Experimental Biochemistry
Prof. Snigdha Maiti
School of Bioscience
Indian Institute of Technology, Kharagpur

Lecture - 33
Quality and Quantity of the Isolated Protein

So, till now what we have done is we have isolated our protein which has a n terminal histidine tag using affinity chromatography and we have used FPLC system to isolated our protein from the other cellular components. There might be other carbohydrates other proteins and nucleic acids and those we have separated out and we got our protein. Now as I mentioned that you need to check how pure your protein is before you go for any kind of other biochemical assay.

So, first we need to do SDS page or gel electrophoresis using SDS page and we need to check the purity of our protein. So, for that what I have done till now is I have collected the fractions that showed a typical peak in the FPLC system.

(Refer Slide Time: 00:59)

And I have collected all the fractions starting from 18 to 24. So, there was almost 15 ml of solution and using this particular centricon, I have centrifuge this those solution and concentrated the sample.

So, as I have mentioned earlier as well this centricon used as diafiltration unit. So, basically what it will do is while centrifugation is happening due to the centrifugal force the buffer or the soluble water what is there will be push to out pushed out from this filtration unit. So, it has a cut of membrane and it will be pushed out of this membrane and also whatever smaller molecules that are not that can be bypassed that can go through this membrane will also come out.

So, this particular membrane has a molecular cut off of 3 kilo Dalton. So, anything which are smaller like different types of ions and proteins which are smaller in size than our protein that will also come out from this particular solution. So, it will push out the buffer the water basically also the ions and the other smaller molecules. Then from 15 ml we have come down to supposed 2 ml or so. So, incredibly what I have done till now is I actually concentrated my sample and now with this sample I will run a SDS page. So, first let us try with the SDS page. So, now we will make a twelve percent SDS page and for that what we have here is the glass plates.

(Refer Slide Time: 02:39)

And we will assemble the glass plates to caster gel. So, there is a bigger plate and also smaller shorter plate and this needs to be assembled in a gel caster.

So, when I assemble these two plate what it will form as a small gap will be bare between these two plate. So, there is a small gap here and in this small gap I will pour the gel matrix basically the acrylamide and polyacrylamide gel will be casted here. So, the

gel matrix will be here and I will pour this matrix and it will form a very fine thin gel for our protein. Now I will put keep this caster in this tray like. So, now, in this tray at the bottom part there is thick material which will stop any kind of leakage.

So, basically it is blocked now and I can pour whatever matrix are water and the buffer here and it will not leak out. So, it will give us the time till the polymerase the acrylamide gets polymerized and then we can take it out and then we will load our sample. So, now, I will make the resolving gel and for that I will take all the components here in this small beaker; ao first I am taking water.

(Refer Slide Time: 04:09)

So, this is deionized water; so to make small gel like this which is almost about 10 ml of volume. So, and the how much water or how much solution you will take will be provided. So, this you can also go and search in the internet or other literature it is already mentioned here for 12 percent gel how much you need to make.

So, the percentage will also varied depending on your protein. So, my protein is almost 15 kilo Daltons I am making 15 12 percent gel if your protein is smaller than that, you have to go for a higher percentage gel like 15 percent or 20 percent also. And if you have a much bigger protein than this suppose your working with 30 kilo Dalton or 50 kilo Dalton.

Now, you can go for 10 percent gel or so; so right now I am taking 30 percent acrylamide and bis acrylamide solution. So, this is already a premix solution, you might also find out that acrylamide and bis acrylamide in a separate container, then you have to make the concentration like 19 is to 1 or 29 is to 1 like that. Now the resolving gel has a buffer of trays which has a pH of 0.8 0.8; so it is highly alkaline. So, right now I am adding the tris. Now I will add 10 percent SDS; so SDS will give us impart the negative charge to our protein and also it will denature the protein.

So, it will be in a polypeptide form basically that is why it is called the denaturing gel. When you give SDS, it become a denaturing. If you do not put SDS, then it will be a native page. But here we are mainly focused on getting the molecular weight of our protein and also the purity of our protein that is why we are going for a denaturing gel. Now I will give 10 percent APS which will provide the free radicals for polymerization.

So, now I in this beaker I have water acrylamide the tris buffer pH 8.8 10 percent SDS and 10 percent APS. At the last, I will add the temed. So, just before you start pouring the gel, you have to add the temed. If you add the temed and wait the gel will polymerize in this beaker and you cannot pour the gel. So, after you add acrylamide temed, you have to be very quick and start loading your sample. So, now I will add the temed. So, temed has a very distinguishing smell of sulfur. So, it has a bad smell; very small volume is needed; just mix quickly and start loading in this sample.

So, leave a small gap where we will caste our stacking gel. Above this on the top of our resolving gel, I will add a small volume of isopropanol. So, this will ensure that our gel is at the top section is not in a touch of air. Basically if there is air oxygen will the free radicals and then the polymerization will be disturbed. So, the isopropanol does to work. Basically it stops the air to get in contact with our gel and also it provide gives clean clean level. So, that our gel is at the same level all over the plates.

If you do not have isopropanol you can just add simple water and then you keep it like that for a 15 to 20 minutes till the polymerization is done. Then after that we will remove this isopropanol or water and then we will pour the stacking gel.

Now there are few tips here; if you want to a very crisp very fine band for your proteins, then you can also add glycerol. 1 percent or 2 percent glycerol will give make the gel quite and the bands protein bands will be from very crisp. Also if you really want a good

gel, you can also the sample the sample which I have poured right now the acrylamide and the tris solution, you can degas it before you at the temed degas pole solution. Then you add the temed and then you the caster gel then what will happen is there will be any kind of oxygen that is trapped inside the solution which your pouring like the buffers and the water and acrylamide that will be that will be out of this halogen solution and the casting or the basically the gel polymerization will be very good and then you can have a very nice gel.

