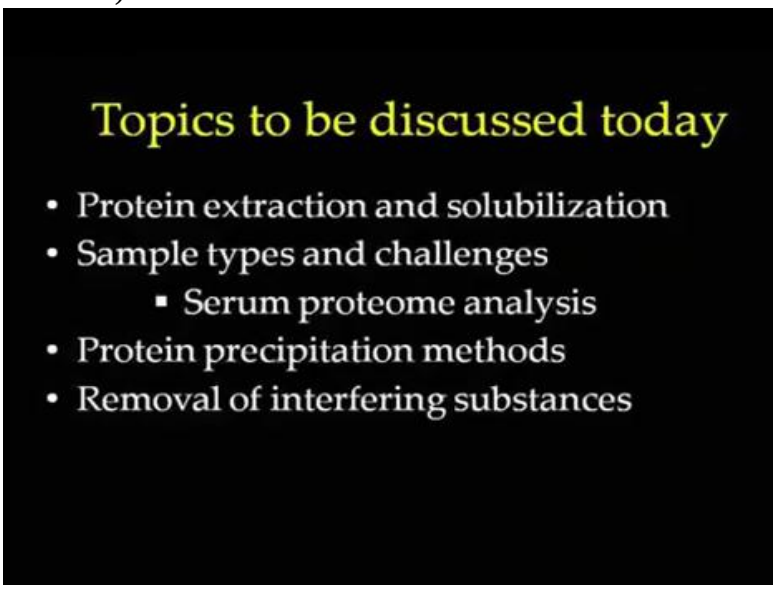
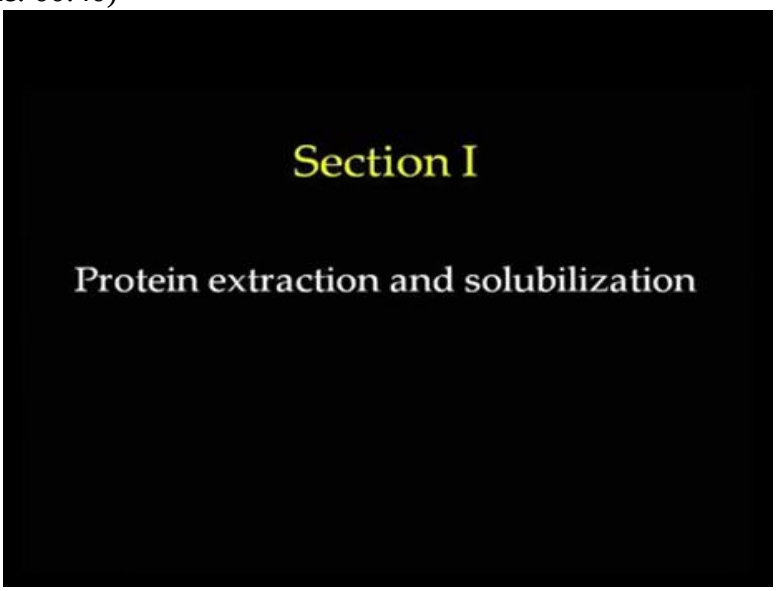
Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 03 Lecture Number 9

(Refer Slide Time: 00:15)



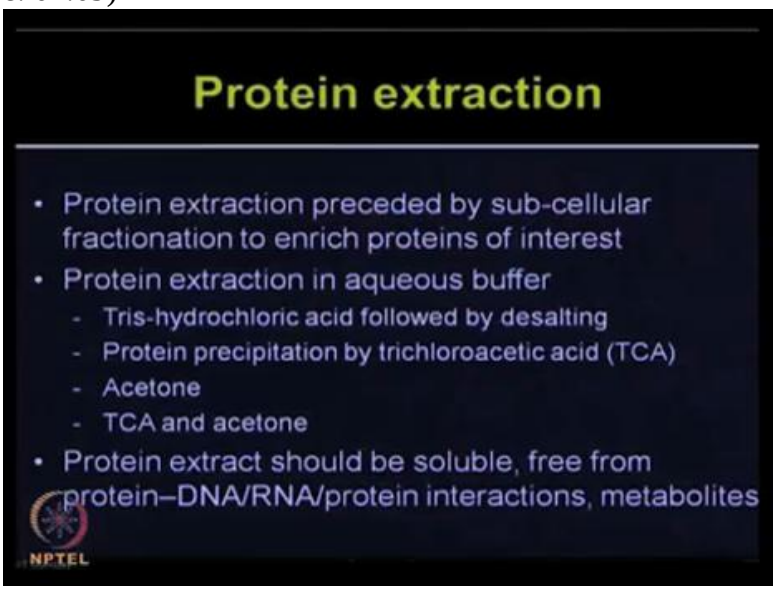
In today's lecture I will first talk about protein extraction and solubilization, few precipitation methods which are commonly used for different types of protein sample preparation. Then we will talk about the removal of interfering substances.

(Refer Slide Time: 00:40)



Let’s talk about protein extraction and solubilization. Now this step will be more towards talking about gel-based proteomics where solubilization will be more important.

(Refer Slide Time: 01:09)

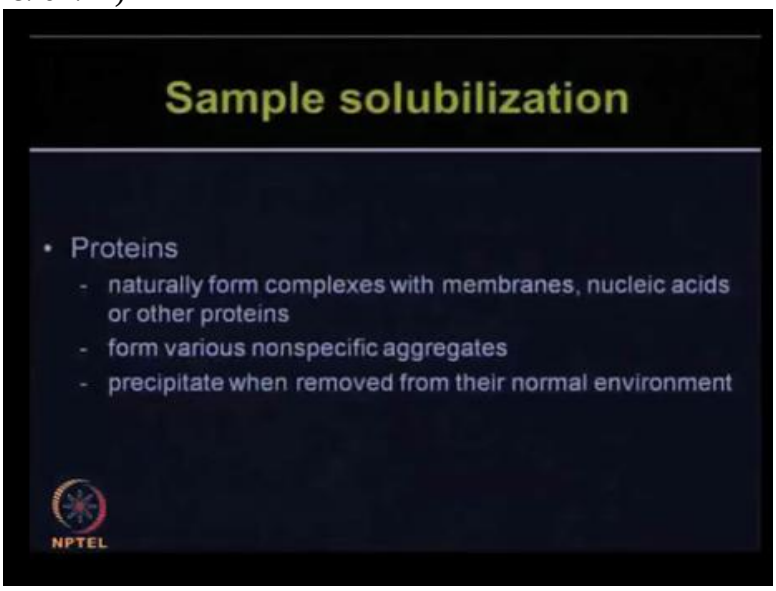


So protein extraction after performing the sub-cellular fractionation so that the proteins can be enriched which is...you are going to be analyzing in your experiment. So protein extraction in the aqueous buffer, one can follow different types of procedure; either use Tris hydrochloric acid followed by the desalting method, protein precipitation by TriChloro acetic Acid or TCA or acetone alone or trichloro acetic acid and acetone.

