Enzyme Science and Technology
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Module - II
Enzyme Structure
Lecture - 08
Secondary Structure of Protein

Hello, everyone. This is Doctor Vishal Trivedi from Department of Biosciences and Bioengineering, IIT, Guwahati. And, in the course Enzyme Structure Enzyme Science and Technology, we are discussing about the different aspects of the enzymes. So, if you recall in the previous module, we have discussed about the General Properties of the Enzymes and then we also discussed about some of the historical aspects as well as the development of the field of enzymology.

And, in the previous lecture, we have discussed about the enzyme structures and in that context we have discussed about the primary structures; we discussed about how you can be able to determine the primary structures and we have also discussed about the different types of methods through which you can be able to determine the primary structures.

So, primary structure is nothing, but the sequence of the amino acid in which the protein is made up of and these primary structures are further get folded into different types of secondary structures. So, in today's lecture, we are going to discuss about the Secondary Structures and how you can be able to determine the Secondary Structures of a Proteins.

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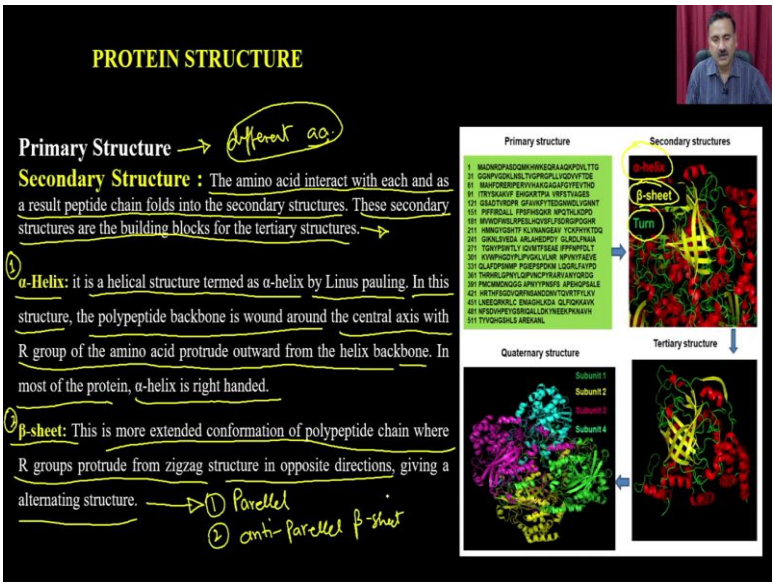

So, this is what we have discussed. We have discussed about the primary structures and while we were discussing about the primary structures, we discussed that the primary structures can be made up of the different types of amino acids and we have discussed various techniques through which you can be able to determine the different types of amino acids, their compositions, their sequence and all that.

Now, primary structures are going to be get folded and they will give you the different types of secondary structures. The most classical structures of the secondary structures are alpha-helix, beta-sheets and as well as the turns. So, what is the secondary structures? The amino acids interact with each other and as a result the peptide chain folds into the secondary structures.

The secondary structures are the building blocks for the tertiary structure. So, this is what we have done, right. Primary structures, secondary structures; secondary structures will you know come together and will give you the tertiary structures, and if the protein has the different subunits, it is actually going to give you the quaternary structures.

So, first primary structure is the alpha-helix. So, alpha-helix it is a helical structure termed as the alpha-helix by the great scientist Linus Pauling in this structure the polypeptide backbone is wound around the central axis with the R group of the amino acid protrudes outward from the helix backbone.

In most of the protein, the alpha-helix is right handed, ok. So, you can have the alpha-helix which is right handed, you can have the alpha-helix which is left handed but all the amino acids are L amino acids mostly the alpha-helix is right handed in the most of the proteins what is present in the biological system.

The second secondary structure is called as the beta-sheets. This is more extended conformation of the polypeptide chain where R groups protrude from the zigzag structure in a opposite direction giving a alternate structures and beta-sheets could be could be parallel beta-sheets or could be anti-parallel beta-sheets.

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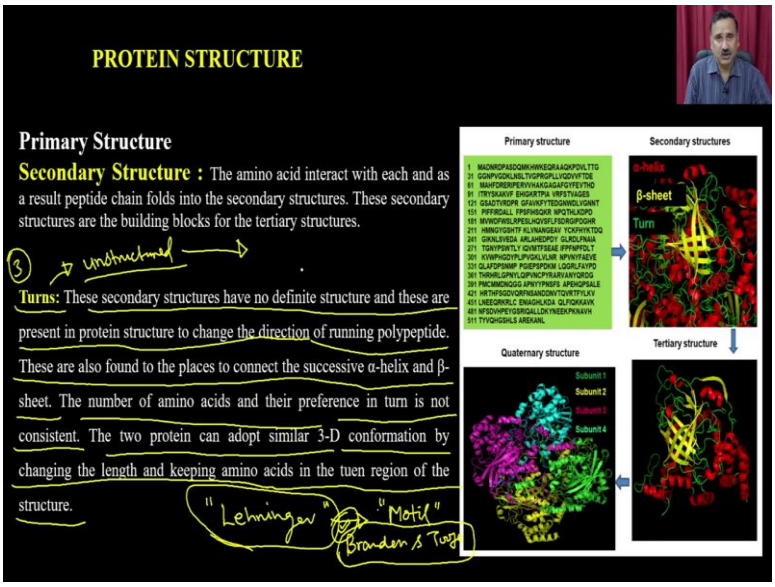

Now, there is a third secondary structure and the third secondary structure is called as the turns. The secondary structures have no definite structures and these are present in the protein structure to change the direction of the running polypeptide. These are also found to be places to connect the successive alpha and beta-sheets. The number of amino acids and their preference in the turn is not consistent and the two protein can adopt similar 3D conformation by changing the length and keeping the amino acid in the turn region of the structures.

So, turn is a unstructured region or I will say very flexible structure. So, it can have the different types of amino acids. It can have no condition that these are the amino acids are not possible or so on. So, this is just an overview of the different types of secondary structures.

If you want to read or if you want to learn more about the secondary structures, what I will suggest is that you can actually be able to consult any of the biochemistry books, so that it will actually go into for example, the Lehninger right or strad or white and white so that they can actually be able to give you the different properties.

So, in this course we are not discussing about the properties of these secondary structures and they will tell you what will be the what will the amino acid which are preferentially being found in the alpha-helix and what are the amino acids which are preferentially will bound in the beta-sheets and turns.

So, there is no preference for amino acids in the turn because turn is actually a structure which connects the alpha sheets to alpha alpha-helix to alpha-helix and that is how it can actually be able to responsible for formation of different types of motifs. And, it can actually connect the alpha-helix to beta-sheets and that also will be responsible for the different types of motifs.

So, all these alpha-helix or beta-sheet when they come together with the help of the turn they are actually going to give you the different types of motifs. And, there is a excellent book which is called as the Protein Structure by the Branden and Tooze. And, this is very good book in case you want to explore or you want to read more about the secondary structures and how the secondary structures are coming together to give you the different types of motifs.

And, they have given you a discrete you know different examples how the different types of the secondary structures or the combination of secondary structures are present. And, what I will suggest is that students could actually go through with these book. There is a single chapter of where they have actually discussed about the motifs and I think it is a very good idea, ok. So, we are not going to discuss or take it into detail about the secondary structures.

Now, the question comes once you have the secondary structures how you can be able to determine the secondary structure in the enzyme structures, ok.

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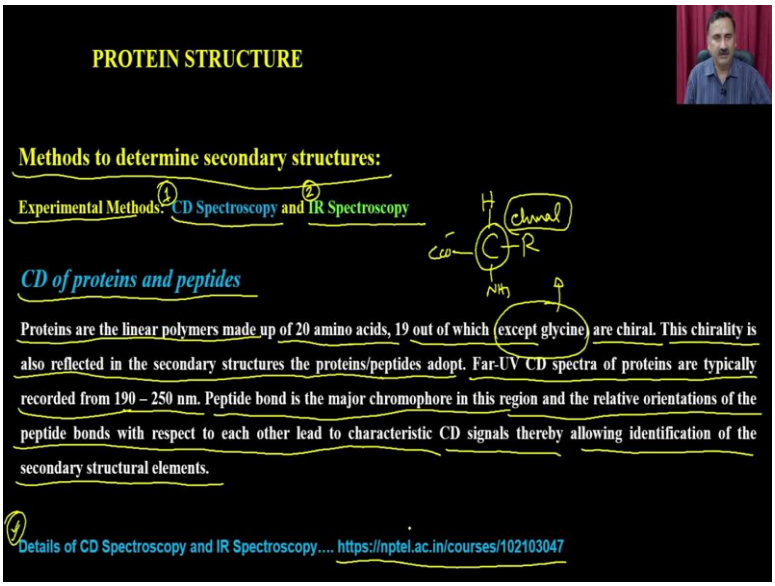

So, methods to determine the secondary structures: so, experimental method you have the two very classical structures which are called CD spectroscopy and IR spectroscopy. So, we have two methods one is IR CD spectroscopy and the second is the IR spectroscopy. CD spectroscopy of the proteins and peptides: proteins are the linear polymers made up of the 20 amino acids 19 out of which except glycine because glycine does not have the side chain are chiral, right.

You know that the you have a protein amino acid structure where you have the four groups, right. You have the carboxyl group and you have the amino group, right. So, all these groups are different and that is why this carbon is a chiral carbon, right and that is why it actually can actually be able to participate into reacting with the circularly polarized light and that is how they are very much sensitive for the CD spectroscopy, but except the glycine.

This chirality is also reflected in the secondary structure the of the polypeptide chain ok. Far-UV CD spectra of proteins are typically recorded from the range of 190 to 250 nanometer. Peptide bond is the major chromophore in this region and the relative orientation of the peptide bond with respect to each other leads to the characteristic CD signal thereby allowing the identification of the secondary structure elements.

So, this is actually I have given you the citations. So, if you want to read more about this CD spectroscopy you can actually be able to see one of our course which is also present on the NPTEL website and you can actually be able to read more about the content.

