Experimental Biotechnology
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Module No # 03
Lecture No # 11
Scientific Questions (Part 1)

(Video Starts: 00:24)
(Video Ends: 01:00)

Hello everybody this is Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And what we were discussing? We were discussing about the electrophoresis so in the previous module we have discuss about the different aspects of the vertical electrophoresis the horizontal gel electrophoresis. And then we have in the previous lecture we have also discuss about the different combinations of the vertical as well as the horizontal electrophoresis.

So that you can be able to understand and tackle the different types of problems related to the, and understanding a particular process. And we have also shown you how you can able to utilize the different vertical or the horizontal electrophoresis gels to answer the different kind of questions. Now in today's lecture we are also going to discuss about the scientific problems what you are going to handle when you are going to how to solve them and how to resolve the different types of problems and how you can be able to utilize the different types of electrophoresis operators?

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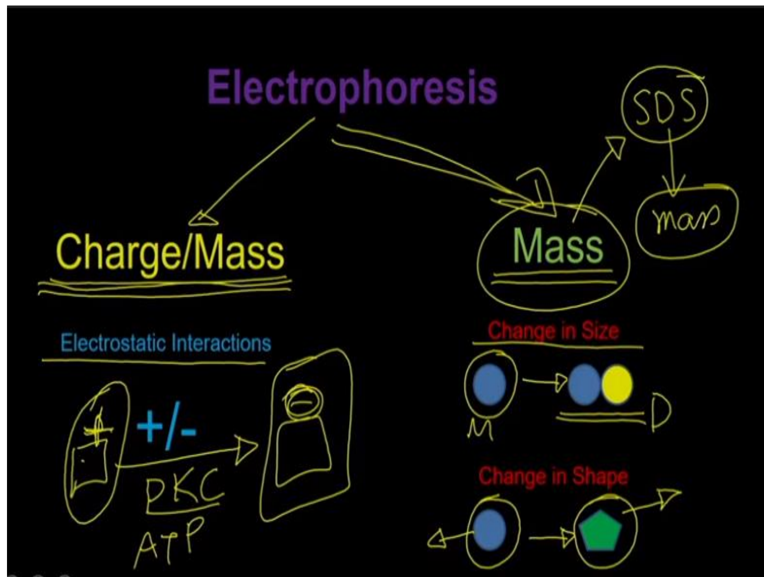

So when we talk about electrophoresis one is sure that the in the case of gel electrophoresis it is actually utilizes the 2 different major techniques or major properties of a molecule. Which means the electrophoresis is either going to exploit the charge by mass ratio or it is actually going to separate the molecule based on the masses. Charge if the molecule are been separated based on the charge by mass ratio which means the process is going to be sensitive to be electrostatic interactions.

Which means the electrophoresis can be utilized to map the interactions where the electrostatic interaction is playing the major roles. Whether it is actually imparting the positive charges to the molecules or whether it is imparting to the negative charge to the molecules or the whether the positive and negative charges are coming together and forming the complexes. These kinds of questions can be addressed because the electrophoresis actually consider the charge by mass ratio as one of the criteria to separate the molecules.

So you can imagine that a molecule is uncharged but if you do some activity or some process because of that if the molecule is actually enquiring the positive or the negative charges. Then this, particularly positively charged molecules can be separated from the neutral molecules or vice versa that you have a positively charged molecule and it is actually changing into a negatively charged molecules.

Which means if you have a mixture for example if you are adding a enzyme like protein (()) (04:00) or PKC then what will happen? If you have a positively charged molecule it will be actually going to impart a negative charge. So if you want to monitor the activity of the PKC what you can do is you can just simply take the complete SA mixture which actually going to contain the positively charge substrate the PKC and then you can add the ATP which is actually going to be the (()) (04:27) agent.

