Experimental Biochemistry
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Lecture - 32
Isolation and Purification of Proteins

Welcome back, today in NPTEL Experimental Biochemistry course we will learn how to isolate and characterize protein. So, if you are working in a biochemistry lab, you will might find out that you need to have some protein for your own experiment and today, we will learn how to isolate that particular protein. So, for today's experiment we will go through the basic steps of protein purification from a source. So, there is no harden first 1 single technique how you can isolate a particular protein. So, depending on the protein you need to change or modify the protocol a little bit, but mostly the basic principle will be same.

So, first we need to have the source of that particular protein. So, if you are using a bacteria, it is a very good source to get a large amount of protein, because you can grow bacteria in a large culture and isolate the protein from that. But then, you need to have the protein inside the bacteria. So, you can do that by recombinantly transforming a particular gene or your protein constructs inside the bacteria. And that we will do today, we have reconstructed the bacteria and put a small plasmid which contains our gene of our protein gene which express our protein of interest. Otherwise if you want to isolate a particular protein from a natural source, then your option is quite limited, because then you need to grow or isolate the particular organ or tissue from where you need to get the protein.

So, as we are isolating the protein from a bacteria first what you need to do is, express the protein inside the bacterial cell. So, for that what the basic steps are that you grow your bacteria in normal LB or in a for specific need M 9 or other minimal media. So, first try to do with in a enriched media like lauria bertoni or LB media. So, if when you grow the natural cell, there will be a huge number of bacterial cells, then you need to express the protein. So, mostly we follow an IPTG or induction based technique. So, there we provide IPTG.

So, I will go through the basic steps how you can express or over express your protein inside the bacterial cell. So, first what you need to do you need to given overnight culture or around 15 to 16 hours culture in LB media, where you grow your cell, bacterial cell mostly ecoli. And the vector which use the strain basically, a of ecoli will be someone which has a expression system. So, we chose BL 21 lambda DL 3 a strain which has this, a lack operon system where you can express your protein, when you provide a particular inducer in our case that is IPTG.

So, basically you grow your cell overnight, then you give a primary inoculum from that particular overnight to a large culture that is suppose 1 liter of LB media. And you grow that particular 1 liter media in the next day for maybe 4 to 5 hours that 37 degrees centigrade, because ecoli mostly grows at 37 degrees centigrade, and half like this or the life cycle is around 20 minutes.

So, when the media reaches are particular ODR point 6 to point 8 that time you know you have enough bacterial cell when you can induce the particular culture with IPTG and the bacteria will start growing or producing the proteins. Do not put IPTG before that, because then you will have end up with a limited number of cells and when you put IPTG it is toxic. So, then the bacteria will stop growing and start producing a protein.

So, first you need to have enough number of bacterial cell and then after that you can start producing a protein. So, when the water reaches at point six to point 8 you induce the bacterial cell to IPTG and then again use do not grow your protein for 4 hours to even 18 hours. So, this techniques or the timings and the temperature will depend on your protein. So, first it is a trial and error based on technique. So, basically you first do something and then if you are not comfortable or happy with that, you change the protocol and so that you get enough number of enough amount of protein.

So, what we did is. So, we have grown our cell in bacteria and when you grow after the induction process, we need to collect the cell pellet. So, basically we do a centrifugation process it and get the cell pellet and the media way which comes as a supernatant we throw it away.

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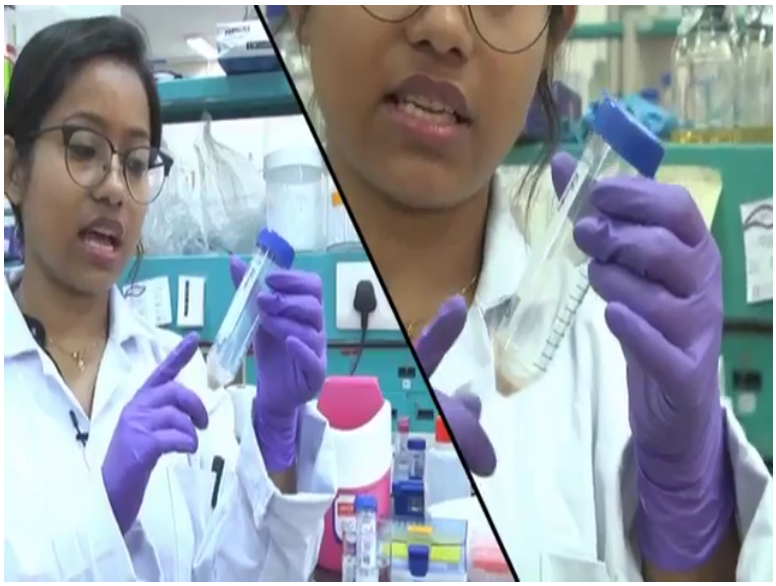

So, when you collect the cell pellet it will look something like this. So, it is a very small amount of culture around 10 ml. So, the cell pellet will be at the bottom of the tube and I have already discarded the media.

So, here this is the cell pellet. Now, you can keep the cell pellet as it is in minus 20 degree or minus 80 degree when and whenever you are comfortable on the next day or some other day when you want to purify the protein you can purify that particular protein from that. Another thing what you can do is, you can get the cell pellet and then we suspend the cell pellet in a lysis buffer.

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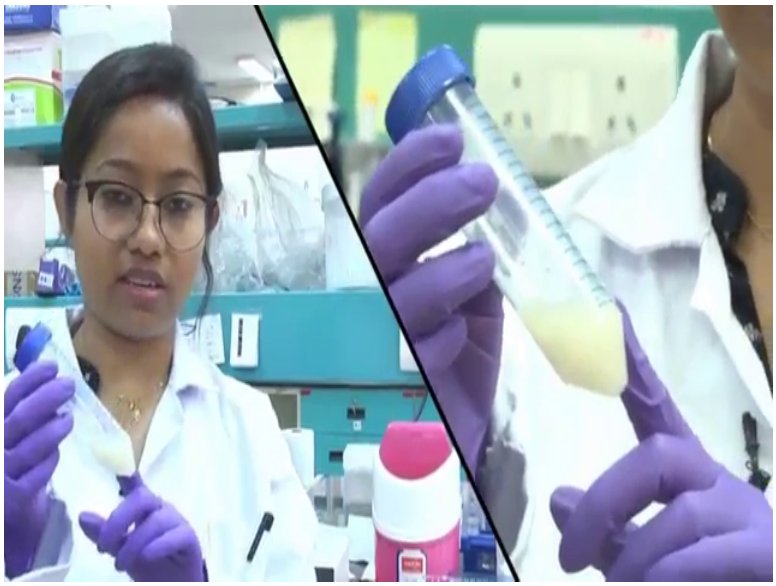

So, here in this case I have re-suspended in the lysis buffer. So, it has around 1 litter of culture, and this pellet I have re-suspended in lysis buffer.

