Introduction to Proteomics
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Lecture - 07
Sample preparation: Pre-analytical factors (contd.)

In today's lecture, I will first talk about protein extraction and solubilization. Few precipitation methods, which are commonly used for different types of protein sample preparation.

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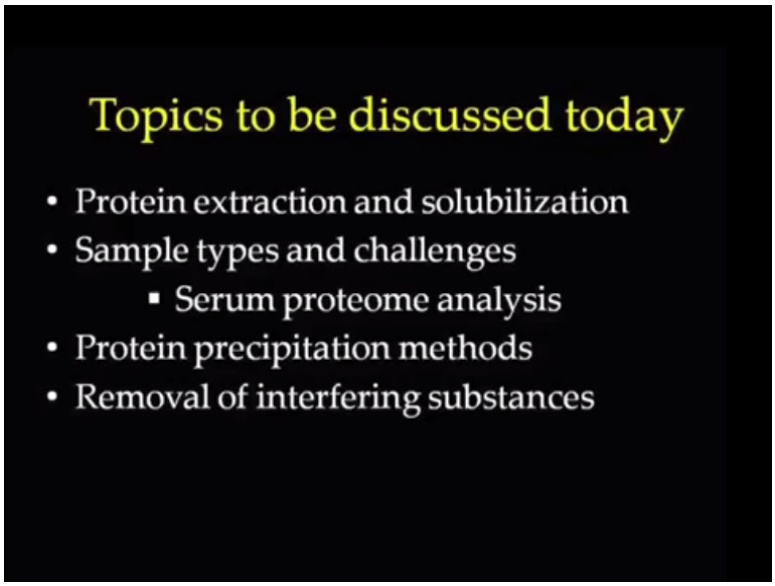

Then we will talk about the removal of interfering substances.

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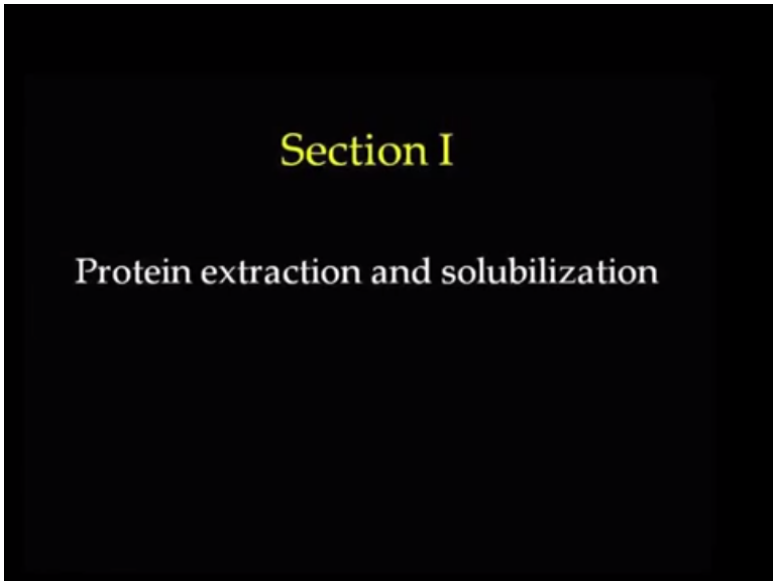

Let us talk about protein extraction and solubilization. Now this step will be more towards talking about gel based proteomics where solubilization will be more important. So protein extraction after performing the subcellular fractionation.

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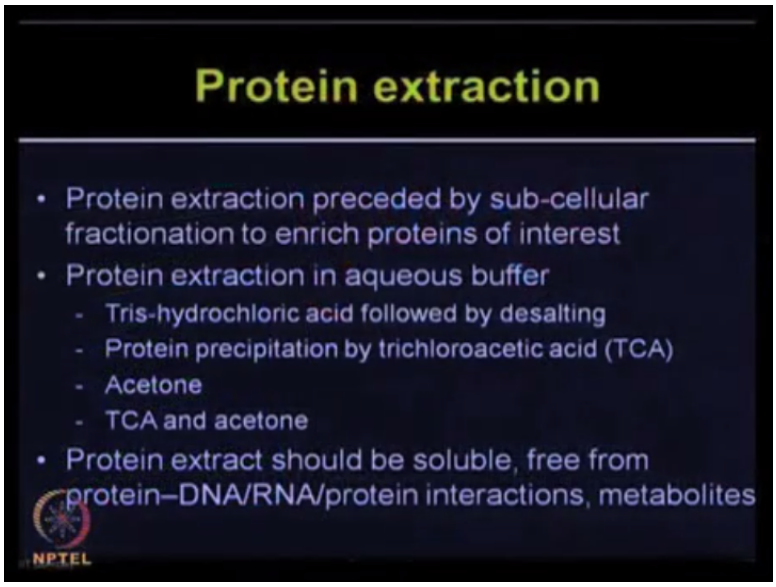

So that the proteins can be enriched, which is we are going to be analyzing in your experiment. So protein extraction in the aqueous buffer, 1 can follow different type of procedure either use Tris-hydrochloric acid followed by the desalting method, protein precipitation by trichloroacetic acid or TCA or acetone alone or trichloroacetic acid and acetone.

I will give you more specific composition and recipe when I will talk to you about a specific type of examples how to perform protein extraction for serum, bacteria and plants? So protein extracts should be soluble. It should be free from protein to protein interaction, protein to DNA or protein to RNA interaction. Similarly, there are different types of other cellular components present and those should be effectively removed.

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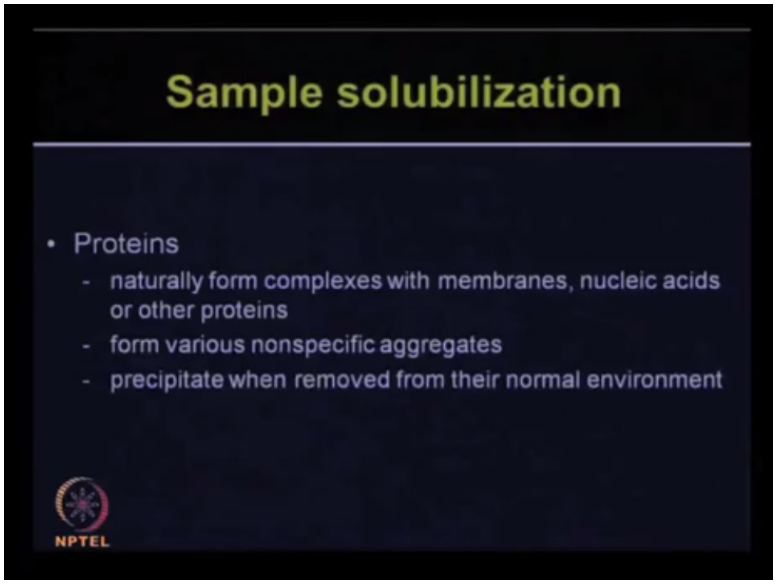

No metabolites should be interfering in your protein extract. Sample solubilization is important because proteins naturally form complexes with membranes, nucleic acids as well as other proteins. So to avoid all of these issues, sample solubilization is very important. There are the different components being used in solubilization. Let us discuss one by one.

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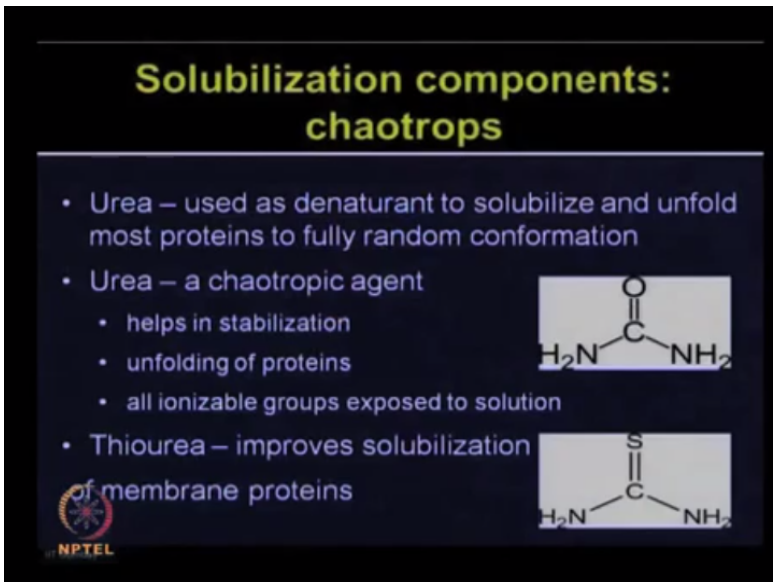

First of all, let us talk about chaotropes, urea, and thiourea. Urea is used as denaturant, which can solubilize an unfold most of the proteins to fully random confirmations. Urea is a chaotropic agent, which helps in a stabilization of the proteins and folding proteins so that all the ionizable groups are exposed to the solutions. Thiourea improves solubilization of membrane proteins more specifically.

Mostly both urea and thiourea are both mixed together during the solubilization step.

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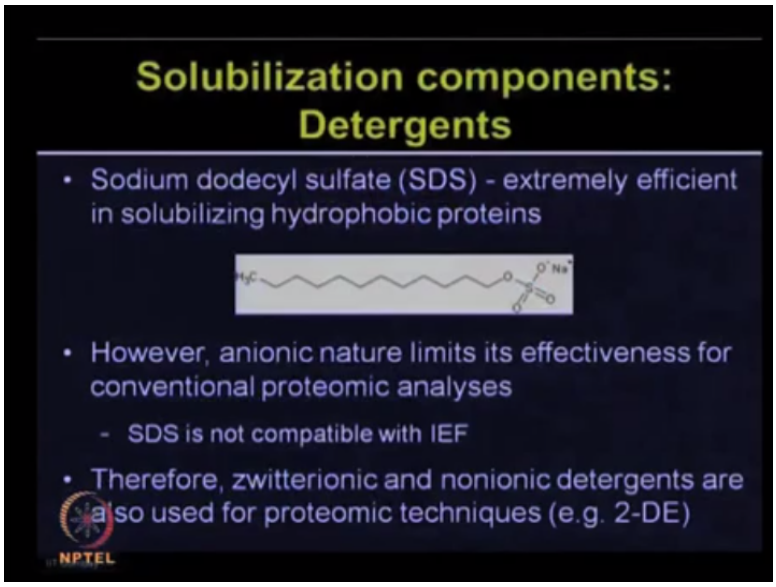

The different type of detergents, which are also used in solubilization such as SDS or sodium dodecyl sulfate, which is a very efficient solubilizing hydrophobic protein. If you want to solubilize hydrophobic protein, SDS can be very effectively used, but due to its anionic nature it limits its effectiveness for the conventional proteomic analysis. The SDS the anionic detergent is not compatible for isoelectric focusing.