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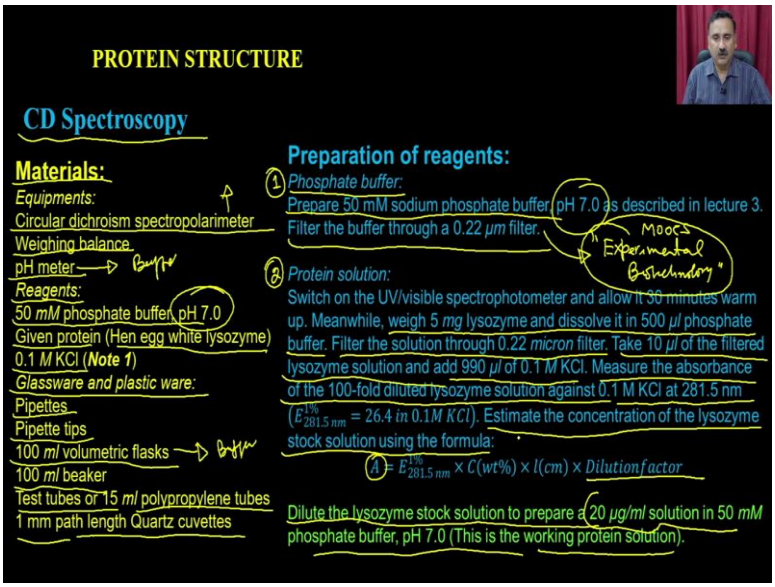

Now, how you can be able to perform the CD spectroscopy? So, what you require? You require the following materials. So, you require CD spectropolarimeter, right, that is the instruments, right. Then you require the weighing balance, you require the pH meter so that you can be able to prepare the buffer, right. Then you require the different types of reagents.

So, you require phosphate buffers like 50 millimolar phosphate buffer pH 7 and then you require proteins. So, for example, in this example we have taken an example of hen egg white lysozyme; then we require a 0.5 0.1 molar K Cl; you require the different types of glass wears and plastic wears such as you require the pipettes, pipette tips, 100 ml volumetric flasks, so that you can be able to use that for preparing the buffer, then you require the 100 ml beaker you require the test tubes or the 15 ml polypropylene tubes and you require a 1 milli mm path lengths the quartz cuvettes.

You have to prepare the different types of reagents. So, for first reagent what you have to prepare? You have to prepare a phosphate buffers. So, prepare the 50 milli ml of sodium phosphate buffer pH 7 and filter the buffer through a 0.22 micron filter. And, if you want to learn more about how to prepare the buffers, what are the different precautions you

should take you can actually be able to see one of my in the MOOCS course which is called as the Experimental Biotechnology.

So, Experimental Biotechnology is a course where I have discussed how you can be able to prepare the buffers because when you prepare the phosphate buffer you have to have a different combinations of the salt and the acid, and that you can actually be able to know from this MOOCS course which is of the called as Experimental Biotechnology.

Then you also require to prepare the protein solutions, right. So, you can weigh the 5 milligrams of lysozyme and dissolve it into a 500 micro liter phosphate buffer. Filter the solution to the 0.22 micron filter. Take the 10 micro liter of the filtered lysozyme solution and add 990 micro liter of 0.1 molar KCl. Measure the absorbance of the 100-fold diluted lysozyme against 0.1 molar KCl. So, that actually is going to tell you what will be the concentration of the protein.

So, you can estimate the concentration of the lysozyme stock solutions using the formula absorbance is equal to station coefficients into the C, right and into the path length and into the dilution factors. So, you can dilute the lysozyme stock solution to prepare a 20 micro gram per ml solution in a 50 millimolar phosphate buffer solutions, ok because this is the working protein solutions.

So, whatever the precision you will take right for example, you weigh the 5 milligrams and dissolve it into 500 micro liter buffer that may or may not be correct. So, to make it more precise what you can do is you can just take the small aliquot of the protein dissolve it into the you know hundred folds or ten folds whatever the case and then you take the absorbance.

And, when you take that absorbance use that absorbance to calculate the concentrations, and that is actually going to be more and more accurate because if you take too much protein it is actually going to interfere in recording the CD spectro CD spectra or if you take very diluted you will not get the signature sequences or signature pattern.

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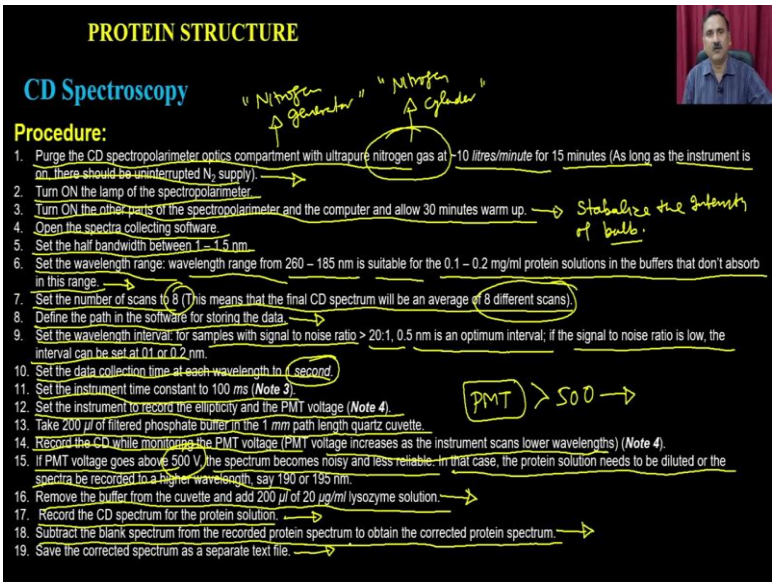

After this you have to follow the complete protocols. So, in the procedure you have to first you have to turn on the spectropolarimeter, right and then you have to purge the spectropolarimeter optical compartment with a nitrogen gas at 10 liter per minute for 15 minutes as long as the instrument is on there should be a uninterrupted nitrogen supply which means, you require either a nitrogen generator, right? If you want to purchase an instrument which will have in built nitrogen generator, or you actually require a nitrogen cylinder, right so that you can be able to connect, right.

Because why we are working in the nitrogen because we want to work in the inert environment then you turn on the lamp of the spectropolarimeter turn on the other parts of the spectropolarimeter and the computer and allow the 30 minutes to warm up. This 30 minute is required because you want to stabilize the intensity of the light intensity of the you know the bulb ok because bulbs normally take some time to get stabilized.

Then you can open the spectra collecting software's. Set the half wavelength between 1 to 1.5 nanometer and then you set the wavelength range. So, wavelength range can be from 260 to 185 nanometer and it is suitable for the 0.1 to 0.2 milligram protein solutions in the buffer and that does not absorb in this range. So, you have to first take the control buffer solution as well, so that you will know what will be the absorptivity of the buffers in that particular range.

You can set the number of scans to 8, ok. This means that the final spectra will be average of the 8 different spectra which means you are going to asking the instrument to do our 8 scans and then show you a average of those 8 scans. Define the path in the software for storing the structure – which means you are going to define where you should actually store the files, right.

Then you set the wavelength interval for example, in the signal to noise ratio 20 is to 1. A 0.5 nanometer is the optimal interval. If the signal to noise ratio is low the interval can be set as 0 1 or 0.2 nanometer. Set the data collection time at each interval to 1 second set the instrument time constant to 100 milliseconds. Then set the instrument to record the electricity and the PMT voltage. Then you take the 200 micro liter of filtered phosphate buffer in the 1 millimolar path length quartz cuvette, right.

Record the CDs while monitoring the PMT voltage. PMT voltage increases as the instrument scans the lower wavelength. If the PMT voltage goes above 500 volt, the spectra becomes noisy and less reliable in that case the protein solution need to be diluted or the spectra be recorded to a higher wavelength such as 190 or 195 nanometer. So, PMT is very important, right and PMT is should not go beyond 500 because if it goes beyond 500, then it is actually going to give you the spectra which is less reliable.

Remove the buffer from the cuvette and add 200 micro liter of 20 micro gram per ml lysozyme solutions. Remember that in the previous step we have discussed about how to prepare the lysozyme solutions. Then you record the CD spectra for the protein solutions. Subtract the blank spectra from the recorded protein spectra to obtain the corrected protein spectra.

So, this is actually in the step 1, we have correct we have for first collected the spectra for the buffer that will be the blank spectra and then we have step 2 we have collected the spectra for the protein. So, that is the you know the protein spectra and then you have to subtract the buffer spectra from the protein spectra and that is going to give you the corrected protein spectra.

Then you save the corrected protein spectra as the excel as a text file, ok, so that you can be able to use that for the in the next step for analysis.

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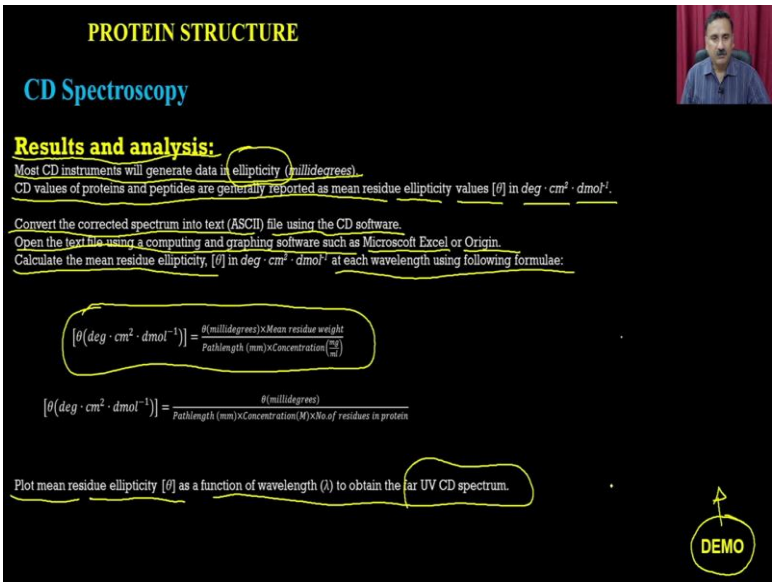

So, in the CD spectra what are the results and analysis? Ok. So, most CD spectra and we will generate the data in the case of in the form of elasticity ok elasticity. So, CD value of the protein and peptides are generally reported as the mean residue elasticity values in degrees centimeter square and per mole ok. Convert the corrected spectra into the text file using the CD spectra software's.