Another result is actually going to generate the negatively charged substrate so as a result if you take this whole mixture and loaded into the horizontal page or the horizontal agarose gel. The positive will go towards the negative electrodes and the negative will go towards the positive electrode. And that is how you can be able to quantitatively say that out of 100 molecules how many molecules are negatively charged and how many molecules are positively charged.

Similarly the electrophoresis is also going to be considered the mass which means it also separates the molecule based on the mass where it is for example when you are adding the SDS into the electrophoresis. The charge is getting utilized and then the molecules are only going to be separated based on the mass which means it actually going to tell you the change in the mass or the change in the size of the molecule.

We have 2 examples where it is actually going to tell you the change in size for example if you have a single protein and if you do some activity and it is actually going to acquire addition proteins or you have monomer which is actually turning into dimer these kind of stuff can be understand or can be addressed simply by using the electrophoresis. The alternate is where they will be no change in mass but actually it is going to change the electrophoretic mobility of that particular molecule because of change in the friction of that molecule is that if the molecule is also going to change its shape.

For example if you have a circular shape object and that actually get converted into a pentagonal object these, surface area of this molecule is going to be smaller compare to the surface area of this molecules or they will be change in the surface area of these 2 molecules. And one of the classical examples is that if you actually monitor the protein unfolding for example if you start with a monomer it is still be, 40 Kda.

The unfolded protein is also 40 Kda but the size of the folded protein is going to be smaller compared to the size of the unfolded proteins. So that can be also be monitored with the help of the native page or the urea page. So these are the 2 main aspects what can be exploited when you are designing a experiment considering the electrophoresis into the picture. So they will going to discuss different type of experiments where we are either going to exploit the charge by mass as a criteria or some places we are also going to exploit the criteria of the mass.

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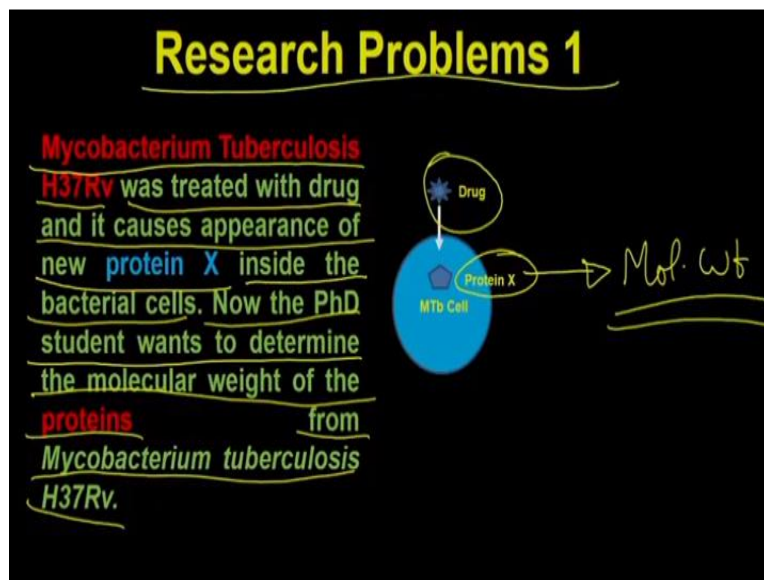

So our research problem 1 is that where we have the going to question is that the mycobacterium Tuberculosis H37RV was treated with a drug and it causes the appearance of a new protein X inside the bacterial cells. Now the PhD students, wants to determine the molecular weight of the proteins from the mycobacterial tuberculosis H37RV. Which means that you are doing to a treatment to the cell and that actually is specifically reducing the protein X and what the student wants to know is what is the molecular weight; of this particular proteins.

And how to do that you can do that simply by the designing in a suitable experiments because here what you are going to do? You are actually wants to know the molecular weight of the protein and that is how you can be able to utilize the electrophoresis. Let us see how to do that?

