Experimental Biochemistry
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Lecture – 30
Isolation and Characterization of Proteins Part- I

Hello, welcome back. So, in today's lecture I will talk about isolation and characterization of proteins. Again this is a two part lecture and I will divide into two lectures and today I will mostly focus on Isolation of Proteins and in the next lecture I will talk about Characterization of Proteins.

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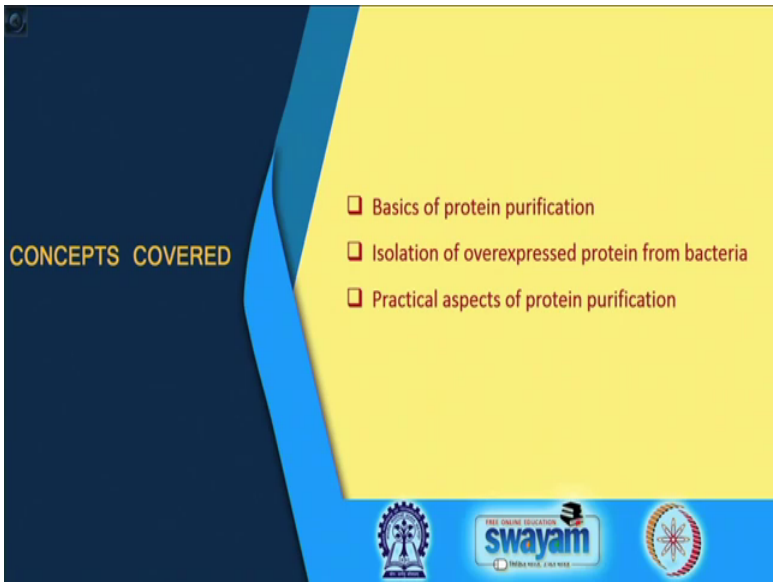

So, what I will cover is the basics of protein purification, isolation of overexpressed proteins from bacteria and practical aspects of protein purification. So, protein can be isolated from various sources such as animal tissue or some microorganism, but in today's science what we mostly do is views recombinant DNA technology to express our protein of interest in bacterial system that has several advantages. We will talk about recombinant DNA technology in some other lecture, but so since we use that we can purify our protein of interest from a bacterial system where the protein is overexpressed. So, today I will mostly focus on isolation of such overexpressed protein from bacteria.

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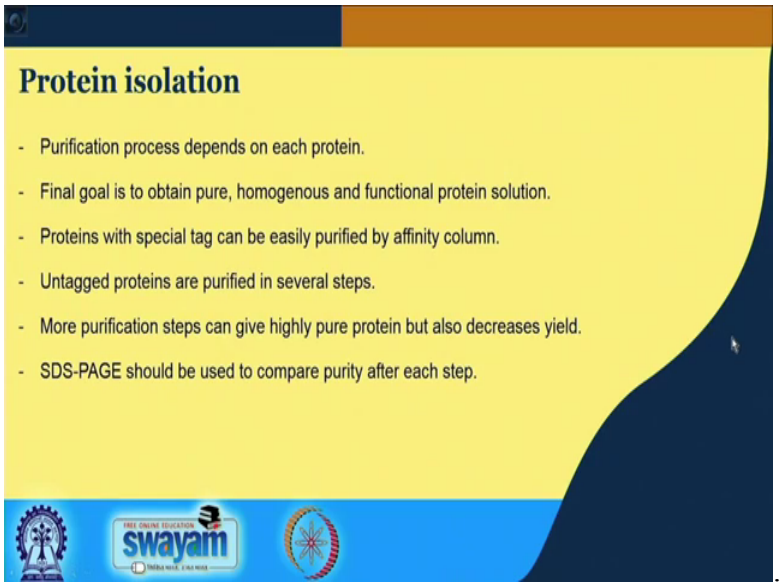

So, protein isolation is even though there are set protocols, but what we will find is that these protocols have to be optimized for every protein. So, the same process cannot be used for all proteins.

So, it is a, so protein purification is something that depends on the type of protein on each protein. The final goal of any such process is to obtain a pure, homogenous and functional protein solution. If it is an enzyme, then it should be a functional enzyme. If it is a ligand binding protein, then it should bind its ligand. So, we need to obtain a functional protein solution and it should be as pure as possible at least 95 percent pure. Proteins since we can use recombinant technology, the recombinant DNA technology we can also introduce some affinity tags to our protein of interest. When something like that is done protein isolation becomes much more easier.

