Enzyme Science and Technology
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Module - II
Enzyme Structure
Lecture - 09
Tertiary Structure of Enzyme (Part-I)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati and in the course Enzyme Science and Technology we are discussing about the different aspects of enzyme and how the enzymes are doing the different types of functions and catalyzing the different types of reactions.

So, in this context so far what we have discussed, we have discussed about the history of the development of the field of enzymology subsequent to that we have also discussed about the enzyme nomenclature and then we also discussed about the enzyme classifications.

And in the current module we are focusing more on the enzyme structures right, because the structure play a very crucial role in identifying the substrates and then catalyzing the reactions and then generating the products and then once the product is being generated it is going to be released from the active site. So, when we were discussing about the enzyme structure in the previous lectures we have discussed about the primary structures, we have discussed about the secondary structures.

So, in today's lecture we are going to discuss about the tertiary structures and how you can be able to use the different techniques to solve the tertiary structures. So, let us start the todays lecture.

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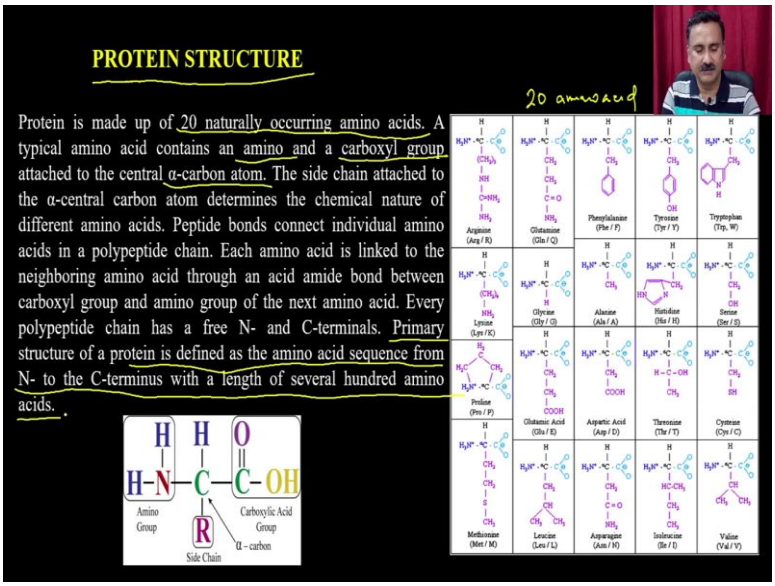

So, what we were discussing we were discussing about the protein structures and what we have discussed so far is that the protein is made up of the 20 naturally occurring amino acids and a typical protein amino acid is containing the two component amino component and a carboxyl group and that is attached to the central alpha carbon.

And what you see here is that the C alpha carbon on that you have the amino group on one side and the carboxyl group on the other side and then it also has the side chains and based on the side chains you can have the 20 different types of amino acids. So, what you see here is the side chain of the different amino acids and as you can see some are positively charged amino acids, some are negatively charged amino acid, some are hydrophobic, some are hydrophilic, some are polar groups and all that.

So, since all these amino acids will come and join together with the help of the peptide bond the that is constituting the primary structure. So, what you see here is that the primary structure of a protein is defined as the amino acid sequence from the N term N to the C terminus with a length of the several hundred amino acids.

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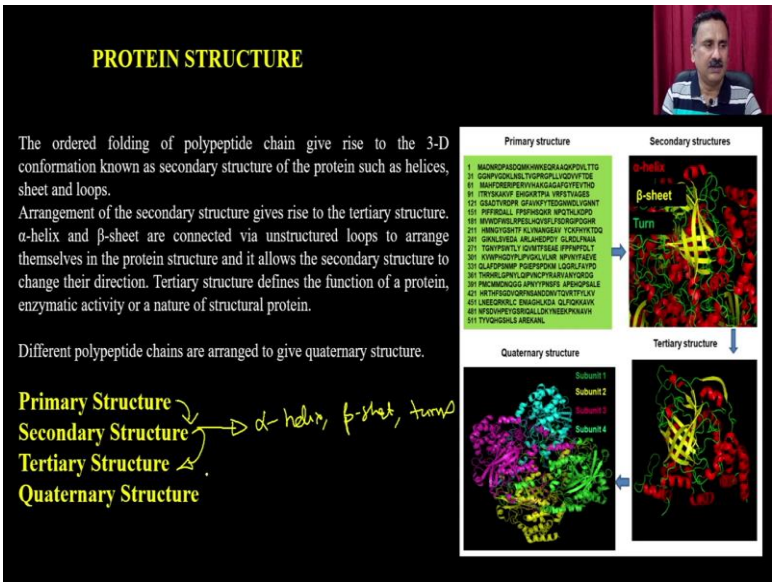

And the primary structure is going to be fold into the secondary structure and then secondary structure is going to be fold into a tertiary structure. So, within the secondary structure we have the alpha helix, we have the beta sheets and we have the turns right.

So, with the help of the turns the alpha helix and the beta sheets can actually be able to form the different types of secondary structures or super secondary structures and these secondary and the super secondary structures actually can come together and they will give you a tertiary structures. So, in today's lecture we will see how we can be able to solve the tertiary structures.