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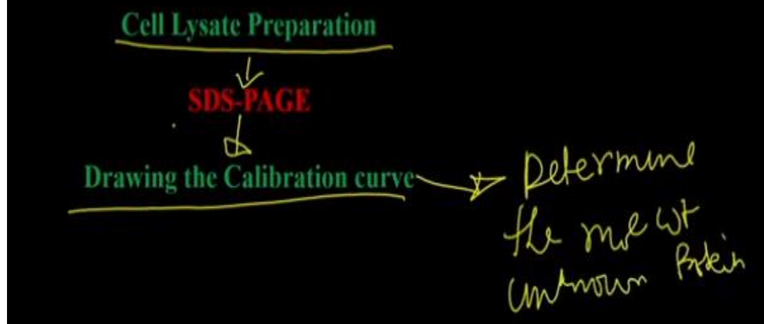

So in the experimental design what you are going to do is you can just take the MTb cells okay and what you do is you first treat it with the drug. And then after that you actually going to prepare this cell-lysate and the cell-lysate is actually containing the protein of your interest which is protein X in this case. And then what you do is you resolve this into a SDS page because you want to know only the molecular way.

You do not want to know the native molecular weight or the so in that case what you do is you run the SDS page. And in the SDS page you also should run the a molecular weight marker okay on the side so that it actually going to help you to know at what position you are going to get what molecular weight and then would you do is you do the analysis of the gel picture with the help of the some of this software's.

If you remember in the previous module we have also discussed how to do the image analysis and how to determine the concentration of a particular protein and how to determine the molecular weight also. So now what you do is you analyzed a gel picture. So these are the things what you have to do first you have to prepare the cell Lysate then you have to prefer perform the SDS page. And then once you have done with a SDS page then you can draw a calibration curve.

The calibration curve is to see what is the how the gel is actually resolving the different sizes of the molecular weight of the proteins. And that actually calibration curve can be used to determine the molecular weight of unknown protein. Let us see how to do that.

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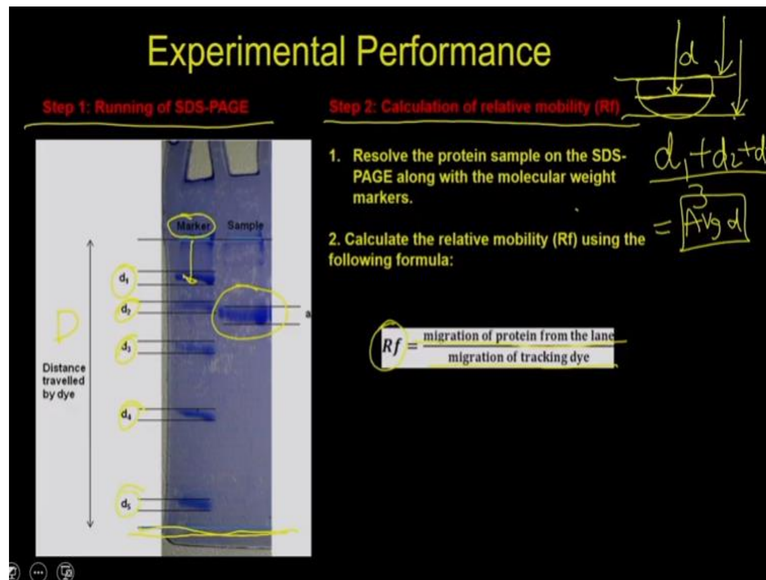

So what we have done? In the step 1 we have done the SDS page so what we done we have actually resolved the protein of your interest which means you the protein which you are interested to identify the molecular weight or you which you are have identified and you want to know the molecular weight along with that we have also run the molecular weight marker. So markers are the commercially available mixture of the proteins which actually have the different types of protein.

And for these all these proteins their molecular weight is already known now what you have to do is in the step 2 you are going to do the calculation of the relative mobility. The relative mobility is been defined as the migration of the protein from the lane verses the migration of the tracking dice. So what you can do is first you determine the position of the tracking die so that is actually going to be the distance d what you are going to put.