So, in that case a special tag is attached to the protein either to its N terminus or to its C terminus and then, an affinity column is used to purify such proteins. We will see one example of such affinity purification. But in many cases it is not practically possible to attach such affinity tags. So, such untagged proteins need to be purified using several methods. So, here what we have a several step purification of protein. More number of steps means the N product will be highly pure, but it also means that we will keep on losing proteins at every step so, the final yield is decreased.

So, there is always a compromise between the yield versus the purity of the protein that we obtain. And throughout all the steps, we need to run SDS-PAGE that is the Denaturing Polyacrylamide gel to check the purity of the protein. We have discussed SDS-PAGE in the previous lecture. So, in this lecture we will see that SDS-PAGE is very useful to detect quickly check whether we have our protein and how pure our protein is.

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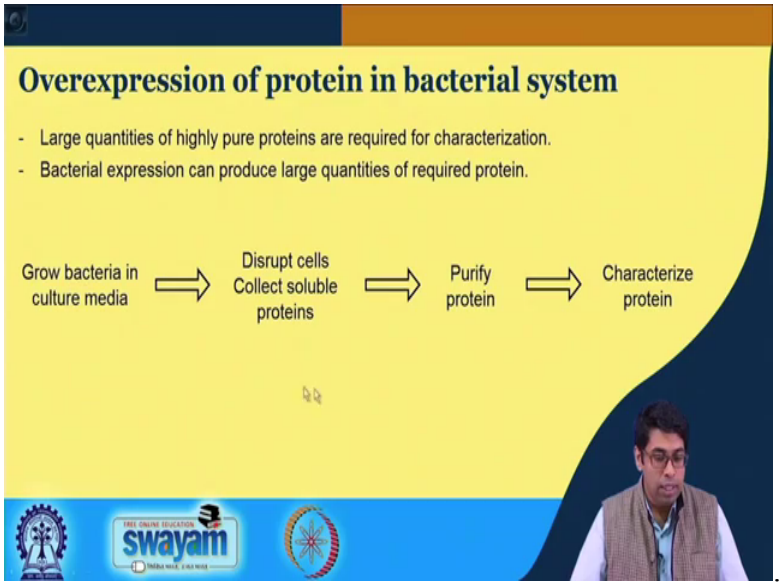

So, this slide gives you a general workflow of protein purification from a bacterial system. As I mentioned before that even though proteins can be purified from different sources, but one of the major sources that we use is purification of protein which are overexpressed in a bacterial system. I will not be going to the details of these overexpression process which will be discussed later.

So, here what you can assume is that somehow we have cloned a protein into a bacteria which over expresses this protein, which means that out of all proteins, our protein of interest has the highest expression level. So, once we obtain such bacteria that bacteria needs to be grown in a culture medium. So, our culture media is nothing but a solution which has all the nutrients and the bacteria happily grows in that media. Once we get enough bacteria, those cells are pelleted down. So, basically they are removed from the solution and then, these proteins are then these cells are disrupted, so that all the proteins that are inside the cell come out into solution. So, at this step what we have is a mixture

of all the proteins that are expressed by the bacteria in which our protein of interest is also there plus all the DNA and RNA and other cell debris.

So, from this huge mixture we have to isolate one protein that we need. Once we have that protein, then we can go on to characterize our protein using biophysical and biochemical methods. So, today's lecture we will mostly focus on this purification process where we want to isolate a single protein from a mixture of more than 1000 different proteins.

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So, we start with a bacterial culture. This is an example of a typical bacterial culture that is used. So, this yellow color solution that you see is a Lysogeny broth. This is the broth which has all the nutrients required for the growth of a bacteria and it is kept in this 2 liter culture flask and it is covered with a aluminium foil.

So, this is how the broth looks. It is transparent. You can see through it. Once we add bacteria and growth is in an incubator, after several hours it looks like this. So, when it becomes completely opaque and you will also have some frothing you know that we can easily tell that your bacteria is growing in this media. This is a typical growth chamber which we use to grow our bacteria.

So, you can see these holders where these flasks can be immobilized and then this whole platform shakes at a particular rate. It can be set at 150 to 250 rotations per minute and

then, the temperature of this chamber can also be said anything between 10 degree centigrade to 37 degree centigrade. The optimal temperature for bacterial growth is 37 degree centigrade, but in some cases some proteins might not be expressed well at higher temperature or they might be expressed and get degraded. So, the whole process needs to be slowed down in such cases are lower temperature is used. So, we can even though the normal temperature is 37 degree centigrade, we can also grow bacteria at 20 degree centigrade or even lower for certain special proteins.

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So, again this is the growth media. Typically a growth media has all the nutrients that are required for the growth of bacteria. So, these are the four important parameters that one should take care of when growing bacteria. The first one is obviously the nutrients. We have to supply all the nutrients that are required. If we miss a critical component, we will see that there will be no growth even after hours of shaking your media with bacteria, pH is very important.

