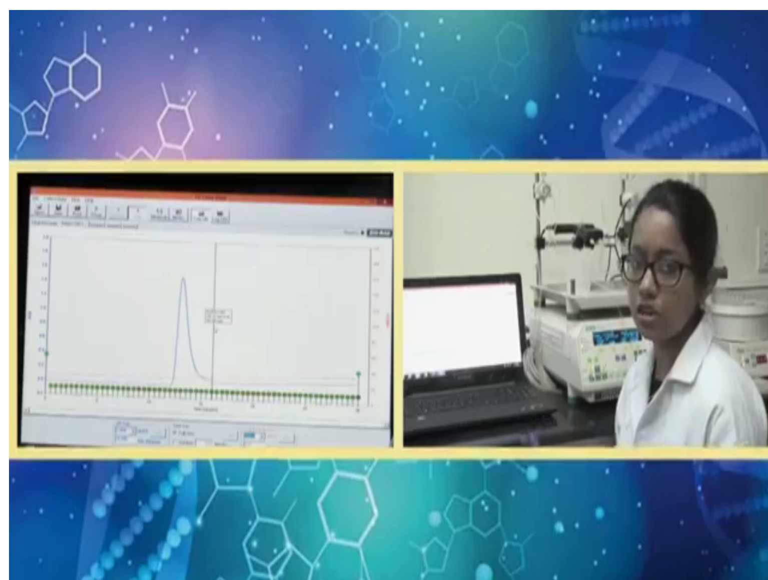

As I mentioned before the whole procedure can be monitored via this computer where it will collect the data from the absorbance also the conductivity. Here, I am showing already a graph where I have increased the concentration of imidazole at the particular gradient and you can see this numbers means at which fraction there is a spike at the absorbance level.

So, the blue line actually denotes the absorbance value at 280 nanometer. Now, whenever you see a spike at that a 280 nanometer, it means that this fractions actually contain some protein. In our case it is our own protein of interest which has excess his tag and now you have to run these fractions you collect these fractions and run in SDS page to check the purity of your protein. The red line denotes the conductance of the solution. So, if you here we have attached a nickel NINTA column in this system, you can also attach other columns like ion exchange column, where you might need to increase the salt concentration.

So, from this conductance line you can actually monitor that increase in the salt concentration and value protein of interest is coming from. So, all this things you can monitor through this computer.

Hello, everyone today I will show you how to isolate or purify a particular protein from sterilize it or a mixture of proteins and based on affinity chromatography. So, as the name suggests in case of affinity chromatography, you have a protein and it has a specific affinity to a particular ligand or a molecule and based on that affinity you can isolate your protein of interest from other molecules like other proteins that are present in the sterilizer.

So, now I have a protein which has 6 x histidine tag. So that means, 6 histidine molecules are present consecutively at the in terminal of the protein. So, this histidine tag protein will help me to isolate this particular protein from other contaminant protein from this cell lysate which does not have this consecutive histidine tag. Now, why it will get isolated from the other molecules? Because 6 x histidine tags has an affinity towards the nickel ions that are that we will use to isolate our protein.

So, it will get attached to the nickel ions while other proteins will flow out from our column and this will fall used to purify our protein of interest. So, now, what we have here is a FPLC system. So, this is semi automatic FPLC system where based on the

pressure, it will take some amount of buffer at a particular flow rate and it will pass the buffer through a column which has the nickel beads in it. So, this is the column which we have.

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So, this is a 5 ml column volume NINTA column. So, this is also known as IMAC or affinity chromatography column. So, in this column there are nickel base that are attached with the NINTA nickel ions that are attached with a NINTA beads and this beads will help me to isolate my protein of interest. So, basically the nickel ions are charged and it will capture the histidine molecules. So, my protein will get attached with the in inside the column and other proteins will come out from this column and later, after it gets attached with the column we also need to elude my protein out from the column for further experiments. To elude the protein from this attached nickel ions, we will run above for which has higher amount of imidazole.

So, imidazole also has this ring which will act as a binding ligand to the nickel ions. So, it will compete with the with my protein of interest which is bound to my nickel ions. So, increased amount of imidazole will basically replace my protein from the column and itself get bound to the nickel ions and my protein will come out from the chromatography from this column.

Then, I can use my protein for further experiments. So, before we begin with our purification protocol, first we need to prep the instrument or the machine the FPLC

system. So, while other people when other people use the FPLC system when they are done with it, they store the FPLC system all that tubes in 20 percent ethanol.