And then you can be able to determine the distances of each and every band of the molecular of the markers and that is how you can have the relative mobility of each and every band. And when you are looking at a band actually you see that this band is actually a slightly thicker than what you expect. So in that case what you have to do is you have to just simply take the middle of the point and from here you have to calculate the distance

The ultimate approach is that you can do a 3 measurement you can do the one measurement from this point one you can do another measurement from this point and you can do the third measurement from the center of the band. And that is how you can actually get the d1 + d2 + d3 and divided by 3 and that actually is going to give you the average distance. And average d that average d can be used for each and every band that actually is going to take care of the errors what you are going to have.

Because if you take the lower point your Rf value is going to be on higher side if you take the upper value you are going Rf value is going to be on a smaller side. So that is how it for getting a minimum error what you can do is you can just take both the values and then you can take the average of that and that is actually is going to be the most appropriate way of doing it. So once you have done the Rf value for d1, d2, d3, d4 and d5 you are going to get the Rf values of the individual proteins and then what you can do is in the step 3 you can actually be able to draw a calibration curve between the Rf versus the log molecular weight.

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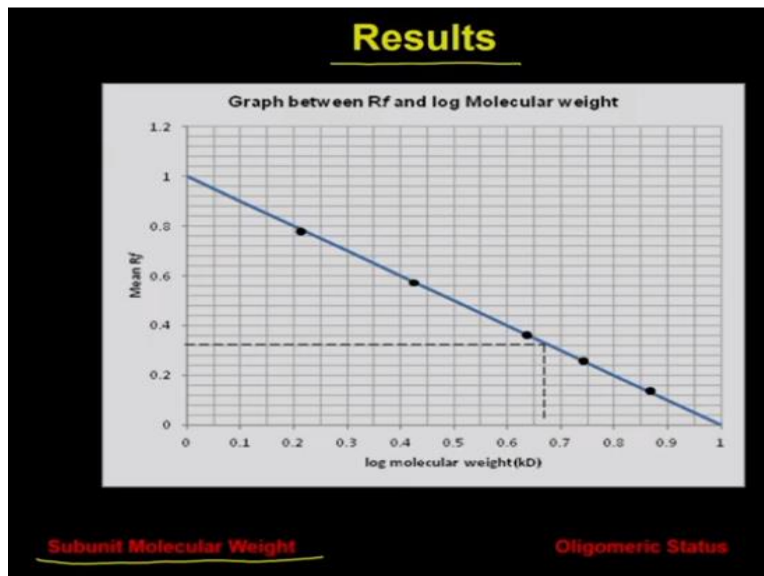

So utilizing this you can plot the log molecular weight on the y axis versus relative mobility on the x axis of the standard. So what you are going to get? You are going to get a negative curve and it is going to give you the equation which is actually going to follow the question of $y = mx + c$. So the using the linear regression equations you can be able to estimate the mass of the unknown protein.

So for example if you have the equation of mx + c where the x value is what you actually required so if you know the y value you can be able to determine the x value because all others are constant and m also you can be able to calculate from this graph. And as a result you will be able to determine the protein the athlete approach is that you draw a perpendicular and that perpendicular wherever the perpendicular hits to this curve you can actually get the value of x.

And since this is a log you can do a antilog of that value and that value of x is going to give the molecule weight of the protein of your interest. So this is the simple method of determining the molecular weight of the protein you utilizing the SDS page ahh what you can also combine with this analysis is that. Because if you determine the molecular weight and you can be able to determine this subunit molecular weight.

But if you are interested and you further want to explore the about the oligomeric status of this particular protein then what you can do is you can the run the similar kind of proteins on to a native page. And then you can be able to determine the oligomeric status which we are going to discuss in the next slide.