Then open the text file using a computing and graphic software such as the microsoft excel or the origin, right and then you calculate the mean residue ellipticity at each wavelength using the following formula. So, you can use the following formula and it is actually going to give you the calculation of the mean residue elasticity at each wavelength, ok. And, then you can actually be able to plot the mean residue velocity as a function of wavelength to obtain the far-UV CD spectra ok.

So far what we have discussed? We have discussed about what are different types of reagent is required to collect the CD spectra of a protein or the enzyme and then what are the different process you have to perform, but this is somewhat virtual. So, I would like to take you to the CD spectrometry facility and where the people are going to show you a demo. This demo is going to be prepared by one of the my colleague like and he is going to show you the CD spectroscopy, ok.

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Welcome to the demonstration of CD spectrometer. I am Doctor Nitin Chaudhary a Professor at the Department of Biosciences and Bioengineering IIT, Guwahati. This is CD spectropolarimeter from Jasco and this is what we will be using for recording the electronic CD spectrum for some proteins or peptides. A far-UV CD spectrum is mostly used for studying the secondary structural elements in a protein or peptide sample.

In the far-UV region it is very essential that you flush out all the oxygen that is present inside the instrument because the ozone lab the Zenon lab would generate ozone gas and ozone is actually detrimental for the optics of this method.

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So, with this CD instrument we also have a nitrogen generator which will filter out the oxygen from the atmosphere and generate nitrogen. So, you can flush that nitrogen gas through the instrument and then only you turn on the lamp of the instrument.

The flow rate of the nitrogen gas can be controlled from here and typically a 5 liters per minute of nitrogen flow is essential. So, what we do is we turn on the nitrogen generator, leave it to flow through the instrument at 5 liters per minute for at least 20 minutes and then only we turn on this (Refer Time: 21:09) Once ready, we will open the software that is used for recording the spectrum. This is the spectra manager software from Jasco.

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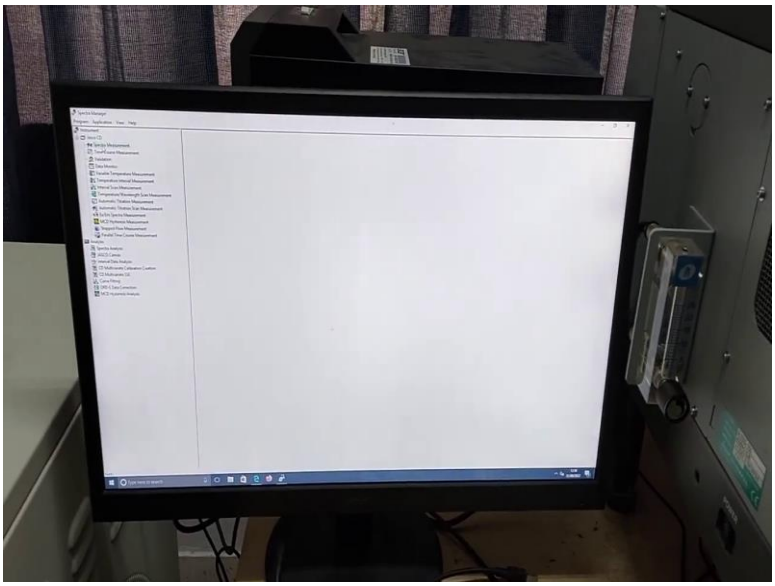

So, what we do? We go to spectra measurement, here open this up ok. So, you set up all the parameters in this software spectrum manager. You set up what range of wavelength you are going to record your spectrum, you select the slip weights, you select the scan speed and you also select the digital integration time for your data. Once you are done with that, you also have to select the number of accumulations.

CD spectra are generally low they have low signal to noise ratio. So, to improve the signal to noise ratio you record multiple spectra and the average of those spectra is presented as the final data.

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So, this is the chamber where you place your cuvette. The cuvettes used for CD spectra spectroscopy are quartz cuvettes because you are working in the UV range and glass strongly absorbs the UV radiation. So, you need quartz.

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This is the cuvette chamber. So, what you are seeing here these are the adapters which I am taking out right now. You can see them. So, once you take out the adapters you will see that you can easily fit a 1 centimeter path length cuvette in this sample chamber. A 1 centimeter path length cuvette is generally used for near UV CD which is weaken

intensity; for far-UV signal for far-UV CD you typically use a 1 millimeter path length set.

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So, you have these two adapters. You place them in the cuvette chamber and you simply sandwich your 1 millimeter paths and quartz towards between them and put it here. The volume of the 1 centimeter pathway towards cuvette is roughly around 200 microliters; that means, you need 200 micro liters of sample for doing the five UV CD spectroscopy, ok.

So, you place your sample here and start recording the CD spectrum. This is the instrument and CD spectropolarimeter is controlled by this software Spectra Manager. You just click on this shortcut for the Spectra Manager and there are different options available for recording CD. What we are going to do is we are going to record spectrum.

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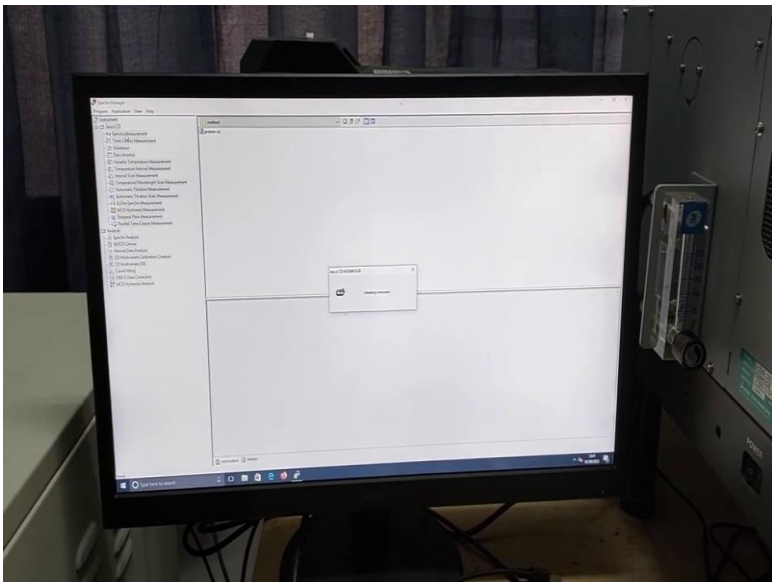

So, we click on Spectra Measurement.

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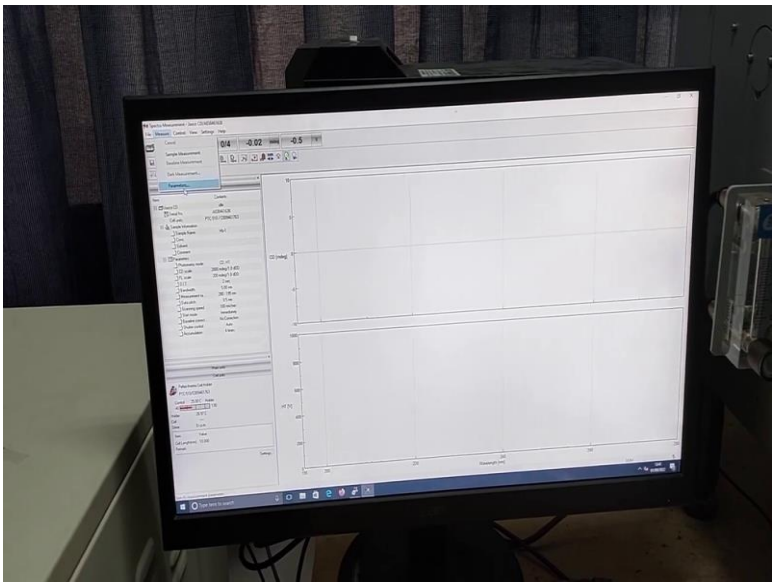

So, here you will set up the parameters that we use for recording CD spectrum. You click on Measure and go to Parameters.

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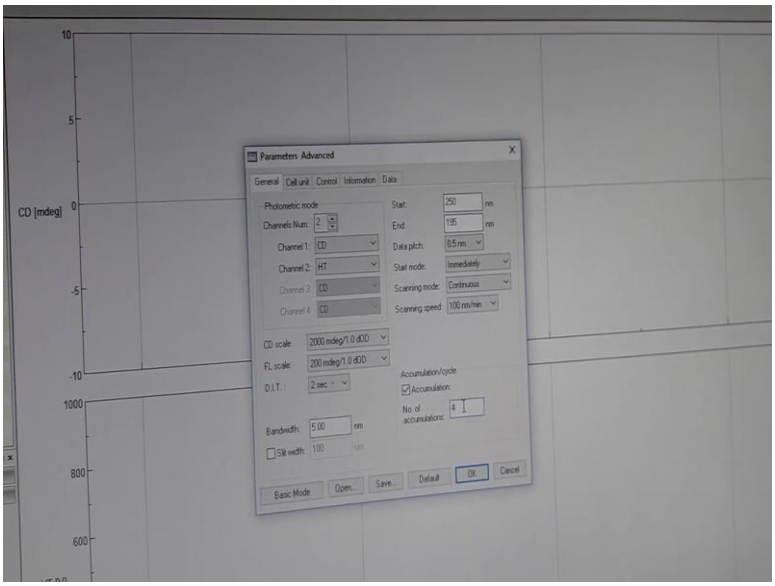

So, generally when you record CD spectrum you record two channels. Channel 1 is CD and Channel 2 is High Tension HT voltage. We can set up the wavelengths here we will be recording the CD spectrum from 250 to 195 nanometers. This is the far-UV region where absorbance is largely because of the peptide bond.

And far-UV region of CD we can give you information about the secondary structure of (Refer Time: 24:55). We will be keeping the Data pitch 0.5 nanometers; that means, you will be having CD values after every 0.5 nanometers. You can set up the Scanning speed which is 100 nanometers per minute. We are recording in Continuous mode and you can have this Digital Integration Time as 2 seconds.

