Fundamentals of Protein Chemistry
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Module - 04
Protein isolation and characterization
Lecture - 20
Biophysical Methods - III

In our final discussion on module 4, that deals with protein isolation and characterization, we will be looking at some other biophysical methods that are commonly used for structural analysis, mostly for molecular weight determination and other methods by which we can characterize the protein.

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Now in the discussion for this class, we will be looking at the specific aspects of lyophilization, electrophoresis, isoelectric focusing, a bit about microscopy and other AFM techniques; which are important in understanding the protein as a whole and the protein in the form of aggregate, something that we will be studying later on as well.

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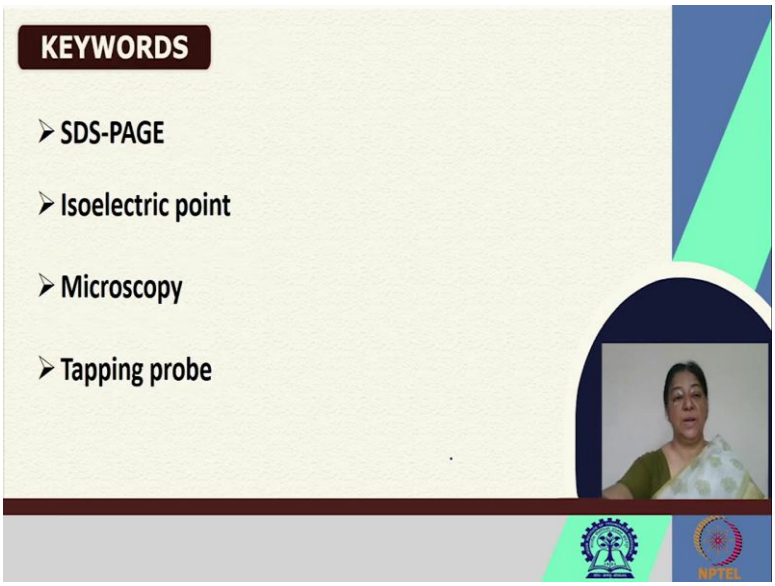

As we look at the specific keywords, we will revisit the concept of isoelectric point as well.

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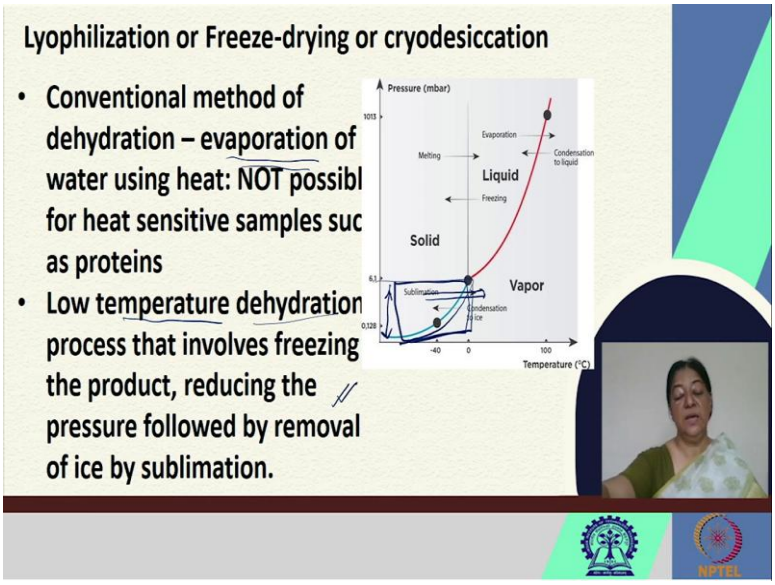

When we talk about lyophilization, the idea here is that when we isolate the protein, we know that we have our protein from the different peaks that we learnt in our chromatography class, that there are the proteins dissolved in the buffer solution. Now, the idea here in lyophilization, is to remove the water or the excess ions that we have present in the solution and retrieve the protein of interest.

The conventional method of dehydration uses evaporation. The evaporation technique which is something that we cannot use for any heat sensitive sample such as proteins. So, in this case, the concept is that we use low temperature dehydration, so that involves a reduction of the pressure which essentially reduces the boiling point of water and then we can remove the water at this low boiling point.

For example when we look at a specific phase diagram; solid, liquid, water phase diagram, where we know that if we are within this [refer to slide] range along the blue line, we have sublimation from the solid phase to the vapor phase, considering that our pressure and temperature are within this range.

So if we can reduce the pressure to a level that would be within this range, then we can reduce the boiling point of water and we would have a sublimation process, taking us directly to the vapor state.

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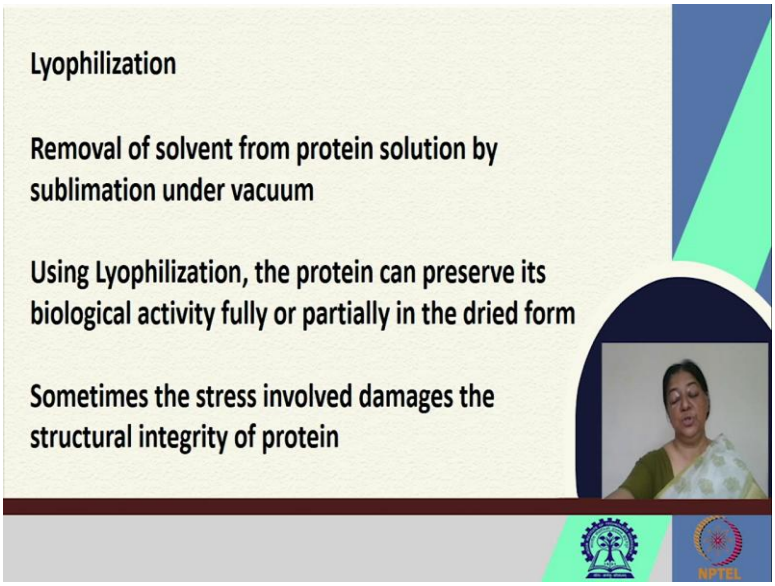

The lyophilization process involves the removal of water from the protein solution by sublimation under vacuum, at a reduced pressure. Using lyophilization, the protein can preserve its biological activity usually fully, but sometimes there is partial loss due to the reduction of the water that is present because of these stress involved damages, the structural integrity of the protein, where we are vaporizing the water that is present. It is a very common technique used.