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Determination of Oligomeric Status of the Protein

The polyacrylamide gel electrophoresis can be use to determine the oligomeric status of the protein. A protein sample can be run under the denaturating as well as in the native conditions in two separate gel. The protein of the known molecular weight runs on both gels and a Rf value is calculated for the standard proteins as described. A calibration curve from native and denaturating gel is used to determine the molecular weight (native and denaturating) of the protein. The oligomeric status of the protein is calculated from the formula given below:

$$\text{Oligomeric Status} = \frac{\text{Molecular Weight (Native)}}{\text{Molecular Weight (SDS – PAGE)}}$$

So how to determine the oligomeric status of the protein the polyacrylamide gel electrophoresis can be used to determine the oligomeric status of the protein. A protein sample can be run under the denaturating as well as the in the native condition in the 2 separate gels. A protein of the

known molecular weight runs on both the gel and then you are going to do a Rf calculation for the standard protein as we just discussed.

A calibration curve can be drawn from the native as well as the denaturating gel and it is used to determine the molecular weight of that particular protein under the native condition as well as the denaturating conditions. The oligomeric status of the protein is calculated from the formula oligomeric status is equivalent to the molecular weight of the protein what you are going to get from the native page divided by the oligomeric status what you are going to get from the SDS page.

So the process remains the same except that you might have to run the same sample on the 2 gels one is native gel where you are not going to add the SDS or the beta (()) (16:40). And then you also, going to run additional gels where you are going to run the protein under the denaturating conditions. Now you have to run the markers as well so marker proteins are also very different when you run it for the native page versus the SDS page.

So the marker protein what I have shown you just now is only for the SDS page so then what you the exactly the same you are going to calculate the Rf value of the standard protein. You are going to draw the vibration of the standard protein in both the condition the native page as well as the SDS page. And then you are going to determine the molecular weight of this particular protein under the native condition as well as the denaturating conditions.

And then what you are going to do is you are going to calculate the oligomeric status simply by dividing the molecular weight what you are going to get from the native conditions versus the SDS. The only thing what you have to worry is or what you do not have too much worry about is that imagine that you got the native molecular weight or else 46 okay. And then you got a SDS molecular weight as 25 okay.

Now if I have to calculate the oligomeric status what I will do is? I will just divide the 46 divided by 25 which is actually going to be lesser than the 2 which is actually going to lesser than 2 because ideally it should be 50 or this should could have been 23. But as you know that the oligomeric status is a perfect number it cannot be 1.75 or 2.25 or any other number. It can be

either 1 it can be 2 it can be 3 it can be 4 it cannot be a middle number so that is why whatever the number you get you have to make it to the next round figure.

For example if you are getting a 1.75 you can make it 2 if you getting the 1.2 then you can make it 1 so that is how that is the general understanding that you have to adopt if you want to determine the oligomeric status in a more and more perfect way of doing it. Because the oligomeric status cannot be a partial number it can be a whole number. Now let us move on to the next problem.

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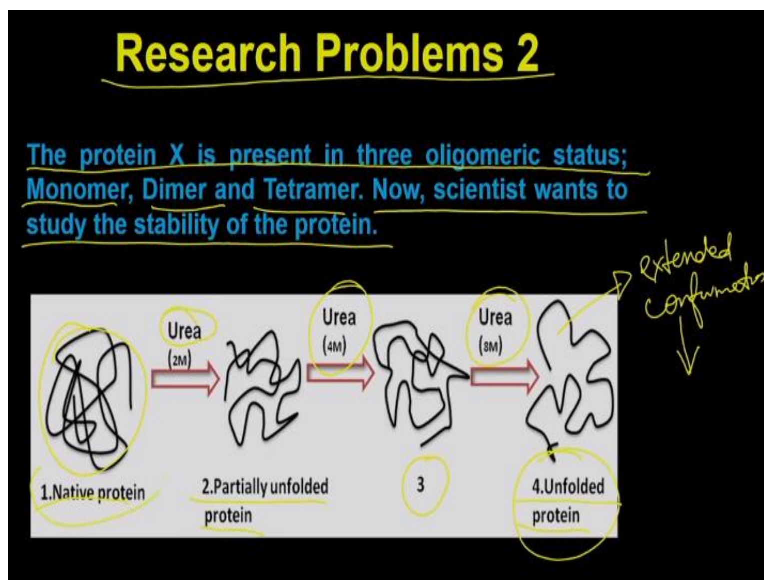