So, what the lysis buffer contains. So, lysis buffer contains a particular; it is a buffer system. So, at; obviously, it has a particular P H range. So, in our case it is around 0.8. So, depending on your protein you need to check the P H range, because you should not be going closer to the PI of a particular protein. So, at the particular PI the protein will be neutral and it might get precipitated out or coagulate.

So, for that you need to check the page mostly the in our case mama protein is at PH 8.2 its happy at PH 8.2 because my proteins PI is around 11 or more. So, it is not closer to its PI. So, along with the PH; so, basically we have this trace and. So, to basically confirm the PH. So, as I have mentioned that your buffer should have a particular PH. So, in our case we use trace to regulate the PH of this particular solution, because this has a basic PH value.

So, the PH is 8.2, along with the trace it has some NaCl or salt basically. So, the salt solution is a important for a particular protein, because if the salt is too low are too high in the protein also might get precipitated out. So, you need to have a proper buffer system. So, in our case the salt solution is around 200 mille molar NaCl. Along with that you might have some other ingredients in the buffering system depending on your need on your need.

So, basically if a protein you are using it if it has a specific need go for a literature survey and see what other people are using for that particular protein, and depending on that you can add those things inside it. So, sometimes people use EDTA; so, that other divalent ion search elated out, and it does not hamper the protein purification, but since we will use FPSC system or a basically a column purification with using a nickel Ni NTA column. So, we do not use EDTA here, because then the column it hampers the later purification process inside the column.

So, as I mentioned the; check the literature and based on your protein you need to choose what kind of buffering system you want to use. So, along with this other 2 important ingredients that we use here is 1 is protease inhibitor. So, when you lyse the cell, all the cell components come out inside from the solution.

So, there will be carbohydrates, there will be membranes and, there will be other proteins also there will be proteases; like, different proteases that are inside the cell. So, if the proteases and the proteins are present inside in a solution, the protease might cleave our chop up the protein of your interest. So, to stop the action of those protease, some people use protease inhibitor as well as EDTA. So, here we are using protease inhibitor, it is named as PMSF. So, PMSF we will use around 2 nano molar concentration.

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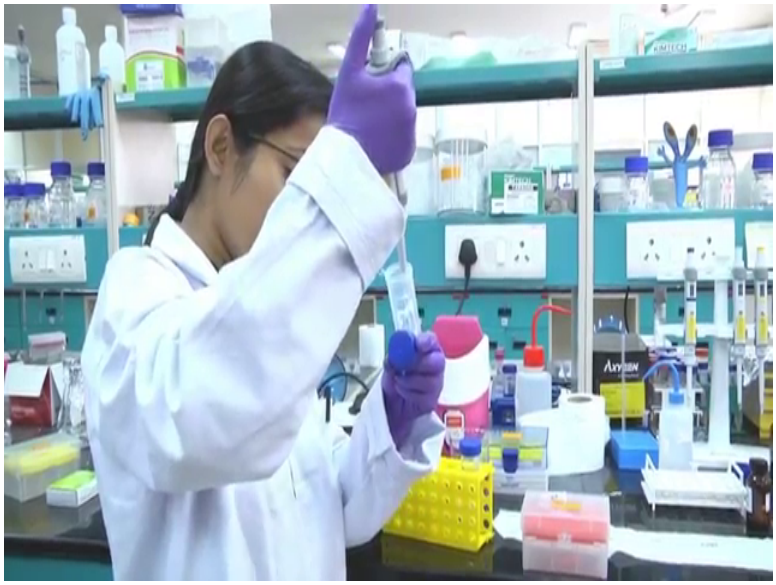

So, I will add some amount of protease inhibitor so, that whenever I want to isolate my protein or start lysing my cell, the proteases will be inactive and it will not chop of my

protein. So, when you dissolve this in our lysis buffer, if you are doing it in a previous day you can keep this dissolved and keep it in a minus 20 or minus 80 degree centigrade temperature. So, that it freezes out. So, the next day you thaw it and then you start the purification. So, there is a freeze thaw cycle once.

So, that also helps in isolating or basically lysing the cells because the proteins are inside the cell. So, you need to break down the cells and to so, that the protein comes out in the solution. So, 1 freeze thaw cycle is good. So, you it is best that you express the protein, then get the pellet dissolve it in lysis buffer and keep it in minus 20 or minus 80 and whenever next day or some other day when you have time you start characterize or isolating your protein of interest.

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Now, when you add another, this is a vortex machine. So, basically we will vortex it out. So, that everything is mixed properly and this also helps sometimes in isolating or basically lysing the cells. So, now; what we are going through is a lysing procedure, before you start lysing you add your protease inhibitor and you also mix it properly. So, what it does is, it is kind of a vortex machine it gently or basically vigorously mix the solution.

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So, it vigorously mix the solution you can go down the speed as well if you are not comfortable with that much of speed sometimes protein might start degraded. So, depending on your need, and your proteins requirement you choose the speed. But here as we are using the crude extract right now we want to be as little bit harsh. So, that is why we are going at a high speed and that is, this will ensure that a my protein is getting mixed up.

So, I have used a vortex machine to properly dissolve the cell pellet whatever the pellet or the cells are there inside the solution to mix it up properly also the PMSF are other ingredients that I am putting inside the solution. So, that it thoroughly mixed. So, sometimes also we use sodium azide to keep for longer storage. So, when you are keeping the pellet or the cell suspension in minus 20 or minus 80 for longer period, here there is a chance of fungal contamination, because the cells or the medias and the proteins are quite good growth medium for the fungus.

So, then you need to add something which inhibits the growth of fungus and sodium azide is the fungal growth inhibitor it is a toxic substance, and it will inhibit such growth and the later when you use it or start isolating or purifying your protein it will not hamper any more. So, now, if you see the solution. So, I am here using a Pasteur pipettes. So, is a plastic Pasteur pipettes, the solution is quite runny.

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So, its forming a proper drops.

So, it is not viscous or anything. So, in this case basically the cells are not lysed, to help with the lysing procedure of the cells. So, basically the membrane the cell membrane or the bacteria memes and cell membrane has to be lysed and the cell walls. So, for that we will use something called lysozyme. So, lysozyme is an enzyme which will hydrolyze the polysaccharide the membrane of the bacteria and then all the cellular components of the bacterial cell will come out inside the outside the cells.

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So, you can make 10 mg per ml stock lysozyme solution. So, lysozyme can be purchased from a company as a powder. So, it is a (Refer Time: 13:07) lyse crystalline powder of lysozyme or you can use the way we are using, basically a pinch of lysozymeis enough to hydrolize all the bacterial cellular membrane.

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So, I am taking a pinch of lysozyme and then I will add the lysozyme to my solution. Now, as it is a powder you need to mix it up properly. So, again we will use this vortex. So, vortex will ensure that the lysozyme or anything you provide is mixed up properly and we are going a little bit harsh, because now it is only cells that are intact and we need to lyse the cell. But if otherwise specified suppose you are using a particular protein or tissue system which does not need or it is not required for the harsh treatment you should avoid that.