So, the tubes are meant to be stored at 20 percent ethanol because it will inhibit any kind of contamination or microbial growth. So, first of all what we need to do is we need to replace this 20 percent ethanol on the tubes with deionized water. So, first I will begin with running the water, so, therefore, around 10 minutes. So, that the ethanol is replaced and my machine will be prep for running my buffer.

So, in the machine there is 2 in inlet, so, a and b. So, this two inlet what it does is with the help of a peristaltic pump, it will take some amount of buffer at a particular flow rate which I will generate; I will tell the machine to do it will take the buffer and it will pass through all this channel and to this chromatographic column. Then, from this column there is a outlet tube and this tube will go directly to this particular holder. Here, the UV lamp is there, it will monitor the absorbance at 280 nanometer. Since, we are using this for protein chromatography; this machine is this machine is told to get the absorbance at 280 nanometers after it gives the reading for 280 nanometer, this flow will come out and the reading this is a salt or conductivity inlet.

So, this will get the conductivity measurement and then, it will come out and it can go either to the west; we do not need to collect the west or it can go to this column collection. So, from this 2 inlet it will take up some amount of water and it will pass through all this tube and then, go through this column. From the column, there is an outlet tube is it will go to this UV lamp. So, this black box has UV lamp, it will measure the absorbance at 280 nanometer because we are working with protein and this is a machine for protein chromatography. We have the UV lamp at the absorbance of 280 nanometer because protein has absorbance at 280 nanometer.

So, it will take the measurement at 280 nanometer of the flow tube and then, it will take go out and then, it will go to this small black holder. It has a conductivity measurement instruments. It will get the conductivity of the solution and then, it will go out and it might get went to the bypass system or west which we will not collect or if you want to collect the flow through you can go for this column collection, where it will be collected in the fraction collectors which we will provide later.

So, right now what we are running is only 1 water we do not need to collect the water because we are basically rinsing the all the tubes and my column and so here all the both the inlet tubes are inside the deionized water. So, I will run this start the machine and the column is already attached to the machine. So, here in this is the particular monitor system, where you can tell the machine at a particular rate of flow rate at what pressure you want to flow the buffer or water. So, here the floor it is mentioned as 5 ml per minute basically depending on the column size and the volume and see the manufacturer protocol; what is the pressure it can withhold or what is a flow rate it can stand.

So, my column is a 5 ml column volume has 5 ml column volume and it can go up to 5 ml per minute. So, I have mentioned it here as a flow rate is 5 ml per minute and from the both inlets. So, buffer is 50 percent a and 50 percent b. So, basically it will take from the both the channel, it will take same amount of water and mix it and then, pass through my column and the tubes. So, in by this way my everything will be cleaned up. So, I will start and it will take out this water and start raising the tubes and my column.

So, how will we know when to stop? So, basically based on our column size and the protocol which you are following, we will start around 10 column volumes. As I mentioned my column is 5 ml per at volume. So, I will run around 50 ml of water. So, for at a flow rate of 500 per minute; that means, I have to run it for 10 minute. Another thing which is attached to this machine is the computer system, where can you where you can check what is the absorbance and the conductivity. So, I have mentioned from this column the flow is coming out the solution or whatever it is it is coming out and going through passing through this UV lamp and the conductivity measurement lamp instrument, so, this 2 values you can check.

So, we have a monitor or computer attached to this system. So, I have been running water through this machine. So, as I mentioned that you can monitor the absorbance and the conductivity, what is flow coming out from your column by using this computer. So, this is the basically the computer which is linked with this instrument.

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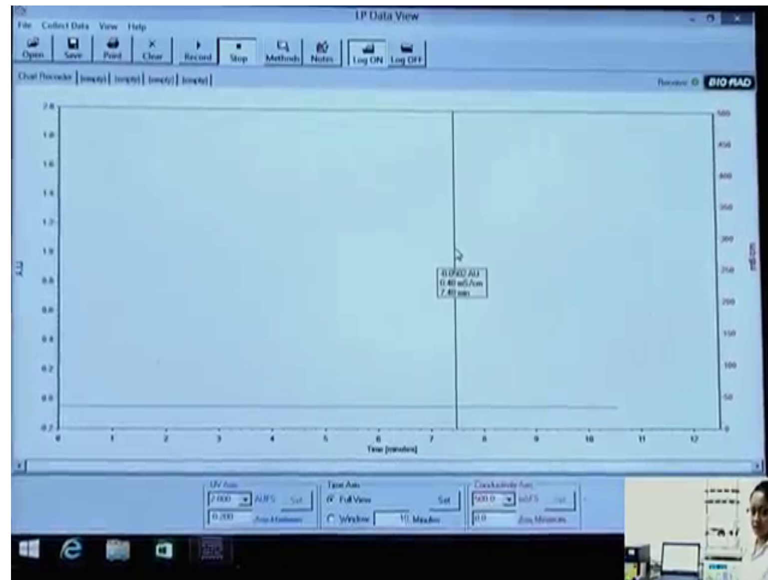