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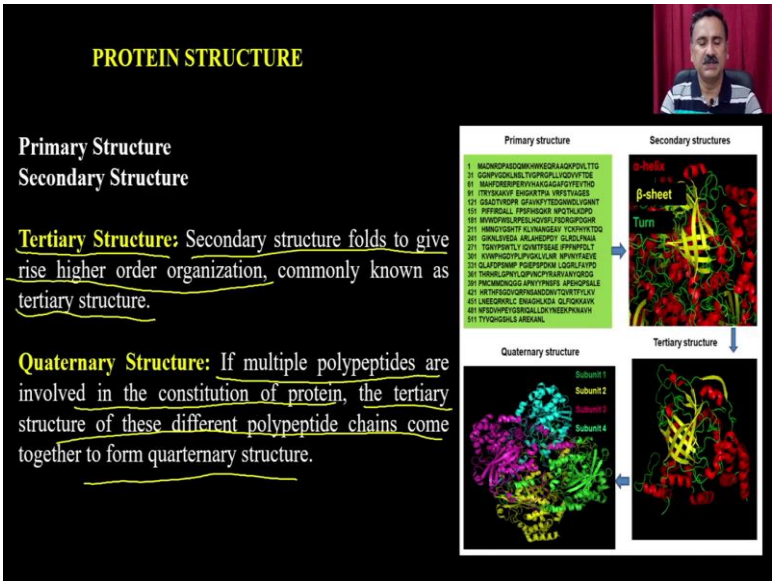

So, in the tertiary structure, the secondary structures are going to fold to give the rise the higher order organization, commonly known as the tertiary structures whereas, the quaternary structure is going to be present if the multiple polypeptides are involved in the constitution of the protein and the tertiary structure of these different polypeptide chain come together to form the quaternary structure.

Now, when we talk about the tertiary structures tertiary structure is a very very complicated structure of the protein, where the many of the secondary structure and as well as the super secondary structures are you know coming together and giving rise due to tertiary structures.

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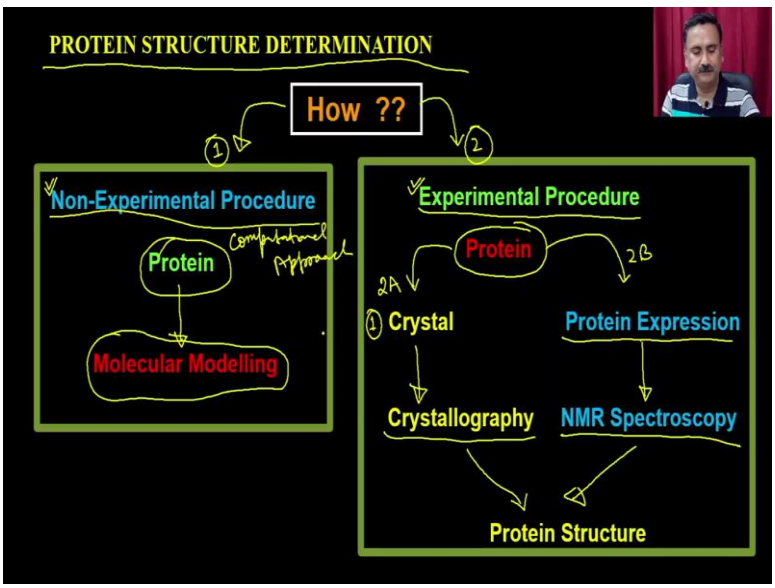

So, when we talk about these protein structure determinations or the tertiary structure determinations we have first question is that how we are going to perform the protein structure determinations? So, we have two options option 1 and the option 2. So, option 2 is more of the experimental method for which you are interested to solve the three dimensional structures and you have the two pathways one is this pathway which is going to be called as 2 A and this pathway is going to be called as 2 B.

So, this pathway what you are going to have is you are going to first going to this is called as the X- ray crystallography and in this the major pathway is that you are going to generate a crystal of this particular protein whereas, in the case of NMR spectroscopy.

So, you have two options experimental options which you can use to solve the protein structure one is the X- ray crystallography the other is NMR spectroscopy, in the X-ray crystallography you might have to generate the crystals of that particular protein and then these crystals can be studied in the X-ray crystallography and that is going to give you the protein structures.

Similarly, in the case of NMR spectroscopy you can express the proteins and then you can actually be able to record the NMR spectras and you can be able to solve that the structure of the protein with the help of the NMR spectroscopy. So, both of these experimental method are going to give you the protein structures. So, what we are going to do is we are going to first discuss about the experimental method and then we are actually going to focus about the non-experimental method.

So, in the non-experimental method these are all so called as the computational method you are actually going to use the protein sequence and that you are going to use different computational tool to do the molecular modelling. So, what we are going to do is first going to discuss about the experimental procedures and then we are going to discuss about the non-experimental procedure when I say non experimental means I am talking about the computational approaches.

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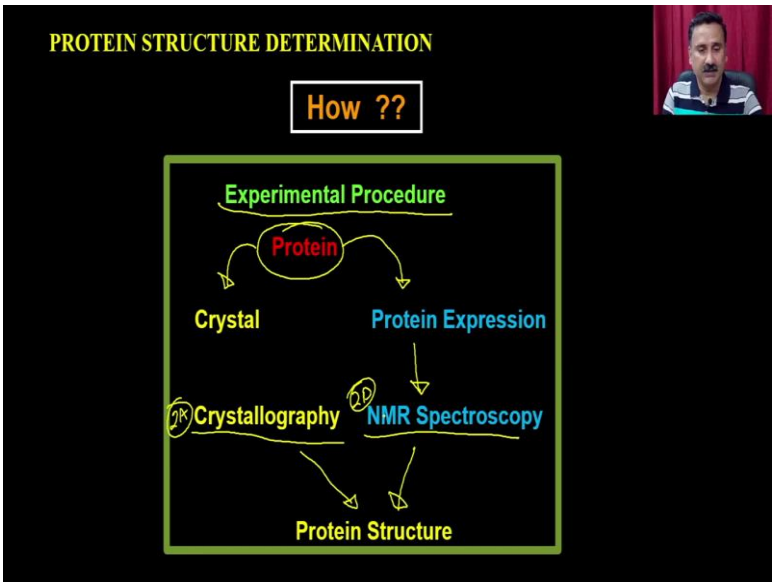

Now, as far as the experimental approaches is concerned once you have the proteins you can actually be able to generate either the crystal or you can express the protein and that will give you the NMR spectroscopy. So, you have two options 2 A that is the X- ray crystallography or 2 B that is the NMR spectroscopy and both of these are actually going to give you protein structures. So, let us start with the crystallography.