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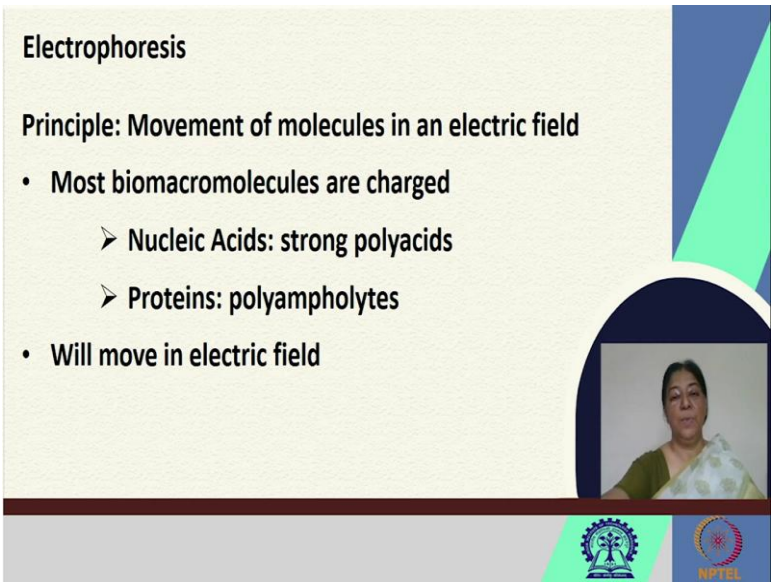

Another very common technique used is electrophoresis, where we are looking at the movement of molecules in an electric field. This is extensively used to determine the molecular weight of proteins and we will see how this is achieved.

Most biomacromolecules are charged and because of their charge they will move in an electric field, if they are subjected to an electric field. For example, nucleic acids are strong polyacids. We have proteins that are polyampholytes. We know that each protein has a isoelectric point.

This isoelectric point and the pH of the buffer will determine the charge on the protein and this is going to be a very important aspect of our understanding, as we saw in ion exchange chromatography where the pH of our eluting buffer, would depend upon the isolation or the characterization of the protein. So, because of these charges we will have the biomacromolecule move in the electric field.

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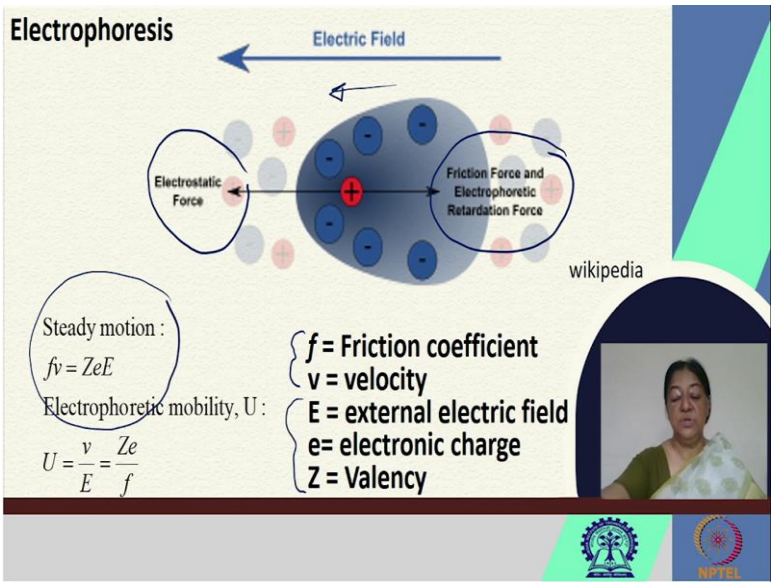

So, we have an electric field and we have two opposing forces here [refer to slide]. One is the movement due to the electrostatic force, the other is due to the frictional force and a retardation force that is there because of the buffer or because of the fluid, in this case it is going to be a gel network. So that retards the motion of the biological macromolecule, but the electrostatic force will pull it into a specific direction and it is the balance of these forces that is important in electrophoresis.

So what happens is we have a steady motion and this steady motion is given by the product of the frictional coefficient, the velocity, the external electric field, the electronic charge and the valence. This electrophoric mobility is important in telling us how the molecule is going to move when it is subjected to this electric field.

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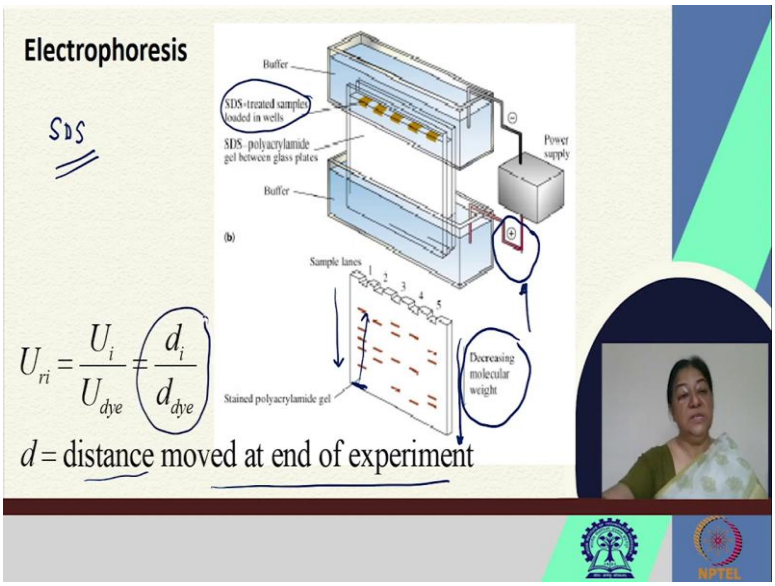

In a typical experiment, we have sodium dodecyl sulfate treated samples that are loaded into the wells. What sodium dodecyl sulfate does is, it renders the molecules negatively charged. So all the protein molecules become negatively charged, as a result of which they all move towards the positive electrode.

Now the movement towards the positive electrode through the gel network, is therefore going to be according to their size, because all of them have a rendered negative charge. All of them will move to the positive electrode and we would have decreasing molecular weight in this [refer to slide] direction, because the larger molecules would have more retardation and would be sluggish in their movement towards the positive electrode.