So, as you can see this is a kind of a chart like a presentation and here there are 2 axis; one is the blue one which basically tells you about the absorbance. So, this is the AU or arbitrary unit. So, this is the absorbance value at 280 nanometers. So, if any kind of protein come is coming out from your column, you can monitor that and this rate there is another rate access which is the MS per centimeter.

So, this is the conductivity measurement. So, if there is any kind of salt differentiation between different buffers or something is coming on which has a charge that also you can monitor. So, as I have been running water for almost 10 minutes which I have mentioned almost 10 column volume. So, you can see there is a blue line is kind of flat and also the radius flat which you might not be able to see because it is kind of mixed with the x axis. So, this flat means the instrument and the column is stabilized in that particular solution; there is no up and down.

So, if there is any up and down in this 2 reading; that means, there is might be some bubble or some precipitation of which you need to clear it out. So, do not stop until you get a flat line in this 2 absorbance and conductivity measurement. So, now, that I have a flat line and the system is almost clean with water deionized water. I will stop it now and then I will start running a buffer. So, what is this buffer?

So, basically we need 2 buffer for these his tag affinity chromatography; one is the wash buffer. So, in case of wash buffer, it is basically the same buffer in which your protein is

present right now. So, where when you use a licensed buffer for isolating your protein from cellulites; so, basically we have trace around 100 millimolar which has a pH of around 8.2 and 200 millimolar of salt. So, in this condition there is opportunities happy based on your depend it will depend on your own protein.

So, in this condition your protein is there. Along with this trace and NACL we also have 10 millimolar of imidazole. So, why we have 10 millimolar of Imidazole? So, basically in wash buffer, we have this particular small amount of imidazole to avoid any kind of non specific binding. So, as I mentioned that my protein has 6 x histidine tag. So, basically 6 consecutive histidine residues are present at the n terminal portion or it might be at the c terminal portion, but there might be some proteins inside the cell which also have a few histidine tags not necessarily 6 x, but it might be 2 or 3 residues of histidine consecutively and they can also buy into this nickel beads..

But we do not want those proteins. So, recombinant recombinantly we prepare our protein to have this 6 x histidine tag, while other protein is not our protein of choice and we do not want that. But they also might get attached to this nickel beads. So, to remove those proteins from this column, we run a small concentration of imidazole which will remove those proteins at the very low; they have very low affinity because they have 2 or maximum 3 histidine residues simultaneously.

So, those will remove our other protein while our protein which has 6 x histidine tag is very tightly bound to this nickel beads because it has a high amount of histidine and they will not come out. So, by having this 10 millimolar Imidazole, it will ensure that nonspecific binding does not occur. So, before we load our protein samples which is present in that particular wash buffer, we first need to equilibrate the column and all the tubes with that same buffer.

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So, for that before we load that our sample we need to run the wash buffer which has this trace and NACL and imidazole for almost again for 10 minutes or so. So, now, I will take the wash buffer and put this to inlet tube which are were present inside the water which we are passing through and then, I will put this to inlet into the wash buffer and again at the same flow rate that is 5 ml per minute, I will start running this particular wash buffer which has trace and NACL and around 10 millimolar of imidazole.

So, it will ensure that my column before am I load my protein is equilibrated at that particular wash buffer and simultaneously, I will start recording here. So, after sometimes we will see that it will go up the conductivity will go up a little bit because I was running water which does not have any conductivity, but this buffer will have NACL. This will go up because NACL has a salt and it will have a higher conductivity.

So, again we will they it will the value will go up and again we will wait for some time so that it goes as a flat line. Because then, we will know it is equilibrated are saturated with this particular buffer, then we will start loading our sample. Now, why it is running the wash buffer is running, I need to check if there is any precipitation or not.