So, even though even after you add all the components make sure that you check the pH of your media and it should be somewhere neutral, we again you somewhere around 7.4 pH. The osmotic conditions have to be optimized. Osmosis is where solutions can water can move from a low concentration to a higher concentration of salt.

So, if your media has low salt concentration compared to the bacteria, then there will be a osmotic pressure where water will start flowing inside the bacteria and it will start

swelling, the bacteria can ultimately basted. If it is the river, then the bacteria will start shrinking neither are optimal for bacterial growth. So, balancing the osmotic conditions is very important for optimal growth of a bacteria. And as I discussed temperature is also an important parameter.

We normally go by 37 degree centigrade temperature, but it can be lowered to 20 or even lower up to 16 degree centigrade. In this media the major elements are this carbon, oxygen, proton, nitrogen, sulphur, phosphorus, sodium, potassium, calcium, magnesium and iron. These six elements are used to make the macromolecules such as protein, nucleic acids and sugars, polysaccharides. These five elements are used by different processes in the bacteria and also they maintain the osmosis osmotic pressure of the media. Some of these elements that are listed here are required in trace amount. So, they also need to be added to the media.

Finally, the major component of a media like the lysogeny media are tryptone and yeast extract. Tryptone is nothing but a trypsin digested casein protein. So, casein is one of the abandoned proteins found in milk and it is digested by trypsin protease into small peptides. So, tipton becomes a very good source of amino acid and yeast extract is again it is extracted from yeast from dead yeast cells and those are fed as a source of nutrient. So, when you have all these elements, you can mix them in water and what we typically do is, we take this mixture and put it into an instrument called an autoclave. So, it is basically a pressure cooker where you put your media and you put it at a pressure of 100 kilo pascal and a temperature of 121 degree centigrade.

So, using this high pressure and temperature, all the bacteria are heat killed that are there in this. All the microbes that are there are heat killed. So, this solution becomes sterilized and now you can add your bacteria of interest and grow that in the presence of some antibiotic.

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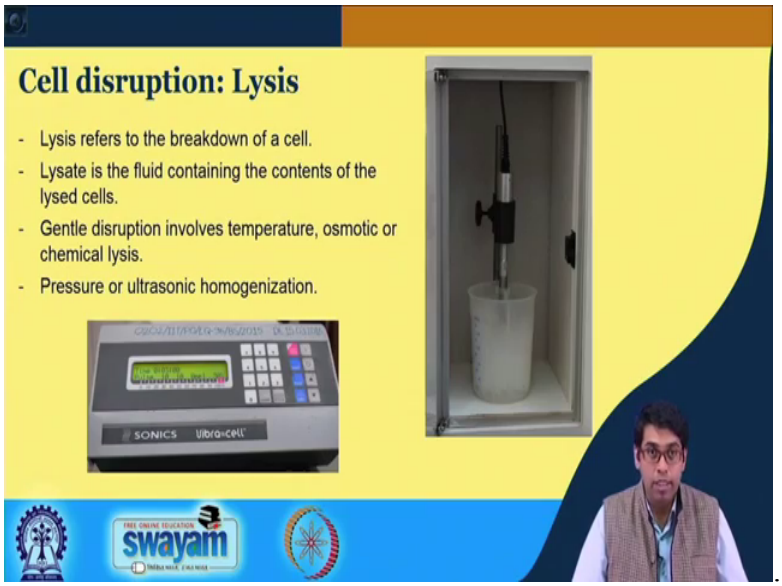

Ones we have the bacteria grown in that case, I will go back one slide it looks like this. So, from here we need to fish out all the bacteria and it is very easily done. This whole solution is put into a centrifuge and it is fun at a high speed something in the range of 15000 rotations per minute and then all the bacteria are precipitated at the bottom and the solution, the media remains on the top.

So, you can pour out the media and you will have your bacteria has a cell pellet. That cell pellet is then lysed to obtain the proteins that are inside the bacteria. So, lysis refers to the breakdown of a cell and when all the proteins and all the DNA and RNA and all the components which are inside the cell come out into the solution, that is referred to as the lysate. So, there are typically two methods by which a cell can be lysed. One is the gentle disruption method which uses temperature osmotic pressure or chemical and then, the other one is mechanical disruption which can use high pressure or ultrasound.