So if you are preparing your protein preparation to perform 2-dimensional electrophoresis SDS should be avoided from the sample solubilization. If your objective is to extract the protein and separate that on SDS-PAGE, then SDS is very useful. So when if you want to do the 2-DE or DIGE or different type of other advanced gel based proteomic applications where you cannot use SDS so zwitterionic and nonionic detergents are used for such applications.

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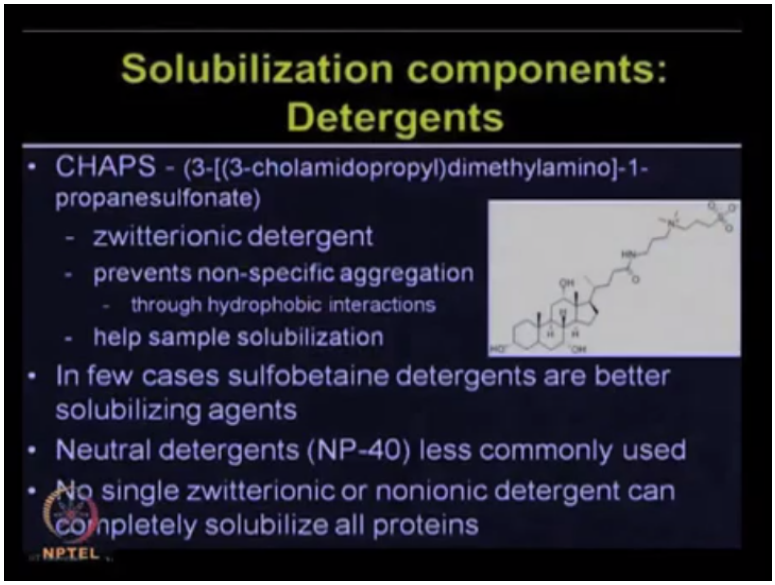

CHAPS, one of the zwitterionic detergent is most commonly used detergent used in protein solubilization when your objective is to perform 2-dimensional electrophoresis experiments. It prevents non-specific aggregations through the hydrophobic interactions and it helps in sample solubilization. Depending upon your sample type, different type of detergents could be useful.

In few cases, ESB-14 or sulfobetaine detergents, they are better solubilizing agents. You also have options of using neutral detergents such as NP-40 although they are less commonly used. So one cannot provide you a list of most effective solubilization agents. No single zwitterionic or nonionic detergent can completely solubilize all the proteins. So depending upon your sample type and if you know your sample is rich in a specific type of proteins, you need to try different type of detergents.

Now let us talk about reductants. In the solubilization deducing agents cleave the disulfide bonds, which are present between and within the protein chains and it prevents the disulfide bond formation.

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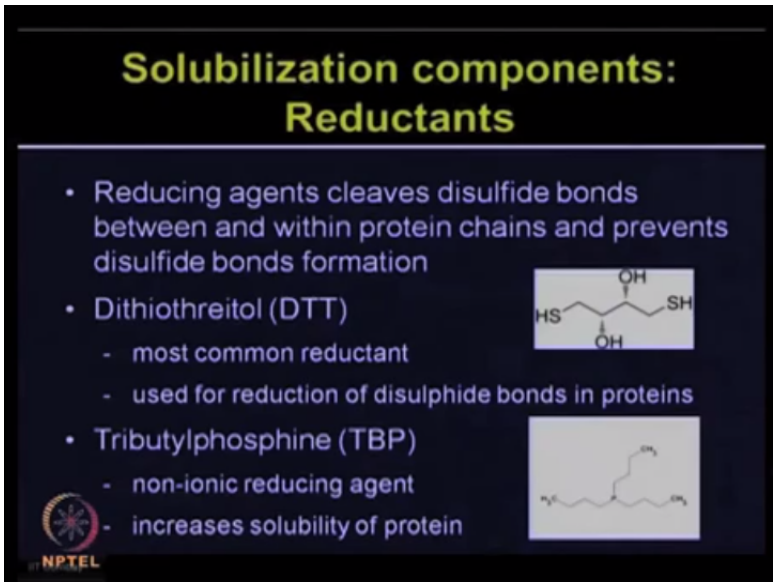

Most commonly used reductants are dithiothreitol DTT or beta mercaptoethanol. These are used for reduction of disulfide bonds, which are present in the proteins. Tributylphosphine or TBP, it is one of the nonionic reducing agent, another very commonly used reducing agent when the aim is to increase solubility of the proteins, often it is used in the 2-DE based gel based proteomic applications.

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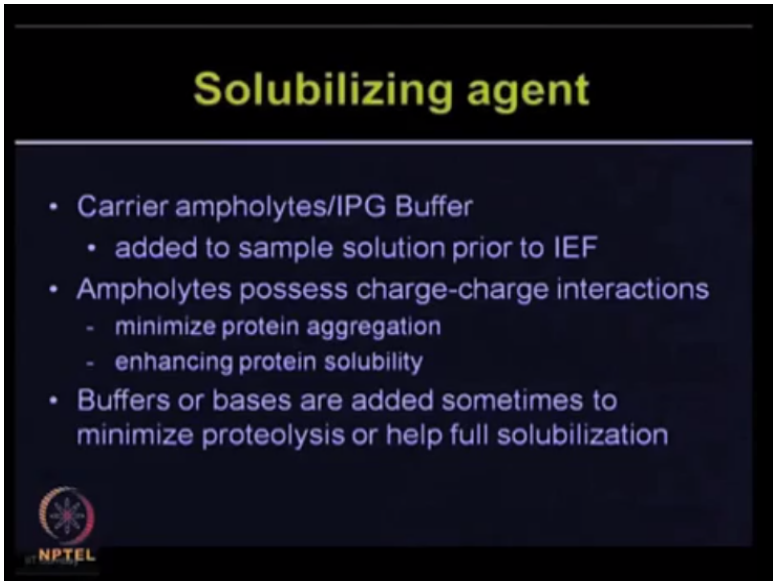

If your aim is to perform isoelectric focusing from your samples, the solubilizing agent should include the carrier ampholytes or immobilized pH gradient buffers. These are added in the sample solutions prior to the isoelectric focusing step, which will talk in the next lectures when we talk about different steps involved in performing a gel based proteomic experiments.

The ampholytes possesses charge to charge interactions. They minimize protein aggregation and enhance the protein solubility. Different buffers or bases are added, which some time minimize proteolysis and also help in the complete solubilization of proteins.

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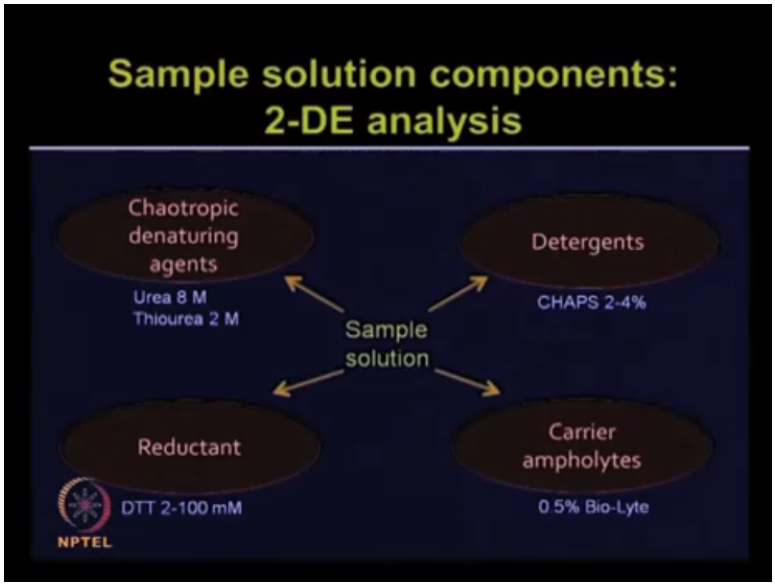

So if your aim is to perform a 2-dimensional electrophoresis experiment, the sample solution involves chaotropic denaturing agents such as urea 8 molar, thiourea 2 molar. Detergents such as CHAPS are most commonly used. It could be between 2 to 4%, commonly used reductant include DTT or beta mercaptoethanol 2 to 100 millimolar and carrier ampholytes in the concentration of 0.5% of Bio-Lyte.

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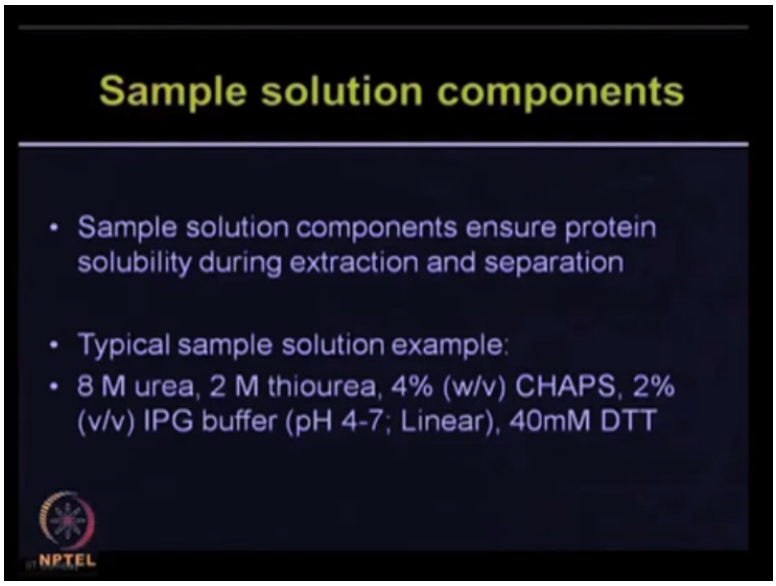

So the sample solution components ensure that the protein solubility is good during the extraction and protein separation. A typical sample solution for the gel based 2-DE

application includes 8 molar of urea, 2 molar of thiourea, 4% CHAPS, 2% IPG buffer, 40 millimolar of DTT as well as few other small components depending upon your sample type.