I will give you more specific composition and recipe when I will talk to you about specific types of examples how to perform protein extraction for serum, bacteria and plants.

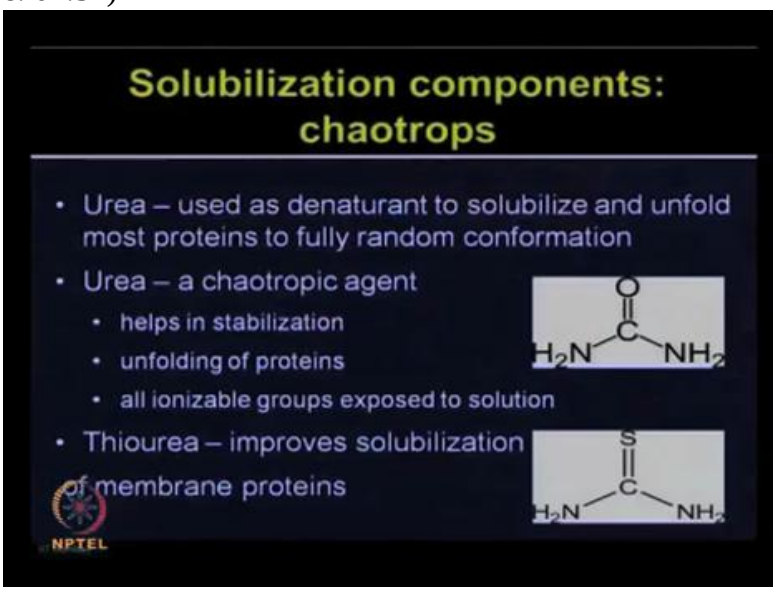
So protein extracts should be soluble. It should be free from protein to protein interactions, protein to DNA or protein to RNA interactions. Similarly there are different types of other cellular components present and those should be effectively removed. No metabolites should be interfering in your protein extract.

(Refer Slide Time: 02:27)



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 different components being used in solubilization. Let's discuss one by one.

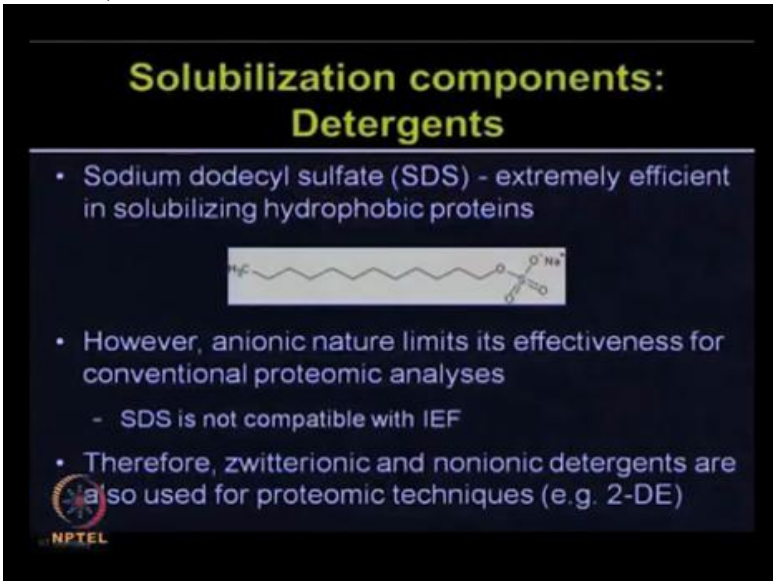
(Refer Slide Time: 02:52)



First of all, let's talk about chaotrops, urea and thiourea. Urea is used as denaturant which can solubilize and unfold most of the proteins to fully random conformations. Urea is a chaotropic agent which helps in stabilization of the proteins, unfolding proteins so that all the ionizable groups are exposed to the solution.

Thiourea improves solubilization of membrane proteins more specifically. Mostly both urea and thiourea are both mixed together during the solubilization step.

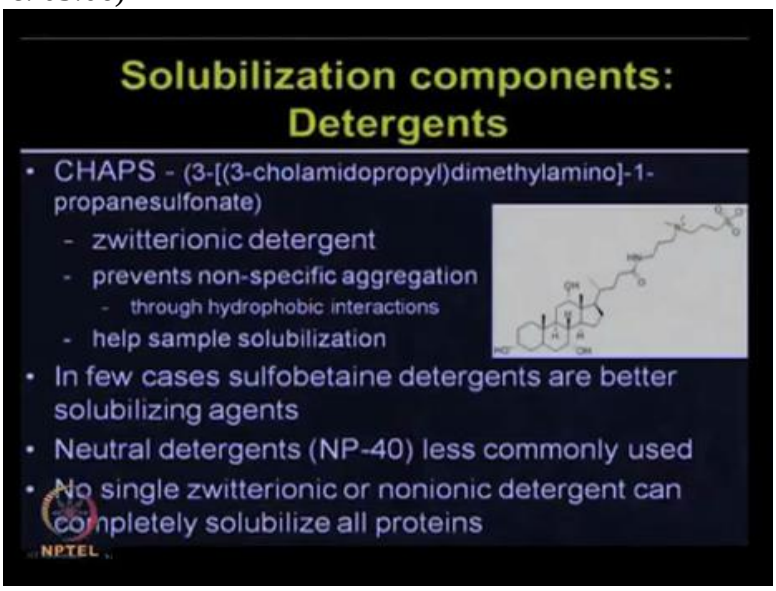
(Refer Slide Time: 03:51)



There are different types of detergents which are also used in solubilization such as SDS or Sodium Dodecyl Sulfate which is very efficient in solubilizing hydrophobic proteins; if you want to solubilize hydrophobic proteins 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 preparing your protein preparation to perform two-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 2DE or DIGE or different types of other advanced gel-based proteomic applications where you cannot use SDS, zwitterionic and non-ionic detergents are used for such applications.

(Refer Slide Time: 05:06)



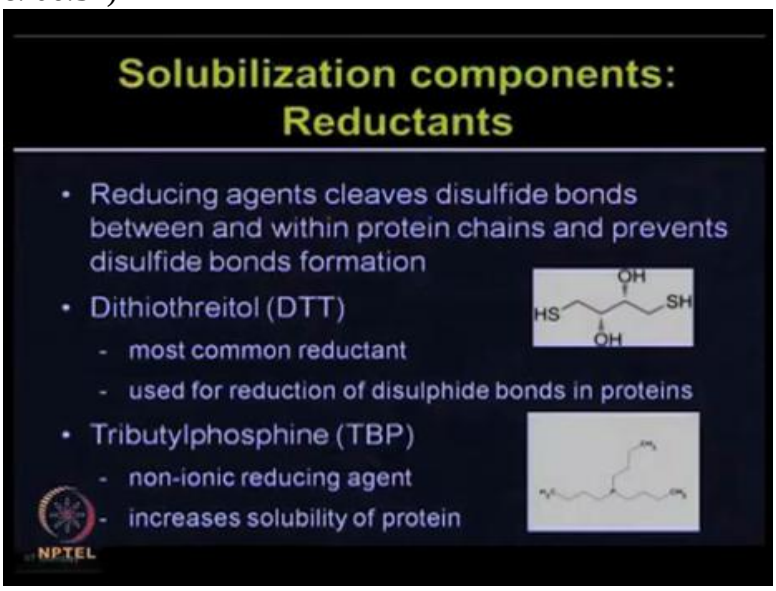
CHAPS, one of the zwitterionic detergents is most commonly used detergent used in protein solubilization when your objective is to perform two-dimensional electrophoresis experiments. It prevents non-specific aggregations through hydrophobic interactions and it helps in sample solubilization.