Now, lysozyme acts best at 37 degree centigrade temperature. So, we will keep it this solution at 37 degree centigrade temperature for 20 to 30 minutes. How we will know that if the lysine has been completed lysis part is completed. So, that part we will show you later. So, now, we will keep this at 37 degree centigrade to incubate it for the lysis procedure. And we will wait and after 20 minutes I will show you what it happens.

So, after 20 to 30 minutes the lysozyme has been inside the solution. You will realize that the solution becomes a little bit more viscous. So, it will try to stick to the air to the this upend of tube and when you try to do this you will unrealize that it becomes a little bit viscous.

So, before that it was kind of liquidity. So, the viscosity was less. So, why it becomes viscous ? Because, after the lysozyme lyse the cells, though all the cellular components including the DNA and RNA comes out from the cell or the nucleus mainly.

So, the DNA are highly negatively charged molecule and they are quite large. So, those DNA molecules make a makes the solution a lot of viscous and they try to stick to the wall of this upend of tube. And that is how you know that the cell has been lysed properly. Now, after the lysis has been done. Now, you need to degrade or basically remove the other cellular components.

So, in this procedure you can go for different machines or you can choose depending on what kind of cells you are using? So, basically here we will use a sonicator machine. So, that generates ultrasonic sound vibrations that will basically degrade the cellular membranes and other DNA and RNAs that are big molecules.

So, other than that if you are using fungus you can use a French press or a different homogenizer or if you are using some kind of tissue system, you can use liquid nitrogen or electric blender system. So, here we are using ultrasonic some sonicator machine which will create or basically degrade the cellular our membranes and the DNA and RNA. So, after the sonication is done we will say the solution becomes again more clear and it the changes a little bit color as well and it becomes again solution or liquid light.

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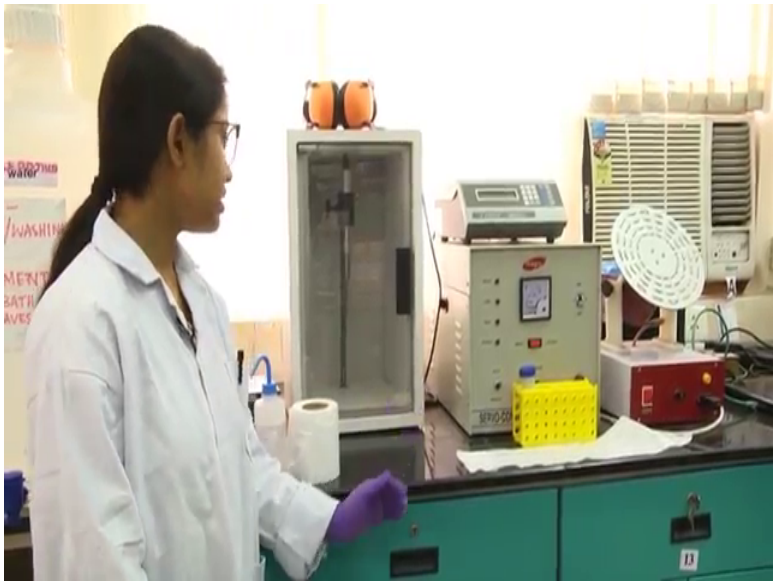

So, this is a sonicator machine. So, it the sonicator machine basically generates a ultrasonic sound by vibration and because of those vibrations the cellular membranes and the DNA RNA of the big molecules becomes degraded. And after that we will go for a centrifugation. So, there will be separation of other insoluble fragments and the solution.

So, if your protein is in the solution then can you can easily isolate the protein from the solution or if it is in the cell the insoluble fraction, then the steps are different.

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So, in a particular sonicator machine basically you have a cell system, where you can choose the pulses and the amplitude at particular which the vibration will occur, as well as you have a probe.

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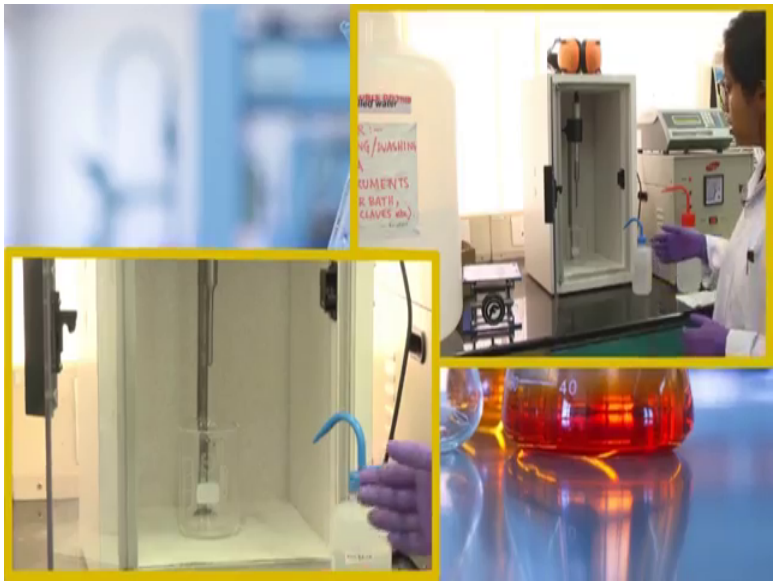

So, in this box. So, this is a basically the sound proof box this because the it generates ultrasonic sound and you should close their have this in a enclosed soundproof box

otherwise it might hamper or it might create a problem for you your own ears. Inside the box there is a metallic probe. So, this probe you can change it based on your sample requirement.

So, if you have a smaller sample volume like 500 micro liter or 1 ml. You can change it to a smaller probe and or if you have a largest volume like 100 ml or 200 ml, then you can also choose a bigger proof than this. So, this probe is good for around 5 ml to 50 ml sample.

So, as we have around 10 ml of sample. So, we are using this medium size probe. Also this particular machine which has a particular sound wave it can generate. So, this machines capacity also differs. So, based on your requirement it might have different machines you can have a smaller one or also a bigger one. So, now, that our cell has been lysed and you can see it become viscous. So, we will go for this sonication.

So, before you put them solution for sonication you need to clean the probe of this instrument. Because people might be using for their own culture and it is a good practice to always clean whatever you are using. So, it is a good practice to always clean the probe before you start using it, because other people might have used it and although they have cleaned it after using it. There might be some dust particle and other particle which might contaminate your proteins or your solution. So, first we will clean it with seventy percent alcohol.

So, this is a metallic probe, you can clean it with alcohol as well as water we will wipe it.

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Then we will clean it with deionized water or double autoclaved water. We can wipe it with the tissue paper. So, now, the probe has been cleaned and it is ready to use.

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So, there is other in some instrument this is a platform and on the top of which we will keep our sample. So, as you can see in a beaker I have taken amount of ice basically where we use the sonicator, when it generates the ultrasonic sound it vibrates and it creates and heat. So, a large amount of heat has been will be generated and you do not want your sample to be degraded, because proteins are heat labile.