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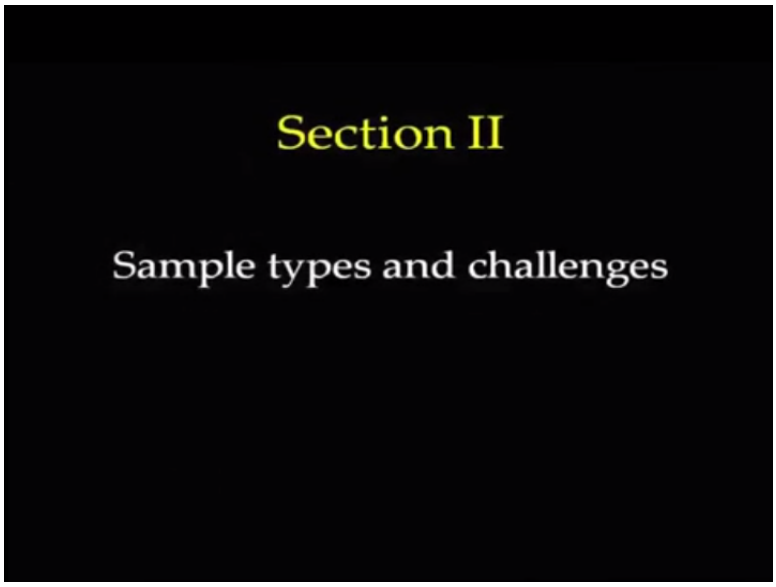

So as I am giving you an overview of how to prepare a very good sample. Let me also introduce you to different type of challenges being imposed by different sample types. So if you are using the tissue culture growing cells, you have to grow in a medium, which will be rich in different components including salts and serum proteins.

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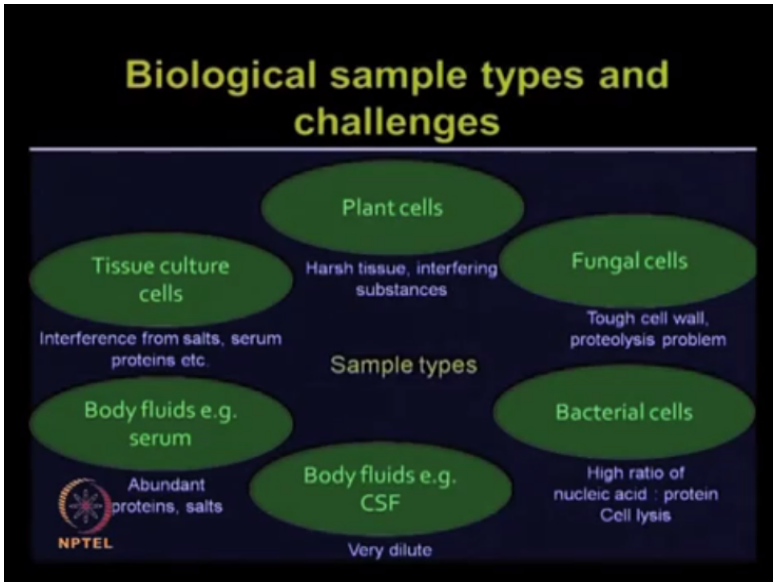

So one need to get rid of those components if you want to perform a good sample preparation from the tissue culture cells. If you are interested in plant cells to extract the protein, those are very harsh tissue and there are various interfering substance present there, phenolic and other salts. Now you need to get rid of those interfering substances. Fungal cell such as yeast or

other type of fungus if you are interested in performing proteomic applications on these samples you need to break open the very tough cells.

So the proteolysis problem also occurs in the samples. The bacterial cells, they have high ratio of nucleic acid to protein and cell lysis is also very tedious. Body fluids such as cerebrospinal fluid, they are very dilute so if you want to perform proteomic experiments on CSF, you need to concentrate your samples. Body fluids such as serum, those are very rich in abundant proteins as well as salt.

So you need to get rid of those abundant proteins such as serum albumin protein and remove the interfering salts. So although the sample diversities very much, samples are very complex. I will still try to take to (()) (11:42) example.

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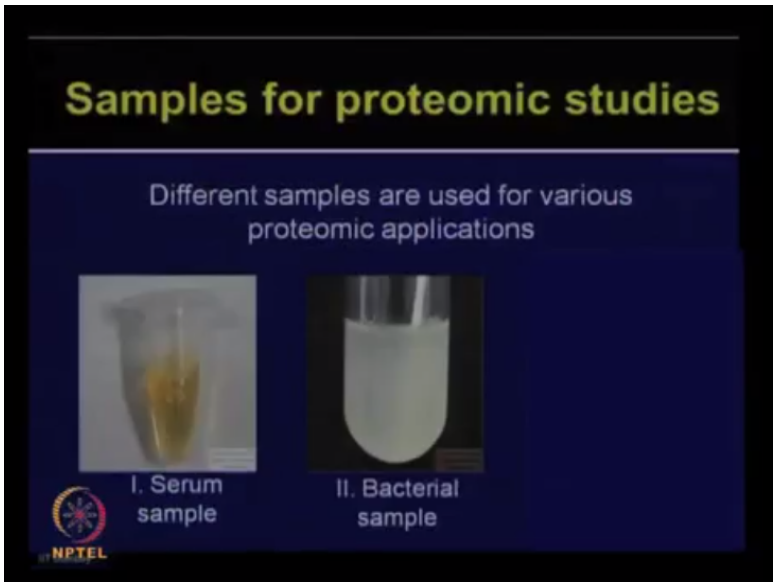

One, the serum sample obtained from human, second the bacterial sample, which will be taken from bacillus species by showing you the protein extraction and solubilization methods. I will try to give you the diversity and different type of methods being used to perform various types of proteomic applications.

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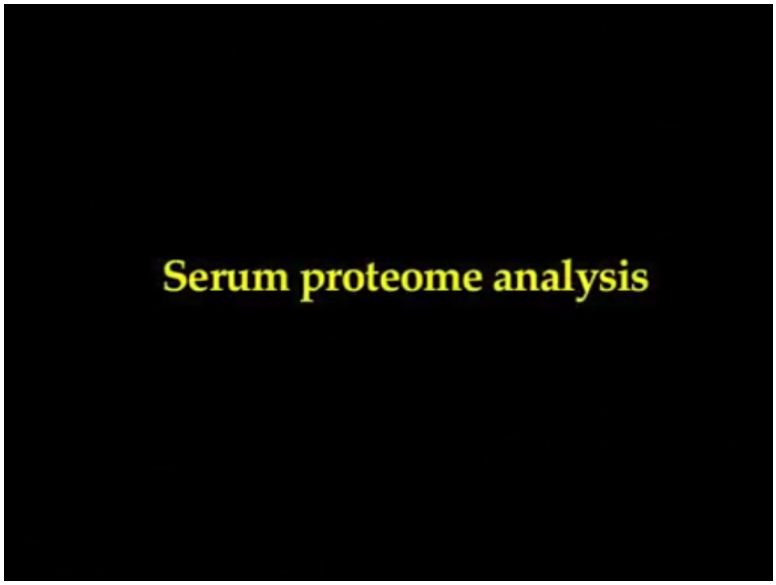

So first start with the serum proteome analysis, but before we talk about how to perform the serum proteome analysis it means all the proteins which are present in the human serum. First of all, how to obtain the samples? How to tore these samples? How to minimize various types of pre-analytical variations? That is one of the very important consideration.

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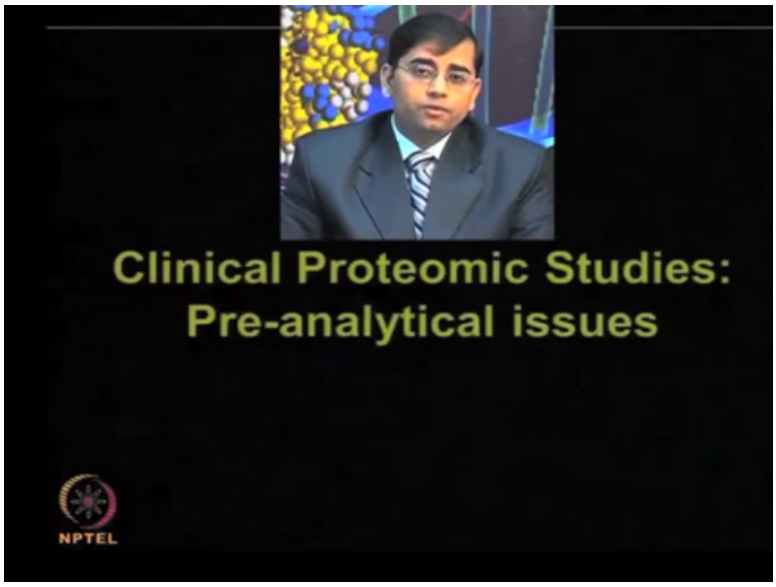

So before we talk about how to really process the sample let us talk about different type of clinical issues involved in these types of samples for the pre-analytical factors.