Depending upon your sample type, different types 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 NP40 although they are less commonly used.

So, one cannot provide you a list of most effective solubilization agents. No single zwitterionic or non-ionic detergent can completely solubilize all the proteins. So depending upon your sample type and if you know your sample is enriched in specific types of proteins you need to try different types of detergents.

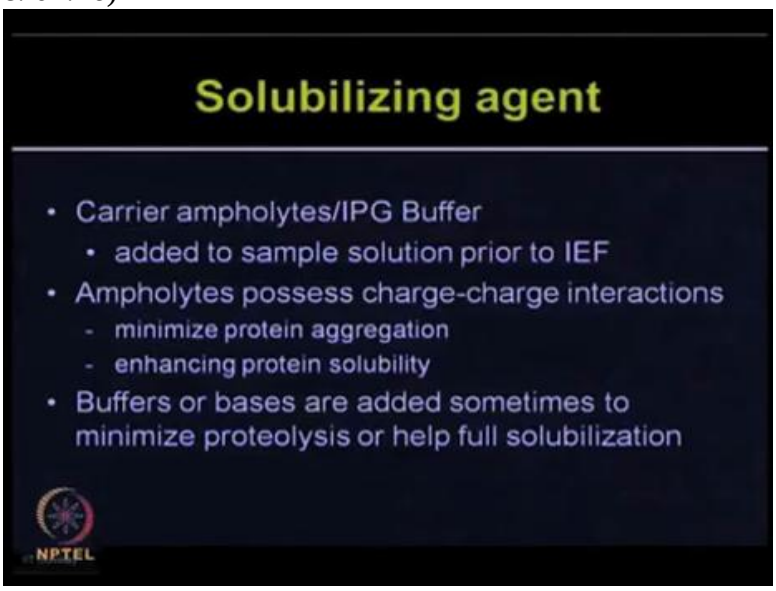
Now let's talk about reductants. In the solubilization, reducing agents cleave the disulfide bonds which are present between and within the protein chains; and it prevents the disulfide bonds formation.

(Refer Slide Time: 06:37)



Most commonly used reductants are dithothreotal 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 non-ionic reducing agents, another very commonly used reducing agent when one's aim is to increase solubility of the proteins. Often it is used in the 2DE based, gel-based proteomic applications.

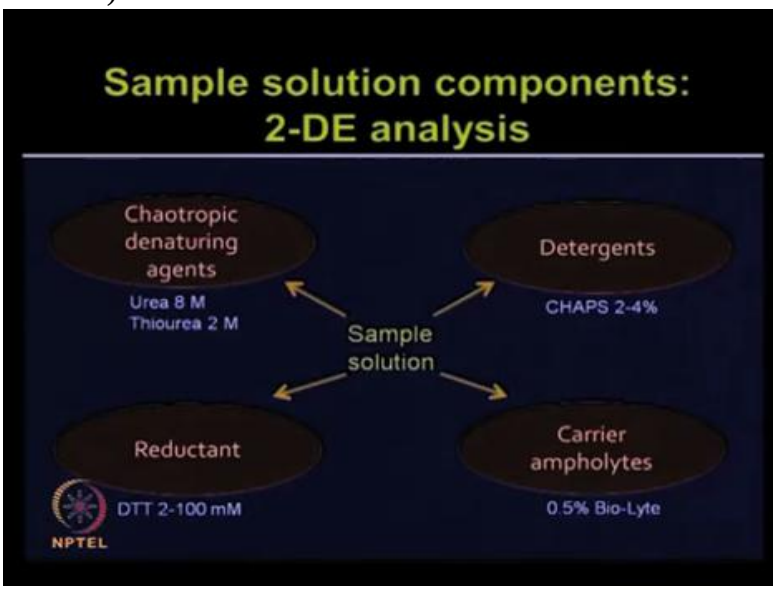
(Refer Slide Time: 07:26)



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

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

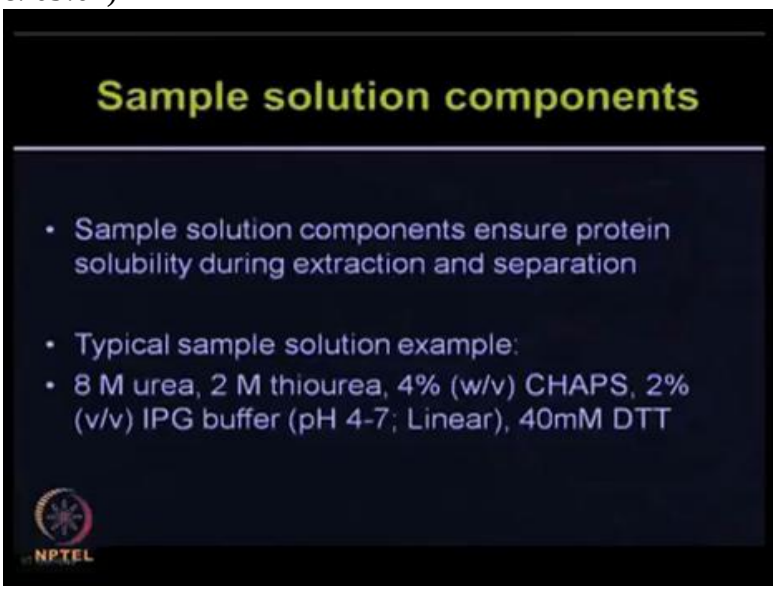
(Refer Slide Time: 08:25)



So if your aim is to perform a two-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%.