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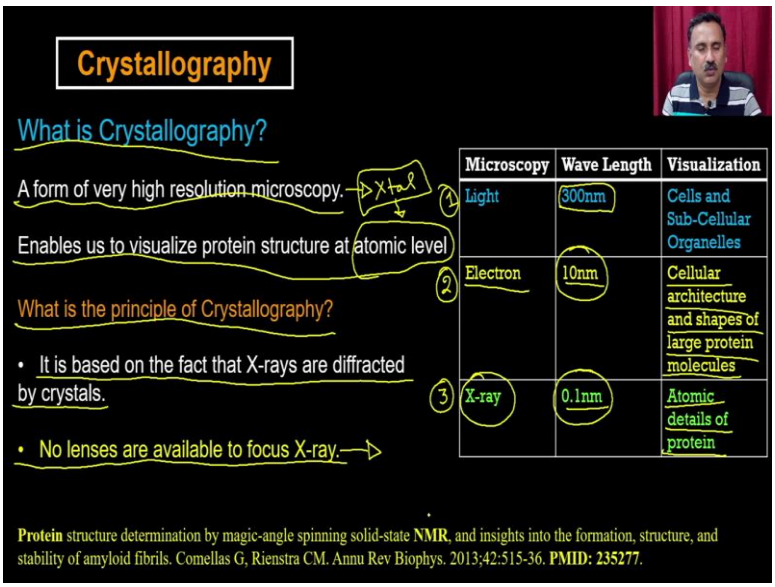

So, first question is what is crystallography? And before we discuss any of these methods I just want to make it sure that we are not going to extensively going to deal either of

these methods whether the X-ray crystallography or NMR spectroscopy. The purpose of this todays lecture is that we are going to very briefly going to tell you about the X-ray crystallography or as well as the NMR spectroscopy.

So, that you will be able to follow the content and you will be able to understand the potential of these techniques, if you are interested you want to explore some of these techniques then it is better to go with the you know well developed MOOC courses where they have actually discussed about the X-ray crystallography. So, this these topics are relatively big. So, that cannot be covered in one or two lectures.

So, what is X-ray crystallography? X-ray crystallography is a form of very high resolution microscopy, so that you can be able to see the crystal and you can be able to see the protein structure inside right just like as we actually see the slides under the normal light microscope, you can also be able to see the protein structures if you have the tools and that is what the tool is X-ray crystallography.

So, X-ray crystallography is a very high resolution microscopy which actually will allow you or enable you to see the protein structures. So, what you see the potential right, what you see here is the potential of different types of microscopy? You have the light microscopy, so light microscopy is going to use the light which is actually the length of the 300 nanometers and that actually is going to give you the visualization of the cells or the subcellular structures with the help of the phase contrast.

Whereas, when you are going to talk about the electrons so, you can actually be able to have the electron microscopy and the wavelength what you are going to use is 10 nanometers. So, light source what you are going to use is 10 nanometer and that is actually going to allow you to see the cellular structure and as well as the shape of the larger protein molecules, in some cases you can be able to see the DNA structures and you can be able to do all those kind.

Whereas, when we use the X-ray as the illumination source the wavelength is going to be 0.1 nanometer and it is actually going to be allow you to see the atomic details of the protein. So, the difference between the electron microscopy versus X-ray is that electron microscopy is only going to give you a gross structure of the proteins whereas, the X-ray crystallography is going to be even finer and it is actually going to give you the atomic details of the proteins.

And that is basically because the wavelength what they are using to visualize the object right. In the case of electron microscopy it is going to be 10 nanometer whereas, in the case of X-ray crystallography it is going to be 0.1 nanometer. So, since it is a form of the high resolution microscopy it enable us to visualize the protein structure at the atomic level which means you can be able to very precisely be able to see the different atoms.

What is the principle of the X-ray crystallography? So, it is based on the fact that X- rays are diffracted by the crystals right. What is the difference between the conventional microscopy versus X-ray crystallography is that here there is no lens involved which means you cannot just use the X-ray crystallography and visualize the object just like as we are visualizing the cells or the subcellular structures in the slide. So, if you are in trusted more about the studying the X-ray crystallography you can be able to go through with this particular articles.

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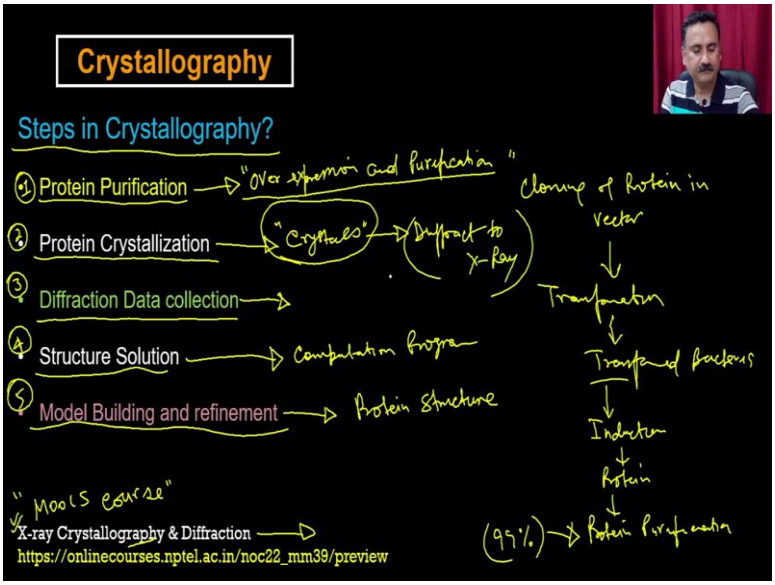