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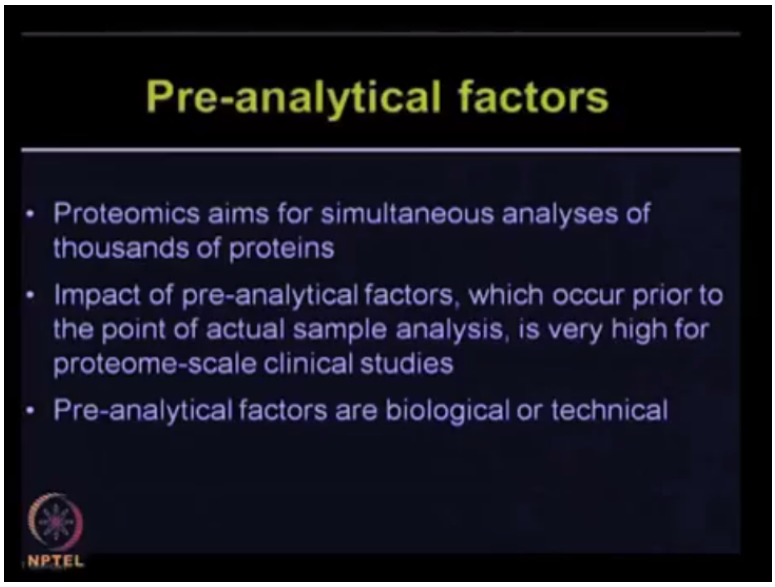

So proteomics, most of the applications are going to aim for simultaneous analysis of thousands of proteins of given clinical sample whether it is serum, saliva, urine, CSF or tissue. The impact of pre-analytical factors, which occur prior to the point of actual sample analysis is very high for the proteome scale clinical studies. The pre-analytical factors could be due to biological variations or it could be due to the technical artifacts.

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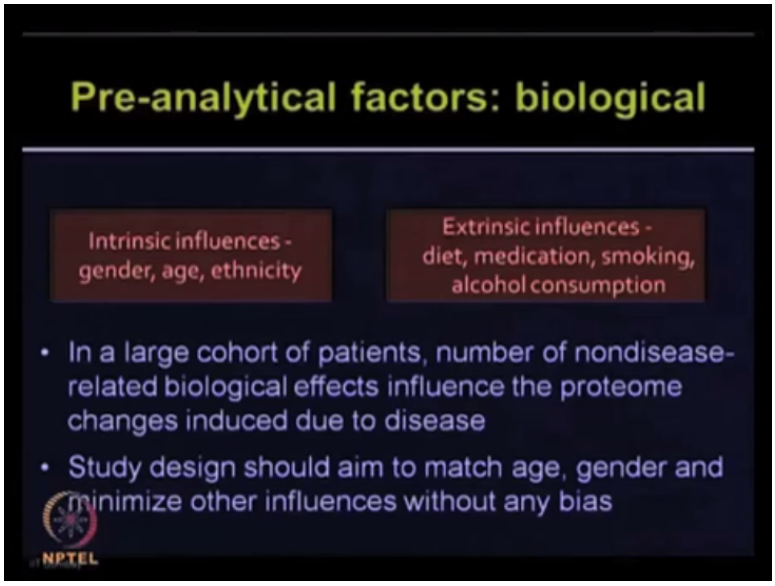

So your studies could be influenced due to intrinsic factors or due to the extrinsic factors. The intrinsic influences include gender, age, ethnicity. The extrinsic influences include diet, medication, smoking, alcohol consumption etc. So when you are designing clinical study you need to ensure that you have no bias with the intrinsic factors. You should try to segregate your population with the different type of age, gender.

In the discovery phase, try to minimize these types of variations and try to perform your analysis with a narrow range of age group and different type of gender groups in the same ethnicity, but when you want to validate your samples then you need to extend your analysis to the different age type, ethnicity and gender. Try to avoid the extrinsic influences such as diet, smoking, alcohol, different type of drug medication.

These are going to alter the proteome and your discovery process will be influenced by these factors. So in the large cohort of patients, the number of non-disease related biological effects will influence the proteome changes induced due to the disease. So the study design should aim to match the age, gender and minimize the other influences without any bias.

Often it is very useful to involve statistician before you are designing these type of experiments and thinking about different pre-analytical factors before you actually perform the experiment will often going to determine how successful your analysis going to be. Now we have looked at different type of biological pre-analytical factors.

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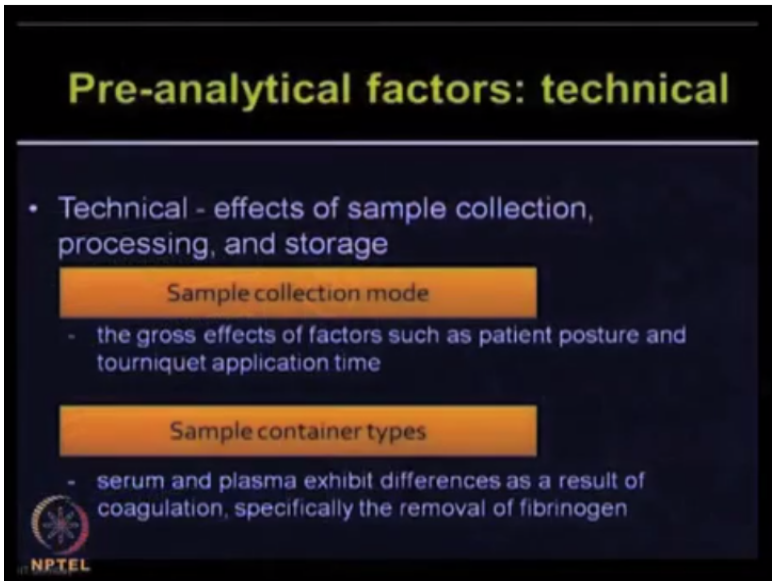

Then there are different types of technical artifacts facts. How to collect the samples? How to process the sample and how to store those? The sample collection mode, the gross effect of factors such as the patient posture and the tourniquet application time, these are very important. Sample container types, when you are collecting the samples such as serum and plasma, they exhibit differences as a result of coagulation.

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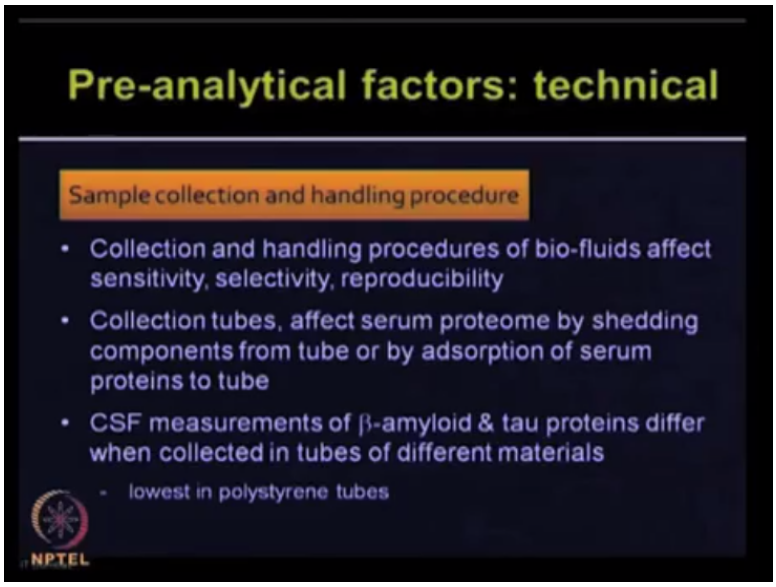

Specifically, the removal of the fibrinogen, so sample collection and handling procedure one has to pay attention. The collection and handling procedure of bio-fluids will affect the sensitivity, selectivity and the reproducibility of the experiments. Collection tubes in which you are collecting your serum sample often is going to influence the analysis if you are using different type of tubes material.

The shedding components of the tube are adsorption of the serum proteins to the tubes will in some way influence the proteome analysis. The cerebrospinal fluid measurement of different proteins such as beta amyloid and tau proteins. When people analyze these in different tubes of different materials, they found that they have different type of effects and the effects were lowest in the polystyrene tubes.

So with this discussion it is very important to understand that one need to avoid different type of sample tubes being used for the collecting your biological samples.

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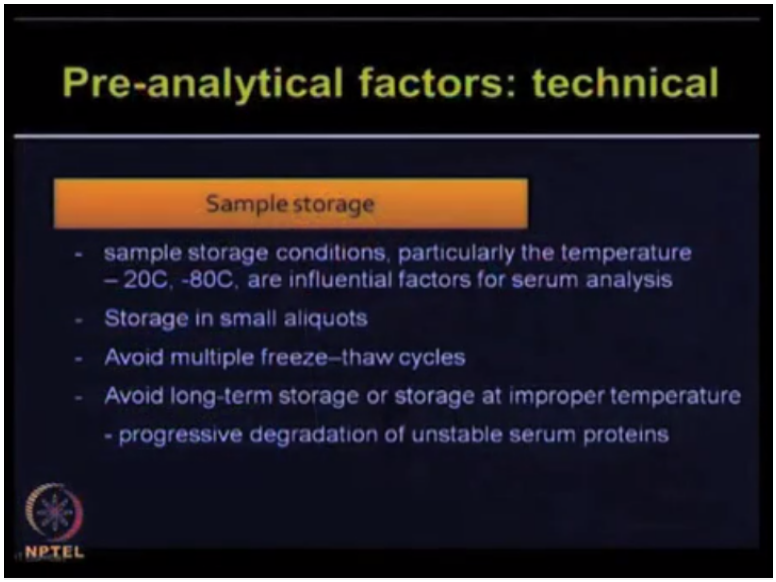

Sample storage is another very crucial factor whether you are storing your clinical samples in -20 degree centigrade or -80 degrees. How quickly have you saved all the clinical samples or how much delay was there before the sample was collected and is stored? All of these small variations actually influence the sample analysis, the proteome analysis later on. So avoid multiple freeze thawing of your samples, store the samples in a small aliquot.