So, if you experimental your gel is very important you are going for a publication also, you can make a you can degas the whole solution. Then pour the gel also you might add some amount of glycerol 1 percent or 2 percent in your solution and then you can have a very fine band of the gel. And you have to wait 15 minutes or to so 20 minutes before the whole system is polymerized, then only we can start for the stacking gel. So, after almost 15 to 20 minutes the acrylamide gel will be polymerized and it will be solid. So, now I will remove the alcohol or the isopropanol which I have put on the top of this gel. So, I will just soak it up with the help of a tissue paper

So, just tilt the whole system. Do not the assembled the system before you have caster the whole thing. Now to clear out the whole propanal, I will just pour some water and then I can I will just tilt the system and just soak it up. So, as you can see the gel is the resolving gel has been prepared and it is solid and there is a small gap where we will put the stacking gel.

Now, if you fill that your gel is not getting solidified even after a long time either you wait for more time or if you fill like you can add some more temed and it will help to polymerize the gel quickly, but do not put too much of temed again. If you put like more temed, then you might be feeling that while you are casting the gel is getting polymerize, then it will make a very bad gel.

So, there should be a balance between the temed and the time, but if you want to do quicker you can add some more temed as well. Now I will start with the casting the stacking gel. Again I will start with water. So, stacking gel will be or almost 1.5 ml or so and volume, it is a small thing it will ensure that all the proteins come at the same front line and starts from the same place. I will add the same acrylamide, but in a very small volume.

So, the stacking gel has larger pour size and in the stacking gel the protein even with the difference of size will run the similar rate. So, it does not work as a molecular sieve for so. So, pour size are very big. So, protein gets to run at the same side. Now I will add the tris buffer and here the tris buffer has a pH of around 6.8 and the concentration of the tris is also very low. So, there in the resolving gel the tris was around 1.5 molar tris pH 8.8 here, the tris is 0.5 molar tris and the pH is 6.8.

Now, again I will add the APS and the SDS first. So, in this particular denaturing gel, there will be always SDS in all the gel for resolving as well as the stacking. So, I have taken SDS as well as the APS both are of 10 percent, then at last I will put temed. The moment I put temed as gel will start polymerizing. So, again be careful just before you or ready you just add the temed. Do not wait after adding the temed, just mix it gently and then pour on the top of this resolving gel.

Now, before you start solidifying just add the comb which will make the wells where we will load our sample. So, I am using a ten well comb, just press it in that particular gap and it will make small holes or wells. There will be some spilite. So, it will go up because it will comb will display some amount of volume of the stacking gel, do not worry about it. Do not pour little in to avoid this spilite because if you pour little there might be a chance that a bubble gets trapped there and if a bubble is there inside your gel the protein there at the particular local environment will not run properly. Because again the current or voltage during the during the electrophoresis will vary and if there is a bubble the, the whole gel will be bad.

So, fill up the a stacking gel at the brink of this particular line and then you adjust the comb and put it there before the gel solidifies. Now you again have to wait for sometime after that the second gel gets properly polymerized. So, again we will wait for around 15 to 20 minutes. In the meanwhile what you can do is you can prepare a sample. So, for SDS page or in case of denaturing gel preparation what we do is, we have a particular loading dye.

(Refer Slide Time: 17:37)

So, you cannot load your samples just as it is; so have a dye. Now, what is the importance of this dye is it has first of all it has a dying which is the bromophenol blue. So, it will run very fast in the gel and you can actually seen where your sample leaves basically. So, you can monitor the run during the electrophoresis using this dye. So, it will not interact with your protein it will not create any trouble during the run or electrophoresis. But it will help you to monitor how long it has been run and when you have to stop the electrophoresis also there are another important ingredients inside this dye out this gel loading dye.

So, basically it has glycerol. So, when you have a sample that is a protein it is quite light. So, basically it is in a buffering system which is mostly made up of water and it when you try to load your sample insight of well, it will float out because it is not heavy or the viscosity is not high. So, it will try to float out. But when you add glycerol because glycerol is a heavy molecular weight substances, it will make the sample make your sample to go inside the well and see that the bottom of the well. So, the glycerol will help your sample to go at the bottom of the well. If you do not have glycerol your sample loading will be hampered and your whole sample whole protein will come out from well.

Another thing is in this particular denaturing condition, there is also SDS in this dye. So, what during gel preparation or basically sample preparation for the gel what you have to do is suppose you have a protein solutions or I have made the protein and it is in the

liquid form. So, it is a white or basically colorless liquid. So, you take small volume of this protein. Suppose four micro liter of this protein and then you add that particular sample in this gel loading dye. So, this gel loading dye is 4x; that means, it is 4 times what you are supposed to use. So, the whole concentration is 4 times more.

So, here what you have to do while loading the dye will be around 1 x. So, basically you have to make the adjustment of the concentration by adding by your sample and if needed the water or something else.

So, you have to make it 1 x. So, basically I will take around suppose if I want to make a 16 micro liter of sample for loading, I will take around 12 micro liter of my sample and 4 micro liter of this loading dye and then the whole sample volume will be 16 micro liter where the dye concentration will be 1 x. So, you have to make it 1 x. You cannot make this dye 1 x because then what will happen you cannot add anything to this particular dye. If you add anything to the 1 x dye, then the dye concentration will go down to 1 x and that is not right.