Now, what are the different steps you can be able to use in doing the X-ray crystallography. So, in the step 1 you are going to first express the protein. So, majority of the time when you are actually going to do the X-ray crystallography it is going to be an over expression of the protein and over expression and purification. And that you is very important because if otherwise you are not going to achieve the required amount of the protein and that is how it is actually going to be a problem for getting the crystal.

Then the step 2 is going to be you have to do a protein crystallization so, that is actually going to give you the crystals right that is what is the prime requirement of the X-ray crystallography. Then the step 3 is going to be diffraction data collections right that you are going to use with the help of the X-ray diffractometers and then step 4 you are going to get the diffraction data.

And then based on that you are actually going to generate the electron density map and you are going to do the structure solution with the help of the computational programs. And then step 5 you are going to use the refinements and you are going to do a model building, so that you are going to get the protein structures.

Now, this is what I was talking about so, there is an excellent MOOC course which is available on the NPTEL website and that is actually you can be able to take. So, if you are interested to understand all of these steps in detail right because here we are just briefly overview we are giving you overview. So, you can actually be able to go through with this particular NPTEL course and that will actually going to give you the detail inside about each of these steps.

Now, let us talk about the first step. So, first step is the over expression and purification. So, in this you are going to do the cloning of the protein into the suitable vector right. So, cloning of the protein or enzyme in the vector then you are actually going to do the transformation. So, once you do the transformation you are going to get the transformed colonies, transform bacteria or whatever the expressions host you are going to do. These all we are going to discuss when we are going to discuss about the production of the enzyme.

Transform bacteria and then that is if you do the induction right. So, that is going to give you the protein and then ultimately you are actually going to do the protein purifications, because protein purification is required because you are supposed to have the 99 percent pure protein for crystallizations. In the step 2 you are going to do the protein crystallization, so that you can be able to develop a crystal and that crystal is actually going to be diffract to X-ray and that is how that going to give you the diffraction data.

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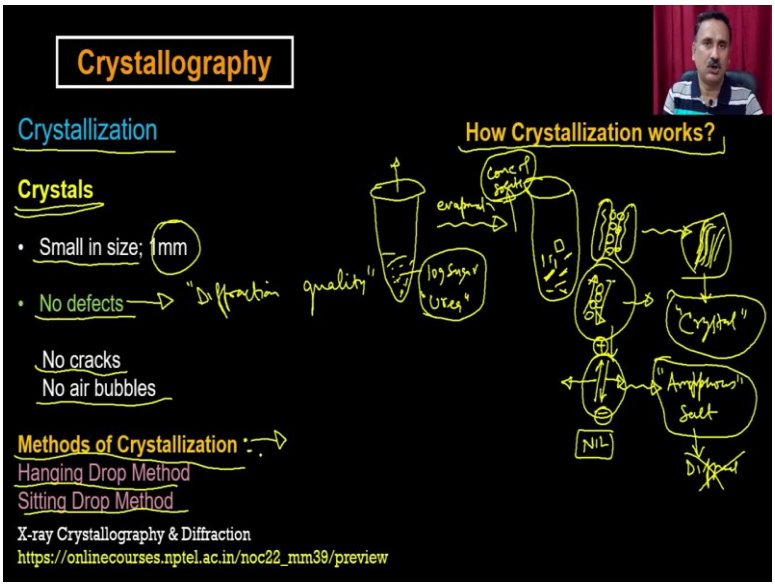

Now, let us talk about the crystallization the step 2 right. So, first question is how the crystallization works? Right, I am sure if you want to understand the process of crystallization what you can do is or you can even do that in your home also do one thing right you take a glass right and just add small amount of sugar. So, for example, you take 10 grams of sugar right. So, that is going to be there right or you can use some amount of urea right. So, these are the solutions right. So, you prepare the solution.

And let this glass to be remain open. So, if you take the steel glass and let it be open. So, what will happen after some time is that all the water what you have added into this is going to evaporate. So, after the evaporation what you are going to see is that in the glass you will see the you know spikes you will see the spikes type of crystals and that these are the actually the crystal.

So, you when you take the sugar it will form the sugar crystal whereas, if you take the urea it is actually going to give you the spike shape crystals. So, how it is actually happening? It is happening because when you are evaporating the water you are basically increasing the concentration of the solute right. So, you are increasing the concentration of the solute right and in that process what will happen is that the molecules are going to come together.

So, for example, you have one molecule like this, the other molecule is very far away but when the water molecules so, water molecules are present in between right. So, once

after evaporation what will happen is these two molecules will come together right and you can imagine that many many such molecule when they will come together eventually that is going to give you the crystal right. These crystals could be produced for the inorganic molecules or organic molecules or these crystals can be produced for the protein.

So, under what condition the molecule or the solute is going to give you the crystal, when the similar kind of proteins like similar kind of molecules are going to come together and will give you a homogeneous stacking. So, if there will be a homogeneous stacking they will actually going to come together and will give you the crystal. If it is a heterogeneous stacking for example, first molecule is going to be placed like this the second molecule is going to be placed like this.