In our lab we use some combination of chemical lysis and ultrasonication. So, for chemical lysis what we do is, we add an enzyme called lysozyme which is found in tears of our eye. So, this enzyme breaks down the peptidoglycan layer of a bacteria which we can see it cell wall and makes it prone to rupture. Ones the cell wall is we can the osmotic pressure itself can lyse the cell or we put the solution where the bacteria is suspended into a chamber like this. This is the sonicator probe which is dipped inside the solution and a very high sound a very high frequency is applied.

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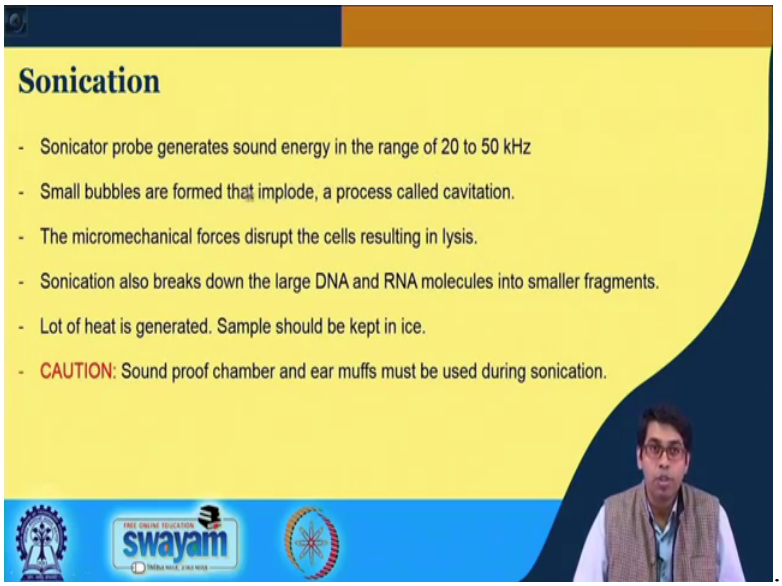

So, this sonicator probe generate sound energy in the range of 20 to 50 kilo hertz. This high frequency sound creates small bubbles of microbubbles which spontaneously implode and that creates a lot of micromechanical forces which disrupts the cell wall and breaks open the cell. Ones the cell is broken, all the components that are inside the cell come out. It is very easy to tell when the lysis happens because before lysis the bacteria are suspended in a solution and it is a free flowing solution. When all these bacterial cells are broken, the DNA, RNA and all other proteins and all other molecules are into the solution and the solution becomes highly viscous. If you take a pipette and if you try to form a droplet, you will see that you cannot form a droplet and also it flows like a slimy solution and it will try to stick to the side of your vessel.

If you keep on sonicating, further these sound waves we will start shearing the large molecules such as DNA and RNA and will break them into smaller pieces. When these are broken into smaller pieces, the solution again becomes highly mobile. So, the viscosity is gone and you can again check that using a pipette and you will see that you can form nice droplets. So, there are few things that one should keep in mind during sonication. The first one is that a lot of energy is put in to the solution using this sonicator probe. So, naturally the sample will heat up. Now heating the sample can destroy your protein; it can denature the protein.

So, it is very important to keep your sample on ice throughout sonication and again sonication is not done continuously. It is done using something in the range of 50 percent due to cycle which means that if the sonicator is on for 3 seconds, it will be off for 3 seconds and then again it will be on for 3 seconds and so on. Another word of caution is that a sonicator should be kept in a soundproof chamber and students were using the sonicators should wear ear muffs even though the sonicator is inside the soundproof chamber, otherwise it can damage your hearing.

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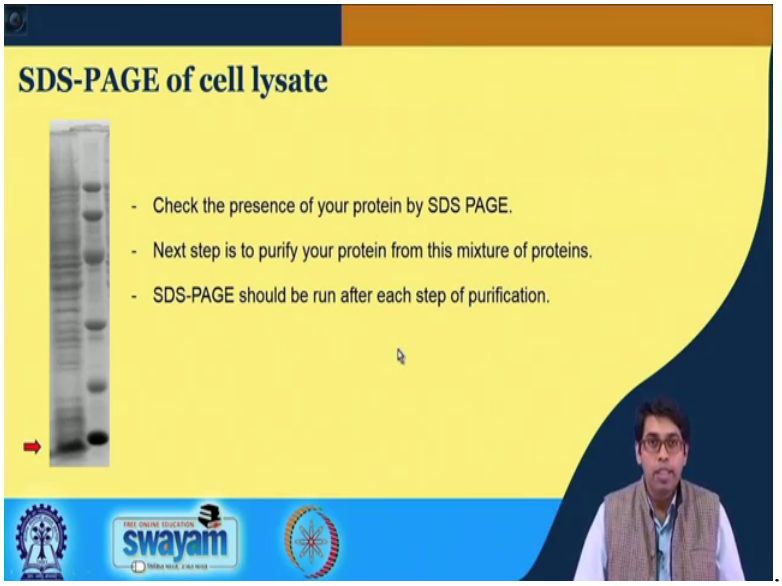

So, once the sonication is done again you have your protein of interest in the solution and you also have certain cell debris plus sheared DNA and RNA and other molecules.