So we have a certain distance that is going to be moved at the end of the experiment. And we have at the end a tracking dye, that has a very low molecular weight, that reaches say at the end of the gel and then anything relative to its movement is going to give us a ratio of the mobility of our molecule.

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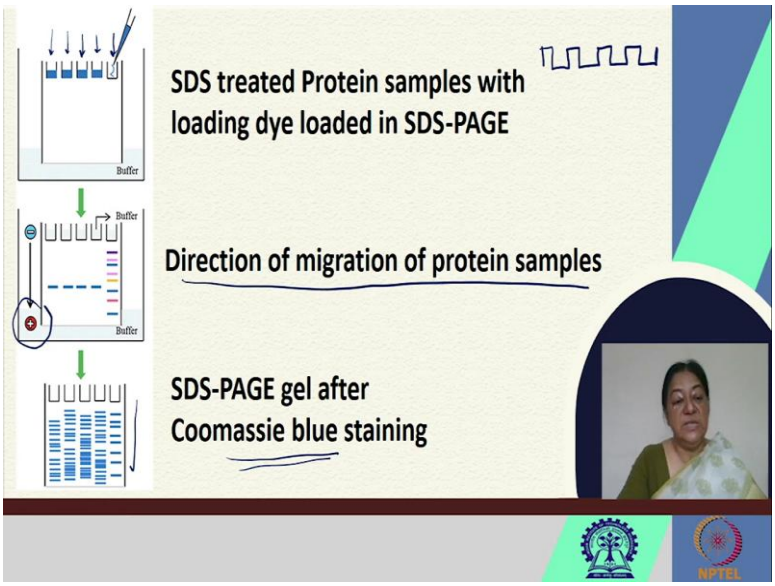

This is a typical experiment. The SDS treated protein samples with loading dye, are loaded in the SDS-PAGE. These specific vessels are called the well. When it is set we have a specific comb as it is called, which holds the protein molecules mixture in space. And the SDS treated protein samples with loaded dye are loaded onto the SDS-PAGE.

Since all of them are negatively charged we would have a direction of migration of the protein samples. And based on the SDS-PAGE, after we stain the sample with coomassie blue a standard staining technique, we will have blue bands of where the proteins lie in our gel.

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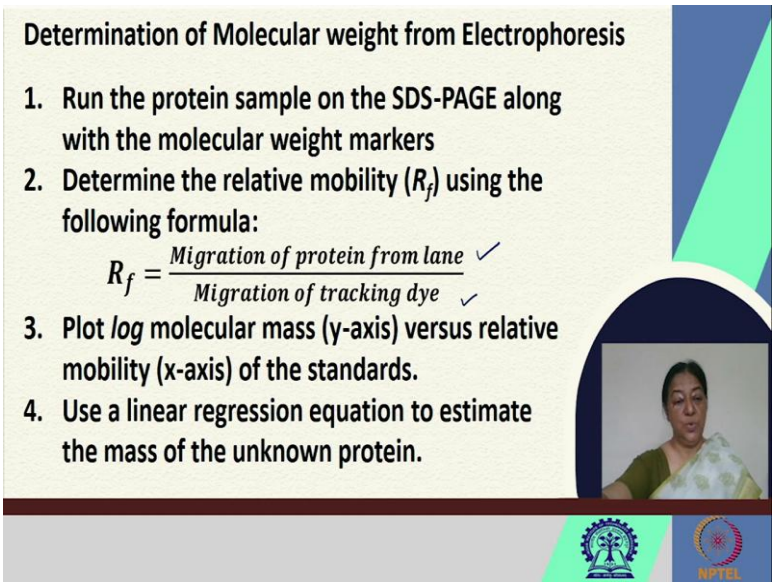

Now how do we determine the molecular weight from the electrophoresis. We run the protein sample on the SDS-PAGE along with standard molecular weight markers. We determine the relative mobility using the following formula;

R_f = Migration of protein from lane/Migration of tracking dye

Following that we plot the log of the molecular mass versus the relative mobility of the standard samples that we have. We have a linear regression equation that determines the mass of the unknown protein.

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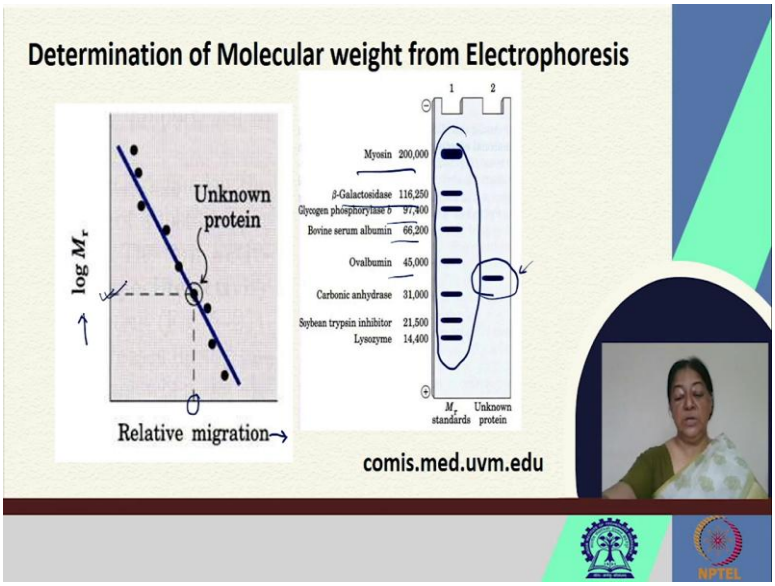

So when we are looking at a standard set which we see here [refer to slide] in lane 1 and we have their corresponding molecular weights part here and this is the unknown protein. Based on this, we can have a calibration curve. The log of the molecular weight along the y-axis, the relative migration along the x-axis. And based on the relative migration of the unknown protein given from the gel, we can find out the molecular weight of the protein of interest.