The Bandwidth for this experiment we are choosing 5 nanometers. Now, CD signals are weak. So, signal to noise ratio is not very good. So, you generally need to record a number of scans and CD instrument gives to the average of those scans. Here we are taking 4 scan 4 recommendations for each CD spectrum. So, we set it, OK.

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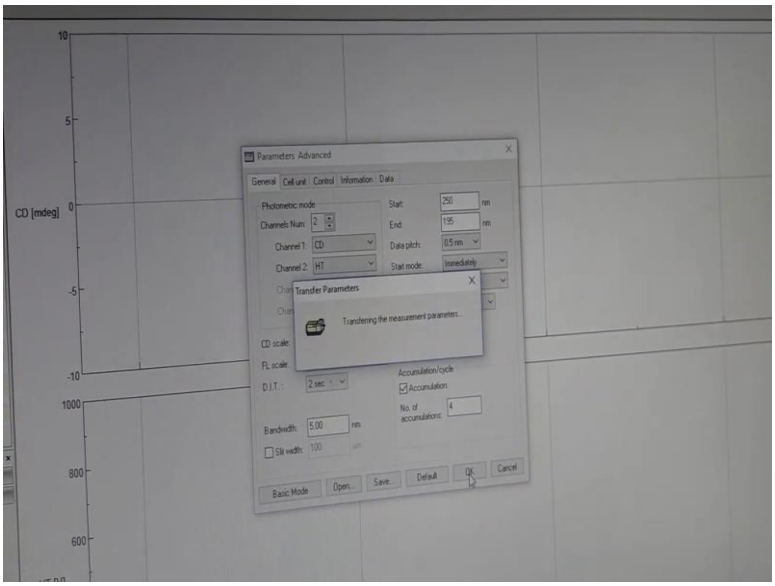

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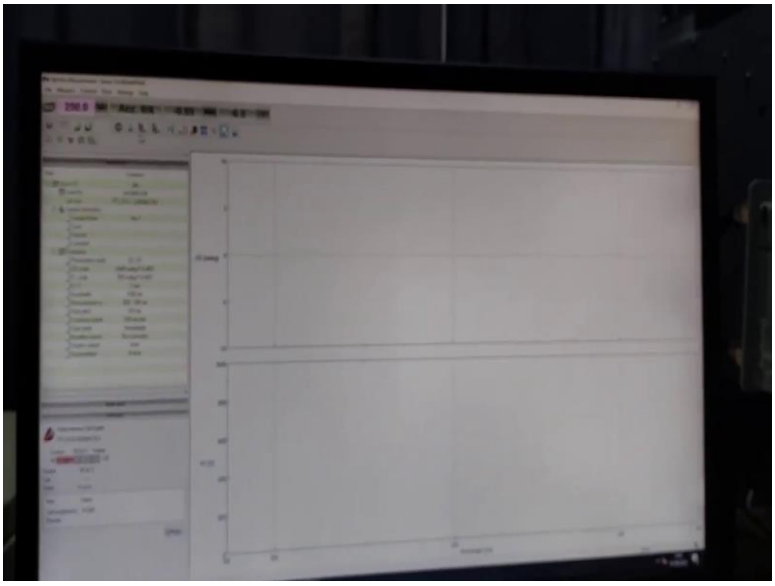

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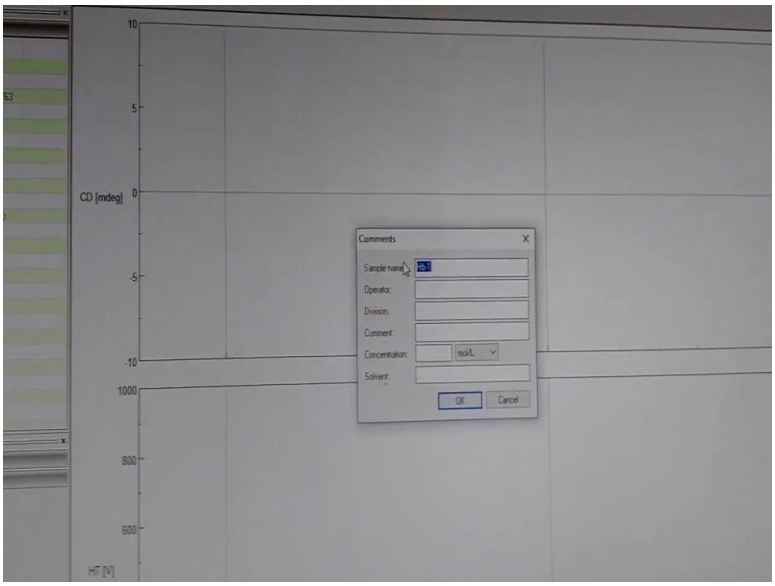

Now, when you click on Start Measurement it asks you to give the Sample name. So, I am putting this as sample 1. In fact, first you have to record blank which will be your solvent or buffer whatever you have dissolved your you for dissolving your (Refer Time: 26:10) blank I say OK. As soon as I say OK, the spectrum will be recorded.

So, now what I will do is, I will be placing the solvent which is placed in water (Refer Time: 26:21).

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So, how far-UV CD spectrum will use quartz cuvette which has a 1 millimeter pathway? We use quartz cuvette because we are working in the UV region and we cannot use blast as blast strongly absorbs the UV radiatiom. So, this is the cuvette for 1 millimeter pathway.

I will now be adding buffer or the solvent whatever solvent we are using in this cuvette. At 200 microliter volume is generally sufficient for recording CD sprectrum.

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So, I am taking 200 microliters of water for our experiment.

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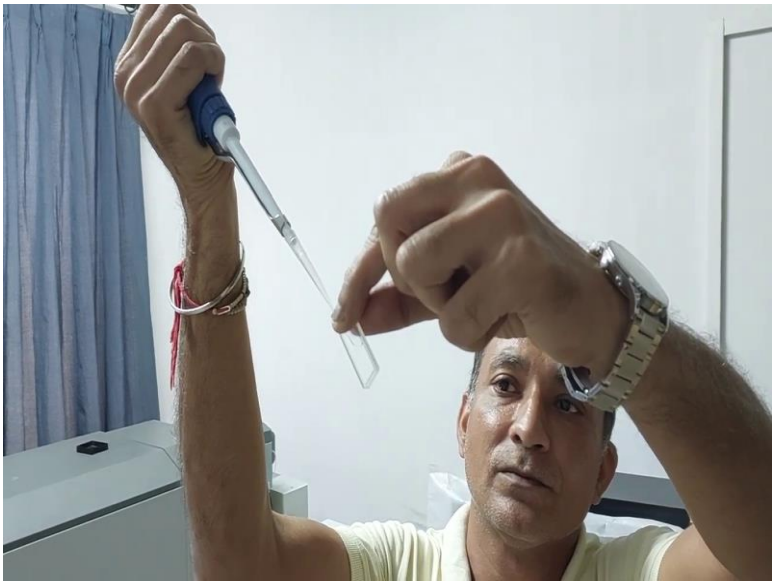

This will be our blank. You add the blank or whatever sample you are adding to the edge of the cuvette ensuring that no bubbles enter inside the cuvette. So get the cuvette and gently push down the solvent ensuring that no bubble enters in the cuvette. You can gently tap the cuvette to get a nice meniscus on the top.

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You can then wipe the surfaces using a link free tissue paper.

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We place this cuvette now in the sample chamber like this. Cover the sample chamber, close the lid.

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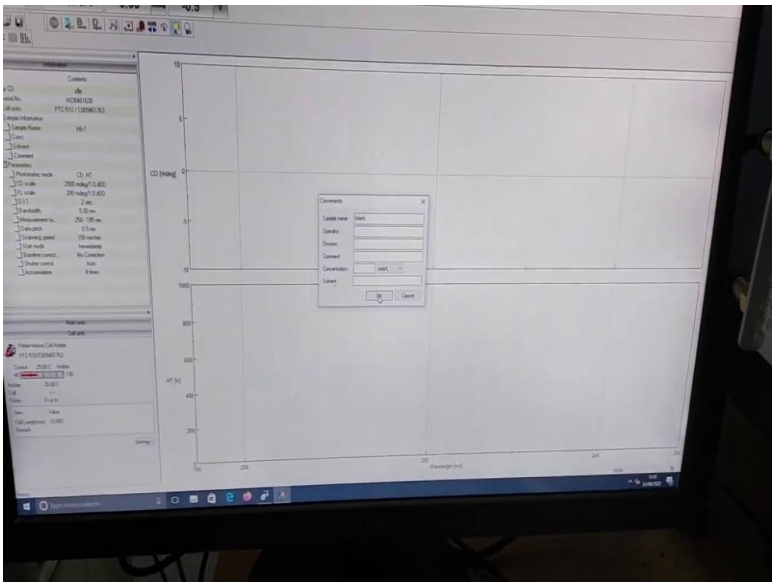

And, click on OK.

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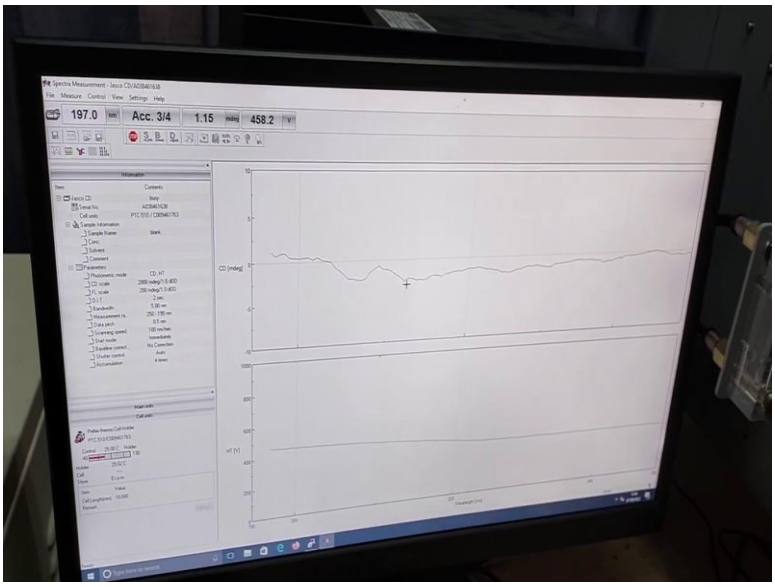

Now, the spectrum will start getting recorded and we have done 4 accumulations for each spectrum. So, you can see the instrument is recording accumulation number 1 out of 4, and you can see the spectrum getting recorded in real time. So, this is the first accumulation.