So, to remove several things from earth bigger particles that are there in the solution, the cell lysate is against fun at a high speed. So, centrifugation is done and all the insoluble fractions get precipitated. So, you will get two fractions. One is the high speed pellet and the other one is the high speed supernatant to check whether your protein is in the pellet or supernatant. You will have to run both on a SDS-PAGE.

So, this is an example of an SDS-Page. This lane has the molecular weight marker and this lane has the supernatant. You can see that in this lane there are several batch. This is the band which is the thickest and it's molecular weight also matches with the molecular weight expected molecular weight of our protein of interest. So, this is the protein that we have overexpressed and you can see that, it is the thickest band. So, it is the

overexpressed protein, but we also see all these different bands which are coming from different proteins. So, now the challenges to isolate this one protein from a mixture of all this different proteins.

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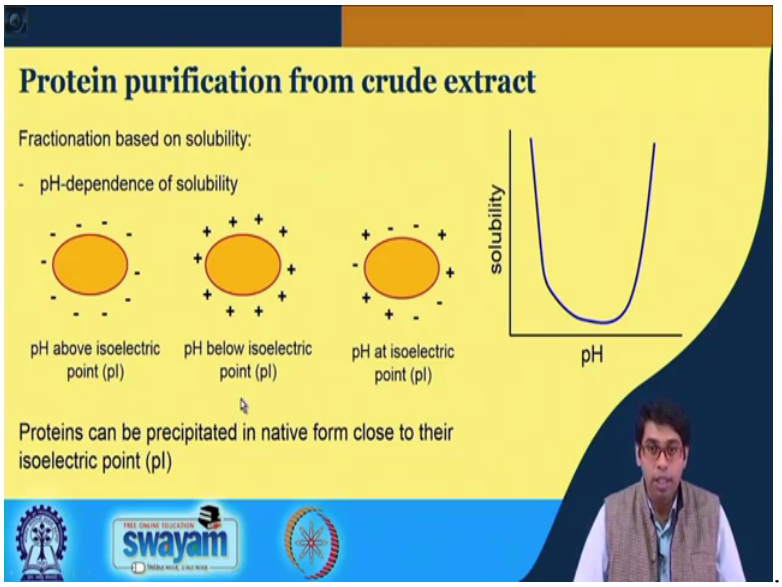

So, as I said before if there is no affinity tag, then we have to do the purification through several steps. One of the variables that we can easily tweak is the pH. So, every protein have their pI. The pI of a protein depends on the number of charged amino acids they have. So, you can count the number of glutamic acids and aspartic acids and the basic amino acids such as arginine, lysine and histidine and you can calculate the pI of your protein.

So, if the pH of your buffer in which the protein is dissolved is above the pI, the net charge on the protein will be a negative charge. If the pH is below the pI, the protein will have a net positive charge and if the pH is very close to the isoelectric point, then the protein will have an equal distribution of positive and negative charge, resulting in an almost neutral protein. Under this charge states the protein is highly soluble, but when these charges are neutralized, then these proteins can approach each other and the non-polar interactions can become prominent.

So, if you plot the solubility of a protein as a function of pH, you will see that the solubility will be lower where the isoelectric point of the protein is. So, one purification step can be that we adjust the pH of the buffer in such a way that it matches very closely

to the isoelectric point of our protein and at that point the protein we will start precipitating in its native form. So, you can spin it down, connect the precipitate and you will have a relatively pure protein.

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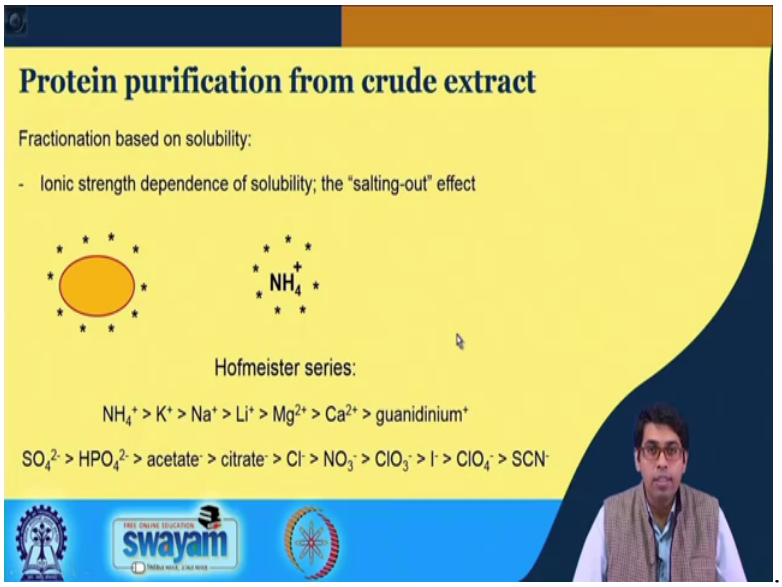