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If it is there is any kind of precipitation you see any whitish or any kind of precipitation, you just centrifuge it and remove the precipitant and take only the solution which is kind of clear and again, you might not be able to see small particles that might be present inside the solution and it might clog up your column. So, to avoid that what we will do is we will pass it through a syringe filter. So, this is a 0.22 micron syringe filter, it will ensure any kind of cell debris or particular matter will stop and get clogged up and whatever you get after passing through this column will be pure solution which does not have any particular matter.

Here, I have a 10 ml syringe. So, first I will take the sample in the syringe. So, now, my sample or whatever the cellulites it might be you be having is in my syringe and then, I will remove this needle. Be careful with the needle. Then, I will put it in this syringe filter. So, a filter looks like this. You can put it here inside this; you can attach this to the syringe and then, I will take a fresh tube and pass this solution through this syringe filter.

So, now, my solution will not have any particulate matter. So, this is the sample we did not load into our column. So, another thing you should observe or make sure is that before you run or load your cellulite into this column, you should run first and SDS page gel to check if at all your protein of interest is present inside that is cellulite or not. So, I have to make sure my protein is ready. Now, I will wait till my column gets equilibrated with the wash buffer.

So, now we are running buffer a through this column all for almost 10 minutes.

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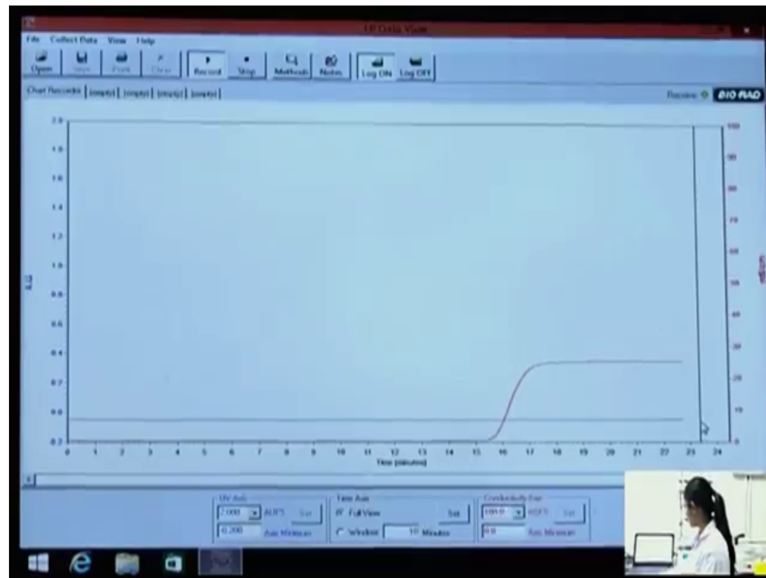

So, see in case of this monitor the red line is going up and it became stable at the particular point. So, this is the conductivity detector measurement. So, basically as I mentioned the buffer has an NACL. So, it went up, but if you see the blue line is constant as the water because we in the buffer there is no protein or anything which absorbs at 280 nanometers. So, there is no change and this 2 line lines are flat. So, basically my column is now stabilized in the particular buffer and it is ready to load get load by the sample. So, I have prepared my sample already. So, it is passed through this 0.22 micron syringe filter.

So, again I will take this sample in to a syringe trying to remove the bubbles and also remove the needle. There is small amount of bubble if you like do this taking, it will go come out. So, try to avoid inserting bubbles to your column because then there you will see there is spikes in the conductivity as well as the UV. So, I have around 5 ml of the sample, where my protein and also the other contaminant proteins are present. In the machine there is a valve where there is a point, where it is mentioned to inject the sample.

So, this is a position where I will be able to inject my sample and while I inject the sample, it will go into our loop. This loop has a 10 ml of volume. Basically, I can load at

once up to 10 ml, but as I have only 5 ml I can load directly and at one go. So, I will remove this small syringe and then, put my syringe here.

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And then, carefully and slowly I will start injecting the sample. So, my sample is going into this sample loop that is of 10 ml of volume. When am done with this injection I will rotate this valve. So, now, this loop is connected with the other inlet that is here. So, after load my sample, I will start running the same buffer that is the wash buffer a which has 100 millimolar trace, 200 millimolar NACL and 10 millimolar of imidazole. So, it will force the sample into my going through my column when my protein will get stuck and other protein will come out from this column.