So that you do not have to freeze-thaw the whole samples together. Avoid very long-term storage or storage at the improper temperature. Try to use as freshly stored samples as possible because if the samples are used and stored for the long time, the progressive degradation of unstable serum proteins may occur.

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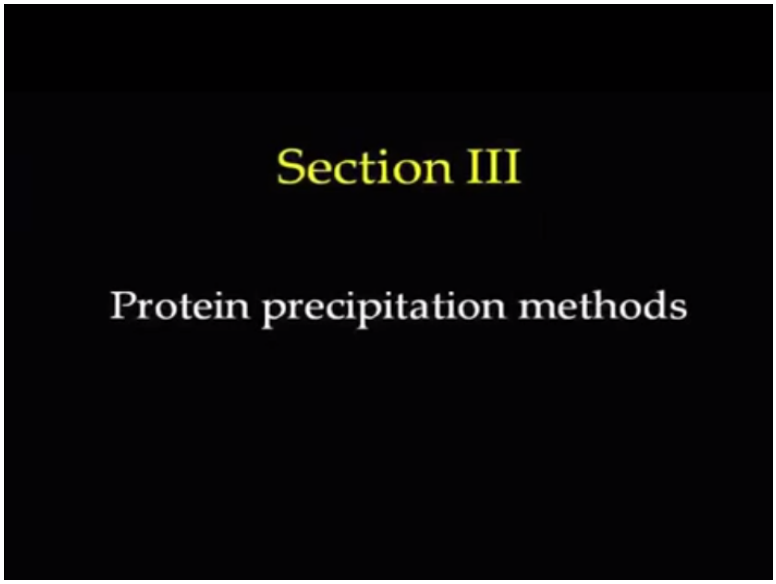

So let us first talk about the precipitation procedures. There are different types of precipitation methods available. I will go through one by one and then we can give you some recommendation about which ones can be more commonly used.

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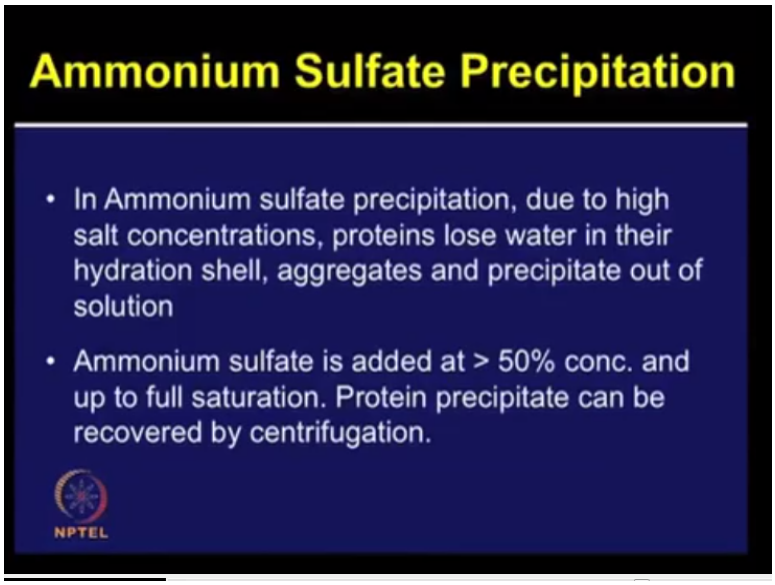

So let us talk about ammonium sulfate precipitation, which is one of the most previously used method from the classical way of performing the experiments. The ammonium sulfate precipitation was used although its use is not so common when you are preparing the samples for the proteomic application, but this one still remains a good choice.

So ammonium sulfate precipitation due to the high salt concentration, the proteins lose water in their hydration shell. They aggregate and precipitate out of the solution. So if you add ammonium sulfate, add greater than 50% of concentration and up to its full saturation, the protein precipitation will occur and then by performing the centrifugation step, this can be recovered.

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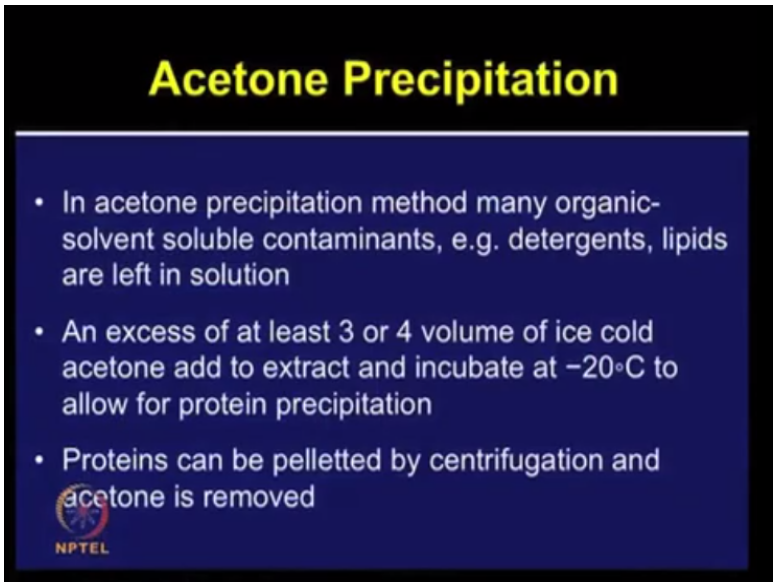

Now let us talk about the acetone precipitation. In this method, many organic-solvent soluble contaminants such as detergents, lipids they are left in solution so it is very effective. If you add an excess of at least 3 or 4 volume of ice cold acetone in your extract, incubate it in -20 degree for 1 to 2 hours and allow the proteins to precipitate. By performing this step, the proteins can be pelleted down during the centrifugation step.

And then subsequently you can remove the acetone and dry it out. So acetone precipitation is very easily performed method and it is very effective.

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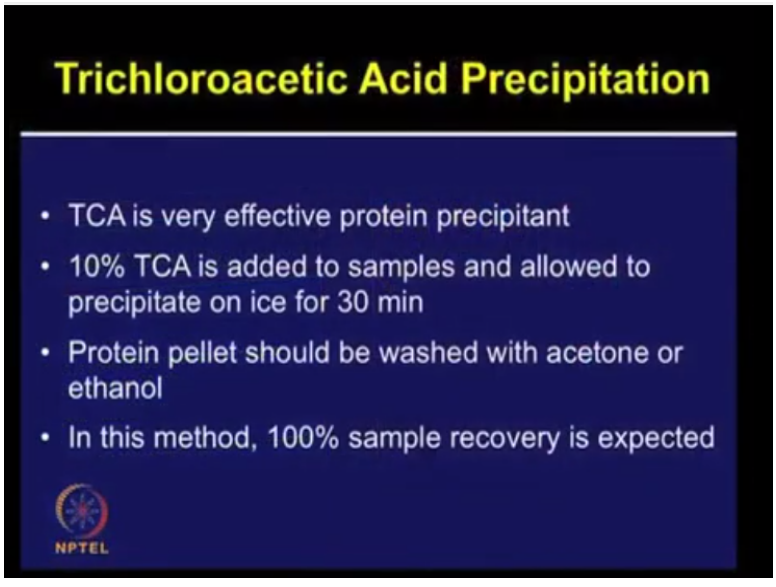

Now let us talk about TCA or trichloroacetic acid precipitation. TCA is one of the very effective protein precipitant. One can use 10 to 20% of TCA (()) (21:21) TCA is commonly added to the samples and then allowed to precipitate in the ice condition for almost half an

hour to one hour. Protein pellet should be washed by adding acetone or other organic solvents such as ethanol.

This method is very effective for the sample recovery point of view. Almost 99 to 100% sample recovery can be expected in this method. Now since both TCA and acetone alone are very effective, people have tried combining both the methods together, it means addition of both TCA and acetone.

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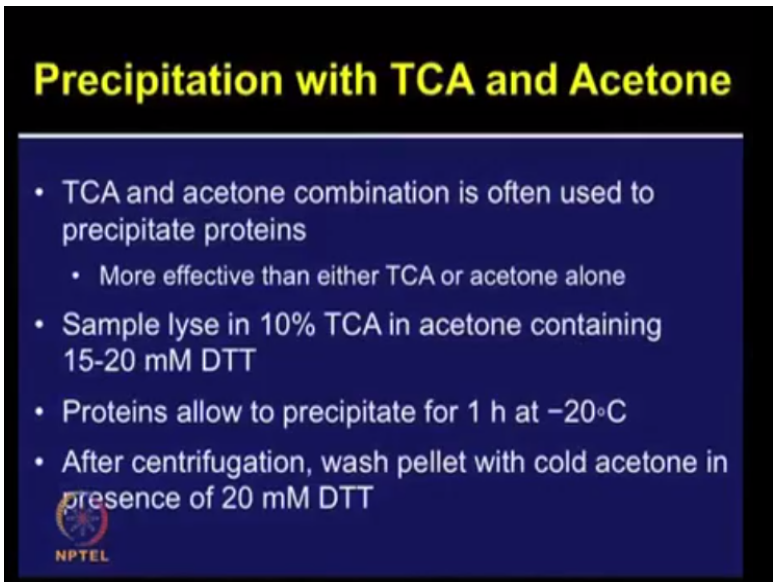

So this combination has demonstrated that it can precipitate the proteins more efficiently, which could not be achieved either by using TCA alone or by using acetone alone. So a recommended concentration one can try lyse the samples in 10% TCA made in acetone and also add 15 to 20 millimolar of DTT. Now allow the protein samples to precipitate for an hour or 2 hours at -20 degrees.

Centrifuge and the pellet can be further washed with acetone alone, try performing the whole step in the cold condition so that you can avoid the proteolysis degradation. Even this step the acetone with 20 millimolar DTT will be effective. So this is very easy method to precipitate out the protein, first add 10% TCA with acetone and after centrifugation, wash the pellet to remove the TCA, which could be present there.

And then further wash 3 or 4 times with acetone containing DTT. After that you need to dry out your pellet so that any residual amount of acetone is not remaining in this pellet.