Another step of purification can involve the salting out effect. So, it turns out that protein solubility changes with change in salt concentration and also depending on which salt ions are used in the solution. So, I am not going to going to the details, but there is this series all the Hofmeister series which lists the cations and the anions. And the ions on the left hand side are the once which are the most efficient salting out agent. Briefly the way it works is that when you have a protein it is surrounded by water molecules. So, there is a hydration cell of your protein .

If we dissolve in ion at a sufficiently high concentration, then this ion will also have a hydration cell and if the concentration of this ion keeps on increasing, then the hydration cells then water molecules which were previously free will keep on moving into this hydration cells, so that less or less water molecules will be available around the protein which increases the non-polar interactions between two protein molecules. And the result will be that ones this protein molecules interact through their non-polar interactions, they can start precipitating out.

So, it has been found that ammonium sulphate is one of the best salting out agents. So, again using of concentration of ammonium sulphate, you can precipitate out proteins

from the mixture which have lower solubility compared to your protein of interest. One example of that is shown in this slide.

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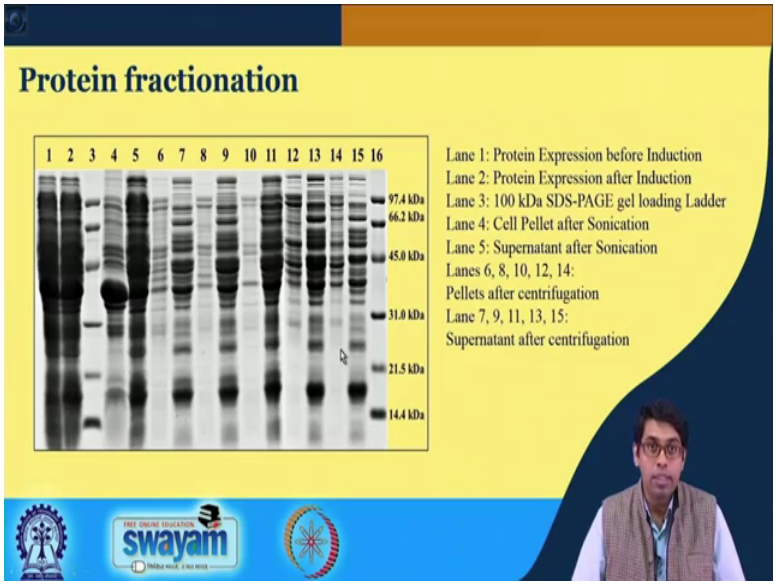

So, again this is a slide where we have used SDS-PAGE gel electrophoresis to optimize our process. The first two lanes are the whole cell lysates. The first lane is where the cells were not induced and the second lane is where the cells are induced.

So, you can see this big band that is present here, but it is so tough absent here. This is the molecular weight marker, this is the cell pellet after sonication and this lane 5 is the supernatant. So, we can see that very small amount of protein precipitates most of it is in the supernatant. Now, in these lanes 6 to 15, different conditions were used to see whether the protein remains in solution or is precipitated out. So, 6 and 7 are one condition and 6 is where the proteins where precipitated and 7 is where the proteins remained in solution.

So, you can see that under this condition our protein of interest remained in solution, but there were some proteins which precipitated out. In another condition again we see the same thing our protein remains in solution, all these proteins remain in solution, but some more proteins precipitate out.

So, if you see like that this combination of 12 and 13, under this condition we have the maximum proteins which are precipitating out and still our protein remains in solution.

So, again using this condition we have eliminated some proteins, but still we have our protein and some other proteins in the solution. So, these are step purifications and after every step of purification we get more and more pure protein, but it is still not 100 percent pure.