So, in those case they will come together still they will come together right. So, you can see this still they will come together right and they will stick to each other because as soon as you remove the water molecule they will come together they will have no option, but in this case they will not going to give you the crystal instead they are actually going to give you the amorphous salt ok, which means the they will give you a precipitate or they will give you the amorphous salt and that is actually not going to diffract. So, they will not going to diffract to the X-ray whereas, a crystal is going to diffract.

So, what I am trying to explain is that when you are actually going to bring the molecules together they will either be present in the same conformation or they will be present in the different conformations. So, when they will be present in the same conformation they will stick to each other, they will achieve the lowest energy and that is how they will going to form the crystal, but if they will be in different orientations or different conformations they will come together, but they will going to form the amorphous powder and that amorphous powder is not going to diffract.

Why they it will not diffract, because the diffraction pattern from this conformation and diffraction pattern from this conformation is going to be opposite. So, we can imagine that if I got the positive diffraction with this guy and negative diffraction by this guy. So, ultimately I am going to get the nil diffraction when there is molecules are going to be present in the amorphous salt.

The other issue why the crystal is also diffracting is because it actually keep all the you know all the protein molecules or inorganic molecules in the same orientation. So, you are actually going to get a positive diffraction. Now, when we talk about the crystals so, there is a defined size and there is a defined clarity there is a defined parameters which are actually need to be followed. So, when we produce the crystals they are supposed to be small in size which means they should be of 1 mm in size then they should not have any defects ok.

So, which means when I said they should be of diffraction quality which means they should not have any kind of defects. What are defects, you should not have any cracks, it should not have any air bubbles or it should not have the mosaicity ok which means mosaicity means hetero heterogeneous nature of the proteins present in the particular crystals.

Now, when we talk about the methods of crystallization so, there are tons of methods what you can actually be able to use for producing the protein crystals, but what we are going to discuss in this particular lecture is the hanging drop method and actually also the sitting drop methods.

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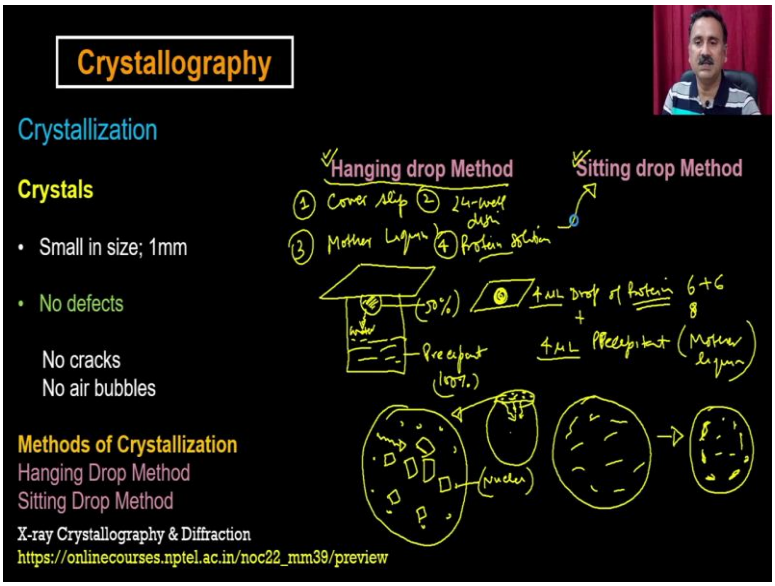

So, let us first talk about the hanging drop method ok. So, in a hanging drop method what are the material you require you require a cover slip, you require 24 well dish and you also require the mother liquor ok.

Now, how this hanging drop method works is that you are going to have the well right, you are going to have the well on top of this you are going to have and fourth is you also require the protein solution. So, this protein solution is purified ok. So, what you are going to do is you are going to have the well right and on top of this well you are going to have the cover slips ok. And the on this cover slip you first what you are going to do is you are going to have a cover slip on this cover slip you are going to make the drop of the proteins.

So you are going to make a 4 micro liter drop of the protein molecules right and then you are going to add the 4 micro liter of the precipitant right or I will say mother liquor ok. So, then you mix them together and make a drop on this cover slip and then you actually invert this invert in such a way that the drop should face the well ok. So, what will happen is you will see the if drop is going to be hang like this ok and then you are going to seal this with the help of a vacuum grease.

And then ultimately it is going to be a isolated chamber and then you keep it and then you can observe the these crystallization apparatus or crystallization setup with the help of the microscopes. Now, what will happen is that eventually the water is going to come out. So, you this well is actually being filled with the same precipitant what you have added into this protein molecule. So, you are going to have the precipitant into this ok.

So, what will happen is that when you do this the water molecule is actually going to be taken up by the this downstream liquid right, because the amount of precipitant is going to be on a higher side into this right. So, it is going to be 100 percent here whereas, it is going to be 50 percent in the case of the drop right.

So, because of this change in the concentration of the precipitant the water is going to come out from the drop and it will fall into the precipitants ok. So, once it happens it is actually going to do exactly the same what we have just now discussed in the glass right in the steel glass or in the case of salt.

So, what will happen is you can imagine that if you have a drop like this right and you have protein molecules. So, what will happen is the initially the protein molecules are going to be very far away right, but once the water will evaporate these protein molecules are going to come together right and that is how they are actually going to start forming the crystals right.

Although, this is not true in most of the cases right it is not that only the removal of water is actually going to bring the protein molecules together and they will you will going to be the crystal, crystal no, it is not that easy. What is the requirement? Requirement is that the protein molecules when they come together they are actually should be attending the minimum energy and they should all be attending the same similar confirmation. So, that they will be sticking to each other and they will give rise to the crystal.