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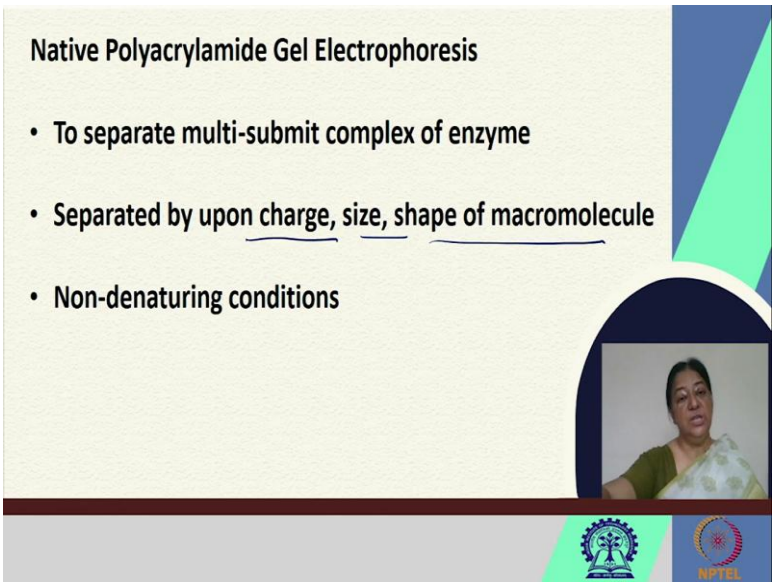

Now, when we use SDS for this particular experiment in SDS-PAGE, there is one thing that we need to be concerned about is, SDS denatures proteins. So, if we have a multi-subunit protein, what will happen is, these subunits will disintegrate into their relative monomeric units.

This disruption is going to be a cause for concern if we do not know our actual protein multi-subunit structure. So, to separate the multi-subunit complex of the enzyme, we separate them upon charge, size and the shape of the molecule. In this case we can do that, where we are using non-denaturing conditions. These non-denaturing conditions result from an understanding of the fact that we are not adding SDS to this particular gel.

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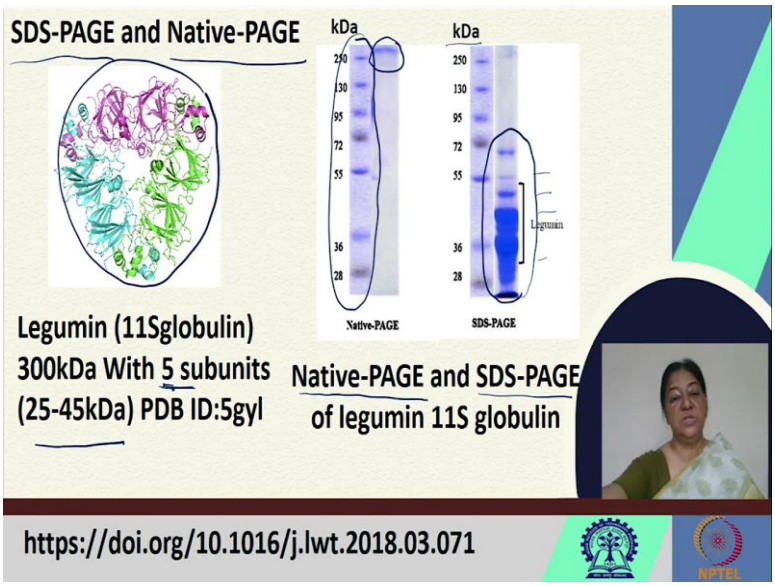

So when we are looking at SDS-PAGE versus Native-PAGE, and we have 5 subunits belonging to this particular protein and we run a Native-PAGE that is without SDS and one with SDS, we will reach a case. So, this [refer to slide] is the Native-PAGE which will give us the multimeric protein molecular weight, because we have not added SDS-PAGE to this.

However when we add SDS-PAGE, we have the subunit molecular weights because the subunits are no longer in their multimeric form, the protein is no longer in its multimeric form and we look at the different bands corresponding to the different subunits in this case 5 of them, that range in molecular weight from 25 to 45 kDa.

So this is a typical method by which we can determine the molecular weights of proteins. And it is extensively used in protein chemistry laboratories to find out the molecular weight of proteins.

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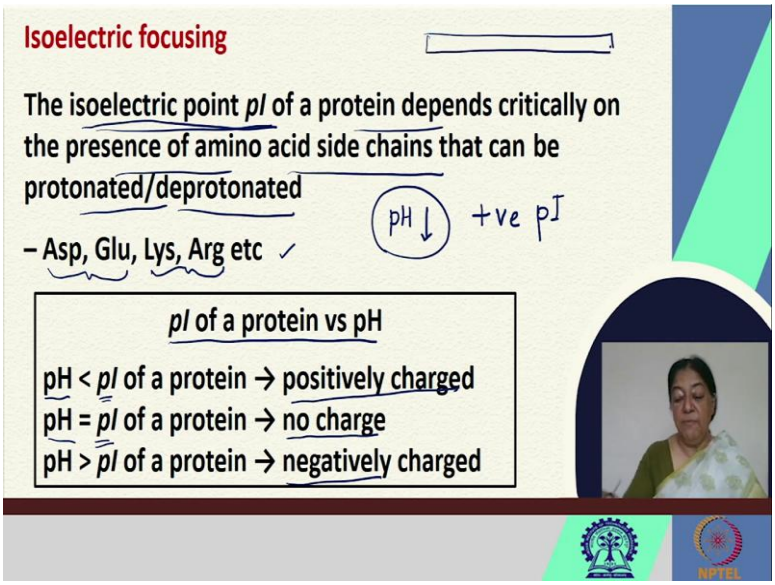

Another experiment where we can also identify the pI of a protein, depends upon the isoelectric point. We know that the isoelectric point depends critically on the presence of the amino acid side chains that can be protonated or deprotonated.

So depending on this, we need to know which are these particular amino acids that will have a charge at a specific pH. We know that we have aspartic acid, glutamic acid, lysine and arginine. Lysine and arginine have positive charges. Aspartic acid and glutamic acid have negative charges. Depending upon the pH if we have a low pH, if we reduce the pH, then we expect our molecule to be positively charged.

This then leads to an interesting idea for the experiment, that if we are looking at the pI of the protein based on its particular charge, then if the pH is less than the pI of the protein that has a low pH, the protein is positively charged. When the pH is at the pI of the protein, there is no charge and when the pH is greater than the pI of the protein, we have a negative charge.