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So, for that purpose we will keep over sample solution inside this ice box. So, basically it is inside the ice box. So, and the probe will go inside this solution. So, we will open this cap and it will go inside the solution. So, whenever the heat has been is will be generated and it will be lost because we are keeping it in the ice.

So, there are wont be too much of heat inside the solution which might degrade our protein.

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So, for that purpose we will put the whole thing here, and we put the platform here. As a sample is at the bottom of this tube we might need to increase the height of this platform. So, that we can do using this particular lever. So, the probe now; tip of the probe must be inside the solution. Now, this setup is complete; we will close that door, always close the door while using the sonicator machine because as I mentioned earlier this door is soundproof. So, it will not irritate your ears or other peoples.

So, now, we will come back to this particular sample instrument. So, this generates the pulses and also you can modify the stuff here. So, there are time; so, how long you want to sonicate your sample, there are also pulse. So, pulse means the pulse on and off. So, basically what we usually do is we go for 15 second of pulse.

So, whatever sonication or vibration it will generate it will be on for 15 second, then we will give a lag period that will be around again 15 seconds. So, this lag period the pulse will be off. So, it will help the sample to cool down. So, that ah; so, that much heat does not generate. So, as we have provided ice. So, it will lower the temperature of the sample, because when the pulses on it will start generating the heat, and when the pulse is off it will stop generating the heat as well as the vibration. And the sample and get the chance to cool down. Then we go for 15 minutes, then we go for amplitude. So, in this machine you can go from 35 to 65 percent amplitude. So, it is basically how much vibration it will generate we will go for the highest one.

So, I will start this particular sonication. So, the timing and the pulse will depend on what kind of tissue or the cells you are using, and the best way to judge is that your solution becomes again liquidity and viscosity will decrease. And also the solution will turn into a pale yellow color. So, it will change a little bit of color.

So, it is a trial and error method. So, you go for it and then if you are not comfortable or a happy with it we can change the methods or the protocols here, and then some starting now.

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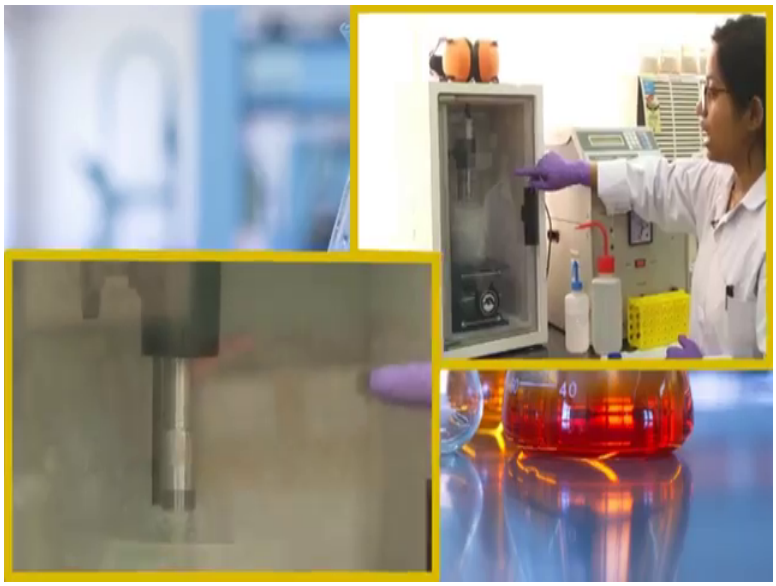

So, there will be a buzz noise coming out from this solution. So, basically it is generating the vibration right now. So, it is on and I have put it for 25 minutes.

So, it will be on 15 seconds and then it will be off for a 15 second, like this the sonication will happen in a stepwise manner. And after 25 minutes it will be closed and then we will go for the next step. So, our sonication is done now. So, it will be stopped by the machine, because it is over 25 minutes, after that we will take out the sample towards the platform, and the sample will be again liquidity.

So, after this we will go for the centrifugation of this particular sample where we will separate the insoluble fractions as well as the solution soluble fraction. But, before we do that again, we need to follow some good lab practice and we will clean up the probe.

So, first we will clean it with normal deionized water. So, the solution gets stick to the probe it is always good for the long storage of this probe to clean it, otherwise there might be some rust.

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So, clean it with water first, and then with 70 percent alcohol. So, if there is any kind of proteins or other molecules get stuck to the probe, it will be cleaned and there will be no bacterial or fungal growth. So, before and after using this machine you should always clean up the probe. If there is any kind of spillage you should always clean it here, close the door and we will switch off the machine.

So, now we will go for this gross fractionation of our solution.

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So, I have taken and the solution from this tube to this particular tube falcon tube because we will go for a high speed centrifugation process and that particular speed is with held by this particular centrifugation falcon tube or centrifugation tube. So, what it will ensure is that, the all big chunk of cell membranes are insoluble fractions will precipitated out from the solution and the if our protein is soluble inside in the solution, it will come out as a supernatant.

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So, we will use a big centrifugation, centrifuge machine for this and this is a high speed rotor. So, this machine can also have basically a low speed rotor also as a high speed rotor.

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So, this we will switch it on, and we will make the speed highest.

So, this is 11000 RPM basically it is 16000 G value, and the temperature we have set it at 4 degree centigrade because proteins are mostly stable at low temperature. If you do not require that you can omit that as well we can go for 12 degree or lower temperature as well. And the time we will go for let us say 40 minutes.

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So, another thing we have to make sure is that, we have an equal weight of this particular solution, because when we do go, when we go for centrifugation it is advisable to always

have this equal weight at the opposite side; otherwise the rotor when the rotor will go or the centrifugation will start it will hamper there will be weight imbalance. And there might be a chance of accident.

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So, put this sample and the weight balance basically at the opposite side. So, it should be exactly opposite, close the rotor head and we will start the centrifugation.

If your sample is too much heat sensitive, if you want to go for a absolutely cold temperature. You should first go for a first temperature change, we make the centrifuge at 4 degree centigrade and then you put your sample and go for this centrifugation process. Our protein is not that heat sensitive. So, it can withstand to normal temperature like 37 or so on. So, we are not doing that, but if you need you can do that as well. So, there is a mode for fast temperature.

So, basically without your sample, it will go it will start rotating and make the rotor at 4 degree centigrade, then you open it and put your sample inside it. There is also short spin basically, if you want to spin down something you can use that part, and multiple program you can set. So, the temperature you can monitor regulate the program, you can set a particular program for multiple use; the speed which can be in RPM, but is the rotational or evolution per minute or the RC about the G value.

So, basically the G value is important, because that is the actual fold that is that has been generated that will be generated by the RPM value; and then the time. So, it can go up to a minute and second. So, it can go for 1 hour or maybe more even that. So, the centrifugation is running now. So, after 40 minutes we will divide the solution whole solution into a supernatant and into a pellet fraction, and we will see what we will do.