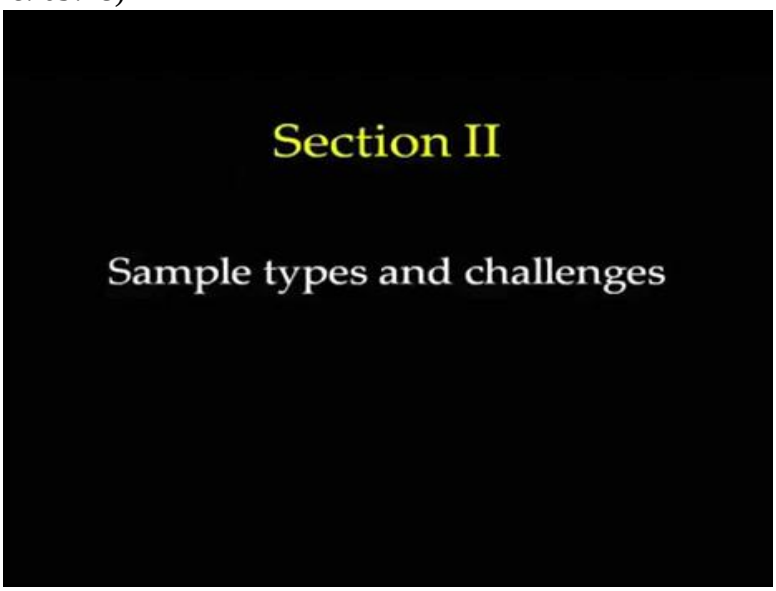
Commonly used reductant include DTT or beta Mercaptoethanol 2 to 100 milli molar, and carrier ampholytes in the concentration of 0.5% of biolyte.

(Refer Slide Time: 09:01)



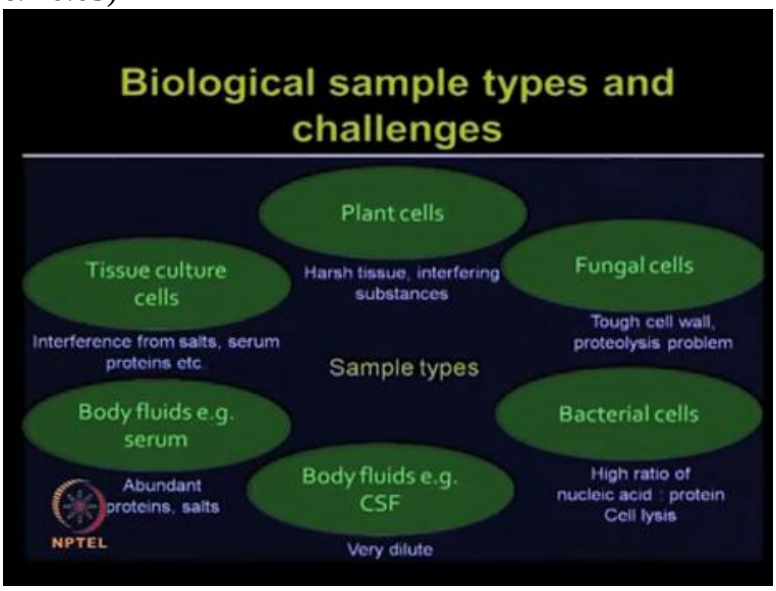
So the sample solution components ensure that the protein solubility is good during the extraction and protein separation. A typical sample solution for the gel-based 2DE application includes 8 molar of urea, 2 molar of thiourea, 4% CHAPS, 2% IPG buffer, 40 milli molar of DDT as well as few other small components depending upon your sample type.

(Refer Slide Time: 09:28)



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

(Refer Slide Time: 10:05)

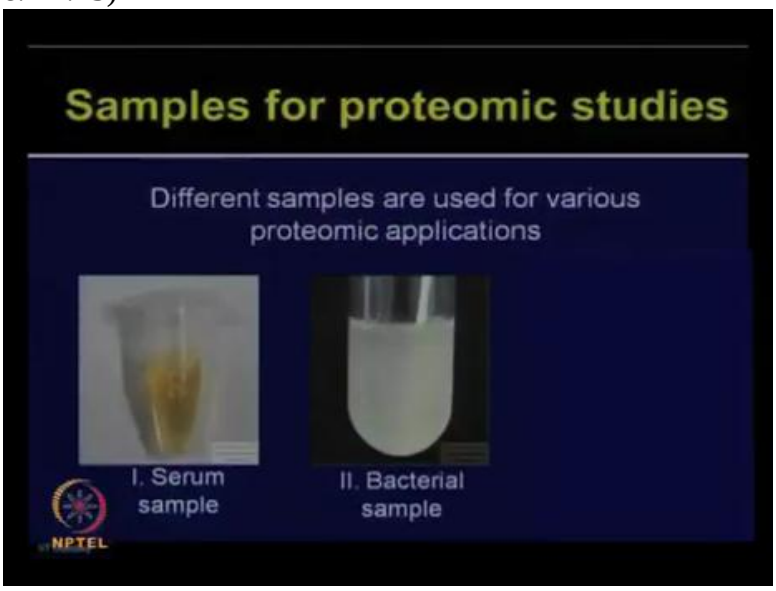


So, one needs to get rid of those components if you want to perform a good sample preparation from the tissue culture cells. If you are interested in plant cells to extract the protein, those are very hard tissue and there are various interfering substances present there… phenolics and other salts. Now you need to get rid of those interfering substances.

Fungal cells such as yeast or other types 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 these samples.

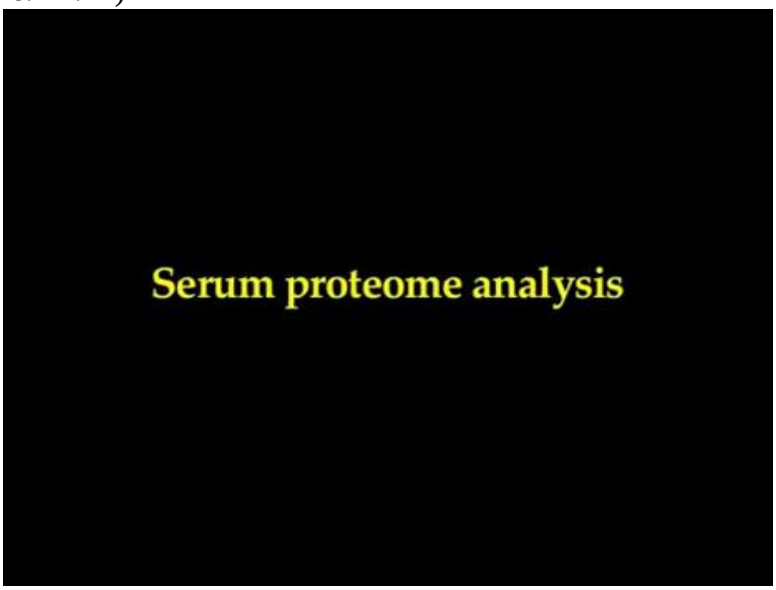
The bacterial cells, they have high ratio of nucleic acid to protein and cell lysis is also very tedious. Body fluids such as cerebrospinal fluid, they are very dilute...so if you want to perform proteomic experiments on CSF, you need to concentrate your samples. Body fluids such as serum, those are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

(Refer Slide Time: 11:45)