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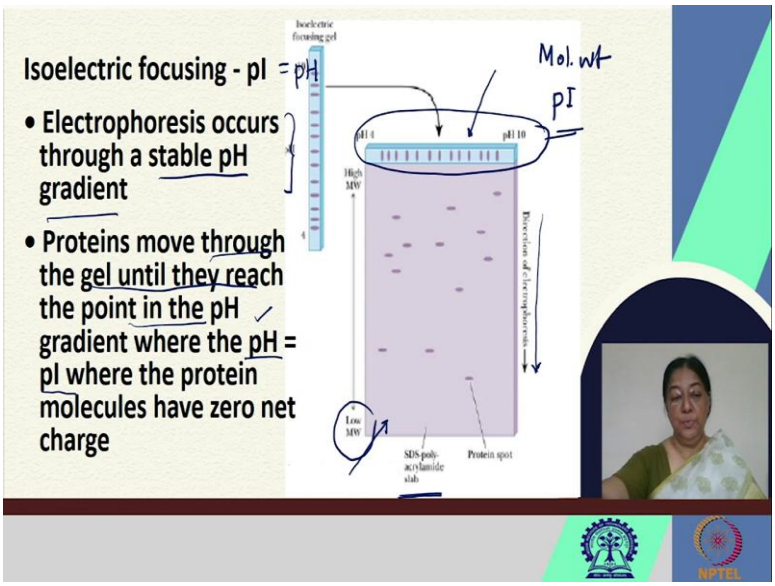

If we subject our protein of interest through a field or through a gel that has what is called a specific strip, an ampholyte strip that looks at a pH stable gradient, where we have the electrophoresis conducted through the stable pH gradient. The movement of the protein will occur as long as the protein has a charge to it. As soon as the pH becomes equal to the pI, the protein will no longer move because it does not have a charge it has zero charge.

In this case, the proteins will move through the gel until they reach the point in which the pH gradient is present, where the pH is equal to the pI, as the protein is going to have a net negative charge at this particular point.

So, this is [refer to slide] our strip, ampholyte strip as it is called that has a particular pH range. This is our isoelectric focusing gel. And when we look at the movement of the proteins, what happens is our proteins move in the direction of that particular charge, depending upon their size in the SDS polyacrylamide gel and then according to their charge along the isoelectric focusing gel.

So what happens here is the movement will continue. We will have a spot that will correspond to the movement, that will be stopped because of the pH being equal to the pI of the protein. And what will happen is, we will have a specific direction of electrophoresis dependent on the molecular weight.

It is a combination of two techniques, where we will be looking at a molecular weight difference as well as a pI or charge difference, which will render the protein immovable in the gel. So, any spot here where the protein does not move anymore, depending upon the pI and the pH, we will have that specific location of the protein.

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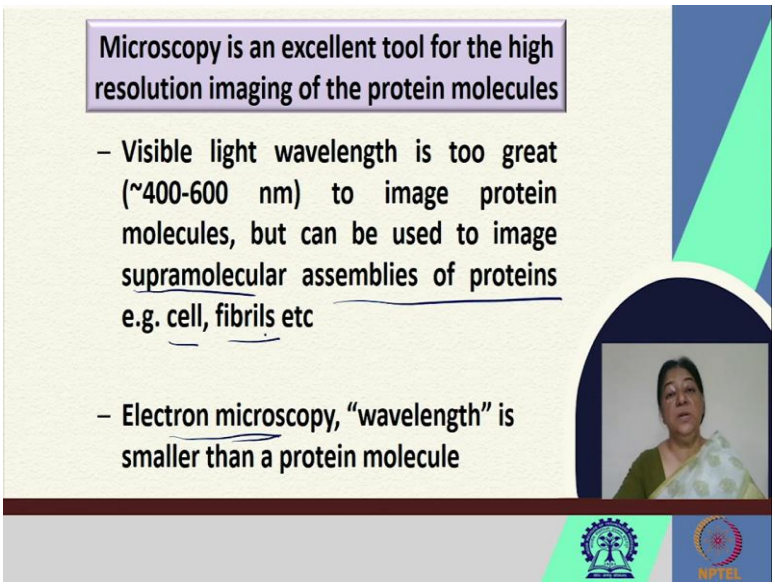

Another very commonly used technique is a microscopy. Microscopy is a very important tool for the high resolution imaging of protein molecules. For example, in many research labs around the world today, they look at cryo electron microscopy. A very new method, a relatively new method to find out the structures of protein molecules.

We will not go into the techniques of cryo electron microscopy. But nevertheless an understanding of how the molecule will behave under this field, where we have the microscopic techniques that we can use. We can look at supramolecular assemblies of proteins in the cell or fibrils that are important components of disease.

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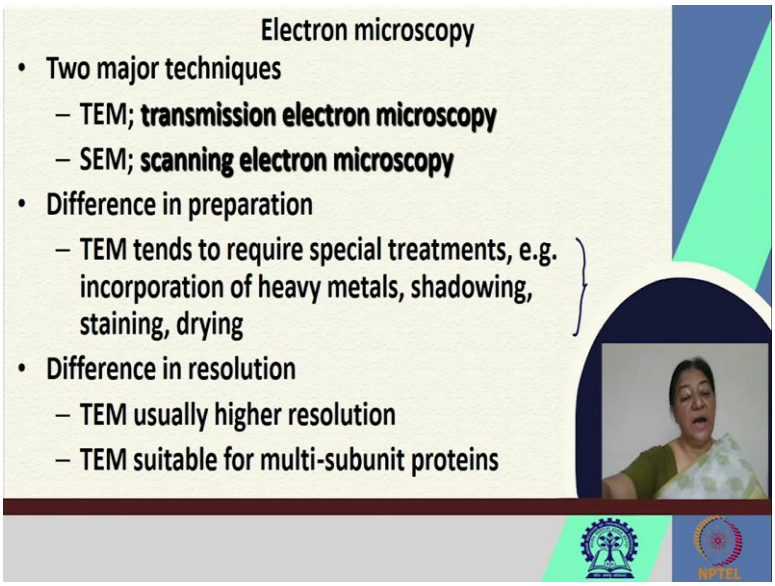

In electron microscopy there are two major techniques, transmission electron microscopy and scanning electron microscopy. In these specific techniques, the difference lies in the preparation and there are special treatments by which we can define our methodologies in this case. And the difference in resolution as well because transmission electron microscopy, in the method that it uses, has higher resolution and is useful for multi-subunit proteins.