So, make it in a 2 x 6 x or 4 x concentration. So, higher concentration and when you add your sample, then the concentration will be go down to 1 x. So, this you have to keep in mind that you have when you start loading you have to make the whole dye as 1 x and then you make the sample like that. So, again I will take 12 micro liter of the sample and 4 micro liter of this loading dye and then the whole sample concentration will be 4 16 micro liter and the dye will be 1 x. Now that is not over, now you have to boil the sample.

So, boiling what will it do is it will denature the whole protein systems. So, protein has tertiary structures or quaternary structures and it will break down. Because SDS the sodium dodecyl sulfate which is an anionic detergent is present and it will break down the whole system and the protein complex will be broken and the whole protein will be remain as a polypeptide chain basically. And that will ensure that depending on the molecular weight, it will move at the certain rate.

So, if you have a protein which is of 20 kilo Dalton and if you have a protein which is a 40 kilo Dalton, the 20 kilo Dalton will be running almost 2 x faster than the 40 kilo Dalton because it is of lower molecular weight. But if you have suppose you do not enter the is SDS page or SDS detergent what will happen that if your protein is folded in a

certain conformation or might it might be forming a dimer or it is making complex with another protein, then that particular complex will have a bigger molecular weight and you cannot estimate the actual molecular weight of that protein.

So, to get the actual molecular weight of a protein and with respective to a particular molecular marker, you have to put SDS in the dye and also in all the system so, that the protein breaks down in a polypeptide chain instance will also ensure that protein is properly negatively charged throughout. So, it will it does not basically choose or selectively make negative charge it just randomly it just put a negative charge in all over the place. So, protein will be negative charge and it will run from negative charged side to positive charge side.

So, then what we have done is we have added the sample and then make this particular sample and then boiled in a either a water bath which has around 100 degree centigrade or heat block dry heat block you can also use which is around 100 degree centigrade. So, 96 degree centigrade to 100 degree centigrade, I have to boil for almost ten to 15 minutes and after that you just spin it down or centrifuge it at the highest speed. So, that any kind of particular matter or present in the dye or your sample will be precipitate out and then basically as a plate form as it will come out from the solution and then you add the this solvent in the well and then it start the running.

So, I have boiled my sample for 15 minutes. Another trip tip is so, suppose you have whole cell lazier. So, make taking suppose your taking 1 ml of lb culture and then you take the palate and then you take took the palate and as a resuspended in a buffer and then you added the dye. So, in that case you what you have to do is you have to boil for a longer time because in that particular cell palate DNA RNA and other carbohydrate other molecules will be present. Then you have to go for a higher temperature or boil for a longer time. So, that the DNA RNA whatever it is present will be broken down in a smaller piece and it will not hamper the gel running.

So, if you have a purifier sample the boiling time can be reduced for 5 to 10 minute is fine. But if you have a taking a cell palate crude lazier, then you have to make a boiler for a longer time 15 to 20 minutes for 25 minutes. So, that everything is broken down and then you have to centrifuge it and then you load this only the supernatant. Another important thing is as I mentioned earlier as well that if you have a system single system

residue and either might be chance that it forms system assisting dimer in that case, you need to run in a reducing need to run in a reducing environment. So, for that you can add 1 millimolar of BME like the. Final concentration of BME will be 1 millimolar in the loading this particular sample.

So, basically you just add BME in the sample and then you boil it for some time and then you loaded. So, what it will do is if the BME is a reducing agent, it will make cliff the or basically do not may for make the formation of disulfide bond and it will cliff the disulfide bond and make it the SH only. So, system residues will not from disulfide bond and it will have reducing agent and then you will can say on your single band. It might be you can see that sometimes when you running when you are running a gel and you are seeing two bands and equally or suppose your protein is 15 kilo Dalton your seeing in a brand at the 15 kilo Dalton as well as 30 kilo Dalton. Then might be because of dimer formation if you add BME, then only you can see a 15 kilo Dalton band and then particular dimer formation will be hamper.

But suppose you have multiple system and that particular system important for your flooding, then do not proceed during the biochemical acid do not to give this BME. Only when you are running as gel and you want to calculate the molecular weight and see how much pure protein you have then only you add this BME sample which is around 1 millimloar at final concentration. So, now, we have prepared our sample we have boil the sample and the sample is ready. Another thing during loading what you need is a molecular weight markers.

So, this is also called the protein ladder. So, in this particular sample it is a provide or so, we have bought it from a manufacturing company. So, what it does us it has a different molecular weight proteins. So, suppose 15, 30 50 like that. And when you run, it it is already priston you can see that all these protein it will form a ladder system. And with respect to this ladder, you can actually measure or estimate what is a molecular weight of your protein.

So, it is important to run a ladder otherwise you cannot judge where your protein is coming because then you do not know which at what molecular weight your getting your protein. So, suppose you have 15 kilo Dalton protein then that 15 kilo Dalton protein

should match with this particular molecular weight marker. Otherwise you will never know at what size which or where it is coming in the gel.

So, we have several taken all this things here. Another thing you will need is a running buffer. So, basically that is SDS page running buffer has trace glycine and SDS basically. So, the running buffer is also alkaline around pH 8.2 also and it also has glazing molecule and this glycine and tris and the chloride ions will help for the migration of your protein. And also it has SDS because in the denaturing page everything contains SDS to basically denature the protein complex and inside you have the denature protein and you can actually estimate the molecular size of the protein.

So, now our stacking gel has been polymerized and then I will remove this whole system in this caster. Then I will open or take out the gel plate from this casting cassette.