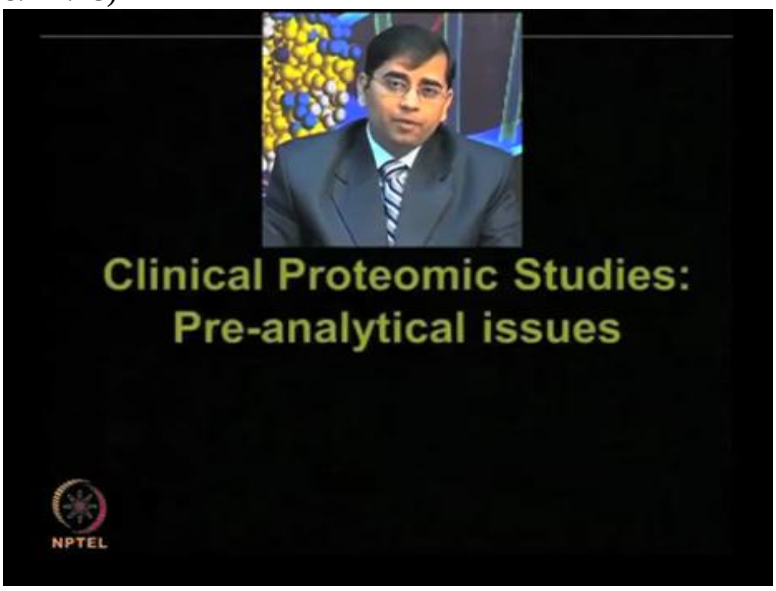
So although the sample diversity is very much, samples are very complex, I will still try to take two representative examples, one the serum sample obtained from human, second the bacterial sample which will be taken from Bacillus species by showing you the protein extraction and solubilization methods. I will try to give you the diversity and different types of methods being used to perform various types of proteomic applications.

(Refer Slide Time: 12:11)



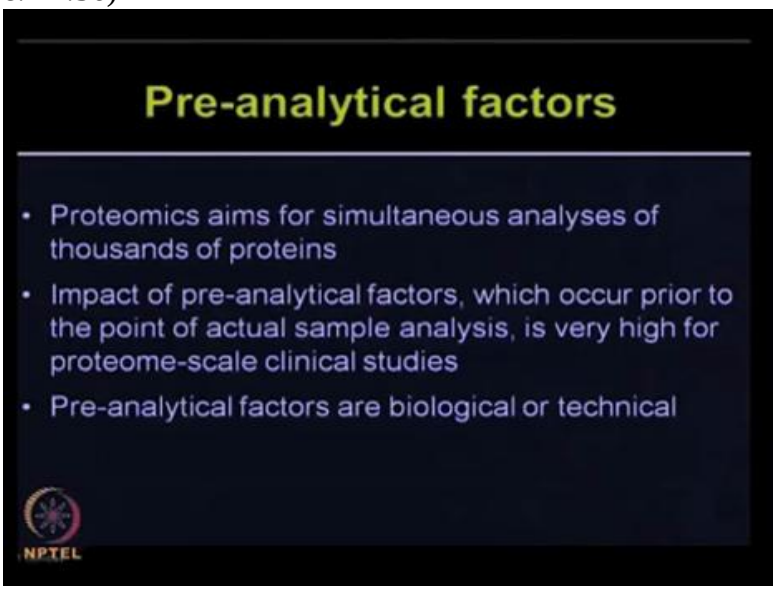
So first I will start with the serum proteome analysis. But before we talk about how to perform serum proteome analysis, it means all the proteins which are present in the human serum. First of all, how to obtain the samples, how to store these samples, how to minimize various types of pre-analytical variations, that is one of the various important considerations.

(Refer Slide Time: 12:48)



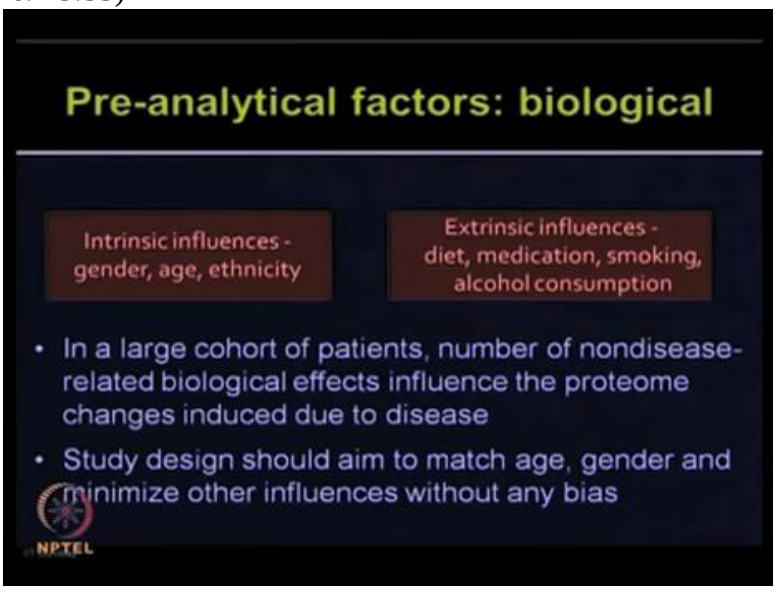
So before we talk about how to really process the sample, let's talk about different types of clinical issues involved in these types of samples for the pre-analytical factors.

(Refer Slide Time: 12:56)



So proteomics, most of the applications are going to aim for simultaneous analysis of thousands of proteins of given clinical samples 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 variation or it could be due to the technical artifacts.

(Refer Slide Time: 13:35)



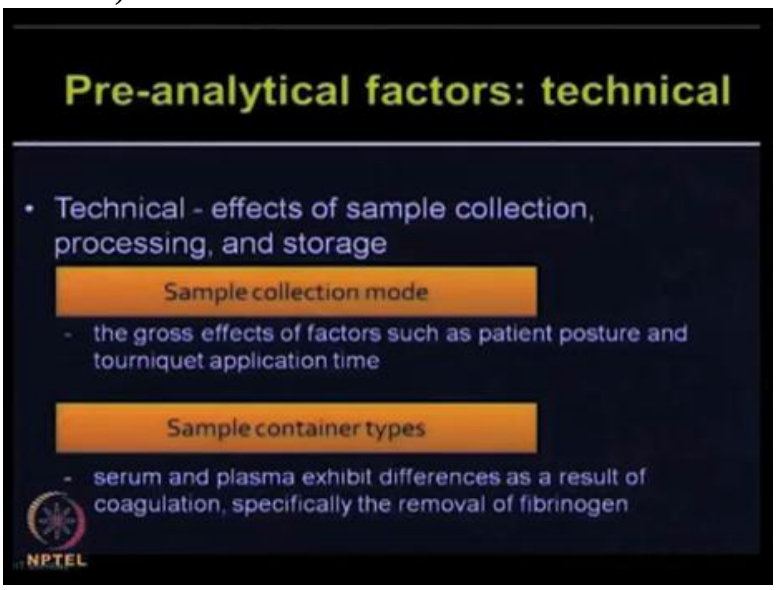
So your studies could be influenced due to intrinsic factors or due to the extrinsic factors. The intrinsic influences include gender, age, ethnicity. The extrinsic influences include diet, medication, smoking, alcohol consumption etc. So when you are designing a clinical study, you need to ensure that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

You should try to segregate your population with the different types of age, gender, try to...in the discovery phase, try to minimize these types of variations and try to perform your analysis with 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 types 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 designed should aim to match the age, gender and minimize the other influences without any bias.