So, how the crystallization is going to appear? The crystallization is going to appear like this ok. So, eventually you are first going to have the nucleation on the corner of this particular drop. So, this is the I am just showing you a drop. So, it is actually hanging like this right. So, you can imagine that this drop is like this. So, what I am showing is actually this cross section ok. So, this cross section is what I am showing.

So, what will happen is in the ideal situation when you have added the precipitant which is stabilizing the protein structure, which is maintaining the minimum energy and which is also bringing removing the water at a very very controlled rate right when you do this protein water removal at a control rate you are going to induce the protein crystallizations.

So, what will happen is that on the corner you are actually going to have the nuclei the protein crystals nuclei are going to be generated. Now, what will happen is that this protein nuclei are going to swim into the center. So, you can imagine that the crystals are you know the nucleis are going to be developed here and then they will actually going to swim into the liquid ok. So, once they swim they are actually going to start collecting the protein molecules ok and eventually what will happen is that they are actually going to form the crystals right.

So, in the second row you are going to get the crystal and as these crystals will go in the center of the tube which means they will actually be in the center they will be start eating or they will be start collecting the protein and that is how you are actually going to get the bigger protein crystals in the center of this particular draw. This is the ideal situation under which you are going to see the crystallizations.

So, this is all about the hanging drop method, you can also do try out the sitting drop method because one of the major limitation of the hanging drop method is that you are

supposed to hang the drop into this particular 24 well dish right. So, in that case you there is a limitation until you can actually be able to keep the protein molecules. So, you cannot keep beyond a certain concentrations which means you can try out the 4 micro liter versus 4 micro liter, you can try out 6 versus 6, once you increase the 6 plus 6 or even if you go beyond that the drop will actually going to fall into the right.

It will not going to hang right because the surface tension of the water is going to be not allowing them to stick to the glass right. So, because you cannot have a desired concentration of the protein into this and some proteins require the even higher concentration. So, you can imagine that if you have got the nuclei, but that nuclei is not growing.

That means there is a shortage of the protein in this drop and how you going to solve that problem, you are going to solve that problem when you are going to try the sitting drop method. So, let us discuss about the sitting drop method. So, in a sitting drop method what you have is you have a bridge on which you can be able to place the protein molecules and that is how it is actually going to work.

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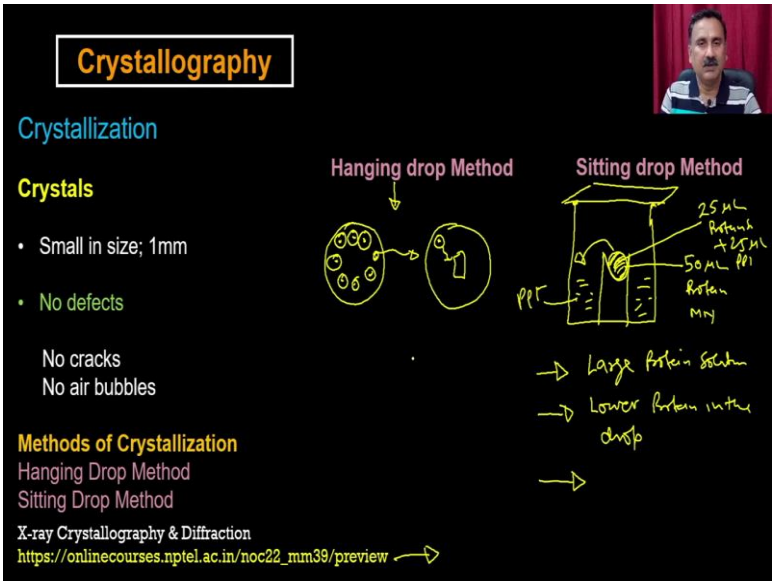

So, sitting drop method you have a you are going to have the well right just like as we have the well in the hanging drop method, but on the hanging in the center instead of you are going to have the cover slip in the in it is actually going to have a bridge like situation ok. So, it is going to have the bridge which is actually going to have the

depression and this depression easily can take up to 50 micro liter of the protein mixture. So, again you are going to fill this tube with the help of the precipitants right whatever the precipitant you want to try out.

And then you are what you are going to add is for example, if I add the protein molecule. So, I can place the 25 micro liter of the protein solution and I will mix it with the 25 micro liter of the precipitants ok and then I am going to seal this with the help of the vacuum grease and I will seal it with the help of the cover slip ok. Now, what will happen is exactly the same thing will happen.

So, for example, if I have added the drop right so, it is a 50 micro liter drop earlier we were just making the 48 micro liter drop now again the same thing will happen the water will evaporate right from this drop in to the liquid and eventually the protein will concentrate and that is how in that process it is actually going to bigger slice. So, with the sitting drop method you can be able to try the large protein molecules or large protein solution that actually is going to reduce or it will take care of the lower protein content in the drop right.

So, that is actually going to increase your chances of getting the protein crystal or bigger protein crystals right, in some cases people also try the using the nucleation. So, for example, if you are trying with the hanging drop method, you are actually going to get the nucleis right, but the nucleis are not growing. So, in that case what they do is with the help of the pipette they actually isolate these nucleis right and then they put these nucleis into another drop ok. So, once they put these nucleis into another drop there is a possibility that they may actually get the bigger crystals.

So, as I said you can actually be able to consult or you know refer this particular online course you can even register for this online course if you are interested to study more about the crystallography.