(Refer Slide Time: 27:57)

And this is another cassette where I will start running my gel. So, the smaller gel part or the smaller plate basically will phase inside of this cassette. And then I will put another cassette here or another plate here. Basically it is a plastic plate to make is a closed vessel kind of thing.

So, this is the total cassette which I have formed. So, this side the actual gel is there and this side this another just cassette, If you have another gel, you can run simultaneously two gel here. So, you can put the other gel here. As I am running only one gel, I have put

a just I have block system basically and this is a buffer chamber. The rate a denotes the negative flying and the black denotes the positive flying. So, basically here also there is a red and black marking and this also have red and black marking So, that you do not confuse yourself.

And just put it red towards red and the black towards the black. After you have put the whole system like this, you have to fill up the inner chamber. So, this whole cassette system makes a inner chamber and there is a outer chamber as well. Suppose you have put it like this and the gel running buffer which I have talked previously, it as tris glycine tris here glycine and SDS. So, I will fill up the inner chamber first. So, just wait for few minutes to see if there is a leakage from the inner chamber. If there is a leakage from the inner chamber, then when you running the gel it will start leaking and the inner chamber will get depleted from the buffer and the whole procedure of the electrophoresis will stop because of the charge distribution; it will be hampered.

So, another I have to fill the outer chamber. There is a marking here it says 2 gels of 4 gel. So, as I am using a two gallic acid I have to fill the outer chamber till the 2 gels. So, it will ensure that this whole system is properly equilibrated and at the same electric field for voltage will be similar. So, the connection will be there, otherwise it will be discontinued. So, I have filled the outer chamber as well.

Now there are two school of method which you can follow you can either remove this comb before hand and then you can wash your wells or you can just pour the buffer and then remove your comb.

So, it will depend on yourself which you are comfortable with I am just pull at the both side the same time. So, that you do not disturb or procedure it is tingle the combs. So, now, you have to see able to see the combs here. So, there will be around ten combs it probably will be difficult in this video. But when you are doing it my hand you can see the combs and their you have to load your sample.

Now we are ready to load our sample. So, I am loading different samples. So, basically I am loading one sample which I made before the purification. So, there will be other proteins or the continent and then after purification which will contain only which is supposed to contain only our protein, but we have to check if it is it will containing only our protein or not.

So, I will make that the small problem. So, I have previously prepared the sample or add at the loading dye as well as the; I have boiled the whole thing. So, just go down put the tip at the top part and slowly just release the sample. When you have done, do not release your ppid inside because I it will take out your sample. Just take it out and then release and then discord the tip. Because of the glycerol and the dye, you can see actually see your sample otherwise you would not be able to see that.

(Refer Slide Time: 33:07)

Now, I am adding the ladder which will be having a different color and it is already pre stain. So, while running you can actually see the different protein fragments. I have made few samples from the fractions as well which we have collected earlier to check all the fractions have pure protein or not.

So, I have only 3-4 samples. There were 10 wells. So, basically what I have done is I did not keep the free wells as it is, I have put some amount of basically the loading dye itself. So, sometimes when you are running SDS page if you have a free well. What will happen is the other the if there is a nearby protein, it will trying to go towards that lane and it will push towards that particular lane the free lane and the band will not be properly crisp or a single band and you might get a weird kind of gel. That is why do not keep any free lane in SDS page gel always filled with some amount of loading dye itself. Now the loading is has been done. You can see there though whole all the samples are actually at the bottom part of the gel so or the wells basically.

So, what ensures that the because of the presence of glycerol it has make the whole sample little bit more weight the weight is more and that is why it is at the bottom part of your sample well. And if you do not have glycerol, it will just come out from the well. So, always at glycerol to your sample and then load now I will just put this cap here. So, the red will go to the red and the black will go to the black.

Now, I will at the whole thing so, I will basically start the running using this power pack basically this is a power pack where you can actually regulate the monitor or the voltage basically. So, I have this power pack here. So, there is also red and black marking, you can see and in this particular code there is also red and black pin. And so, the basically red will go towards the red and the black will go towards the black. And here I can change this as it is showing 0; that means, it is at 0 volt now. It is not showing anything running or pause or anything.

So, I will change the voltage. So, I will make it suppose 100. So, this means it is 100 volt. So, v is on. So, there is a blue or basically yellow light going on. So, the 100 volt and then I will start the run. So, the basic rule of thumb of what voltage, you should run your gel is around like per centimeter you should be a 10 volt. So, if your gel is suppose 10 centimeter bake, then you can run it at 100 volt or so. So, first it is good to run at a lower voltage like 90 or 80 like that while the gel is running other sample is running at the stacking gel. And then after it went to the resolving gel, you can increase the voltage to 120 or 130 to save some time.

So, now that I have started running. You can you will able to see some bubbles going up here; that means, basically the electric field has been generated and it is going from negative to positive side. And if you want you can do all this whole thing in a ice box or in a cold trombe. So, when you do in a cold trombe again the gel will be far better, then this and you can keep the whole system in ice box outside yourself. So, you put just ice all over the place you have to keep the buffer as well in the ice previously. So, it will be chilled.

And when run in chilled condition then gel will be far better. So, if you have a important experiment or important gel which you want you can run in a cool environment which is cold and buffer will also be cooled. And so, basically now it has to be run. So, it will take almost 1 hour or so and you can if you will be able to see how much it has been run

because there is a marker or the basically front dye the bromophenol blue will run as well as we have loaded pre stained marker.