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

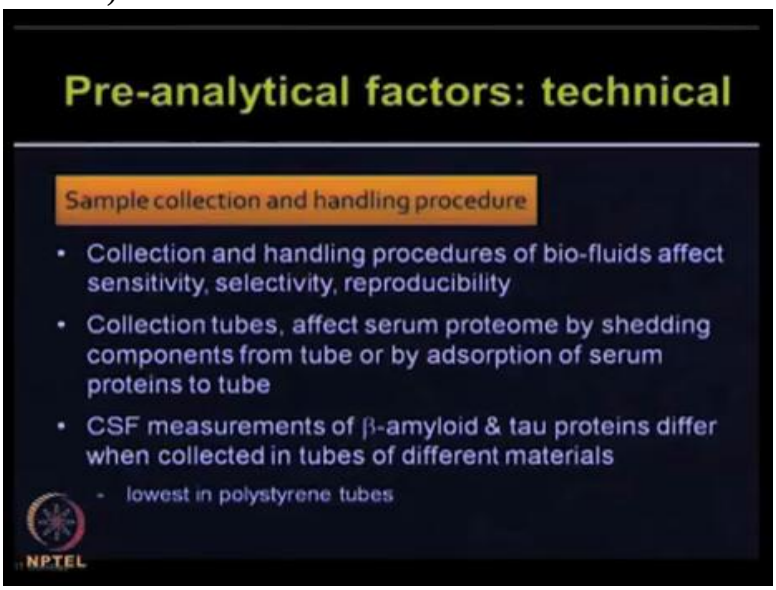
(Refer Slide Time: 15:57)



Then there are different types of technical artifacts. How to collect the samples, how to process the sample and how to store those? The sample collection mode, the gross effects 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, specifically the removal of the fibrinogen. So sample collection and handling procedure, one has to pay attention.

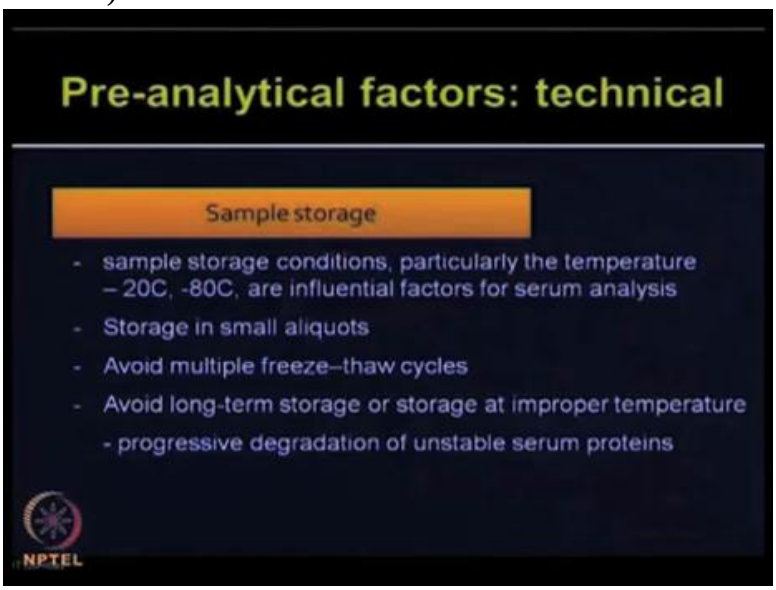
(Refer Slide Time: 16:43)



The collection and handling procedure of biofluids 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 types of tube materials. The shedding components from the tube or adsorption of the serum proteins to the tubes will, in some way, influence the proteome analysis.

The several spinal fluid measurements 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 types 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 types of sample tubes being used for the collecting your biological samples.

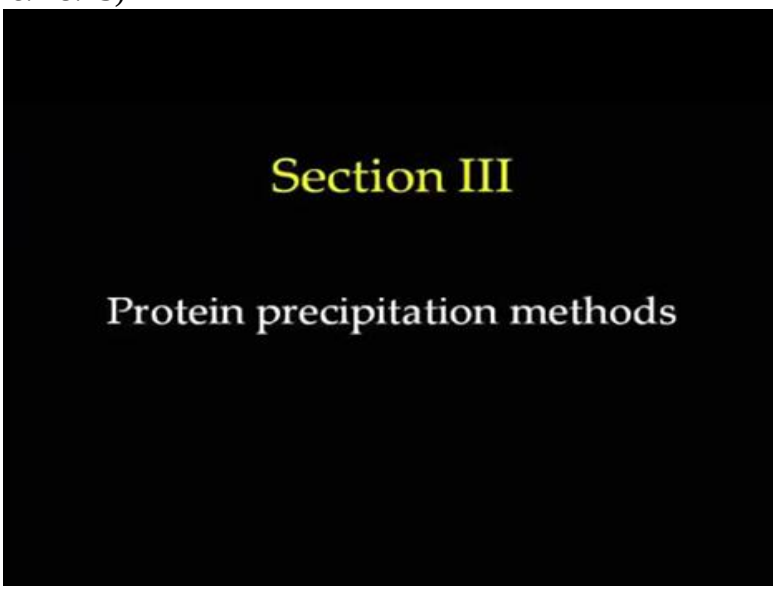
(Refer Slide Time: 17:51)



Sample storage is another very crucial factor. Whether you are storing your clinical samples in -20 degrees 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 stored, all of these small variations actually influence the sample analysis, the proteome analysis later on.

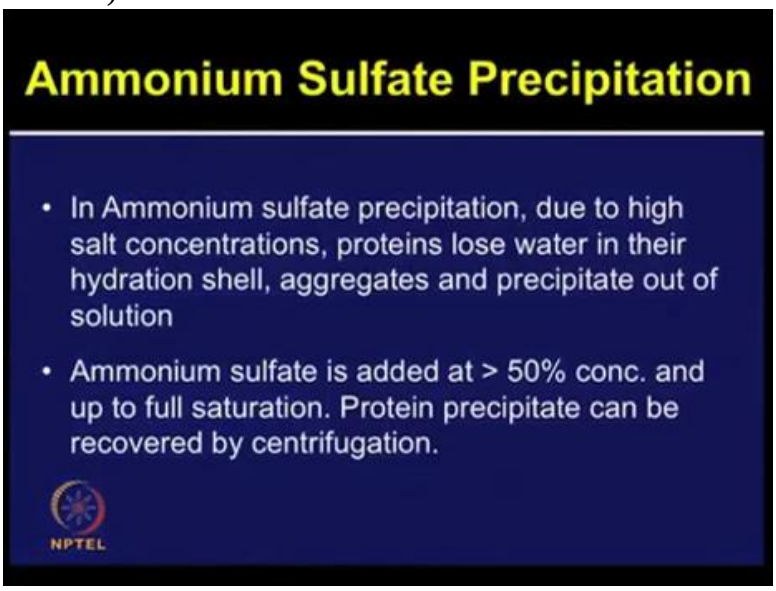
So avoid multiple freeze-thawing of your samples. Store the samples in a small aliquot so that you don't have to freeze-thaw the whole samples together. Avoid very long term storage or storage at the improper temperature. Try to use as freshly stored samples as possible because if the samples are used and stored for the long time, the progressive degradation of unstable serum proteins may occur.