So, while the centrifugation is a happening we will make the preparation for the next step. So, in our case our protein is 6 X histidine tag and, so, basically it has 6 consecutive histidine molecules at the in terminal of the protein. So, for that we can easily separate our protein from other residual protein of the cellular components from the cellular components using Ni NTA column.

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So, this is the Ni NTA column which has this nickel basically beads that are attached to this NTA or the agarose beads. So, while the centrifugation is happening. So, we will make the preparation for our next step. So, in our case our protein is a 6 X histidine tag; basically, it has a 6 consecutive histidine molecules at the in terminal of the protein. So, we can easily separate that particular protein from other cellular components or other proteins. So, we will use Ni NTA a column.

So, basically this column has nickel ions that are attached to this agarose beads and it is packed in a small column. So, this is a pre packed Ni NTA a column that we have

purchased, and we will use our FPLC system. So, in your chromatography lab as well we have mentioned.

So, this is basically a fast performance liquid chromatography system, you can attach any kind of column. So, here I am using affinity chromatography column or Ni NTA, but you can also attach iron exchange column or other different types of column are also compatible. Based on you need you can attach different types of column. So, as I mentioned that our protein is 6 X histidine tag, we will use this particular Ni NTA column.

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So, before you start purification we need to prepare this particular machine and the tubings and the column, before we load the sample.

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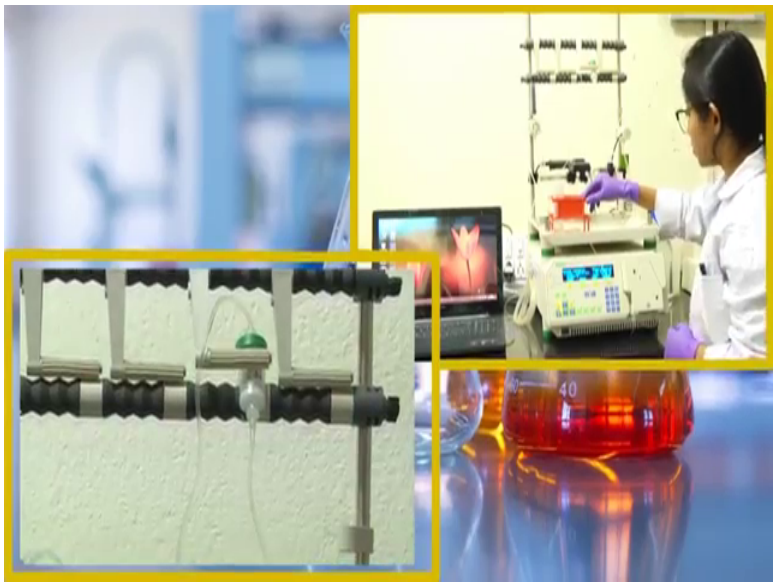

So, for that what we will do is, we will basically attach this column in a particular site. So, we will attach this column to the machine. Now, if the column has a particular orientation always follow that particular orientation which side should be up and which side should be down.

So, now, as we have mentioned earlier as well in the chromatographic lab that all the tubing's and the column are stored at 20 percent ethanol. So, that will basically inhibit any kind of bacterial contamination or fungal contamination. So, before we start, we need to clean all the tubing's and the column with normal deionized water.

So, for that I have put the 2 tubing's the buffer A and B.

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So, in this particular machine we have 2 tubes, buffer A and B in a particular beaker or the container which has only the deionized water.

So, mille Q water we are using.

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And, now we will basically run water all through this channels and the column as well. So, first we need to clear out any kind of precipitation of the salt or any kind of alcohol that are present and get out of the system. So, first we will go for the flow, and this particular column can withstand 5 ml per minute flow.

So, always check the columns requirement and do not go high column or flow, basically it might damage the column as well. So, go for this column what kind of column you are using depending on that particular column you change the flow.

Basically, flow is also dependent on the pressure. So, basically if you go for a higher flow rate the pressure might increase and it can damage your column. So, this column can withstand 5 ml per minute and, in case of buffer we are giving mix basically we want buffer A and buffer B both. So, we have put 2 channel in the same container, you can put it in different container, but we want to run water through all the channels.

So, basically that is why we have put the buffer A and B in the same container and this container contains only mille Q water. So, we will go for mix. So, it will take from this 2 tube it will take both water and then mix it and it will channelize this water through all the tubes. So, now, what we will go is, we will run water right now what is happening is taking water from this tube channel and it is passing through the water from all this channel, and it will go to from this and it will go through the column as well.

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And everything what is happening we can monitor it in this LP date of view basically it will show what is happening. How we can see what is happening? Basically, when the any sample or any solution is passing through this column it will come out from this particular tubing and it will go here. So, this is UV light chamber. So, it will measure the absorbance at 280 nano meter.

So, this FPLC system as I have mentioned earlier as well. So, this is made for protein purification. So, all most of the proteins which has tryptophan tyrosine residue, they absorb at 280 nano meter. So, it will take the measure absorbance measurement at 280 nano meter, and if any protein particles or any proteins are there, it will measure that particulars observance.

And then it will come out and this is a conductivity measurement instrument. So, it will measure the conductance. So, the conductance means how much salt or conductivity is present; basically, for water it will be low very low, but if you have a high salt concentration of a solution, it will show that what is the conductivity of that particular solution.

So, if you are going for suppose iron exchange like, cation exchange and anion exchange you can monitor that particular exchange as well. So, by using this 2 small instrument it can show what is happening in there.

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So, from then what will go is record. So, it will start recording the absorbance as well as a conductivity measurement. So, right now water is passing and it will clear out any kind of alcohol that are present or any kind of precipitated out.

So, when the last time when someone uses this particular machine there might be some salt precipitation or other components which might be precipitated out inside the tubing's

as well as might begins column. So, it needs to be cleared out properly. Now, another small tips while using this particular instrument a filter system, that while you are using start using it and while you are running waters water anything, you might feel that there are might be a blockage inside the tubing's or the water is not passing as you want like clearly the flow is hamper.

So, then; that means, either your column has been blocked by different precipitations of other molecules or the tubing's also might be clogged. So, for that you need to clean the machine with proper care. So, there are instrumentation the cleaning procedures which you need to follow, those will be provided by the instrument or the manufacturer which where you have purchased the instrument. So, always follow that, mainly what we do is in our case is that, we pass in a weight and a sale at a proper amount.

So, one molar anyways and then, water and then, one molar a sale and then, water. So, that will clear out any kind of salt precipitation or anything which has been precipitated in the column as well as in the tubing's. So, the cleaning of the column and the machine is different. So, always make sure what cleaning you are doing. So, do not, so, if there is suppose a sale has to be passed through your tubing's, but you cannot pass it through their columns.

So, always detach the column beforehand and then clean the FPLC system, and then while you are using the cleaning the column, then you can attach the column again and then clean your column as well.