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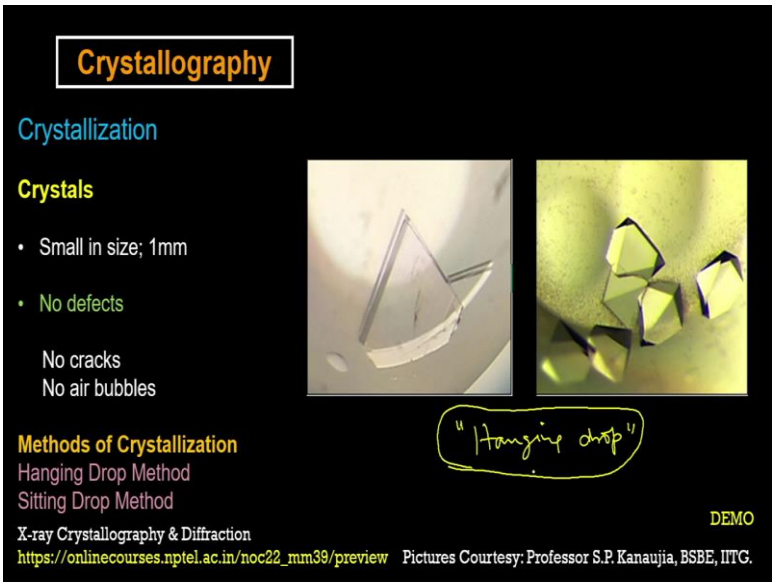

And once you do the crystallization you are actually going to get the crystal like this right. See, these are the protein crystals right and this picture is being provided by the Professor Shankar Prasad Kanaujia from our department only, since he was kind enough we actually took the liberty and that is how we went to his lab. And some of his students actually have demonstrated how you can be able to crystallize the protein using the hanging drop method.

So, let us show you the video where we have discussed about the hanging drop method and how you can be able to perform the hanging drop method in a laboratory. So, the material what you require for the protein crystallization is as follows.

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You require a 24 well dish.

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You require the mother liquor where you would like to test the crystallization conditions.

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You require a forcep.

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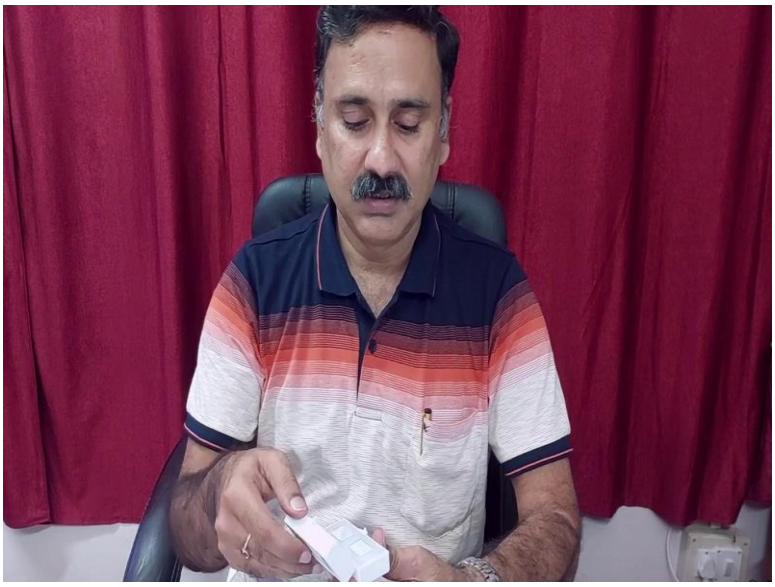

And then you also require a siliconized cover slips. So, you have the two choices to prepare the siliconized cover slips either you can buy the siliconized cover slips from the vendors like the Hampton research or the sigma or you can actually be able to prepare the siliconized cover slips by coating these cover slips with the help of the sigmacote.

So, you can buy the sigmacote from the sigma company and you can actually be able to tip your cover slip into the sigmacote and that will actually go to make the siliconized cover slips. So, let us start the demo.

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So, first thing what you have to do is you have to you have to put a vacuum grease. So, you what you can do is you can just fill the vacuum grease into a syringe like this and then you can actually be able to make the rim on top of the this actually 24 well dish right.

So, that it is actually going to seal when you are going to keep the cover slips, after this you are going to add the 1 ml of the mother liquor or the crystallization conditions. So, for example, in this case since we are trying to do the crystallization for the lysozyme, we are going to do the we are going to use the mother liquor which is the 1.5 molar Nacl and 100 millimolar sodium acetate pH 4.6. So, let us put the mother liquor and ok.

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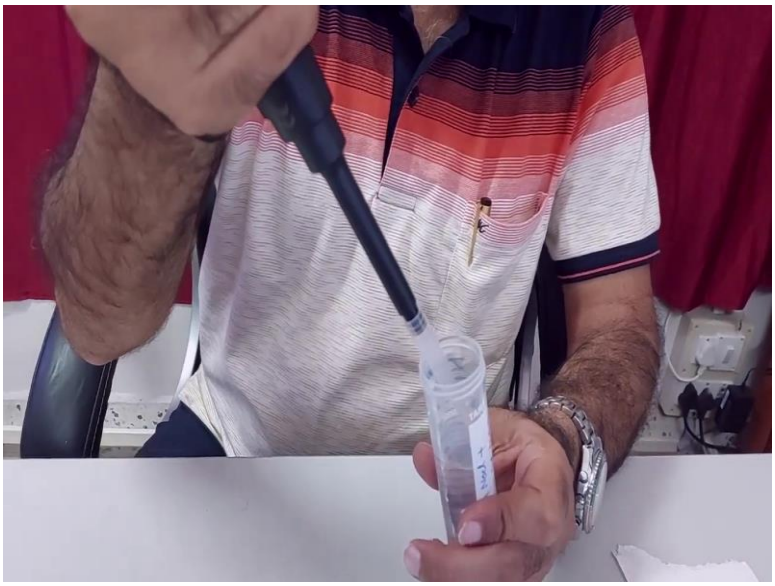

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Now, you have to ensure that when you prepare the mother liquor it should be filter sterile and it should be filtered. So, that there should be no suspended particles. Now, what you are going to do is you are going to take out one siliconized cover slips with the help of the forceps.