(Refer Slide Time: 18:45)



So let's talk about the first, 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 recommendations about which ones can be more commonly used.

(Refer Slide Time: 19:10)

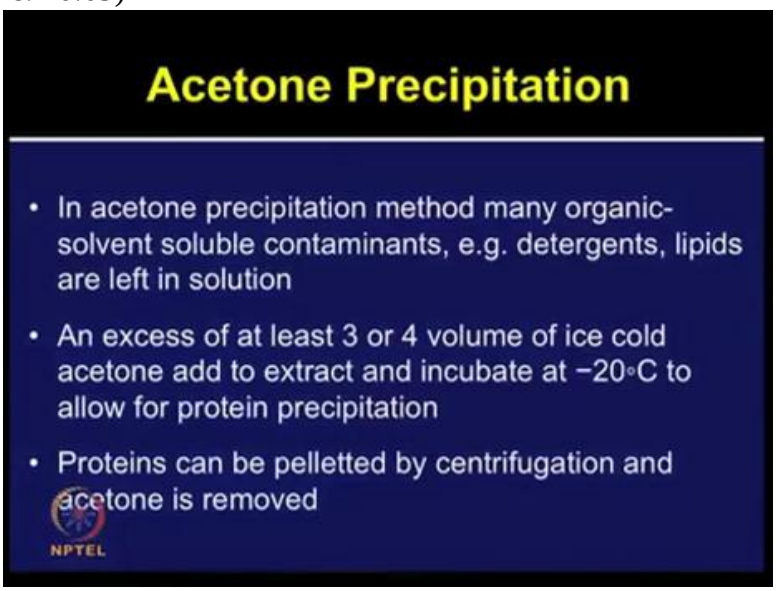


So let's talk about ammonium sulfate precipitation which is one of the most previously used methods for the classical way of performing the experiments. The ammonium sulfate precipitation was used, although its use is not so common when you are preparing samples for the proteomic application but this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

So ammonium sulfate precipitation due to high salt concentration, the proteins lose water. In the hydration cell, they aggregate and precipitate out of the solution. So if you add

ammonium sulfate, add greater than 50% of concentration and up to full saturation, protein precipitation will occur and then by performing the centrifugation step this can be recovered.

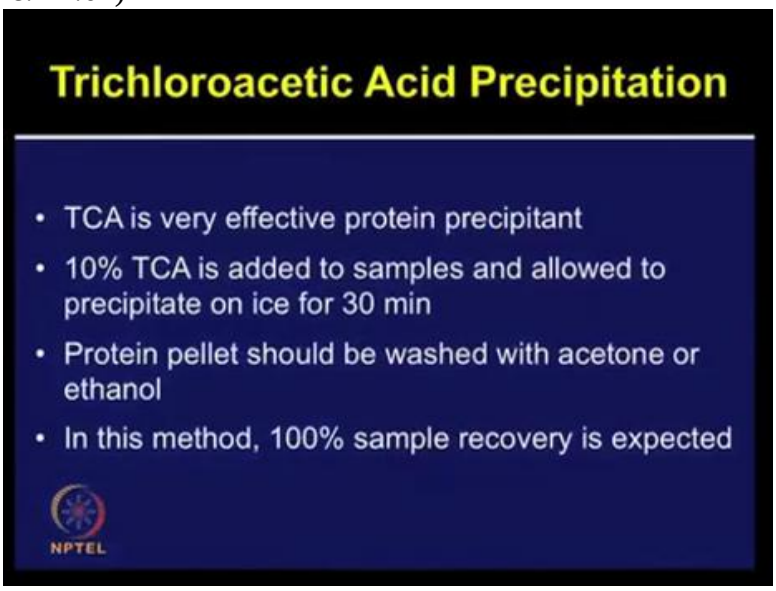
(Refer Slide Time: 20:09)



Now let's talk about the acetone precipitation. In this method, many organic solvents, 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 volumes of ice-cold acetone in your extract incubated 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 centrifugal 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.

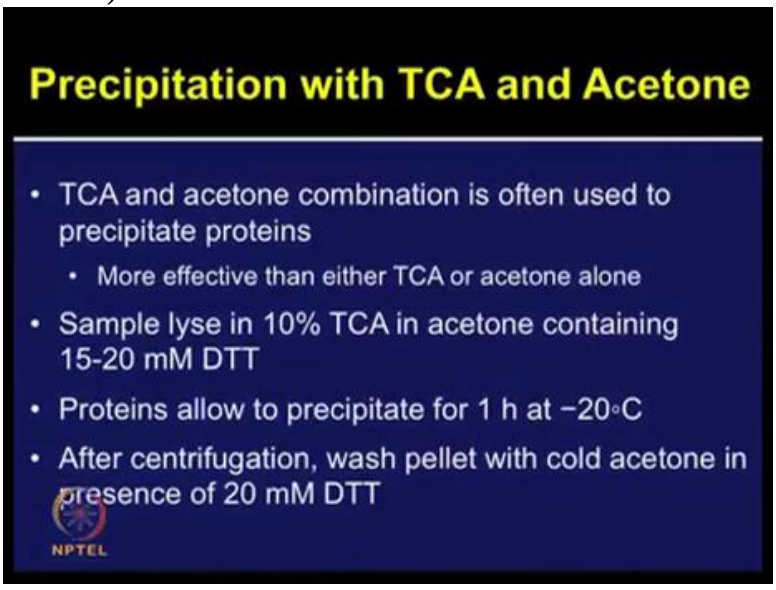
(Refer Slide Time: 21:07)



Now let's talk about TCA or trichloro acetic acid precipitation. TCA is one of the very effective protein precipitant. One can use 10 to 20% of TCA, usually 10% 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 TCS and acetone alone are very effective, people have tried combining both the methods together. It means addition of both TCA and acetone.