So, after I start the sample loading, I will again start the measurement or seeing the monitor as well. So, when if there is any protein coming out from this sample loop or from this column I can monitor it in the UV absorbance detector. So, now it is again flowing at the 5 ml per minute volume and only buffer a is running. So, I will monitor here when the other protein is coming out and then, it getting stabilized. So, will again see a flat line; then, I will start the elution protocol which I will show you later.

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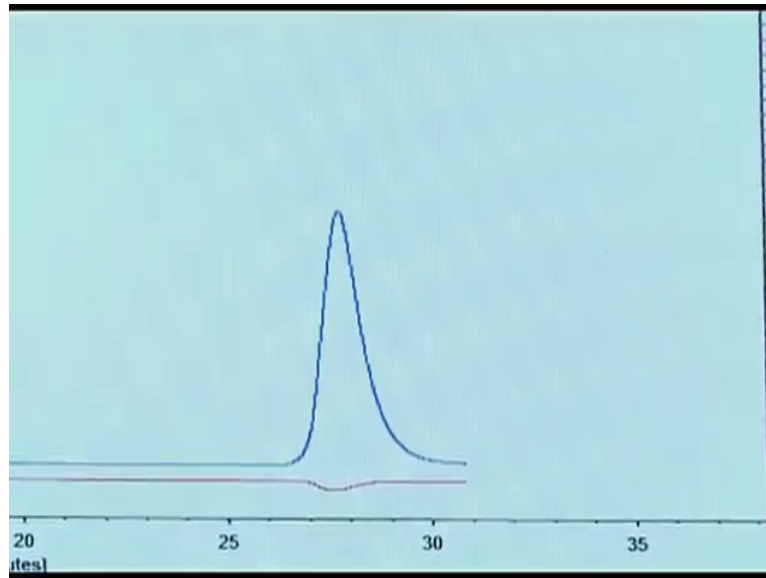

Now we can see in the monitor the blue line is going up. So, as I mentioned earlier that blue line denotes the absorbance detector. So, whatever protein which does not have this consecutive histidine residues will come out right now because it will not get stuck to this nickel column and as we have 10 millimolar of Imidazole, it will displace any kind of non specific or low binding of this other proteins and this proteins after its coming down. So, it now it is going down.

So, anything which has which does not have histidine when it is coming out, we have to wait till it goes again as a straight line. So that means, my protein is inside the column and other protein has come out completely from the column after that happens we will then start for this elution.

So, we need to take out our protein from this column. It might it should not be get struck to the column for eternity. So, then we will run buffer b. So, buffer b has the concentration of imidazole that is 500 millimolar. So, it has same amount of trace that is 100 millimolar of trace page 8.2, 200 millimolar of in NACL and then 500 millimolar of imidazole. So, as we have higher amount of imidazole that will displace the protein of which has this affinity towards a nickel beads that is my protein of choice and it will displace my protein and then, my protein will come out and that we can measure and see when it is coming out. So, that time we will collect all the factions.

So, right now we are not collecting anything that is coming out, but it is advisable if you are doing it for the first time you should collect all everything what is coming out night now or elution because it might happen that your protein is the bacteria or the cell which in where you are isolating of expressing a protein that might be cleaning your his tag or for some reason for buffer condition or something, you protein is not binding to the column. So, at that time your protein also might come out at this stage. So, always collect everything before and if you are doing it for the first time later on and if you as you progress you can also make the judgment if you want to collect it or not.

So, right now am not collecting anything, but when I start the elution procedure, I will start collecting all the fractions were different type of fractions will be there and based on the curve or the peak, where I will get my protein I will select those fractions only and take the sample and then run a SDS page that is a (Refer Time: 34:30) and check if my present protein is present or not and if at all other contaminant protein is present or not. So, now, you can see this peak has gone down completely and it is becoming flat. So, at this particular stage the protein has gone; so, other contaminant protein has come out from this column and my protein is suppose if this stuck inside the column.

So, now, what I will do is I will stop this run and here also I will stop it. Then, as I mentioned earlier there are 2 inlet buffer a and buffer b. So, right now only buffer a was running now I will have buffer b as well and I will take this buffer b inlet and put it inside the buffer b. So, there are 2 inlet. Now it will take a certain volume of buffer b. Now, this is a mixer where it will mix at a proper gradient which I will mention it will mix and then, it will pass that particular gradient buffer through my column. So, now, I will run a particular program. So, right now I am running this manually. So, 5 ml per minute only buffer b or a. Now, I will select a particular program here.