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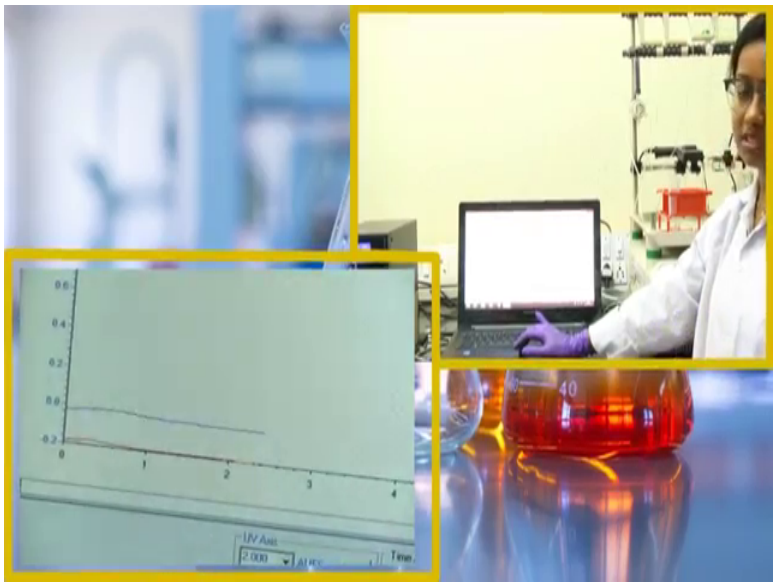

So, you need to when where the particular this blue and the red line is at the stable position. So, it will be flat line basically the blue line denotes the absorbance measurement.

So, this is arbitrary units. So, it does not mean that 0.6 or 1 absorbance at 280 nano meter, this is arbitrary unit. And the red line denotes the conductivity measurement. So, both will be flat a before we start running a buffer. So, first we will run water. So, it will clear out anything, but that might be present in the column or the tubing.

So, after we run it run the water then we will go for this buffer A. So, buffer a or wash buffer is basically the same buffer in which my protein will be present. So, in buffer A the trace will be there which has a PH of 8.2. So, it will regulate the PH of this particular buffer salt will be there as I mentioned earlier the proteins are stable or a particular salty salt environment, if you have too much of low salt or too high salt the protein might be precipitate out from your solution. And if it is precipitate out inside the FPLC system, it might clog up the whole system it might clog up your column as well.

So, 200 mille molar salt is present in our case if you need you can change the salt solution based on your requirements. So, your depending on your protein to protein it might vary we also have 10 mille molar imidazole inside the wash buffer or buffer A, that will ensure that nonspecific binding does not happen. So, this particular procedure has been mentioned in the chromatographic lab.

So, basically if you have a small amount of imidazole, which is another binding partner or ligand for this nickel irons, it will inhibit any kind of nonspecific binding. So, particular other protein might also have histidine moieties which might get bound to this column, but it will hamper and the imidazole will hamper that kind of binding. Since, our protein has a long consecutive which histidine tag, it will get bound to the column and other proteins will come out from the column.

So, if you do not have any histidine tag in your protein or you are then you have to go for different kinds of methods there are other methods which you can follow there is ammonium sulfate precipitation or other methods. But, here we as we harvested in tag molecules, and that is why we are using this particular method.

So, we have been running buffer water basically through this FPLC system for the past 10 minutes at the flow rate of 5 ml per minutes. So, almost 50 ml of water has been run and in this monitor, we can see that the blue and the red line is quite flat.

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So; that means, everything has passed out and nothing is there. So, now, we will stop this water and then we will run buffer A.

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So, basically this will ensure that my column is equilibrated in this particular buffer condition.

So, again through all the channels, I will pass buffer A. So, buffer A has trace NSA and 10 mille molar of imidazole. If your protein has a cysteine residue which is not linked or a single sustained residue, and you want to run a in a denaturing condition then you might also add BME solution that is 1 mille molar 2 millimeter of BME. Do not add DTT, because sometimes it is a hammer this particular Ni NTA a column purification.

So, sometimes you need to add BME for if you have a system residue. So, in the buffer you can add that particular BME. Now, we will start running buffer A through again through all the column, and again when the blue and the red line becomes stable we will know that buffer A has been passed through this column and it is equilibrated in the particular column.

So, as I mentioned the buffer A has higher salt solution. So, basically the red line will shift because the conductivity will increase and by that we can actually measure if the buffer A has been passed or not. So, the blue line will also shift because imidazole also have some absorbance value at 280 nano meter because of this imidazole bring.

So, when this blue line and the red line will shift up it will have a higher absorbance and higher conductivity and if then again it will become flat then early we will stop the

running of the buffer A. Now, after this buffer A has been run then our column has been equilibrated in the buffer A solution and it is ready for the loading of the sample. In the meantime the centrifugation will be over and we will separate out the supernatant and the insoluble pellet fraction and then we will run the soluble fraction through this column.

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So, our centrifugation has been done and you can clearly see there is the supernatant and the pellet fraction. So, right now, after the centrifugation is over we will separate the solution fraction this will be can the solution fraction.

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So, this is the pellet fraction. So, this is in soluble fraction and this is the soluble protein fraction. So, and this particular stage what you can do is, if you are not confident where your protein might be sometimes in case of ecoli big due to this folding problem.

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The low protein might be inside this insoluble pellet fraction.

So, basically it means that your protein is in the inclusion bodies it does not fold properly and it goes in the inclusion bodies or. So, in that case what you can do after running after running the centrifugation you can take some small amount of sample from here, and small amount of sample from here. And then run SDS page gel.

So, that will show you, that where you are protein of interest is present. So, if it is present in the solution fraction then it is easy. So, you just run the solution fraction from the through this column. But if your protein is inside this insoluble fraction or inclusion body, then you need to again dissolve this inclusion body or insoluble fraction in a particular denaturing condition. So, basically you have to put a six molar vanadium hydrochloride or urea solution.

So, that this becomes soluble and then you purify your protein and then you have to go for refolding of your protein. So, if you do not want to do those kind of steps, basically if you want to avoid those refolding steps, because it might be that your refolding is not proper. So, protein needs to have a proper folded structure and it should be the native

structure. If you might if you want to avoid those kind of structure then you might need to change the expression timing or the temperature.

So, that your protein comes under the solution fraction, then you can avoid that kind of situation. So, we know that our protein comes in the solution fractions and that is why we are running this solution fraction through this column, but if you are not confident at this particular stage, you can run both the systems the supernatant as well as the in soluble fraction through SDS page.

And then when you are confirmed that when you confirm that their protein is present in our either of the fraction then you run that particular stage. So, now, I have taken the supernatant solution, but I cannot load this particular solution right now to the column. So, first this has been shown before as well I need to pass through this whole supernatant solution through a filter this is 0.22 micron filter.

So, that will ensure that any kind of cellular debris that might be present in the solution big particles with not clog up will not clog up my column.

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So, I will take a syringe and I will take the whole solution in that particular syringe, very careful with the needle try to avoid making any bubble. So, now, my protein is inside the syringe I will remove this needle, and this is a 0.22 micron syringe filter.