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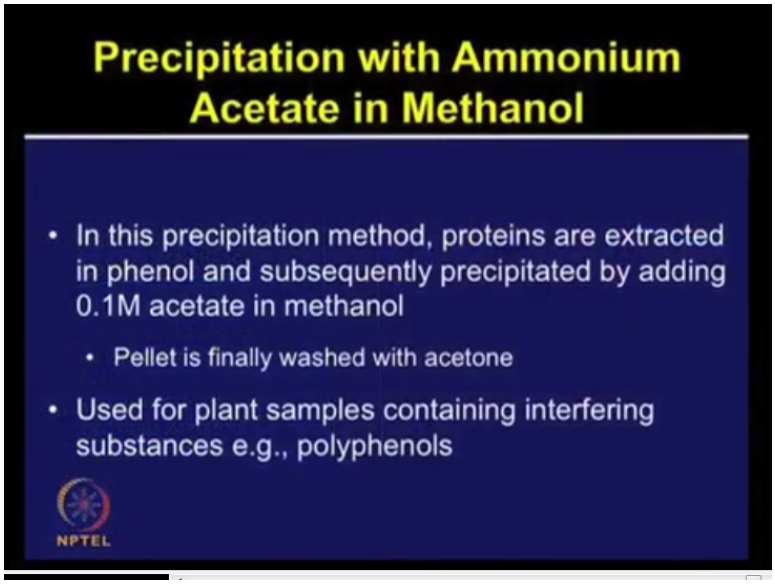

Now let us talk about one of the less commonly used method precipitation with ammonium acetate in methanol. This is more commonly used when you are talking about some plant samples, which are rich in polyphenol another interfering substance. So by using these precipitation proteins are extracted in phenol and subsequently precipitated by addition of 0.1 molar acetate in methanol.

Pellet can be finely washed by adding acetone so as I mentioned this is less commonly used method and for some specific application people try different type of precipitation and washing steps.

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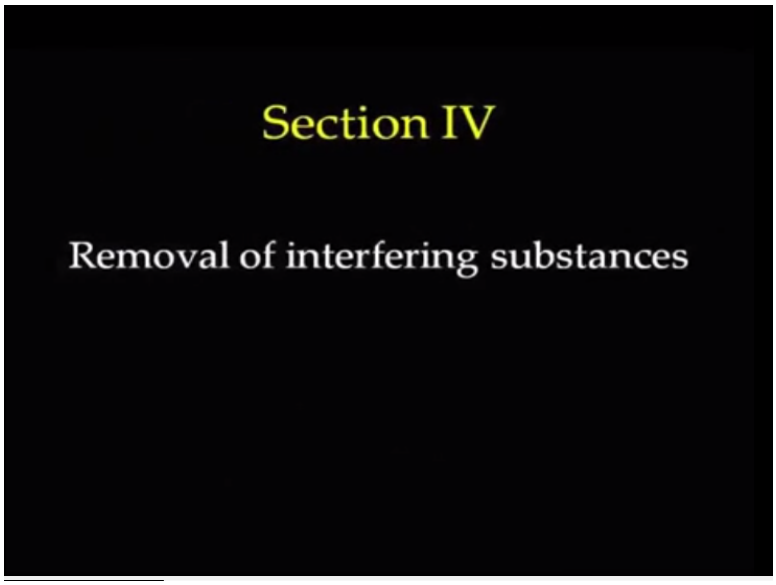

So now let us talk about how to remove the interfering substances because as I mentioned the interfering substances are very detrimental for your any proteomic application whether you

want to perform 2-dimensional gel electrophoresis or you want to go for directly LC-MS based applications or you want to do surface plasmon resonance, label free based proteomic techniques or you want to apply on protein microarrays.

In all of these methods, different type of interfering substances will be very detrimental. So let us talk about what are these different type of interfering substances and how we can get rid of them? It is probably not possible to completely remove these interfering substances, but at least partially if we can remove them that will ensure the success of your further experiment.

So we need to remove the contaminants and these contaminants include salt, a small ionic component, polysaccharide, nucleic acid, lipids and many other small interfering components.

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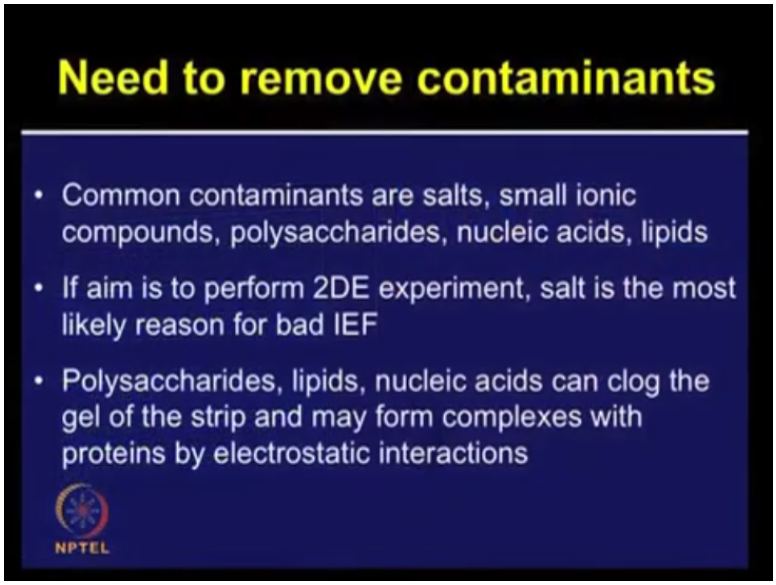

So if your aim is to perform 2-dimensional electrophoresis experiment, please ensure that you have removed salt very efficiently. Otherwise, it is going to interfere in the isoelectric focusing step. Now there are different types of contaminants, which may also affect the quality of your proteomic experiment such as polysaccharides, lipids, nucleic acids. These types of components can form complexes along with the proteins by electrostatic interactions.

And when you are separating the proteins by using gels they can form clog on the gel. So in the gel based proteomic methods, these types of components, these artifacts are going to affect the quality of the experiments very much.

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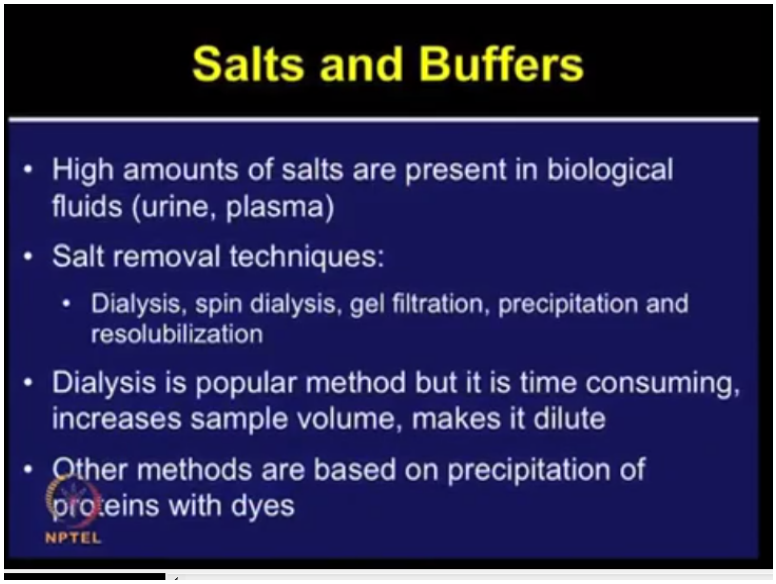

Let us first talk about salts and buffers. During your entire processing, you use different type of buffers and residual buffers are always there, which could affect the overall sample preparation. Now salt is also present due to the sample type, the kind or the nature of the sample itself. For example, if you are talking about biological fluid such are urine, plasma or serum, these samples are already very much rich in the salt content.

Similarly, there are different types of plant cells, which are quite rich in the salt contents. So if you want to remove these salts you have to follow different type of salt removal method. These methods could be dialysis, spin dialysis, gel filtration method, precipitation and resolubilization.

Dialysis is one of the most commonly used method in which in a dialysis membrane you can add your sample, the protein along with salt or other interfering components and in the water or different buffer condition slowly the salt can be eliminated out. Only problem here is your sample volume can be very dilute and it can become very much so if your application requires concentrated solution and with a small quantity.

Then this may not be the very popular choice for doing the application for the proteomics. There are other methods based on the precipitation of proteins with dyes. Those are also commonly used depending upon your proteomic application. Then there are nucleic acid contaminations. Nucleic acids, if they are present as a trace amount or in the more amount in the protein extract, they can increase the sample viscosity.

And later on, if you are separating the proteins on 2-dimensional electrophoresis gels, it is going to show background smear or different type of streaking.

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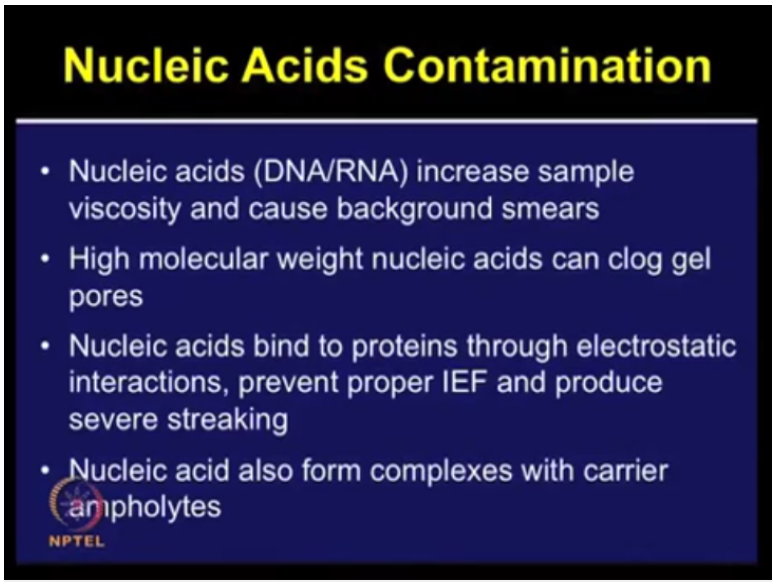

The high molecular weight nucleic acid such as DNA or RNA they can clog the gel pores, which will be used for gel-based proteomic applications. These nucleic acids can bind to the proteins through electrostatic interactions and it will interfere in the isoelectric focusing step and it may ultimately result into severe streaking. The nucleic acid can also form complexes with the carrier ampholytes, which are added during the isoelectric focusing step.