So the next problem is the research problem which we have discussed before also and where if you remember when we were discussing about the gel filtration chromatography we have taken the same problem. And now what I am going to do is I am going to take the same problem but instead of using the gel filtration chromatography now I am going to use the electrophoresis. The protein X is present in 3 oligomeric status; Monomer, Dimer and Tetramer. Now scientist, want to study the stability of the protein.

So you know that the native protein is partially folded it actually forms a compact structures when you express them to the denaturating agent to actually initially gets the partially unfolded protein when you increase the denaturating conditions further then it reduces the it becomes the structure becomes slightly more loose and at the end of the very high consideration of the denaturating agent the protein get completely unfolded.

And this actually forms the extended confirmation which means the radius of this particular structure is going to be very large compare to the native proteins.

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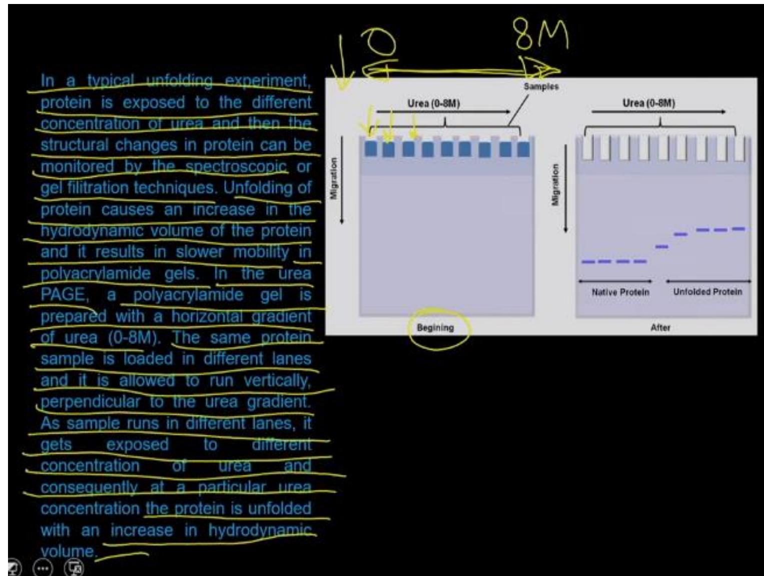

So now, how to solve this you can run a urea page to address or to study these complexes and you study this particular protein unfolding process. So in a typical unfolding experiment the protein is exposed to the different concentration of the urea. And then the structural change in the protein can be monitored by the spectroscopic or the gel filtration technique. Unfolding of the protein causes an increase in the hydrodynamic volume of the protein and it results in the slower mobility in polyacrylamide gels. Why it is so?

Because once the hydrodynamic volume will increase it is actually going to experience the larger and larger friction so that is how the friction component is going to be increased and that is how it is mobility is going to be compromised and that is why it is going to run at a slower rate. In the urea page a polyacrylamide gel is prepared with the horizontal gradient of urea which is 0 to 8 molar. The same protein sample is loaded in the different lane and it is allowed to run vertically perpendicular to the urea gradient.

Which; means you are going to maintain a urea gradient across the vertically okay horizontal gradient from this side to this side. So this side you are going to have the 0 and this side you are going to have 8 molars urea and then you are going to resolve the protein samples in each lane

corresponding to each urea consultation. As sample runs in different lanes it get exposed to the different consultation of the urea and consequently at a particular urea consultation the protein is unfolded with the increase in hydrodynamic volume.

So what will happen is? When you are starting the experiments all, the sample will look same except we are going to get the exposer of the different amount of urea, So what will happen?