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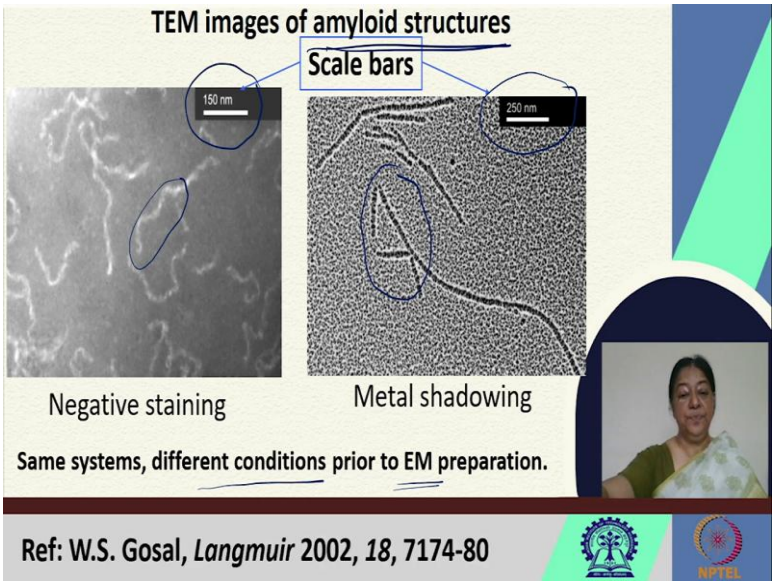

If we look at amyloid structures, a topic that we are going to cover when we study protein aggregation and protein-protein interactions, we will look at a negative staining set and a metal shadowing where you can see a distinctive same systems, but because of the different conditions prior to the electron microscopic preparation; we have distinctive sets where we can actually see the different fibrils in the microscopic image.

The different scale bars are important for the microscopic images as well because this tells us how long our fibers or fibrils are.

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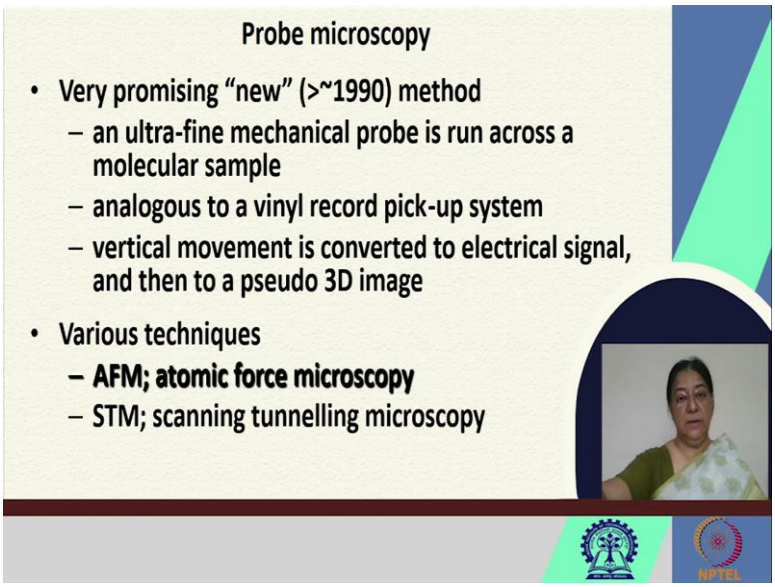

If we look at probe microscopy, probe microscopy is a relatively newer method and it uses an ultra-fine mechanical probe that runs across the molecule sample. Now as it does so, it sees the conversion to an electrical signal and produces a pseudo 3D image. Techniques like this are atomic force microscopy and scanning tunneling microscopy.

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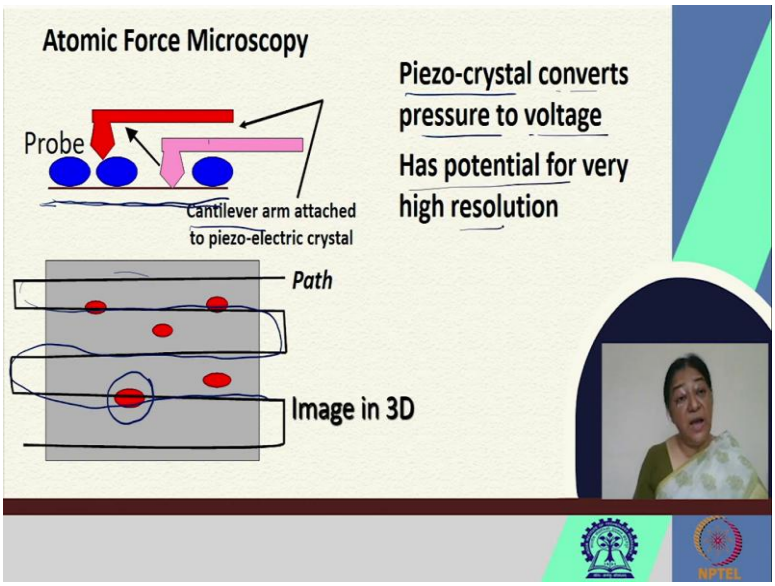

In atomic force microscopy, we have a cantilever arm that is attached to a piezoelectric crystal and we have our mono layer, most preferably on the surface and we have a method where we will have the path of the cantilever follow a specific path. And then when it hits upon a protein, it will give us a 3D profile image. The piezo crystal converts pressure to voltage and has potential for very high resolution, that will tell us or give us some information about the protein.

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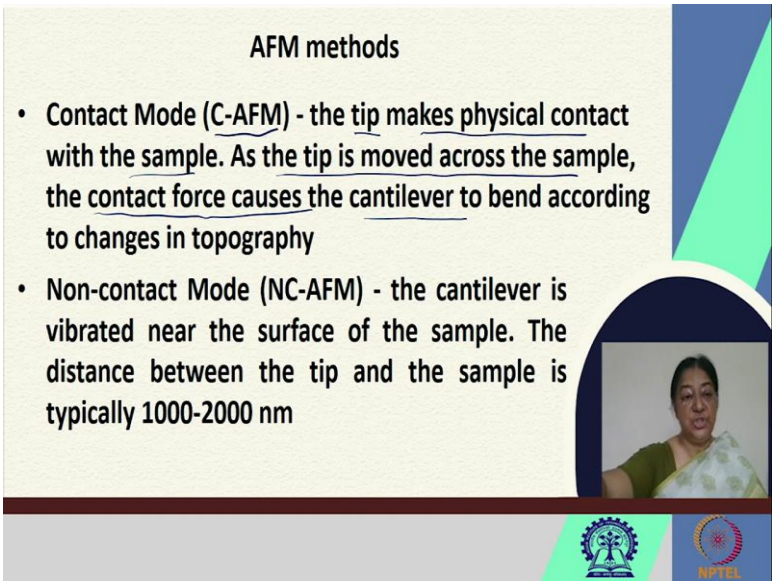

If we look at the different AFM methods that are known, we have what is called the contact mode the C-AFM, where the tip makes physical contact with the sample. And as the tip is moved across the sample the contact force causes the cantilever to bend because of the changes in the topography of the sample that is connected.