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So, you can just take out the cover slip put it into a tissue row tissue paper right.

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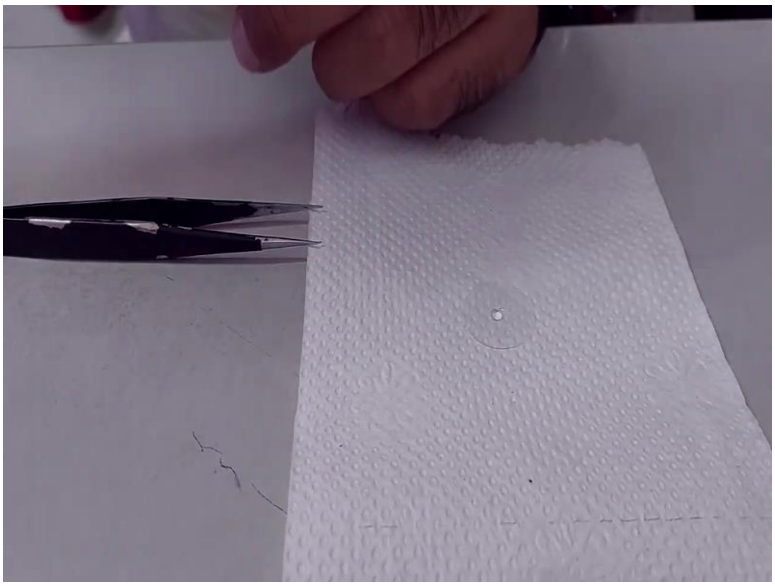

And then you are going to make a drop of the proteins and so, let us do that. So, in this particular experiment we have taken lysozyme as a model protein. So, you know that lysozyme is very easily crystallizable.

So, what I have; what I have we have done is we have pepped the cover slips onto our tissue paper now we are going to add the protein. So, you have many choices of adding the proteins you can add 2 micro liter of protein, you can add 3 micro liter of protein, 4

micro liter and so on. So, in this particular demo we are putting the 3 micro liter or 2 micro liter of the protein. So, we have made 2 micro liter right and now we are going to add the 2 micro liter of the protein.

So, this I have prepared the protein which is filtered also. So, this is the 5 milligram solution and I am going to add a drop in the center of this tube cover slip, when you add the protein you have to ensure that there is no bubble which is going to be formed in the protein solution because the bubble is actually going to deactivate or inactivate the protein and also it is actually going to disturb.

And now we are going to take the 2 micro liter of mother liquor and we are going to add that also into this protein solution then you are actually going to make this as 4 micro liter. So, that you can be able to mix both the protein as well as the mother liquor solution properly and now your drop is ready right. Now, you take the drop into your hand ok and then you have to invert this like this ok and when you invert you have to put this on to the top of the well ok.

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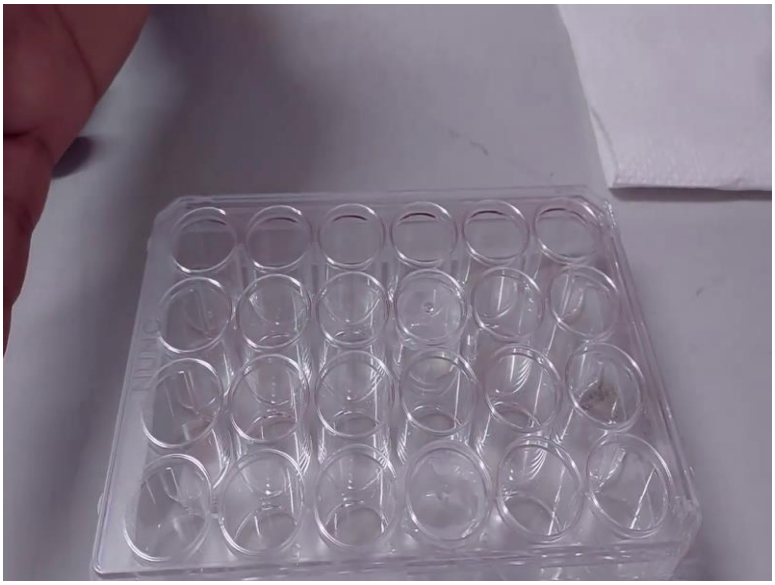

Now, once you have put it on the top of the well you can use the used tip and then you press the slide on this well very carefully from all the ends, so that it should not break the well and it should also not have any air gaps. So, when it is got sealed initially what you have to do is you have to cover it with the lid and then you have to observe this drop under the microscope and that you have to observe to see that there is no precipitate

which is going to be formed into this protein droplets protein drop and because if the precipitate is formed instantly then it is either the protein concentration is very high or the precipitant what you have taken is also withdrawing the water very fast.

So, in either of these cases you have to you have to change the conditions since you see in this particular conditions my drop is still clear. So, this means both of these parameters are perfectly fine. Now, I will keep this under the end in a crystallization incubator and I will observe this plate after every 24 hours and ideally I should see a protein crystals by tomorrow morning.

So, this is all about the protein crystallization. So, with this I would like to conclude my lecture here.

Thank you.