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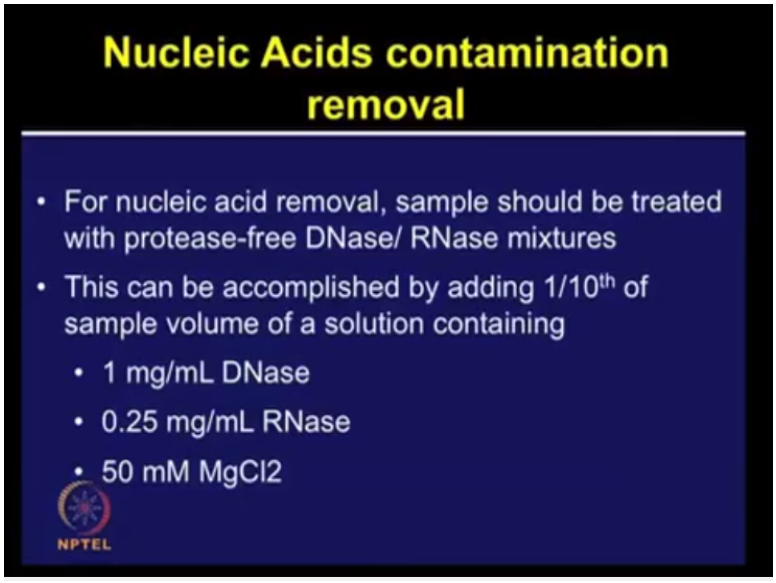

So how to remove the nucleic acid contamination removal? To remove the nucleic acid contamination, your sample should be treated with protease free DNAs or RNAs mixtures and you can accomplish this by addition of 1/10th of the sample volume of the solution

containing 1 mg per ml of DNase, 0.25 mg per ml of RNase and 50 millimolar of magnesium chloride.

Please perform these steps in the cold conditions, try to keep this reaction in ice so that you are effectively performing the nucleic acid contamination removal.

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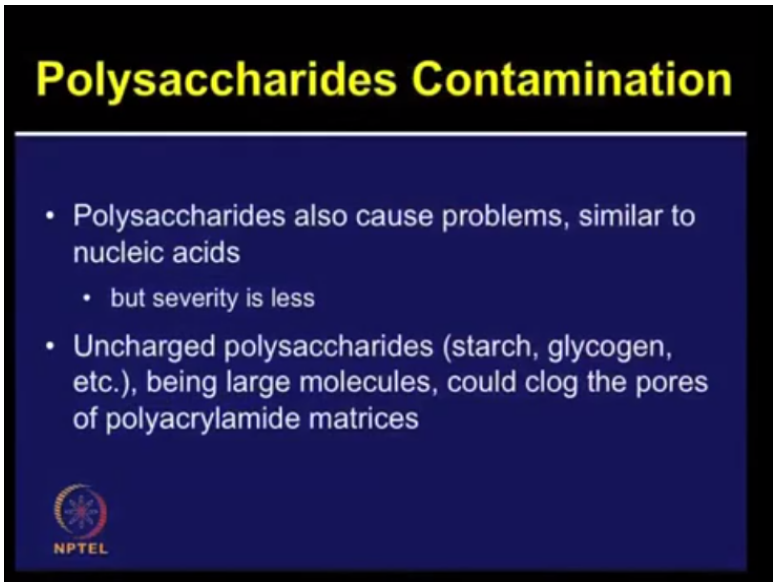

So now let us talk about polysaccharide contamination. Similar to the nucleic acid, polysaccharides they may also cause problem; however, the severity will be less as compared to the nucleic acid contamination. The different type of uncharged polysaccharides such as starch, glycogen, and these are very large molecules so they can clog the pore of polyacrylamide matrices similar to what we talked for the nucleic acid.

So how to remove these type of polysaccharide contamination?

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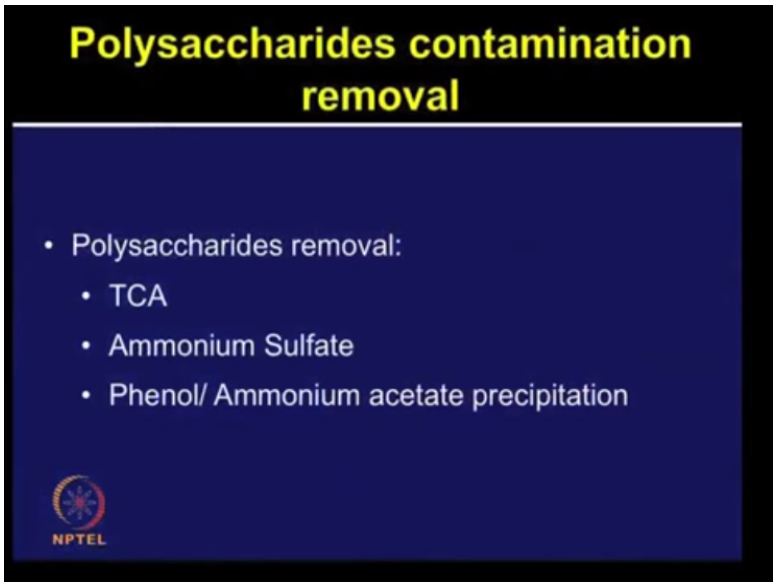

During the precipitation step itself lot of polysaccharides they get removed so TCA trichloroacetic acid, ammonium sulfate or phenol or ammonium acetate precipitation they are efficient ways of removing the polysaccharide contamination. Lipids, they are very important.

They are used for various type of biological problems to probe, but if your context is to study about the proteins you would like to get rid of any lipids or any other nucleic acid or other interfering components because you just want to analyze only proteins. Since we are talking about sample preparation for the proteins and proteomic applications, you would like to get rid of lipids.

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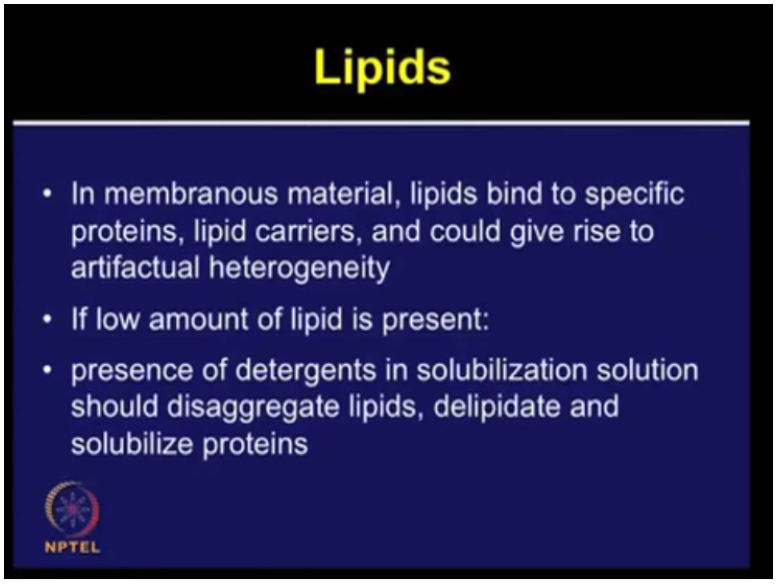

In membranous material, the lipids bind to the specific proteins such as lipid carriers and it could give rise to artifactual heterogeneity. If very low amount of lipid is present in your protein sample, the presence of detergents in solubilization solution should disaggregate the lipids, delipidate and solubilize the proteins, but if your samples are very much rich in lipid contamination.

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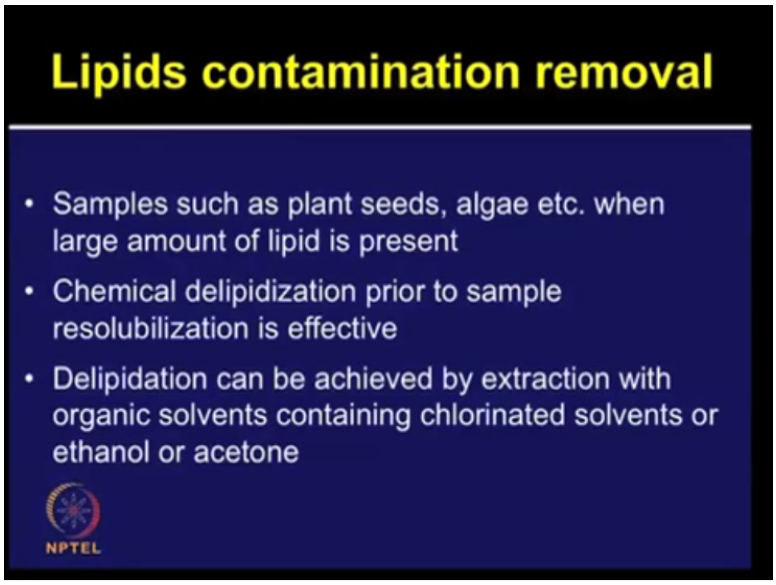

Few samples which are interestingly rich in the lipids such as plant seeds or algae so you need to treat the samples by using chemical delipidizaton prior to the sample resolubilization. This process of delipidation can be achieved by extraction with organic solvents containing chlorinated solvents or ethanol or acetone alone, but this step becomes very crucial if you are analyzing the proteome of those biological samples, which are very rich in lipid components.

Now let us talk about ionic detergents. SDS, sodium dodecyl sulfate, this is one of ionic detergent, which forms very strong complex with proteins. We will talk about SDS and how it can be used for gel electrophoresis such as SDS-PAGE.