As you can see so, this is the channel the top channel is for CD and the bottom channel is for HD. So, what happens is, when you place your sample and when the CD gets

recorded, your sample is actually absorbing photons. As the sample absorbs photon less number of photons will reach the detector, so, a higher voltage is applied to the detector to get more secondary electrons by the PMT. So, essentially the light get extinguished by your samples and a high higher voltage is applied.

So, when the absorbance of the sample becomes too high, very few photons reach the detector because very few electrons reach the detector a higher voltage is applied to amplify the signal and this is where the problem is. A very high voltage is applied when there are very few electrons, very few photons reaching the detector. So, under those conditions when very few photons reaching the detector your signal becomes noisy and it becomes reliable.

So, one has to ensure that the CD voltage never exceeds 600 voltage to get a reliable CD spectrum. It is not problem. So, now this spectra is recorded and we can save this data in some directory.

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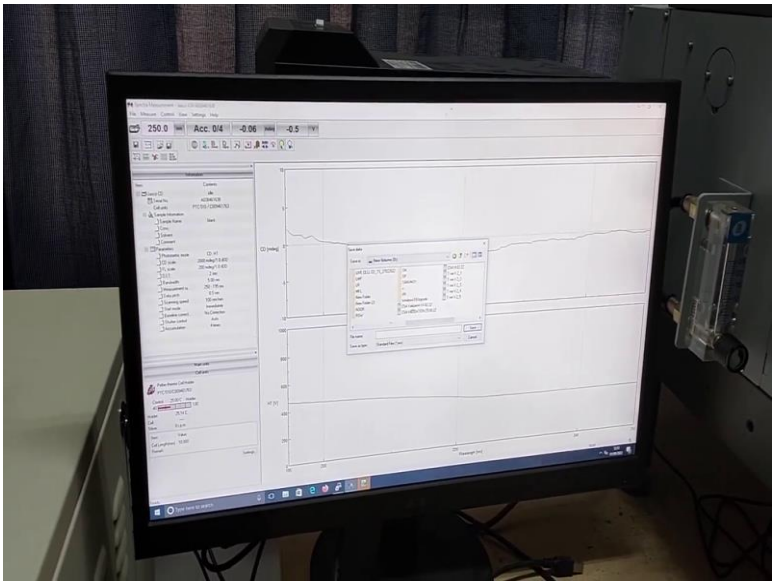

So, you can just save it as blank.

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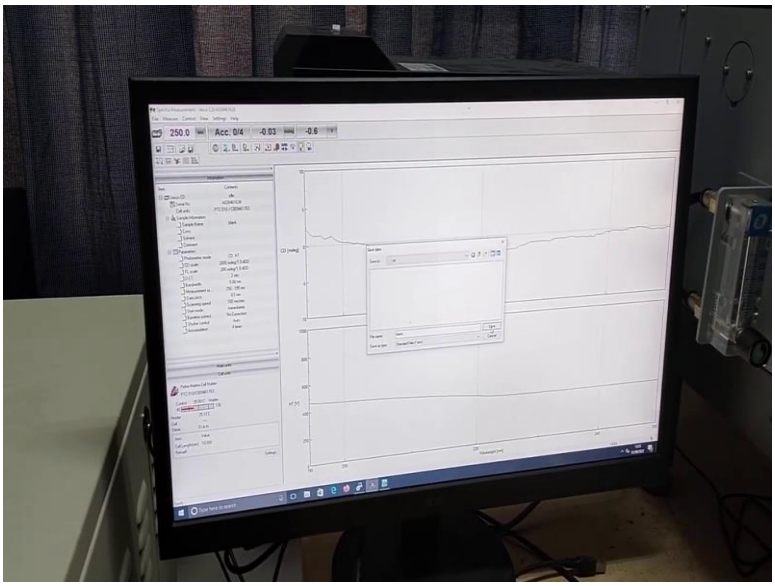

So, now, I will be recording the CD spectrum for a protein which we expect to be largely (Refer Time: 30:20). Let us see how this spectrum works.

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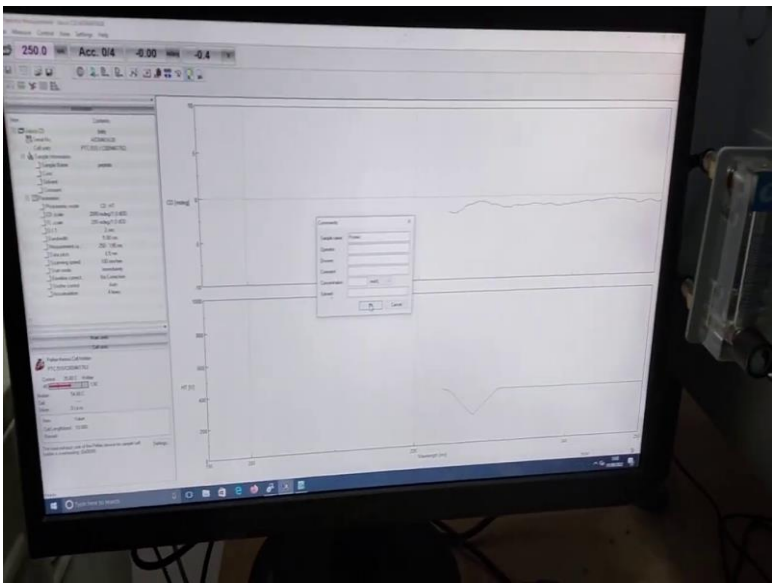

So, this is a protein sample. I am putting Protein, right click on OK and the spectrum will.

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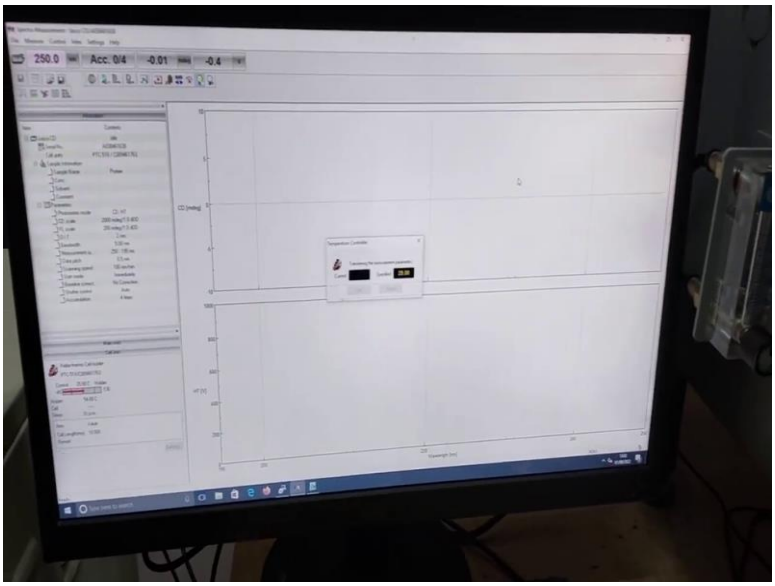

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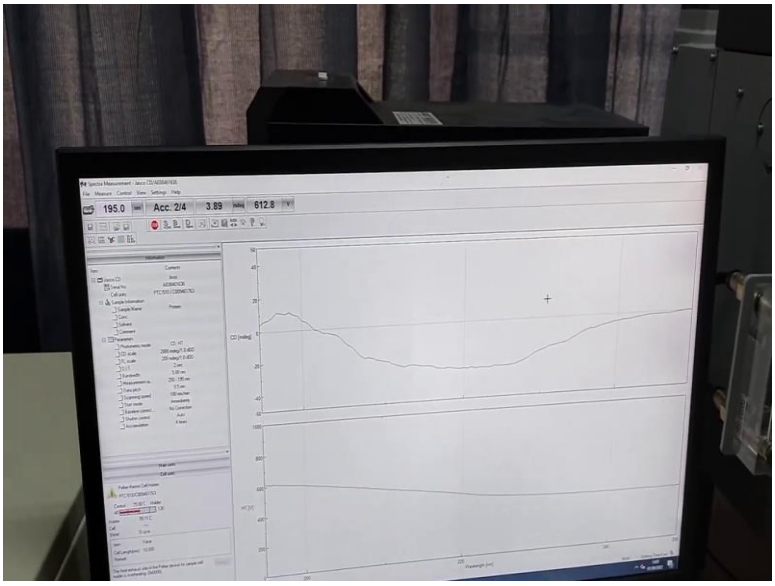

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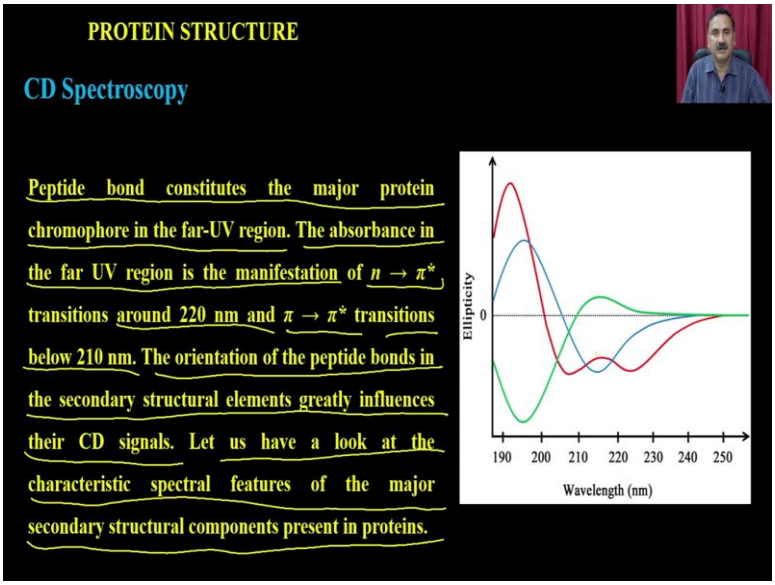

Now, once you collected the CD spectra ok the CD spectra is going to be look like this so, where you have plotted the elasticity and the wavelength on the one side. So, the peptide bond actually constitute the major protein chromophore in the far-UV region. The absorbance in the far-UV region is the manifestation of n to pi transition around 220 nanometer and the pi to pi transitions below the 210 nanometers.