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So, you can attach this filter to this particular syringe like this.

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So, this has this filter membrane and in a clean tube you just take that. So, basically it will filter out any kind of particulate matter. There are beyond 0.22 micron. So, this will ensure our longer storage or a longer life time for your column; do not load any crude sample just right away to the column. Now, this is our filtered solution and we will run pass this solution to the column.

So, you can use this kind of filter just once. So, discard after using it. Now, if we go into this particular FPLC system, you can see that the blue and the red line has gone up. So, basically the conductivity has increased, because our buffer A has 200 mille molar sodium salt before it was just normal water. So, this means and these 2 are in flatline this means in our column has been equilibrated in the buffer a solution we will stop it here and then we will load our solution.

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So, this is the syringe fill; that means, it has a mark of syringe. So, basically now you can here in this particular channel you can load your sample. So, before loading I will take my sample in this syringe again. So, this is the filtered sample, if you have any kind of bubbles try to remove the bubbles.

Now, our solution is in that particular syringe and as you can see there is a marking and they there is a 2 sided arrow here. So, you need before you start loading a sample, I will remove this empty syringe, put the syringe which contains on my sample.

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And, then twist this side. So, this means I can load now. So, this will ensure that my sample is inside the sample tube. So, this sample tube container has a volume of 10 ml. So, at a go I can load 10 ml of sample.

So, right now, I have around 8 to 9 ml. So, I can go for the whole sample loading. So, I will just load the whole thing. So, gently start pushing this syringe., As I have less than 10 ml of sample I can load at a one go, if you have higher volume just load 10 ml then wet, then twist this thing again back to its original position.

So, now, the channel is linked with this buffer system, run buffer A and then after some time you again twisted and load another 10 ml of sample. So, now, what I will do is I will flows start running the buffer A solution, and simultaneously I will start recording. So, then buffer a will run and come and it will push the whole sample in right which is present inside the sample tube to this column. So, the it will go to the column then it will come out.

So, anything that is that does not have the this a histidine tag or a moiety, it will come out because it cannot mind cannot bind to the nickel bits. And the whole thing will come out and if the absorbance will be measured and at 280 nanometers or other proteins you can see that it is coming out. Your protein because our protein has 6 X histidine tag it will get stuck to the column and.

So, there will be huge of increase in the absorbance as well as absorbance on a monitor and then it will again go down. So, when it goes down and becomes stable then you know that you can start running program where buffer A and buffer B will be run.

So, then buffer B which has a higher amount of imidazole will compete with your our protein of interest and it will displace our protein from the column, and then at the elution of fraction our column is coming will our protein will come out. So, right now what will happen is other protein will come out from this column that is also called wash. So, basically you are right now we are washing our column through this wash by using this wash buffer.

So, if you are doing it for the first time save their like take the wash solution and keep it separately and while running an SDS page also run those wash solution, because you need to ensure that your protein is not coming out at this point of stage, it should be there inside the solution.

So, you should see that there is a no protein your protein will not be present at the wash buffer. So, other proteins will be there, but your protein will be absent. So, always save the washing solutions wash solutions in a separate container and then when you have a elute in fraction is where you go and run the elutant solution through an SDS page to see where your protein is present.

So, right now what will happen is this blue line will go up, because the wash buffer is actually pushing the solution or the supernatant solution which we have collected through this column and it has come out from the column and go went passed through this UV monitor.

So, you can say it is the blue line is going up; that means, all the other proteins that are present inside this particular supernatant will is coming out of the solution of the column. Because if whatever is coming out from the column is not bound to the column, because if it is bound then it will not come out in the wash buffer, because wash buffer has only has trace SACL NaCl and 10 millimolar of imidazole. So, it cannot compete with 6 X histidine tag.

So, nonspecific binders and other proteins will come out from this column. And it is getting monitored here in the blue line. So, blue line is going up; that means, all the other

proteins contaminant proteins that are present in the solution is coming out. So, you should also say as I mentioned, there save this particular collection in a different tube and while you run an SDS page you should check if your protein is there or not.

If your protein is in there now in the wash solution; that means, either the histidine tag has been chopped up by the other proteases or your might protein might be getting degraded and that is why your protein is coming out in the wash solution, if that happens then you need to change or modify the purification protocols the steps or which you are following, otherwise you will not get a pure protein, because in the wash solution other contaminant proteins are also present that are bacterial proteins.

So, now it is coming out. So, it is taking some time, after it comes down then only we can start eluting our protein from the column.

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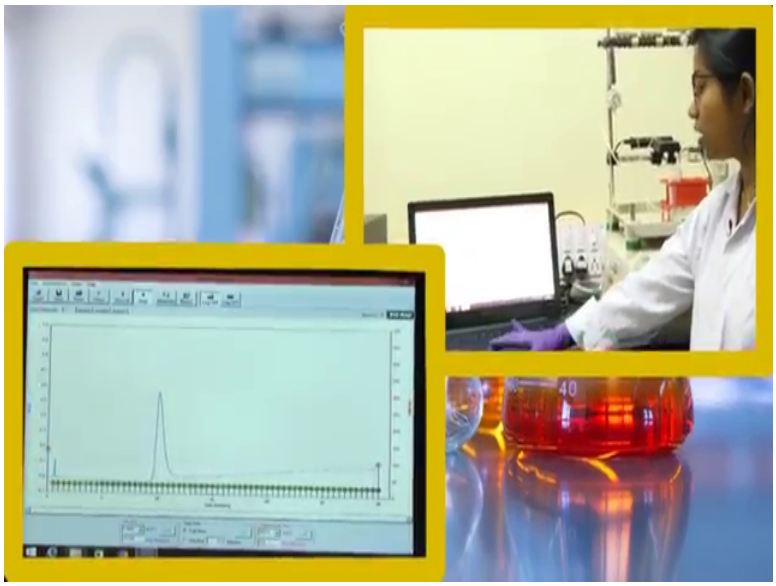

So, now, the program has been start. So, it has been run for 30 minutes and you can see in this particular chart is that there is a peak here at the blue line we notice the absorbance at 280 nano meter, because the protein that were bound inside the column has come out at this particular stage.

So, it is around 40 percent of buffered B. So, 40 percent of buffer B; that means, around 200 to 250 millimolar of imidazole as well. So, as I mentioned 6 X histidine 6 consecutive histidine molecules the around 200 to 250 millimolar of imidazole to come

out from the nickel bits. So, this thing corresponds to this our protein of interest. So, basically we are hoping that our protein is present in this particular peak, and if you zoom into it you can see there the small green plants are basically denotes the fraction number.

So, here you can see, at this particular peak hump the fraction number 18 to 23 is there here. So, now, our job is to connect this particular fractions and then run from this particular fractions on small amount of sample to this SDS page to see what is the purity of our protein. And also they have to concentrate our protein and check what is the actual concentration or amount of protein that are present.