So, you can see the marker will also separate out and you can actually able to see that because it is already pre stained, you want able to see your protein because it does not have the stain. So, after the running, we will put it in a stain and then after the staining and destaining process, you only able to see your protein. Another important aspect of this isolation and purification of protein is to know how much protein you have. So, the amount is also important. So, you need to know how much protein you got in a particular from a single purification protocol. So, the gel will help us to see how pure your protein is an another thing we will do is to check the absorbance at two eighty nanometer to get to know how much protein we have.

So, it will be important during developing any kind of assay where you need to know how much protein you are providing in a in assay system and to measure the activity of the protein. So, further getting the estimation of how much protein, we have we need to check the absorbance. So, protein usually have tryptophan and tyrosine residues which are the this hydrophobic residues have this phenol ring and it absorbance 28 nanometer and that can be monitored in a nanodrop system. So, I can previously also we have shown that the how the nanodrop works here also we will see how we can estimate the amount of protein we have.

(Refer Slide Time: 40:07)

So, as I have got this protein from this also basically from the fractions I have concentrated my protein and that is the fraction I am running here in the gel to check the purity as well as using this particular sample I will see how much protein I have. Now sometimes you might be working with proteins which do not have any tryptophan or tyrosine residues, then it will not show any absorbance at 280 nanometer; then what you can go for is gel estimation.

So, from seeing the gel band and how much you loaded a sample in the gel you have to have a basically sample protein which have a known concentration and relative to that known concentration, you need to estimate or measure how much protein you have. If you do not have any tryptophan tyrosine residues which will be observed at 280 nanometer; another methods which are there like lowry assay or bradford assay for which will be useful to know the estimation of the protein.

So, as here you can see that the sample as already passed the stacking gel and the pre stained marker has already started to separate out. You can at least see the red band here. So, I have also increase the voltage here I have made it from 100 to 120 or you can make at one thirty as well to save some time.

(Refer Slide Time: 41:25)

Otherwise you can keep it 100 and run it for a longer time at the same as same rate. Otherwise after the side passed through the stacking gel, you can increase the voltage to

save your time. So, we will run till it goes at least half way through the gel and then we will stop and then will put it in this stain.

(Refer Slide Time: 41:49)

Now, we will measure the absorbance of our sample at 280 nanometer. So, this is a nanodrop this is a spectrophotometer basically, but it uses nanoscale solution. So, you do not have to have a bigger volume of your sample. You can just use it in a small volume like 1 micro liter, 2 micro liter. So, I will switch it on. It will some it will take some time to initialize the machine. So, in this particular nanodrop, we have a monitor as well as this disk where the handle is there and in that particular point we will load our sample.

So, before we load our sample we have to make the blank as well. So, in this particular sample our protein is there, but it also contains a buffer system.

So, basically the blank will be the same buffer without any kind of protein or our sample. So, here I have used only the same buffer which I have tried to get the purified protein. So, basically it has tris and NaCl. During the purification protocol the buffer which we have used it is the same buffer here. So, I have to use the same blank here always make sure that in whatever buffer your protein is in, you should use that particular buffer as a blank.

So, now it is showing that instrument is initializing and do not lift the arm during the initialize; that means, this arm. So, in that particular arm there is a point where the light

is coming through and there you can get the absorbance. Trop scale solution as well as you can add a cubit there is a point basically there is a portion where you can at the your cubit and where you have can have greater or volume of your sample.

So, in this particular machine you can show or measure the absorbance at different wavelength of light like 600 nanometer 540 nanometer or so.

(Refer Slide Time: 43:51)

But where mostly going to do here right now is for the protein and a proteins absorbed during the tryptophan and tyrosine residues at the 280 nanometer. So, now, in this particular screen you can see there are different options for you.

So, nucleic acid proteins OD600 custom and kinetics; so nucleic acid means DNA or RNA, you can also measure DNA RNA at the particular 260 nanometer wavelength. You can measure OD600 basically when we grow a bacterial cell in lb media or so, the bacterial cell. Basically have a measure absorbance have to OD600. So, that called optical density you can see how much bacterial growth has been there. So, when you just loaded the basically normal lb use as a blank and then when the bacterial growth will be there the OD600 value will be more because the bacteria cells will scatter the light and the absorbance will be more.

But right now what we are going to do is go for the proteins. So, even in the protein section there are different options here. Here you can see in the protein at a 280 proteins

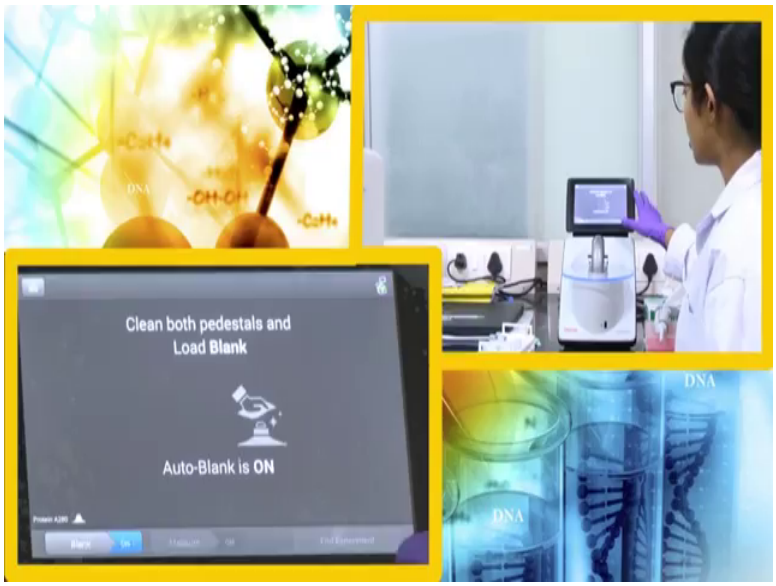
and levels; that means, if you have a particular specific level which has an emission or absorption spectra that you can choose protein at A205. So, that A205 means observance as 205 nanometer. So, if you your protein do not have tryptophan and tyrosine residues, the peptide backbone basically has this absorbance at 190 to 195 nanometer scale. But mostly none of the machine measure the absorbance at 190 because of some noise because mostly at one nineteen almost all of the substances or molecules absorbed.