The orientation of the peptide bond in the secondary structure element greatly influence their CD signals, ok. Let us have a look at the characteristic CD spectra of the major secondary structure component present in the proteins. So, what you see here is that we have shown you the CD spectra of the different secondary structure elements, ok. So, when you do it for the protein, you will not get the exclusive for the alpha-helix, beta-sheets or turns you will actually going to get a complex pattern.

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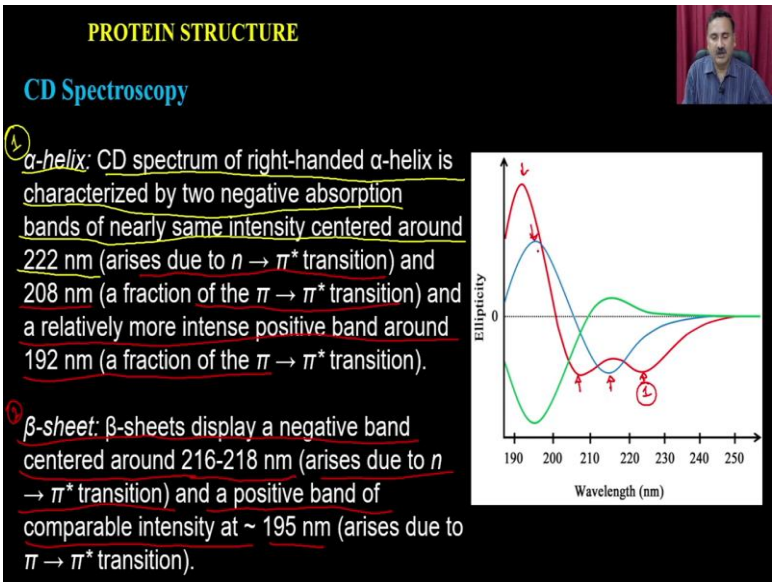

So, what you are going to expect if it is a alpha-helix protein, ok? For the alpha-helix the CD spectrum of a right handed alpha-helix is characterized by the two negative absorption band of nearly same intensity centered around 222 nanometer which means which means this, right. So, this is the negative region of the first peak which you are going to expect in the alpha-helix.

And, then you are going to have this is this is coming because of the n to pi transitions and then you are also going to have another dip which is actually 208 nanometer and that is because of the pi 2 pi transition. So, this is the 208 transitions, ok and a relatively more intense band around 192 nanometer that is going to be because of the pi to pi interactions, ok. So, that is this one ok.

Now, if it is a beta-sheet for example, ok, so, beta-sheets display a negative band centered around 216 to 218 nanometer. This arises due to the n to pi transition and a positive band of the comparable intensity at 195 nanometer arises due to the pi to pi interactions. So, this is the beta-sheets. What you see here is one transition which is one transition which is right this is the 216 to 218 nanometer. This is the and then you are also going to see another transition which is at the 195 nanometer.

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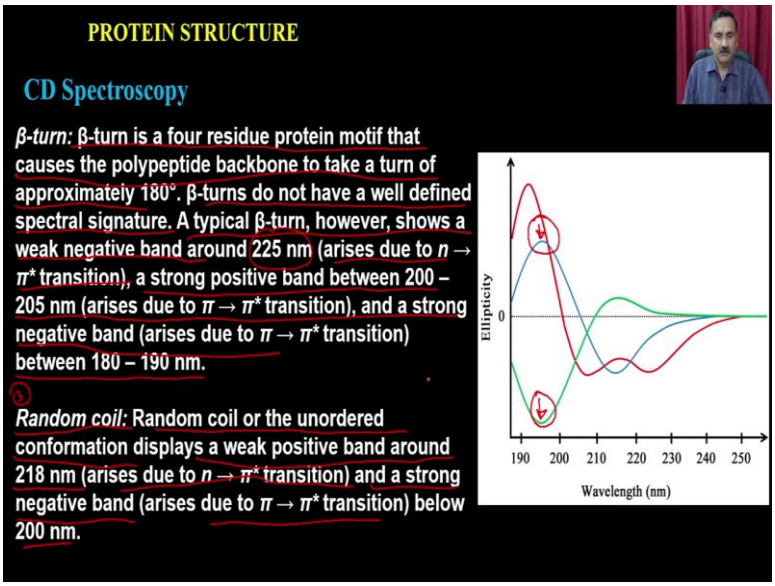

Now, if it is a beta-turn is a four residue protein motif that causes the polypeptide backbone to take a turn of approximately 180 degree. So, beta-turn do not have a well defined spectral signals. A typical beta-turn however, show a weak negative signal around 225 nanometer arises due to the n to pi transitions, a strong positive band between 200 to 205 and a strong negative band which is arises from the pi to pi transitions between the 180 to 190 nanometer.

And, then we have the random coils. So, this is the random coil. So, random coils random coils or the unordered protein structures display a weak positive band around 218 nanometer arises due to the n to pi transitions and a strong negative band which is arises due to the pi to pi transitions below 200 nanometer, ok. So, this is what it is showing this is below 200 nanometer what you are expecting in the unstructured sorry, this is the what you see ok and in the random coils, ok.

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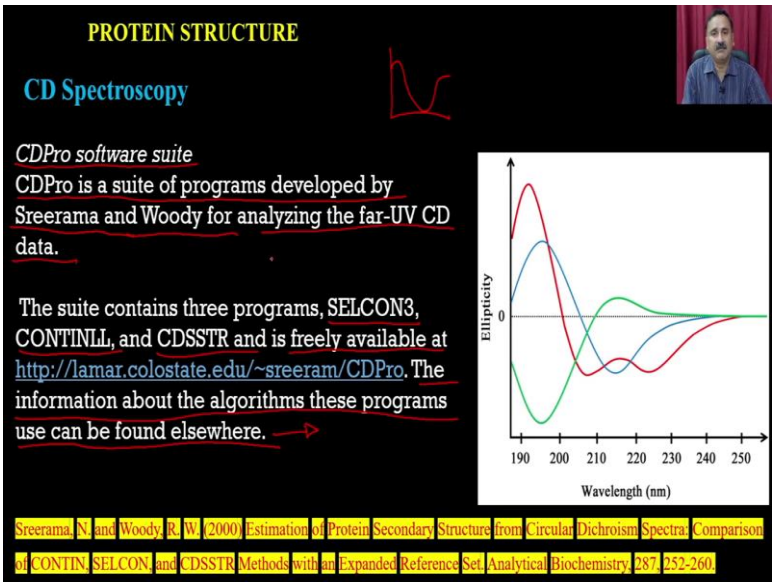

Now, once you got the CD spectra, ok so, once you got the CD spectra of the proteins ok, you are actually going to use that for and suppose you have corrected the blank and all that you are going to get the corrected soft data, right. That corrected one you can actually be able to put into the CD spectra CDPro software suite and that is actually going to tell you the percentage of the alpha-helix percentage of the beta-sheets and so on. So, there is a there is a lot of analysis what you can do.

So, CDPro is a suite of program developed by these scientist and they have analyzed the far-UV CD data. The suite contains the three programs it is called SELCON, CONTINLL and the CDSSTR and it is freely available at this particular link. And, the information about the algorithm and these program use can be found elsewhere, ok. So, this is I have given you the link for you know for this for the reference of this CDpro program.

And, when we were going to show you the CD spectra demo that time we are also going to show you how to use the CDpro software and to analyze the data also. Now, once you are doing the CD spectra ok you have to follow the different types of precautions.

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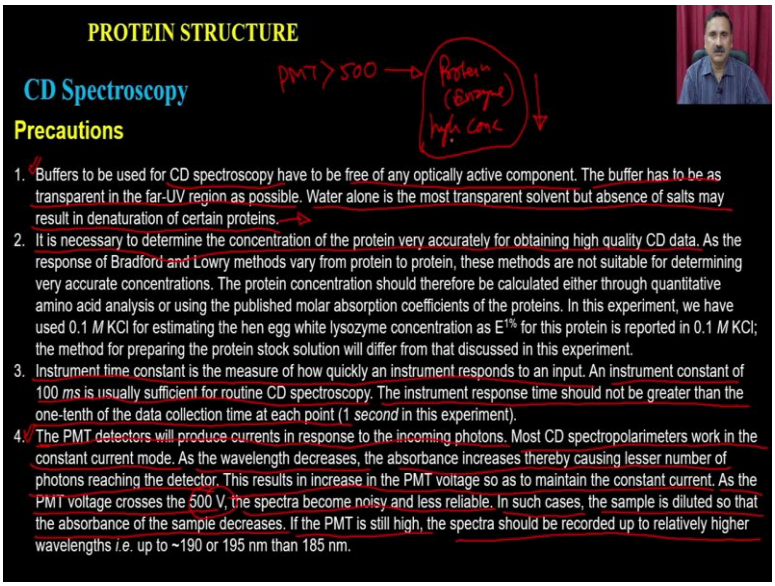

What are the precautions you have to take? Number 1 – you have to use the buffer which is going to be of very high quality and that is going to be used for the CD spectroscopy is free of any optically active elements. Because more and more optically active elements are going to be present, they are actually going to increase your background, ok. So, the buffer has to be transparent in the far-UV region as possible. Water alone is the most transparent solvent, but absence of salt may result in the denaturation of the certain proteins.

It is necessary to determine the concentration of the protein very accurately for obtaining the high quality CD data, ok and we have shown you, right? Even if you have prepared very precisely by dissolving the 5 milligrams of protein in 0.5 ml of phosphate buffer that may or may not correct because the there could be some issue with the weighing and all that. So, whenever you want to do a CD spectroscopy, it is important that you take the absorbance at 280 nanometer and use that with the extinction coefficient of that particular protein and to calculate the accurate concentration of the protein.

Then instrument time constant is the measure of how quickly an instrument respond to an input. The instrument constant of 100 millisecond is usually sufficient for the routine CD spectroscopy. The instrument response time should not be greater than the one-tenth of the data collection time at each time point ok.