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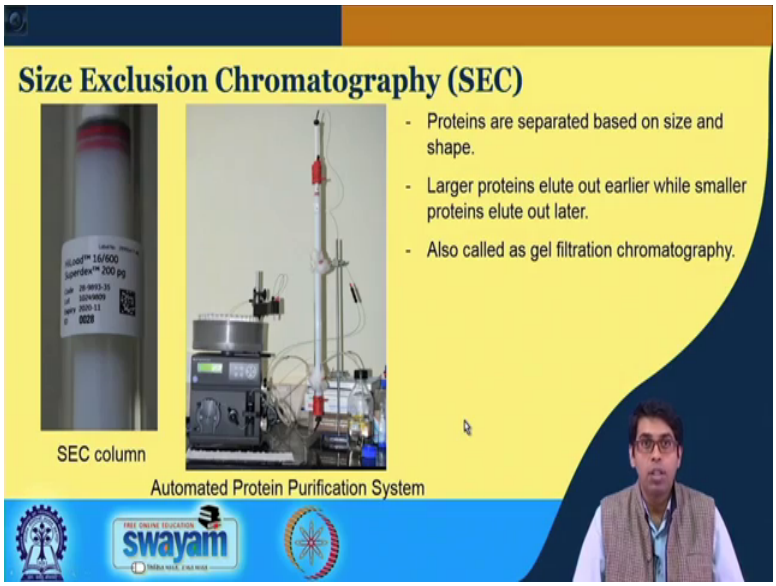

So, after this type, this protein can actually be put into a size exclusion chromatography where proteins are separated based on their size and shape. The larger proteins elute out faster while the smaller proteins elute out later, so it is also called Gel Filtration Chromatography. This is a typical gel filtration column and this is an automated protein purification system which runs the buffer through this protein, through this column and monitors the UV absorption of the solution that is coming out of this column.

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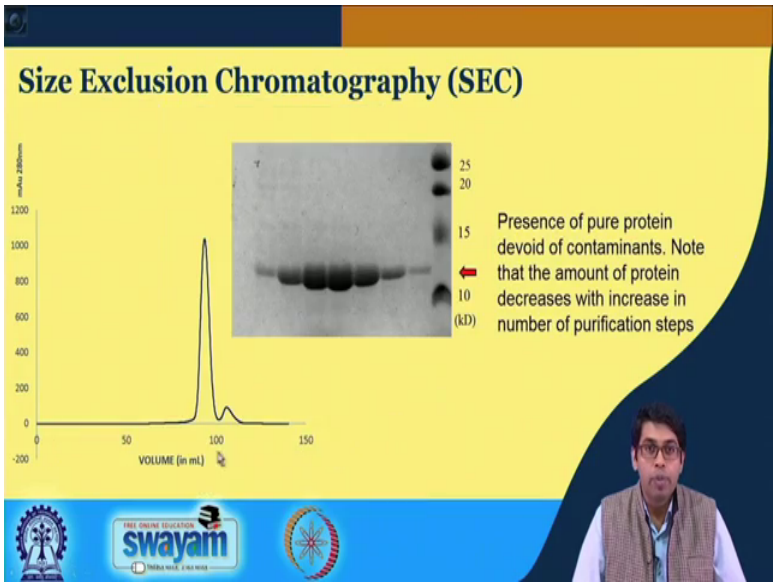

So, if the UV absorption is measured as a function of the illusion volume, what we see is that a sharp peak appears in this range. So, this is our protein of interest because at this point of time most of the protein in the solution is our protein of interest. So, every other protein is either here or here. Now again SDS-PAGE gel was run for the fractions under this peak and we can see that, we have only our protein in these fractions and there are no other proteins in these fractions. So, now after size exclusion chromatography we have a highly pure protein.

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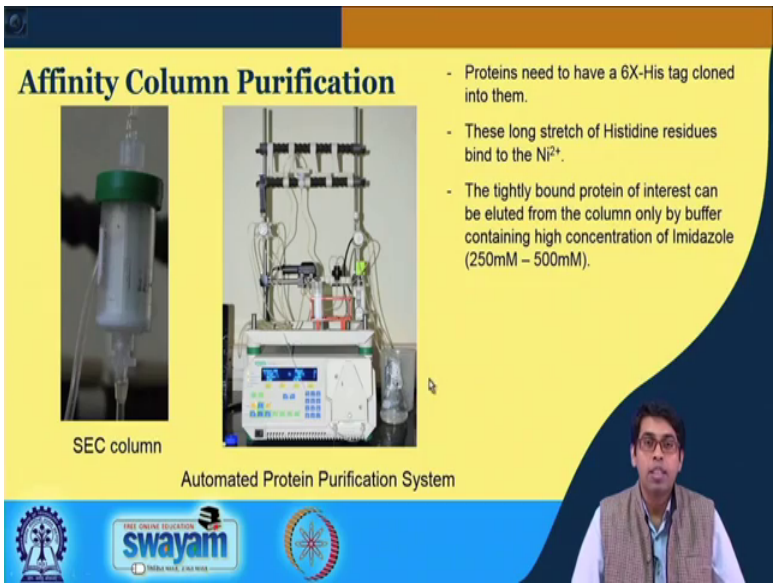

So, if there is no affinity tag, we have to go through this multiple steps of purification. If we have an affinity tag, then the purification becomes much simpler. One example of an affinity tag is a tandem 6X-Histidine tag which can be added to our protein of interest either through the N terminus or to the C terminus. This 6X-Histidine residues bind to nickel.