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So, in this program if I open; so, as I have selected. So, from 0 minute to 3 minute only buffer B will run at a particular flow rate that is 5 ml per minute. So, that will ensure that any kind of contaminant proteins are washed out from the column and only my protein is present. At the next step from 3 to 18 minute 0 to 100 percent B will be there. So, what does it mean 0 to 100 percent? So, basically it will make a constant gradient from 0 to 100 percent of buffer B. So, it will take some suppose for 1 percent it will take 99 microliter or 99 ml of this buffer and mix it with 1 ml of buffer B, so, that means, 1 percent of buffer B.

So, it will take like that it will take make this gradients and then it will flow the that particular buffer through my column and then, until last step we have only buffer these are from 18 minute to 21 minute only buffer B will run. So, all the flow rates of same 5 ml per minute and I have set up this program like this based on your requirement you can change it. If you have higher amount of protein or higher column volume, then you can go and modify this measurements. Now, I am done with this method and I will start running this particular method, where it will take buffer A and buffer B and then we will also monitor what is happening to our protein of interest.

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So, before we start the program another thing we need to do is we need to collect all the fractions. As I have mentioned that in this particular step, my protein will come out from the column and we need to make sure which in which fractions or in which particular volume my protein is coming out. So, this is the fraction collector where I have this collected tubes. So, this is FPLC collected tube.

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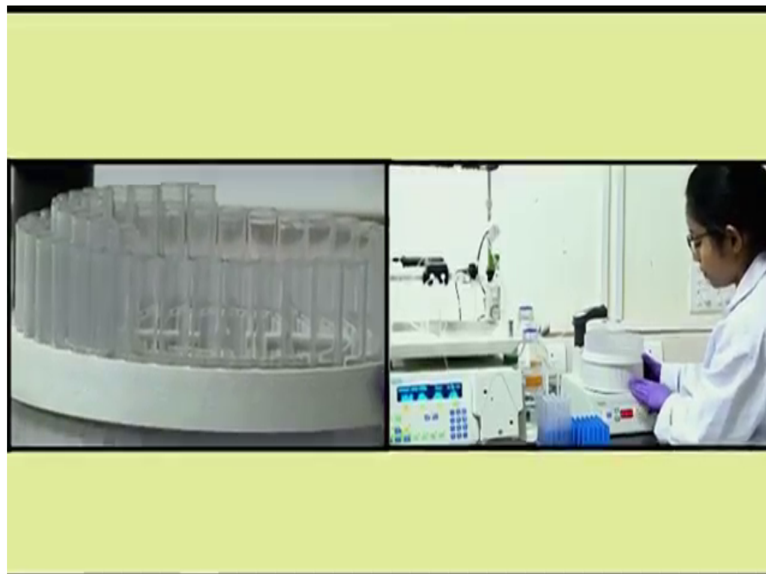

And I will put these tubes here. So, this tubes have been washed properly with deionized water and this tube in each fractions, I will have around 2.5 ml of sample or the elution.

So, this fraction collector will rotate and this will also show at which particular fraction number I will have my protein. So, there are numbers written in this fraction collected tube. So, based on that you can identify the corresponding fraction collection number of the particular tube. So, I need almost 42 of this collection tube. So, the program is running for 21 minute and as I am collecting per half minute particular fraction.

So, in each fraction I will have 2.5 ml of sample because at 1 minute 5 ml of buffer is running. Now, I will set up this particular fraction collector properly. So, there are markings which will be help you to set it up properly and this is the collector tube which is coming from this column going through this UV and then, conductivity and then from here it is coming out and I will put it like this.

So, when I start the program, it will start collecting the whatever flow is coming out of the column. So, now, I will start running the method here and there in the monitor, it will start recording all the parameters that is the UV and the conductivity. So, now, we have to wait and see in which fractions my protein is coming out.