So, then the protein backbone measurement is done at 205; the best nanometer scale at the higher time point which is around 190. So, at two 205, you can measure the absorbance which is due to the protein backbones. But not necessary it is always useful or helpful because at this particular times nanometer ranges I have mentioned a lot of molecules absorbs.

Another thing you can see that there are BSA Bradford and Lowry. As I have mentioned earlier that you can also go for Bradford and Lowry estimation so, it is are the reagents which you add in your sample and the sample changes color. And due to during that changing color how much color is has been changed, you can actually estimate how much protein you have. Also you can have BSA basically BSA is a Bovine Serum Albumin sorry. So, BSA is a Bovine Serum Albumin and there you can have a esteem in as a standard protein. You can make a graph using a standard concentration of BSA and then when you respect to that particular graph where your protein concentration is coming you can measure that.

I will go for protein a 280 nanometers time scale. Here I went there and I will select a type which means I have I am going for one absorbance equal to 1 mg per ml and we will go for done.

(Refer Slide Time: 47:07)



So, now it is giving me instructions and here in this particular monitor like here it is showing that clean both pedestal and load the blank and the auto blank is on. Basically I have to clean the pedestal. So, I will just open up the arm and then first what I need to do, I will take in a clean kimwipe I will take small amount of deionized water and this two portion I will clean it. So, if there is any particulate matter or anything that are present in a dust particle will be cleaned and then I will load first the blank. So, as I mention this is the nanodrop machine. So, I can load this small volume. So, I am right now I am loading around 2 micro liter of blank.

So, this is a blank in which the buffer basically in which my protein is there blank does not contain any protein buffer, it just the simple buffer. Now carefully I will load the blank and then I will lower the arm. Now as the auto blank first on it itself started taking the blank and the next step it will give us the instruction what we have to do. So, here you can see the blank is so; that means, there is no noise or any other absorbance is there. Clean both the pedestal and load the samples.

So, it is giving me telling me that you again have to clean the pedestal remove the blank and then you have to load the sample; so that I will do right now. So, I will again one of the arm. I will remove the blank from here I am taking a nanodrop, I will just clean it. These are very sensitive instrument trying not to make any scratch there all the

spectrophotometers are quite sensitive. If there is any scratch, then the absorbance will be absorbance will vary.

(Refer Slide Time: 49:27)

Now, I will load my sample again I am taking two micro liter of the sample and carefully I will load. Try not to make any bubble in that particular small sample, otherwise the absorption will be different and then I will load the blank.

(Refer Slide Time: 50:03)

Now, it is showing new particular value that is the here you can see mg per ml, it is showing me 9.796. So, do not trust this particular mg per ml. It means that at particular

one absorbance is coming from 1 mg of protein, but that is actually a standardized for a known protein that is mostly BSA, but our protein is different. So, take this value that is A280.

Now, A280 value shows 9.80. Now there is another value A260 by 280. So, A260 by 280 basically shows if you have any particular d n a or r n a contamination because as I have mentioned that DNA RNA all the nucleic acid absorbs at the 260 nanometer wavelength. And if you have higher value like here the it is 0.6, it is fine. If you have suppose 1.2 or 1.8 like that; that means, DNA contamination is there if it is go beyond 2; that means, protein RNA contamination will be there.

So, this is values is ok. So, we have only our protein and there is no DNA or RNA contamination. And since we got this A280 value 9.8 what does it means? It is not tells us how much protein we have. So, for that what we need to know is the extinction coefficient of our protein. So, the extinction coefficient is basically you can have it. If you provide the protein sequence in the Protparam software in the website you go and protparam software, you just upload your proteins sequence and it will calculate the extinction coefficient of the sample of your protein. Here I know my absorption coefficient is around 14.45 millimolar per centimeter. So, that is given in the protparam.

So, now what I will do is I was just divide this particular A280 value. So, that is 9.80 and I will divide by the molar extinction coefficient. So, the molar extinction coefficient as I have mentioned is 14 1450 and then if I divide that, I will have an a molar rings or the how much molar protein I have. Then if I multiplied that by thousands, I will have how much millimolar protein I have. So, now, after doing this calculations so, to just dividing this 9.8 by 14450 and then multiplying by thousand, I am getting around 0.67 67 millimolar protein.

So; that means, in this particular volume of sample it is around 1 m l or so, I am getting around fourteen 0.67 millimolar of protein. So, that is good because 0.6 0.67 millimolar is quite a good. Another thing if you want to calculate how much mg per ml protein you have in that particular protparam file you will see there is another option or given that how much absorbance you will get for one m g per m l protein.

So, in my case it give first that 1.7 observance or A280 value will come for 1 m g per m l. So, now, you can easily calculate how much mg per ml protein you have in a particular

sample and then how ml how much ml protein you have, you can calculate the total amount of protein you got from a single purification protocol.