So, beads are used on which nickel ions are immobilized and if you add your cell lysate into this column, only your protein will bind because it has this 6X-Histidine and all other proteins will flow out. So, this can be exhaustively washed with a wash buffer and finally, when all the proteins are washed out and only your protein is bound, your protein can be eluted using a high concentration of imidazole which is the side chain of a Histidine residue. So, 250 millimolar or 500 millimolar of imidazole can be used to elute the protein.

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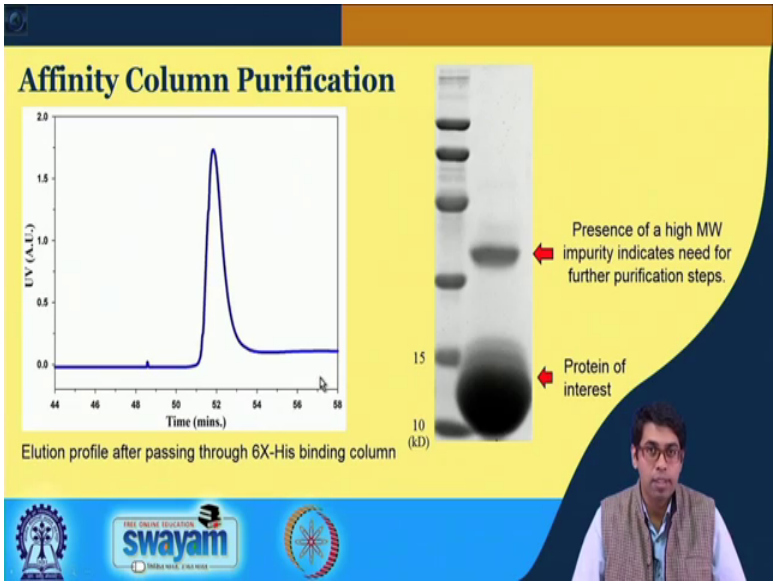

One such example is shown here. Again this is time. It can also be volume and this is the UV absorption in the y axis. So, again we see that after sometime a strong peak comes out which is our protein and there are no other peaks and if we run this one of this fractions on the gel, we see this big band which is our protein of interest and it has a very small impurity. Now there are only two proteins in this mixture and this can be easily separated by size exclusion chromatography. So, another step of ACP, we will give us almost 100 percent pure protein.

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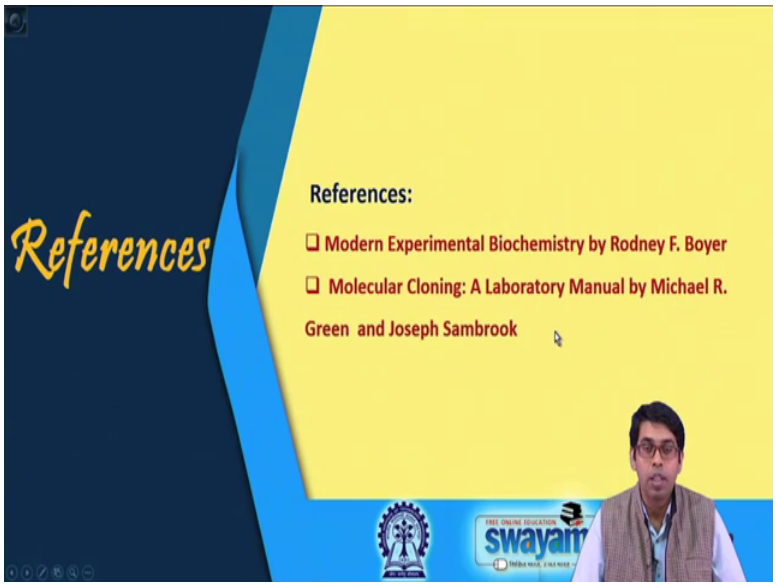

So, this is; so I discussed two processes of a protein purification one when you have an affinity tag and one when you do not have an affinity tag. In the next lecture, I will talk about how you characterize these pure proteins using biophysical and biochemical methods.

Thank you.