In a non-contact mode, the cantilever is vibrated near the sample and we have another kind of detection possible.

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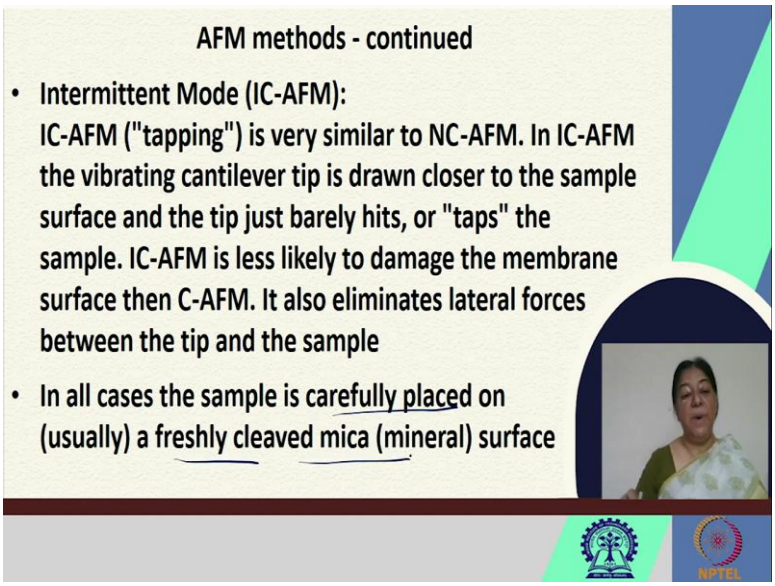

In the intermittent mode, we have a similar non-contact mode and we have it closer, so it taps on the surface. And in all cases the sample is carefully placed on usually a freshly cleaved mica surface for analysis.

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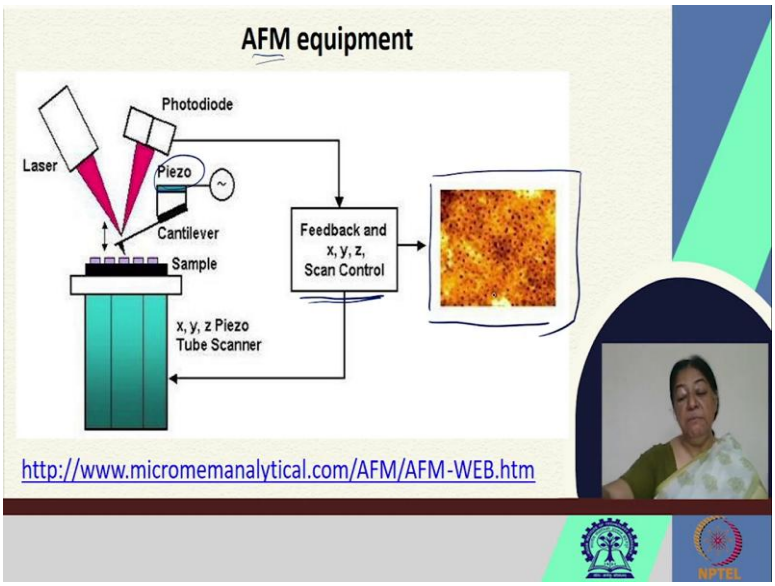

In the typical AFM equipment, what we have is we have a laser beam and we have a cantilever and this is the piezoelectric crystal. As we have our scanning across the sample, we have the feedback of the x, y, z controls here, that give us a scanned image of our specific sample.

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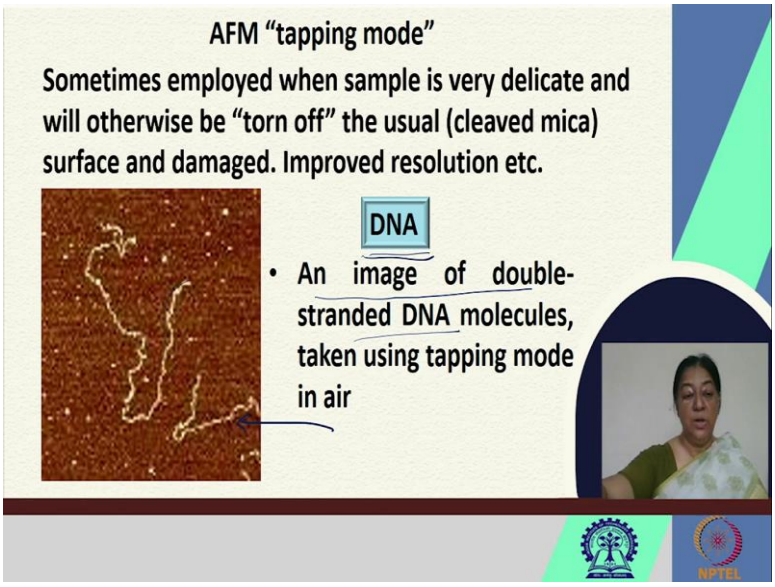

And based on the tapping mode as it is called, we can look at the tapped image of our protein. So this [refer to slide] for example, is a picture of DNA, where we have an image of the double stranded DNA molecules taken during the tapping mode that

actually gives you some idea about the surface of the molecules and how the molecules can aggregate.