The PMT which is very important, right, so, PMT detector will produce current in response to the incoming photons. Most CD spectro polarimeter works in the constant current mode. As the wavelength decreases the absorbance increases thereby causes the lesser number of photon reaching the detector. This results in the increase in the PMT voltage so as to maintain the constant current, ok.

As the PMT voltage crosses these 500 volt; that means, you are actually providing the current externally because the photon which are reaching to the detector is decreasing ok, the spectra becomes noisy and less reliable. Because now what is happening is that the signal from the sample is very less and that is why to make it more detectable, you are providing you are increasing the current, ok. So, if you increase huge amount of current it is becoming the spectra becomes noisy and less reliable.

In such cases the sample is diluted so that the absorbance of the sample decreases. If the PMT is still high the spectra should be recorded up to a relatively higher wavelength, which means if the PMT is more than 500, ok it could be because you are taking a protein or the enzyme which is of very high concentration.

And, because it has a very high concentration it is absorbing very strongly in that particular area and that is why it is actually not allowing the large quantity of the photon to reach to the detector. And, because the detector current is going down as a corrective measurement the PMT is increasing the current. So, that is actually can be avoided if you want to lower down the concentration of the enzyme.

So, this is all about the CD spectroscopy. What we have discussed? We have discussed about the general principle of the CD spectroscopy and I would suggest that if you want to read more about the principles and other things we I have given you the link for the NPTEL web course and there we have given you the complete content.

And, then we also discuss about how to perform the CD spectroscopy and my colleague Professor Choudhary has also shown you a complete demo about how to record the CD spectroscopy and how you can be able to analyze the CD spectra.

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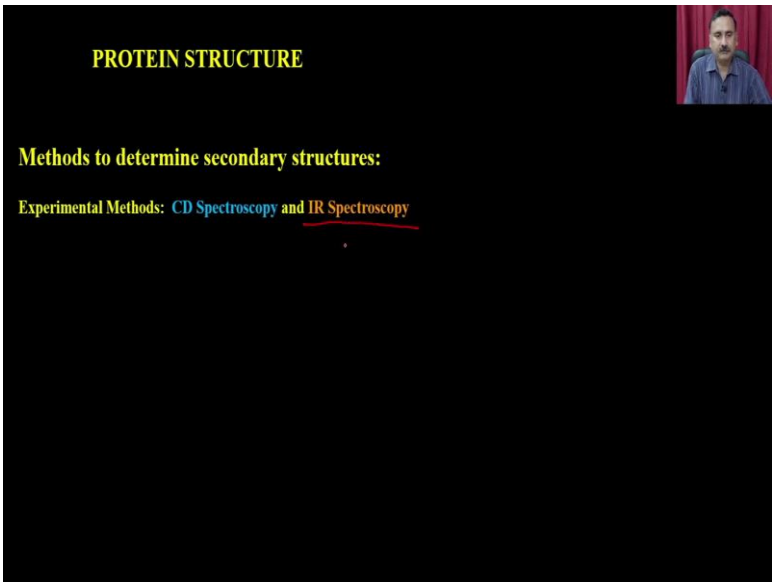

Now, let us move on to the next method and the next method of detecting the determining the secondary structure is the IR spectroscopy.

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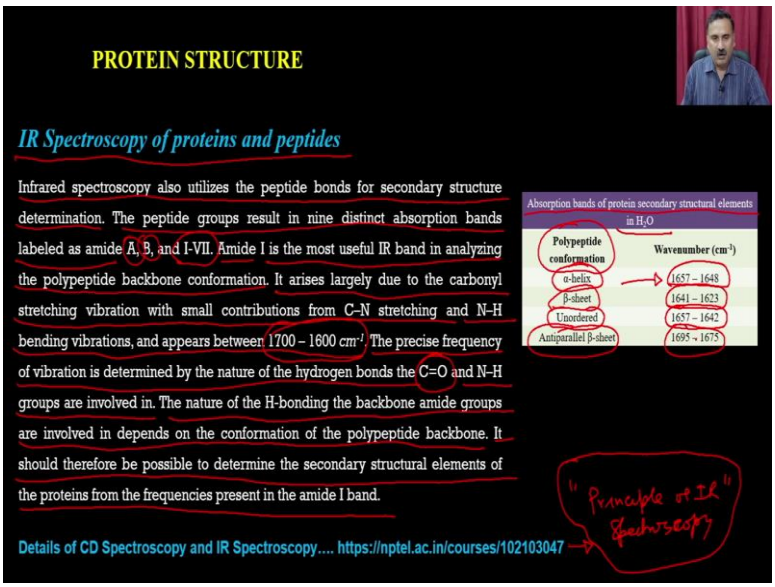

Now, IR spectroscopy of the proteins and peptides: IR spectroscopy also utilizes the peptide bond for the secondary structure determinations. The peptide group results in the nine absorption bands labeled as A, B and I to VII. Amide I is the most useful IR spectra ok band in analyzing the polypeptide backbone conformations. It arises largely due to the

carbonyl stretching vibration with a small contribution from the C-N stretching and the N-H bending and vibration and appear between the 1700 to 1600 centimeter minus 1.

The precise frequency of vibration is determined by the nature of hydrogen bond that is the carbonyl group and the N-H group are involved in. The nature of the hydrogen bond the backbone amide chain are involved in dependent on the conformation of the polypeptide backbone. It should be therefore, possible to determine the secondary structure of the protein from the frequencies present in the amide I band, ok.

And, we have given you the link for this particular course. So, this is the content where we are actually going to discuss about the principle of the IR spectroscopy and it is actually going to give you more detail. What I am discussing right now is little summary of what we have discussed in the NPTEL web courses.

So, what you see here is that the absorption band of the protein secondary structure element in the water. So, polypeptide conformation if it is a alpha-helix, it is actually going to give you absorbance in the range of 1657 to 1648. If it is a beta-sheet, it is going to give you an absorbance at 1641 to 1623. If it is a unordered structure which means it can be the turns or loops, then it is actually going to give you an absorbance in the range of 1657 to 1642. And, if it is a antiparallel beta-sheets it is actually going to give you a absorbance in the range of 1695 to 1675.

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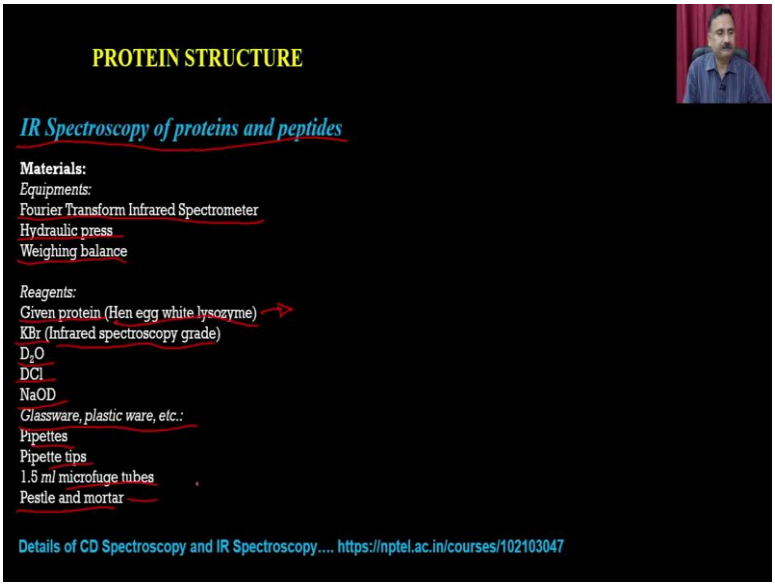

Now, how to perform the IR spectroscopy of the proteins and peptides? What material you require? You require FTIR spectrophotometers. You require the hydraulic press you require the weighing balance. You require a proteins. For example, in this case we have also taken another example where we have used the hen egg white lysozymes.

You require the KBr. So, if KBr should be of very high grade which means the IR grade then you require the D 2 O. You require DCl, you require NaOD and you require the different types of glass wears and plastic wears such as the pipettes, pipette tips, 1.5 ml micro centrifuge tube, pestle and mortar.

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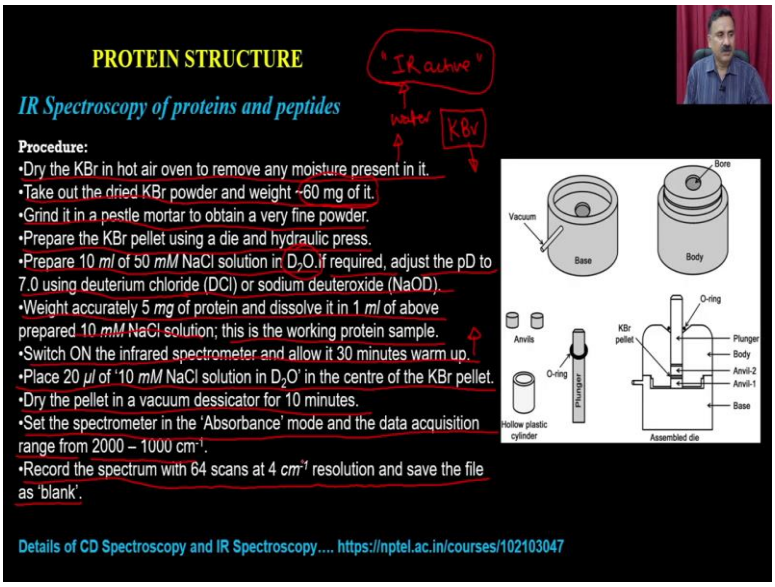

And, this is what you are supposed to do the procedures. So, these are the instrument you require, right. So, you dry the KBr in hot air oven to remove any moisture. Remember that the water is actually very much IR active, ok and that is why the water if the amount of water is present, it is actually going to reduce the signal and it is actually going to make it may mask some of the signal, ok.

So, that is why the water has to be removed and that is why we are using the KBr as the solvent actually or KBr as the medium where we are actually going to prepare the sample because KBr is going to be IR inactive molecule. You take out the dried KBr powder and you weigh the 60 milligrams of KBr, ok. You grind the KBr in a pestle mortar to obtain a very fine powder. Prepare the KBr palette using a die and hydraulic press. So, this is the die and hydraulic press.