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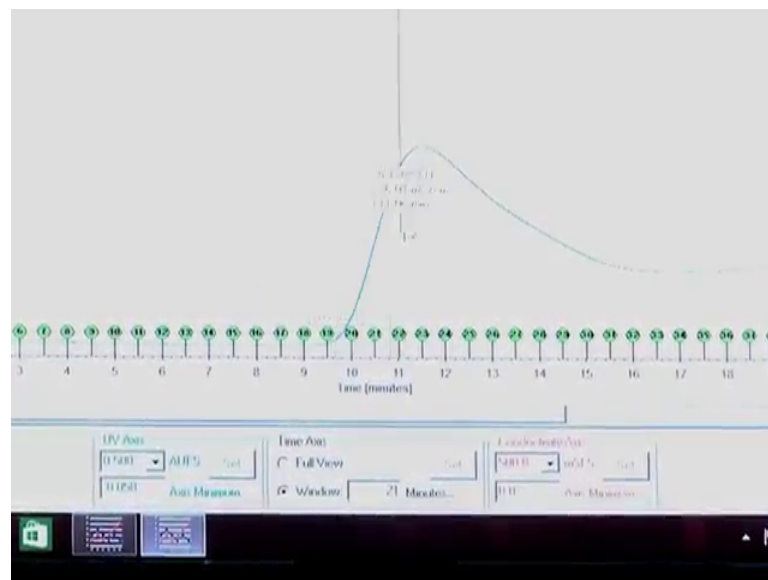


So, right now three things are happening simultaneously fraction collection collector fractions are getting collected. So, almost ninth tube is getting collected. So, it will rotate and then, each tube there will be 2.5 ml of sample. In the monitor mixing has been started. So, basically it is taking buffer some amount of buffer n buffer b and mixing it. So, in this monitor, it will show how much of buffer b is getting passed through. So, it is

around 12 percent b; that means, is taking some the mixing it at the proportion of 12 percent b. As I mentioned simultaneously it will go up. So, as we have mentioned that 0 to 100 percent of buffer b will pass.

So, now, it is going to 14 percent to b and while this is happening here the monitor will show us what particular in which fraction is getting collected. So, this small peak will be there this green colored things. So, there it will be written what kind of what number of fraction is getting collected and the blue and red line denotes the UV as well as the conductivity measurement. So, now, it is still flat because at 17 percent b it is not coming out of my protein. If my protein is coming out, then again it will the blue line or the absorbance will go up and we will identify which particular fractions my protein is there and then, we will isolate or collect those fractions only and then we will run our gel to see if my protein is present or not.

So, now, our program has finished. So, in this monitor you can see. So, it was going as a straight line or constant line; then the blue line went up here. So, basically at this particular point in this peak shows that the my protein has come out from the column and then, it went down as every all the protein has come out and then it became again flat.

So, now as I have mentioned earlier this green ones the balls shows in which fraction my protein or at particular which fraction it is collected. So, if I zoom into this particular peak, here you can see starting from the fraction number 19 and till here at 26 main the base of the peak is there. So, I will collect from 19 to 26 this fractions and pull all the and then, for each fraction I will run the acrylamide gel or SDS page to check if the protein is pure or not or if my protein is there or not.

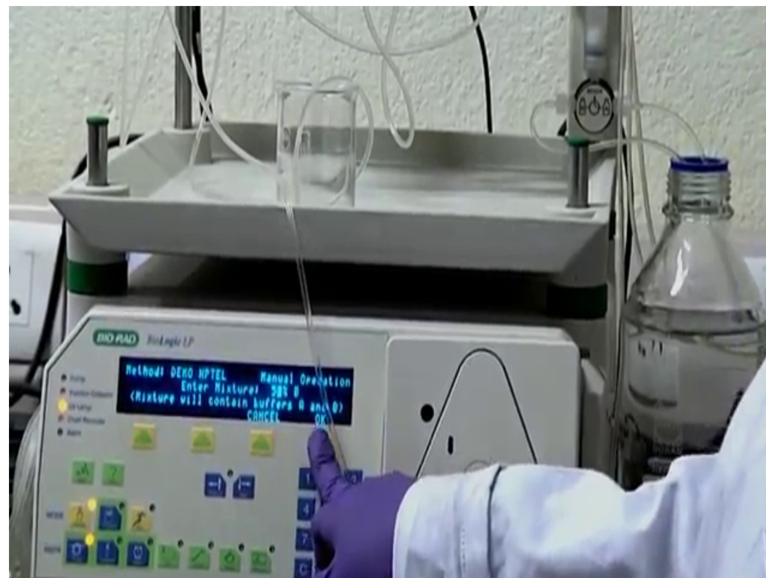
So, from this chromatographic technique, we can see that my I have isolated my protein and it will be confirmed when I run a gel, but I have isolated my protein from other contaminant protein from the cell lysate or other mixture of proteins and this protein has 6 x histidine tag. Now after I collect all the fractions and run the gel and when confirm that my protein is present and pure; then I can go for this his tag I can go for this cleaving of this his tag. So, in my construct I have this his tag and then there is a cleavage site and using an enzyme called (Refer Time: 44:30) I can cleave out the his tag part and then, I can use the protein which does not have the his tag then for further

experiments or I can leave it like that if it does not hamper my proteins function or other further experiments.