So, by doing this we can actually calculate how much protein we have and then it will help you to establish any kind of biological assay system where you need to know how much protein you are giving in a particular experiment. And also you cannot happy with the protein you got in sample during the protocol. You can always change or modify your protocol and then try to get as much protein you have.

Now, if the this particular explained is done so, I have been going to end this experiment. Again it will tell me what to do. So, I have to clean the sample area. I am just opening this, again I will take a tissue paper and soak it with just water. Just clean it do not be rough with this, do not pour any harsh chemicals here and then we lower this area and touch the cut. So, my job is done here; so I will just close this machine.

(Refer Slide Time: 54:35)

Now, you can see that the gel has almost run and we can see that by seeing observing the marker. So, the ladder of the protein marker has separated out. You can see the different fragments of the protein as we are using pre stained marker we can we are able to see that. But in this particular part we cannot see anything because our proteins are not stained in a previously. We have to go for the staining after the running is done.

So, now we can stop it because I am with the run right now. So, I will just stop the voltage, remove the whole system. Now I will dismantle; so you can see the protein marker has quite separated now. So, it those mean; that means, it is has this different fragments and we already know what is the molecular weight of those fragments. And this is basically the front dye which is running this is the bromophenol blue which was there in the loading dye.

Now, remove the buffer and we will dismantle this whole system keep it here. This is the basically the plate or the gel which we were running.

(Refer Slide Time: 56:03)

Now, I will try to separate out the gel from this plate. So, this is basically a gel staining try which we got here a box basically and this is basically a spreader or separate out which was already provided with the whole gel casting tray. And gently we will trying to lift up this small plate from the bigger one. Now you can see the gel is stuck the bigger plate. You may or may not remove this comb or basically without wells. If you comfortable with it, you can just keep it like that then in this staining box I have taken first a small amount of water and I will just dip this plate.

So, the gel will come out in this box. So, we have to do it. So, now, that you can see the gel has come out. I will remove the plate here. So, for storage we need to clean this plates and then keep it. So, I am just washing it once likely with a water and then I will pour out the water. Now I will pour the staining solution.

(Refer Slide Time: 57:55)

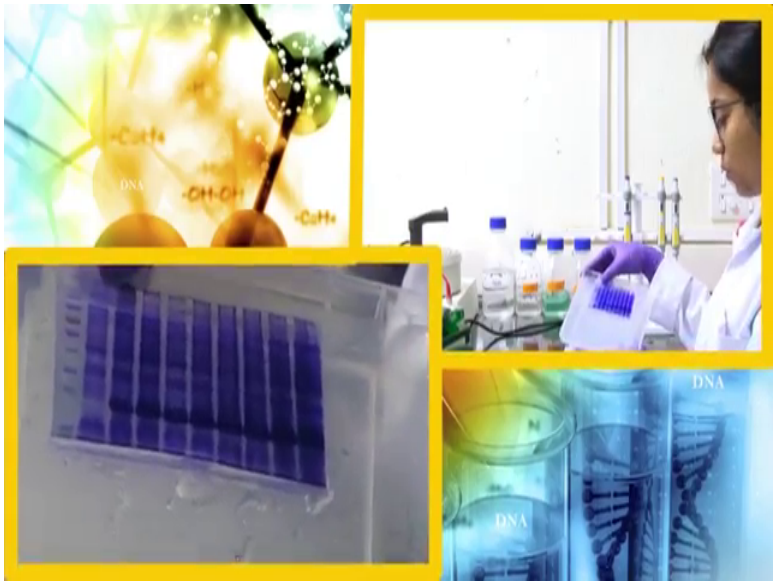
So, this is coomassie brilliant blue which will stain our protein from just pouring the staining solution here closing this and now we will keep this in a rocking motion for almost 1 hour. You can keep it overnight as well and after that we will pour out the stating solution and pour the destining solution.

Now, if you after the staining solution it will stain all over the gel basically you cannot see anything properly, but the destining solution has the same solvent. So, basically in the coomassie brilliant blue solution, but what we have is glycerol acetic acid methanol and water and then coomassie brilliant blue as a powder form we just added and make the staining solution. The destining solution will have the ethanol and acetic acid and what in the methanol basically acetic acid and water, but it will not have the staining solution because we want to avoid. So, any kind of non specific staining of the gel will be avoided and you will only be able to see your protein and the whole gel will be white.

So, now I am you can see there is this whole gel is stained. Most of the time and you would not be able to justify or basically make sure where your protein is coming. So, for that we will keep it for some time and then we will pour it in a destining solution. So, our staining and destining of the gel has been done. So, we kept the staining solution for almost 1 hour and then we kept the then we remove the staining solution and put destining solution the gel and kept it until the background gel becomes clear and we can only able to see the protein part.

So, I have here I am showing two gels basically. So, as a mentioning so, we are right now what we are doing is to isolating and purifying the protein.

(Refer Slide Time: 59:53)



So, first as I mention you need to check if you have your protein in the cell lysate or not. So, here I am showing a test of expression. So, in this whole system basically so, this gel has been run previously before I went for all this protein purification and isolation of this protein. So, first I need to check if I protein is there or not. So, I have this is a ladder and this is the just a normal cell palate where I did not induce my cells to produce my protein. And after a different time point I have taken out 1 ml of culture and loaded the whole system sample

So, here you can see there are lot of protein. So, all these blue lines denotes proteins. So, basically and there are lot of proteins. Along with this lot of protein, there is a particular protein that is over express. So, it is a very fat big band and that is our protein which is coming around 15 kilo Dalton. So, we are getting that particular molecular weight from this marker. We know the molecular weight of all the bands that are present in the marker. So, this is the protein which we are trying to over express and it is this is actually present in the sample

So, after you ensure this that your protein is actually getting over expressed, then only you go for this a big culture and isolation of your protein. Now if you see what we got after isolation are.