Then you prepare the 10 ml of 50 millimolar NaCl solution in D 2 O. If required you adjust the pH to 7 using the using the deuterium chloride or the sodium deuterate deuteroxide. So, it means you are going to use a deuterium derivative of the HCl and NaOH. Weight the accurately 5 milligrams of protein and dissolve it in 1 ml of the prepared NaCl solutions. This is the working protein sample ok.

Then you switch on the IR spectrometer and allow it 30 minutes to warm up. Remember that for every spectro photometer whether it is the CD polarimeter or IR meter or IR spectrometer, minimum amount of the warm up is required, ok. So, for example, most of the time we turn on the instrument for 30 minutes, so that, the intensity of the bulb should be stabilized.

Place the 20 micro litre of ten millimolar NaCl solution in D 2 O in the centre of the KBr pellet. Dry the pellet in a vacuum desiccator for 10 minutes. Set the spectro photo spectrometer in the Absorbance mode and the data acquisition range in the range of 200 to 2000 to 1000 centimeter minus 1. And, record the spectra with 64 scan at 4 centimeter minus resolution and save the file as blank; which means first you are going to take the spectra of the only reagents or only buffer ok.

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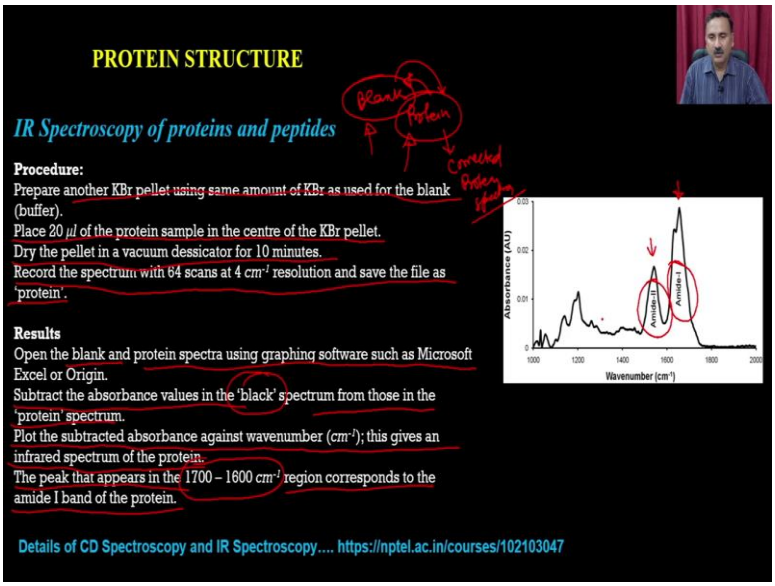

Then what you are going to do is you are going to prepare the KBr pallet, ok. So, you prepare another KBr pallet using the same amount of KBr ok and that will be the blank

then you place the 20 micro liter of protein solution in the centre of the KBr pellet. You dry the pellet in the vacuum desiccator for 10 minutes.

Then you record the spectra with 64 scan and save the file as protein which means you are going to have the two files. You are going to have the blank file which we have generated in the previous file right, this is the blank file what we have generated in the previous step and then you are going to have another file which is going to be the protein file.

Now, how you are going to analyze these files? So, you open the blank and the protein spectra using the any graphing software such as Microsoft Excel or the Origin. You subtract the absorbance value in the blank spectra from those in the protein spectra which means you are actually going to subtract the whatever the value you are getting from the protein and you are going to subtract the blank so that you are actually going to get the corrected protein spectra.

Plot the subtracted absorbance against the wavelength. This will give you the IR spectra of the proteins, ok. The peak that appears in the 1700 to 600 minus 1600 region corresponds to the amide I band of the protein. So, what you see here is that is the typical IR spectra of the proteins, ok and what you see here is that you are going to have the peak these are the peaks, right. So, this is the amide I peak, this is the amide II peak which you are going to get and you are going to get the some more peaks also.

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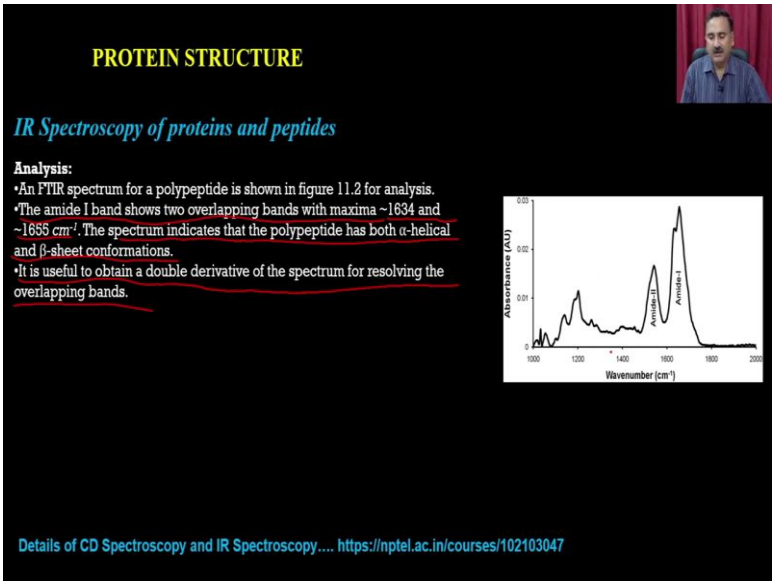

Now, how you are going to analyze this? You are going to analyze this in a. So, the amide band I band will show two overlapping band with a maxima as the 1634 and the 1655. The spectrum indicate that the polypeptide has both alpha-helix and the beta-sheet conformations. It is useful to obtain a double derivative or derivative of the band for resolving the overlapping bands.

Now, once you are done with the IR spectroscopy, you also have to take lot of precautions while you are doing the IR spectroscopy.

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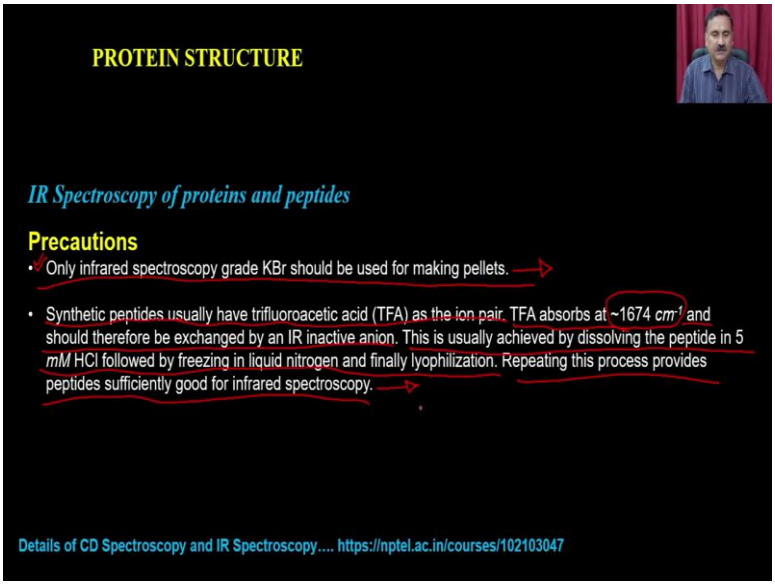

So, what are the precautions? Only IR spectroscopy grade KBr should be used for making the pellets, ok. If you use the KBr of the lower grades it is actually going to give you the IR spectra of the interfering agents. Synthetic peptide which means the small peptide what you are going to synthesize in your laboratory, right usually have the TFA as the lone pair which means it is going to have the bound TFA.

So, TFA absorbed at this particular wavelength that is the 1674 and should be there be exchanged by an IR in active anion so that you can be able to get the values for this particular wavelength as well. Otherwise the TFA is going to interfere in collecting the correct spectra. This is usually achieved by dissolving the peptide in 5 millimolar HCl followed by freezing in liquid nitrogen and finally lyophilization.

Repeating this process provides the peptide sufficiently good for the IR spectroscopy, ok.

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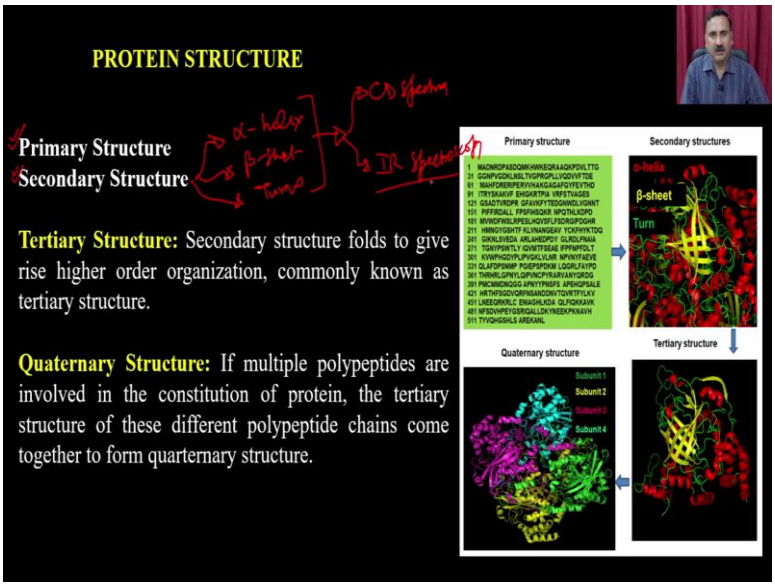

So, what we have discussed? We have discussed about the primary structures; we have discussed about the secondary structures; within the secondary structure we have discussed about the alpha-helix; we have discussed about the beta-sheets and we have also discussed about the turns. And, we have also discussed about the two methods – we have discussed about the CD spectra and we have also discussed about the IR spectroscopy to determine the secondary structures.

So, in this particular lecture, we have discussed about the secondary structures, we have discussed about how you can determine the secondary structures and so on. In our subsequent lecture we are going to discuss about how you can be able to determine the tertiary structures and what are the different methods through which you can be able to determine the tertiary structures.

So, with this I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss more about the tertiary structures of the enzyme.

Thank you.