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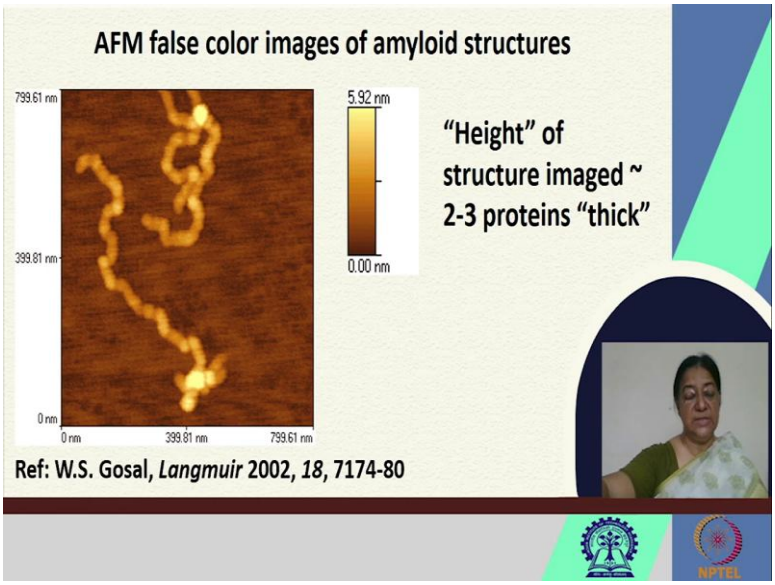

Sometimes we have specific amyloid structures, that are useful for identifying the presence of fibrils, the presence of smaller aggregates of proteins as well.

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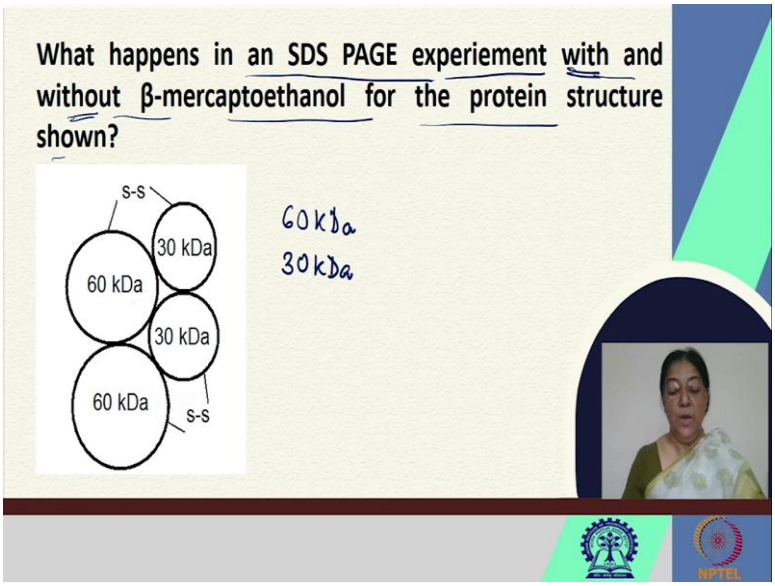

In a specific problem that we will now look at, the question that has been is what happens in an SDS-PAGE experiment with and without β-mercaptoethanol for the protein structure shown [refer to slide].

In this particular protein structure that we see, we see that there is a 60 kDa molecule fragment subunit and we have a 30 kDa subunit. What happens here is if we have the specific gel electrophoresis run, with and without β-mercaptoethanol that is the experiment that is going to be conducted. Now, what we have here is a specific idea based on how to do the experiment.

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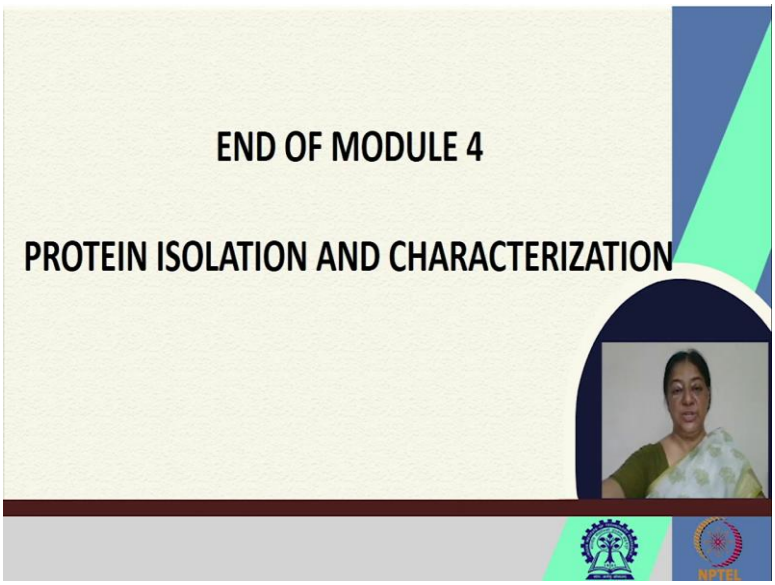

This is the end of module 4 that looked at protein isolation and characterization.

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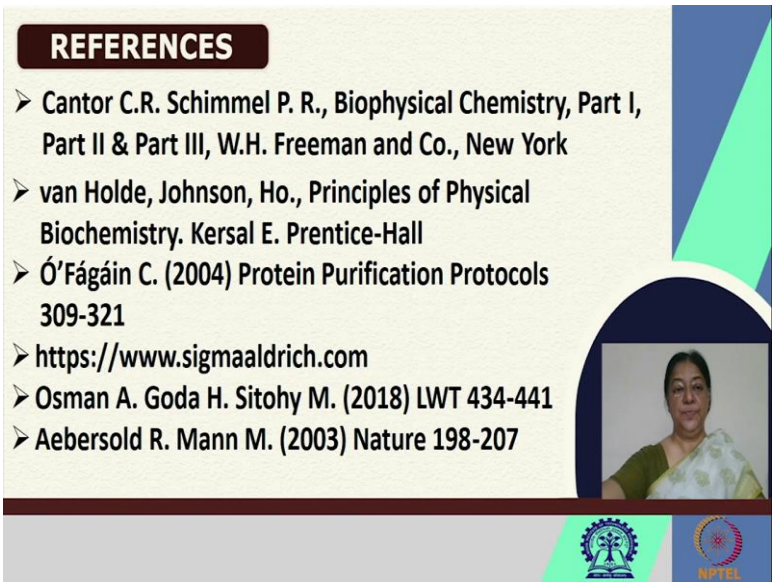

[Refer to slide] these are the references.

Thank you.