(Refer Slide Time: 61:21)

So,. So, first in this particular lane what we run is. So, after getting the cell pellet and dissolving the cell pellet we went for this sonication and then centrifugation and then which have taken only the supernatant part after the centrifugation.

Now, if we load only the supernatant part you will be seeing this there are other different proteins and as well as there are the protein which we wanted to over express and this our basically protein of interest. Along with the protein of interest there are different small bands above and at the door bottom as well which are as a present as a contaminant. This is the different protein this has a lower molecular weight. This is not of our interest.

Now, this is the ladder which we have a loaded here along with our protein and here you can see there are only clean bands presents. So, this is all the fractions that are present in that particular peak. So, basically these are the respective fractions that are we collected from the FPLC system and this shown as a peak. And you can see that there are no other proteins that are present in the upper part or the lower part of this gel; that means, this is the only portion protein that are present in our sample and it is quite clean; that means, a protein is actually pure protein.

So, from this gel you can actually see the qualitative nature of this protein. So, it is pure and from this absorbance we also got the amount of protein we have. So, now, after this whole procedure we got a quite clean protein pure protein as well as the amount of

protein which we got. Now everything the protein right now what is in a buffer that is present is in a tris NaCl. Now for different biological experiment or a biochemical assay or even during storage, you need to have your protein in a different buffering system.

So, further you need to go for a buffer exchange or sometime even go for a have to go for a desalting purpose. So, right now our protein is in a very strong buffering say it just in tris and also the molar range concentration of slat is quite high around two 100 millimolar. Now if you want to buffer exchange your protein as I have shown previously you can use a centricon.

So, basically you load your protein and then you pass you also add some amount of the new buffer that you want to give. Suppose in our case we have keeping it in a phosphate buffer. You load, suppose you have 1 ml of protein and ethane you will load around 15 ml of your new buffer the phosphate buffer and then you concentrate out. So, that will ensure so, you have to go for twice or thrice and that will ensure that your protein has been buffer exchange in the phosphate buffer.

So, you can use this kind of instrument this is the centricon and amicon, but you forcefully due to the centrifugal force buffer will go out the right now whatever present buffer we will go out and the new buffer will come in that place and you will your protein will be in a new buffer another way of doing this is a dialysis. So, it is another most convenient and classical way of doing desalting and the buffer exchange procedure. So, for dialysis what will happen is in a dialysis bag. So, basically it is a semi permeable bag it is mainly made up of cellulars semi permeable bag we will keep our protein and then with the whole bag will be kept in a new buffer system which is in our cases the phosphate buffer. And then we keep it for a overnight or more than 8 to 10 hours.

So, due to the semi permeability of the membrane, it will ensure thus any small molecules or the buffer that a water will go out and distribute in the new buffer and it will make equilibrium among the outer buffer and the inner buffer. So, basically if you have a higher salt solution. Suppose 200 millimolar of salt in your sample and the outer buffer is around 50millimolar or so, then the high concentration or salt will go out from the membrane because it is very small molecule it is permeable for them. It will go out from the solvent and it will create a equilibrium due to the diffusion of the salt.

And then your protein will be in a new buffer system and also the smaller tris and cell will go out as well. And if you have in a smaller protein fragments like that if your or smaller molecules that are bellow the cutoff that particular membrane that will also go out from the membrane, but since your protein is quite big it will not go out. So, like this so, using this you can actually concentrate your sample as well as change your buffer. But dialysis will only ensure that your buffer has been exchange, but it will not go for any kind of concentration

(Refer Slide Time: 65:55)

So, here I have already made a pyrolysis system. So, in this particular beaker what I have is around 800 ml of new phosphate buffer. So, this is a very low concentration of phosphate over 20 millimolar of phosphate buffer and around the salt is around 50 millimolar of NaCl, but our protein was as you quite be a remembering that is in 200 millimolar of salt and tris here.

So, now in that particular buffering system what I have kept it here is my protein. So, you can see there is a small bag or pouch. So, this is the semi permeable membrane basically, it is also called as next skin here. So, basically you can purchase this kind of membrane. This is semi permeable membrane I have cut it out in a small portion and then I was stick this two clip to the either part of this membrane. So, that my protein is inside this particular bag and it does not go outer leakage prevented. So, these are the clips basically and the whole system.

So, here inside this particular I have around two m l of my protein and I have dipped this whole thing inside the new buffer system and there is also magnetic stirrer here I have kept this whole thing in a magnetic stirrer overnight. So, this will ensure that proper diffusion is happening and the salt and other small molecules will go out form the solvent go out from my sample and it will be a may it will create an equilibrium along with this buffering system and my protein will be in the new buffer.

So, right this way I can ensure that the desalting and the buffer exchanges happening. Now the meant when you can choose to go for dialysis because this a very non harsh technique you are not putting directly your protein in a new buffer that might cause some sometimes that might cause a precipitation. So, what your doing is you are gradually changing the buffer that sometimes helps. Otherwise if you have a time issue or you know your protein is quite stable in a new buffer, you can use centricon.

And even after this dialysis if we need to concentrate your sample for different biochemical purpose or different assay or you need to concentrate and keep it store your protein, then also you have to go for concentrated or concentrate to concentrate your sample. After this dialysis is happened, we will take out the sample concentrate using a concentrator.