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So, basically in 1 or 2 fraction we will have around 2.5 ml of sample like this.

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And we will take supposed 10 micro liter or 20 micro liter of sample and then run through SDS page to check the quality or the purity of our sample. So, if there is other protein contaminant that is present. So, you know your molecule the molecular weight of your protein. So, suppose it is a 15 kilodalton protein and you also see something and 20 kilodalton or 7 kilodalton or suppose 50 kilodalton; that means, that is a impure contaminant that has been that is present inside pure solution.

So, then again you have to go for another round of purification procedure. Suppose, if it is too much may relation in a molecular weight you can go for size exclusion chromatography or suppose you have a particular affinity tag or other ion exchange something like that you have to go for a tact to be take up your protein. So, if you have a pure protein right away, because mostly in case of Ni NTA a column I mean purification it is quite PO and you do not need to go for any other purification to go.

So, if you have that; then you can go for this and concentration and check the constant in a sample. So, how we will concentrate your sample?

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So, here we have a device. So, which is called as centric (Refer Time: 58:38) So, basically what this device tool is? So, it looks like a falcon tube like the centrifuge tube which we have been using previously like this.

So, far it has 2 things.

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So, along with this falcon tube it has a small holder like this, and this you can see there is a white patch this has the membrane. So, this has a cut off of 3000 Dalton. So, basically what it does is. So, you can see there is written 3000 MCM WCO (Refer Time: 59:16);

so, molecular way to cut off. So, this particular membrane will let out anything that is below 3 kilodalton. So, basically small molecules are protein that are below 3 kilodalton will come out from this membrane.

So, basically a code your sample here showing this particular holder and then when we while you do centrifugation. So, through this centrifugal force the other small molecules will come out. So, the buffer will also come out, and it will be stored in this particular falcon. So, it goes like this. So, when you rotate the sample it will come out and it will be stored here. As my protein is around 15 kilodalton, it will be remain here inside this particular fold.

So, using this particular; this type of centrifuge instrument, what you can ensure? Is that you can concentrate your sample to your certain volumes, suppose here it is marked as well. So, this is around 50 ml, it can go 15 ml of sample. So, you can go, so, from 15 ml to we can go for 1 ml or so. And also it will ensure if any small contaminants are presently degraded the proteins are present that will also come out, because it has a control. If you are using suppose 25 kilodalton protein or 50 kilodalton protein, then you can have a higher molecular weight cut off. Suppose 10 kilodalton cut off also. So, that anything below 10 kilodalton will come out from this particular coding.

So, you should use a particular cutter which is has a lower range in the than your protein molecular protease molecular weight. So, suppose you have a half of 15 kilodalton protein, you should not use a cut off of 12 for and so. You should use a 3 cutter item, because it might and there might be a chance that would also come out from this for this hot (Refer Time: 60:59) So, by using this kind of machine and the small; this kind of small instrument and also centrifuge machine, we can concentrate our samples and then we can check its purity and the quantity of that particular solution.

So, till now I am mentioning that how you can check the quantities. So, basically you can go for absorbance at 280 nanometer most protein have this tryptophan and tyrosine residues inside the peptide chain, and it observed at 280 nanometer. But if your protein does not have such tryptophan tyrosine residues, then you have to go for other methods to quantify the amount; basically, you can go for this gel estimation.

So, you run a gel and then through the gel band intensity you can estimate how much protein you have. And also another important part of this purification procedure is that,

you need to devise assay system to check how much protein? Or how pure your protein is? So, by or how much active your protein is? As we are going through different kind of steps and it might be harsh for the protein in your protein might be degraded or your protein might get unfolded. If the folding is not proper then the function of that particular protein will be hampered.

So, for a your protein, you need to devise a particular assay system. So, suppose your protein is in enzyme, then you have to have the substrate and check the enzymatic activity of your protein after you purify like this. And suppose you protein binds to a certain particular molecule, then you have to go for this binding assay as well, and you can check the folding in by different techniques likes you can do anymore you can go for CD 2 see how folded your protein is and if you hampering is folding or not.

So, if you hamper the folding or if your enzyme does not show enough enzymatic activity, then you have to change the purification protocol. So, that you do not use that kind of environment, where your protein is getting hampered function is getting hampered. Suppose your protein is get not being active then you have to probably do all this system at a cold temperature.

So, might be a protein might be very heat sensitive. So, you should be using a cold room where the temperature is 4 degrees low, and then you have to go for this FPLC run or centrifugation at 4 degree temperature. My protein is not that heat sensitive that is why I am using it in a outer environment where the temperature around 25 degree. But if your protein is heat lab all then you have to go for a cold room.

Also, you might need to change the buffer composition as well depending on the proteins. So, as I mentioned earlier as well that depending what kind of protein you are using? You need to add those kinds of small molecules or to stabilize your protein. So, the buffer system should be like that. So, that a protein will be as stable as possible in their solution otherwise a protein will precipitate out and you will not get a pure protein or you might not be able to purify that protein from the tissue or the source.

So, as I mentioned again earlier, that this particular purification of your protein is not like DNA purification. So, it is not a straightforward protocol which you can follow. So, it is a trial and error based method you have to do something then if you are not happy with the result you have to change and make some modification in the protocol, then again

redo the whole thing. So, it takes some time to standardize a particular purification protocol for a particular protein.

So, it will vary depending on, on what kind of protein you are using? What kind of expression system you are using? Sometimes it might happen that if your protein is going into the inclusion body. So, I have mentioned that, in the after centrifugation you are getting our protein is getting into the solution fraction and there is a 1 part which is insoluble inclusion body, and that is unfolded basically. So, if your protein is going inside the inclusion body, then during expression and so, as I mentioned the ecoli express at 37 degrees centigrade, but during expression. I you lower down the temperature to suppose 16 degree or 20 degree, it might not go to the inclusion body it might be inside the solution.

So, like this if you have to modify the whole protocol to based on your requirement and see what is best for your protein, so, right now whatever job is to run the SDS page. So, SDS page throw running does SDS gel electrophoresis we can see, what is the purity of our protein? So, we will take small amount of this fraction or do we can concentrate it down and take this particular solution and then we will run through SDS page to check if other contaminant proteins are there or not.

So, for 50 percent, so, will run probably 12 percent or 15 percent SDS page and also we will check the quantity of the protein. So, how much protein we have by using a nano drop. So, we will basically have check the measurement absorbance at a 280 nano meter. And if you know the molecular extension coefficient of a protein you can actually measure the amount that is amount that is present inside the solution by you if you know the extension coefficient as well as the absorbance of that particular protein.

So, by this way you can actually estimate how much protein you have? And what is the purity of your protein? If your protein is not pure then you have to go for another purification step, if it is pure, then you can do the enzymatic activity or any kind of biochemical assay system which you have devised for your protein and say what is a functional.

So, if have to check the purity the amount and you have to go for the some kind of an assay to ensure that whatever protein you are trying to get is pure and it is functional.