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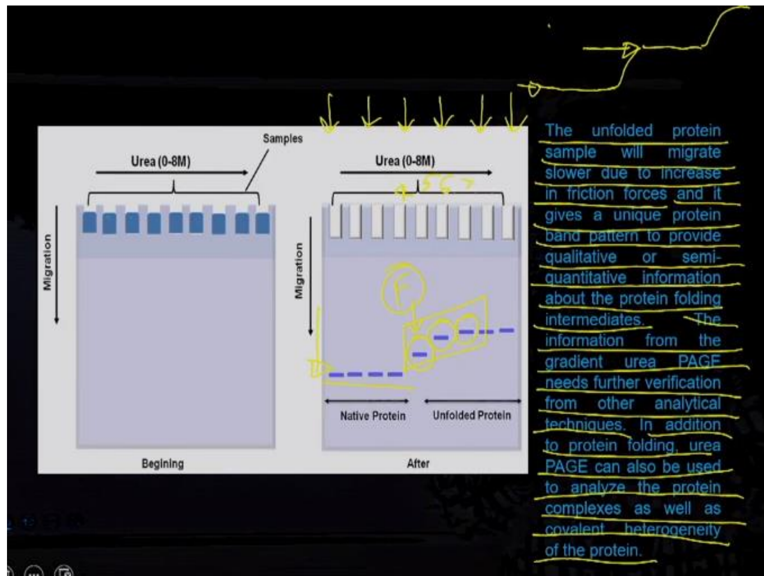

The unfolded samples the unfolded protein sample will migrate slower due to the increase in frictional forces and it gives a unique protein brand pattern to provide qualitative or semi-quantitative information about the protein folding intermediate. So what will happen is? When the proteins are been loaded into these wells and you are allow them to migrate while they are running they are actually getting exposed to the different consultation of urea.

So you can imagine that upto the 4 molar urea the protein, is still maintaining a native structure so that is why the migration is very fast. But as the protein is moving towards the 5, 6 and all that high consultation of the urea the protein are getting unfolding. And at this stage the protein got completely unfolded because as it will unfold it is actually going to experience high consultant of the friction force.

Then because of that it is actually going to oppose the migration within the acrylamide gels and as a result it will its migration is the slower and slower and that slowering is actually going to be

proportional to the increase in the hydrodynamic volume. And that actually is can be mapped to draw the semi quantitative as well as the qualitative information. When the protein is going to be unfolded when the protein is going to be denatured and when its protein is stable so that information can be used to determine the relative stability of the 2 proteins or even the different components of a protein.

Because if the different components of the protein have the different stability what you will see is that it will get unfolded then it remains then it opposes the then it opposes the resistance towards the denaturating agents and then ultimately again it get unfolded. So that is how you are may get a (()) (24:28) behavior where it is actually going to be unfolded it is like this right? It is actually going to be remain native then unfolded then it going to be remain like this.

Then at unfolded so it actually can give the multiple steps in the folding and that actually will say that the protein as the multiple regions which are actually going to be folded at a different kinetics unfolding and their stability is also different for the denaturating agents. The information's from the gradient urea page needs further verification from the other analytical techniques.

In addition to the protein folding urea page can also be used to analyze the protein complexes as well as the covalently heterogeneity of the proteins. So every technique when you perform the technique which is no good enough or it is not absolute on its own. So that is why the, whatever the information you get from the urea page can be verified with the other analytical technique. For example you can use the gel filtration techniques you can use the cross linking experiment you can do the CD based experiment then you can do some you know fluorescence based experiments.

To even further verify that you have the multistep unfolding processes going on in this particular protein because you have the 3 or 4 different domains which are folding in a different way. So with this I would like to conclude our lecture here in the subsequent lecture we are going to take up few more exciting experiments where we are going to use the electrophoresis as a tool to answer those questions and to solve those questions and with this I would like to conclude our lecture here thank you.