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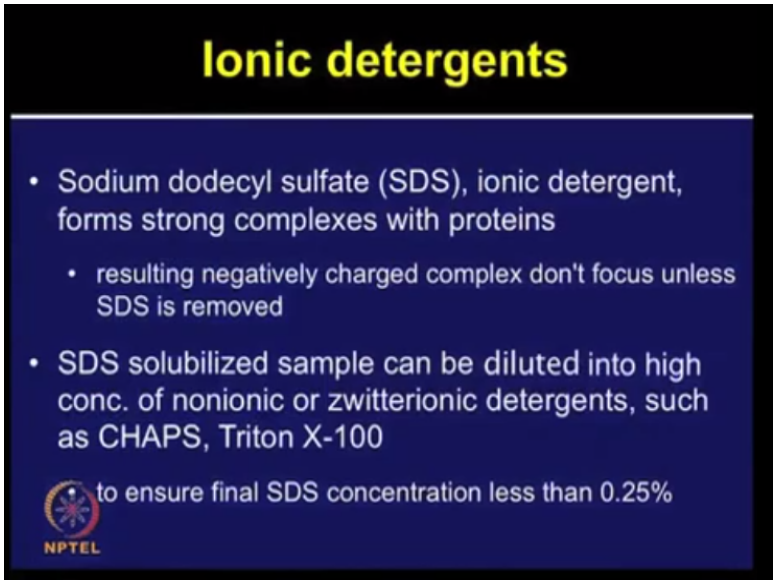

But in this context when you are talking about protein preparation, SDS is one of the very efficient compound; however, if you later on your aim is to perform protein separation by using isoelectric focusing or other gel based methods, it is going to create some problems because it will result into the SDS will bind to the proteins, which will result to the negative charge complex.

And that will not focus unless the SDS is removed from the protein sample mixture. SDS solubilized sample can be diluted by using high concentration of nonionic or zwitterionic detergents, which are CHAPS, Triton X-100 and we have talked about all different type of detergents in the last lecture, so you can try different type of nonionic or zwitterionic detergents.

This step will ensure that the final SDS concentration is less than 0.25% otherwise your isoelectric focusing will be hampered by this excess of SDS molecule.

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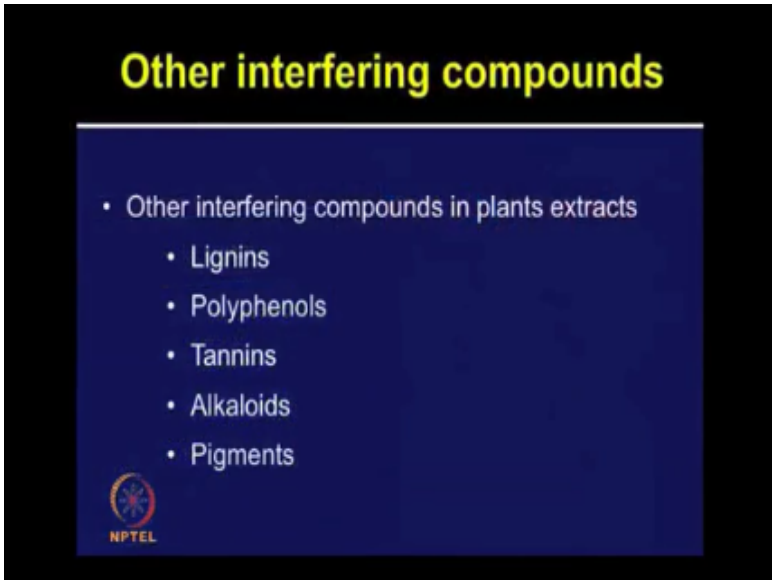

So we have talked about different type of interfering components. In the last (()) (36:19) I will just say that this is not the end of the list. There are many other interfering compounds present and depending upon your unique biological sample, you may encounter more and more interfering compounds and you may have to come up with new creative ways of removing those interferences.

So that your proteomic study can be performed with very high quality. There are few interfering compounds, which are also present in the plant extracts such as Lignins, polyphenol, Tannins, alkaloids, and pigments. I will talk about some of these in more detail when I will talk in the next class about the case study how to perform plant proteome analysis.

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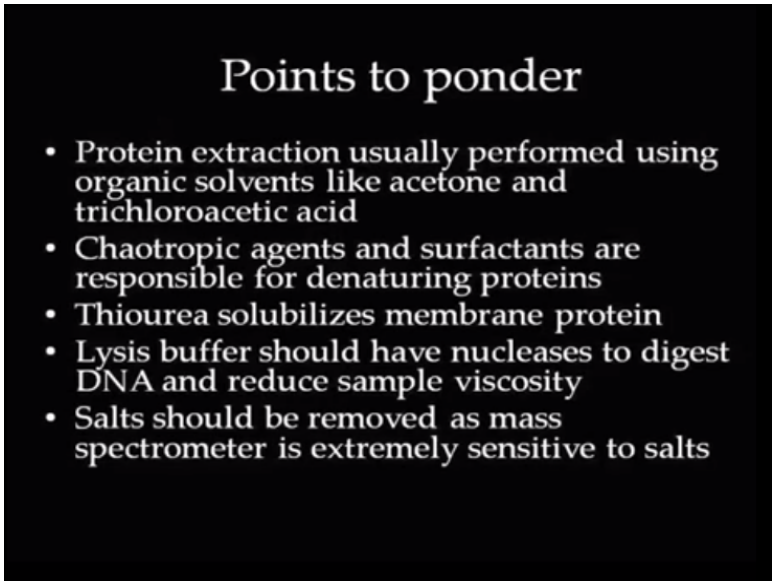

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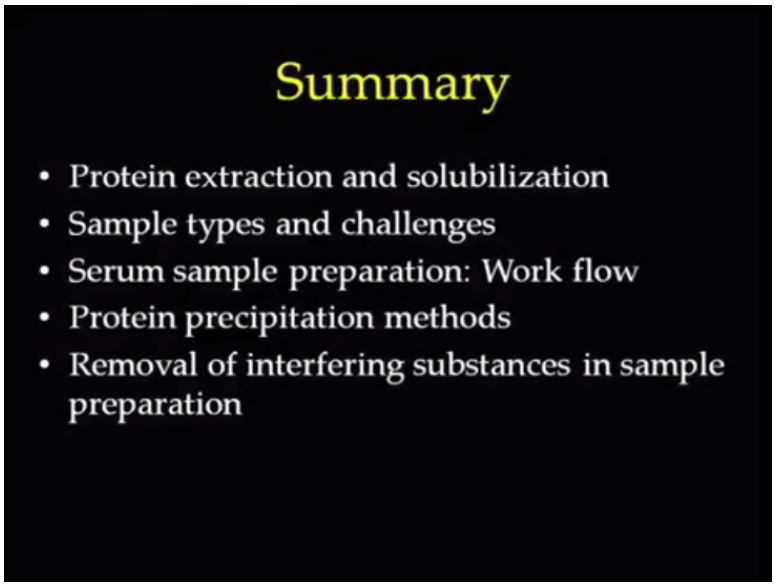

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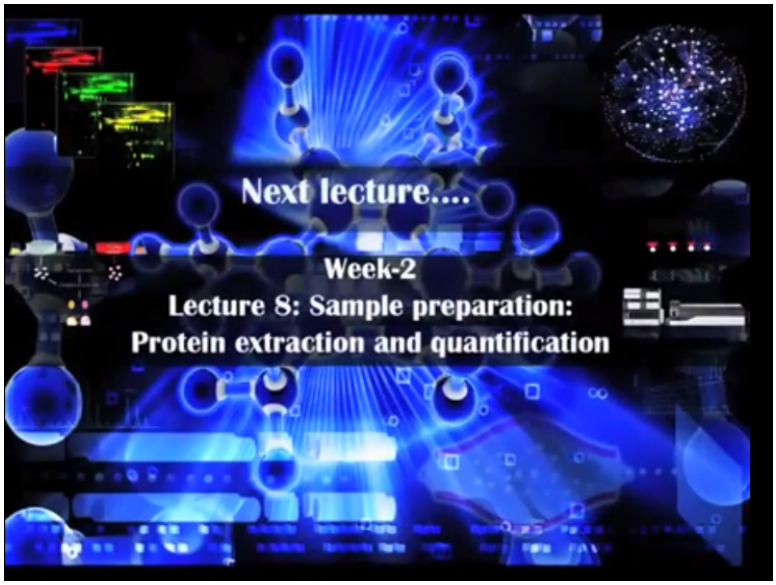

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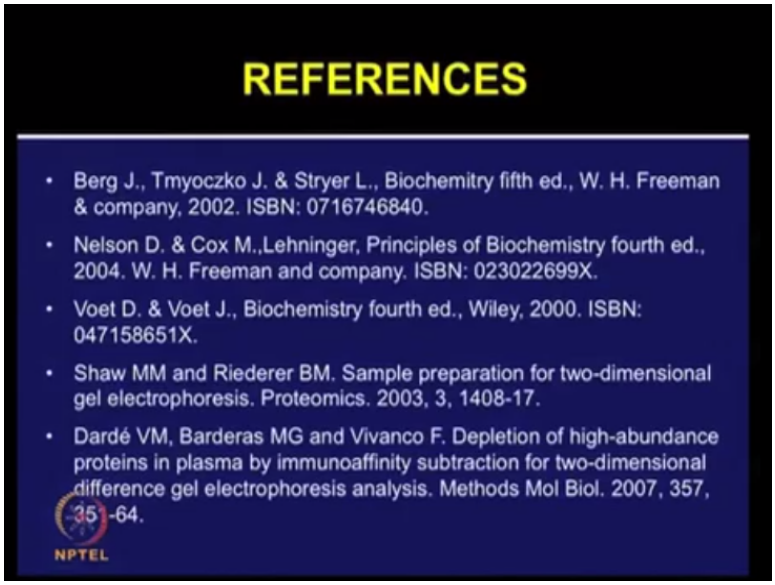

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