So, now, the machine has done his work. So, I have isolated my protein. Now, after you isolate the protein and run all the programs then it is still in the buffer which is the buffer b. After you are done with using this machine for purification of your protein, so, to maintain the machine what you need to do is you need to wash all the tubes and your column with deionized water.

So, basically it will remove all the salts any kind of proteins that are that might be precipitated out in the channels and it will clean up all the all your column and the tubes. So, now, I have put both the inlet in again in the deionized water and I will make the buffer as mix and take 50 percent of a and 50 percent of b.

(Refer Slide Time: 45:29)



And again at the flow rate 5 ml per minute, I will start running only water and start recording here. So, what it will ensure is that the column as well as all the inlets and tubings are getting cleaned up with the help of water. So, sometimes what it might happen is after repeated use of this particular column, you might say see some precipitation. It other salt or proteins might get clogged up inside the column. Also might also see some precipitation or something inside that tubings as well. So, for that we also follow some cleaning procedures.

So, for cleaning this tubing say we need to run 1 molar of NAOH or a high alkali solution and then, cleans rinse it with water and then again 1 molar of hydrochloric acid again highly acidic solution and then rinse it with water. And then for storage purposes you should always store it in 20 percent of ethanol because it will inhibit the growth of any kind of microbial growth inhibit a growth of any kind of microbes for this column or the cartridge here. So, it might also get clogged up with after repeatedly use.

So, if you see any kind of back pressure on the sample or the flow solution is not getting passed through your column or after purification, you are also seeing some kind of contaminant protein inside in the fractions; what do you need to do is you need to regenerate the column. So, for the regeneration of our NINTA column, what you need to follow 3 basic steps; one is first and the foremost step is to clean up with pure or water or deionized water.

Then, you run 1 molar of NAOH for almost suppose 10 column volume that is for this column it is 50 ml. So, that that will help ensure that any kind of protein precipitation or other precipitation will come out from your column and then, when you use it repeatedly some of the nickel ions might be reached out and the column might lose its sensitivity or the activity on of its own.

So, for that what do you need to do is you need to pass through EDTA. So, EDTA will remove all the nickel ions. So, while you pass EDTA it will chelate the nickel ions and it will remove all the nickel ions. So, you will end up with only the NINTA beads or (Refer Time: 47:59) beads which does not have the nickel ions. So, then after you remove all the used about the nickel ions, then you have to regenerate the beads using ni nickel sulphate now.

So, basically nickel sulphate has this ni nickel ions, it will get chelated again in the beads and then you can use the column for further future use. So, by this procedure you can have to regenerate column if you see any kind of clogged about back pressure. So, back pressure means it will or it will somehow inhibit the flow of the solution. So, you might see there is clogging up or something then you need to regenerate your column.

So, by this way you have to regenerate your column and if using NAOH and HCL, you can clean up this valves and as well as this tubings. But the most important thing is you should always store your column as well as this tubes at 20 percent ethanol. So, this

column if I stop it here, it is removable. So, after your cleaning up after you have cleaned up your column, you have to remove this column from here. So, basically you pass 20 percent of ethanol and then this column should be stored at 4 degrees centigrade, so, keep it in the fridge.

Well, this machine is here and everything should be at 20 degree 20 percent of ethanol. So, now, we have cleaned up the column as well as the machine; so, our job is done. So, basically at the end of this purification procedure, you will have a particular recombinant protein which tells us at 6 x histidine tag on its own and that you have to ensure after running a gel and this protein will be readily used before further experiments.

So, all those fractions has 2.5 mls. I will almost collecting fraction of almost 25 ml of sample I will get to increase the concentration I have can go for this concentrating the sample using a centric ones. So, basically it is a centrifuge tube which has a cut off membrane cut off and I can concentrate down my sample and then, measure the absorbance at 280 nanometer to see how much protein I have.