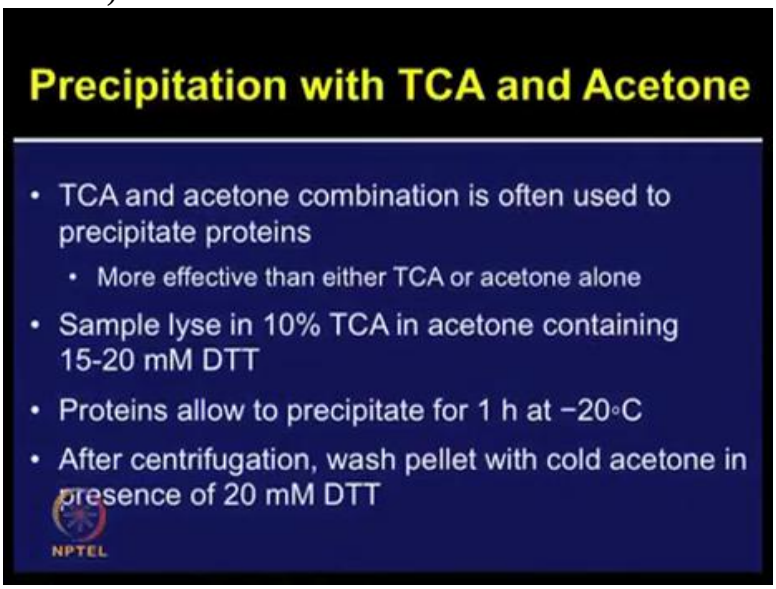
(Refer Slide Time: 22:12)



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 samples in 10% TCA made in acetone, and also add 15-20 milli molar of DTT.

Now allow the protein samples to precipitate for an hour or two hours at -20 degrees, centrifuge and then 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 milli molar DTT will be effective.

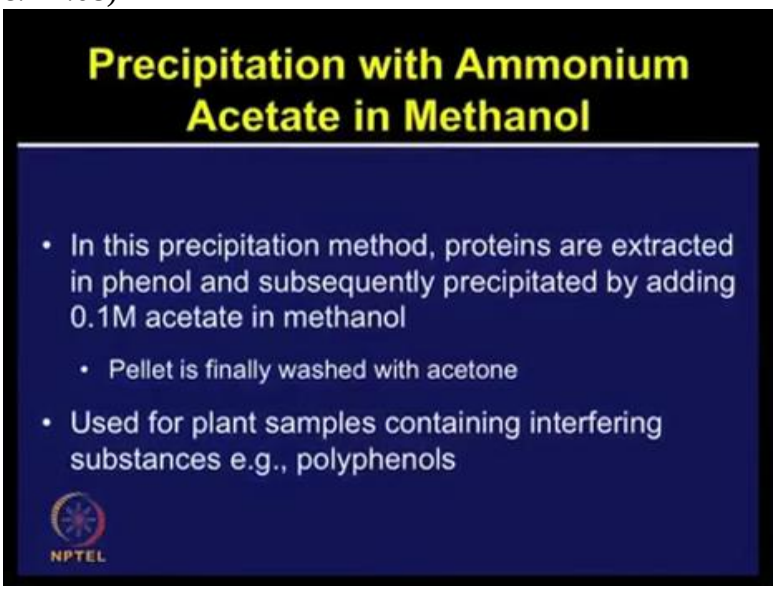
(Refer Slide Time: 23:26)



So this is very easy method to precipitate out the protein. First add 10% TCA with acetine 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 pellet

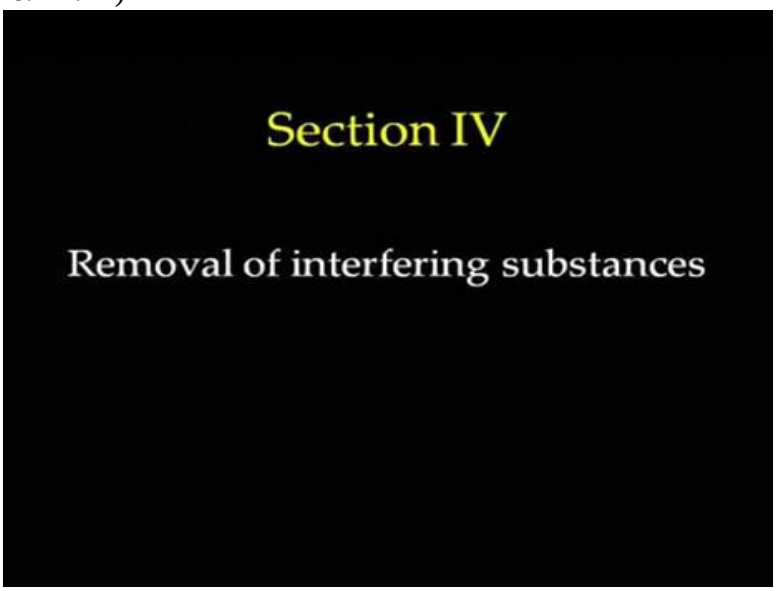
Now let's talk about one of the less commonly used method, precipitation with ammonium acetate in methanol. This is more commonly used when you are talking about some plant samples which are rich in polyphenol and other interfering substances.

(Refer Slide Time: 24:08)



So by using these precipitations, proteins are extracted in phenol and subsequently precipitated by addition of 0.1 molar acetate in methanol. Pellet can be finally washed by adding acetone. As I mentioned less commonly used method and for some specific application, people try different types of precipitation and washing steps.

(Refer Slide Time: 24:42)



So now let's talk about how to remove the interfering substances because as I mentioned the interfering substances are very detrimental for any proteomic application, whether you want to perform two-dimensional gel electrophoresis or you want to go for directly LCMS 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 types of interfering substances will be very detrimental. So let's talk about what are these different types 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.

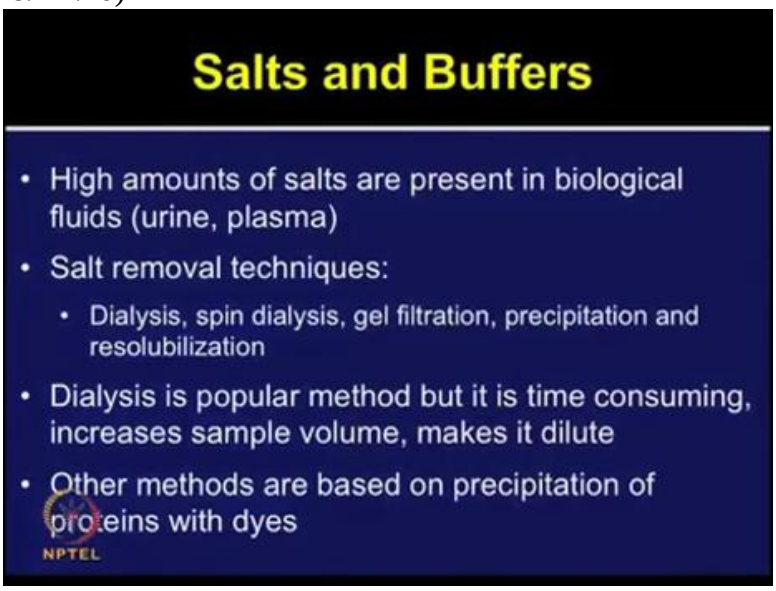
So we need to remove the contaminants. And these contaminants include salt, small ionic component, polysaccharides, nucleic acid, lipids and many other small interfering components.

(Refer Slide Time: 26:07)

So if your aim is to perform two-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 proteomics methods, these types of components, these artifacts are going to affect the quality of experiments very much.

(Refer Slide Time: 27:10)



Let's first talk about salts and buffers. During your entire processing, you use different types 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.

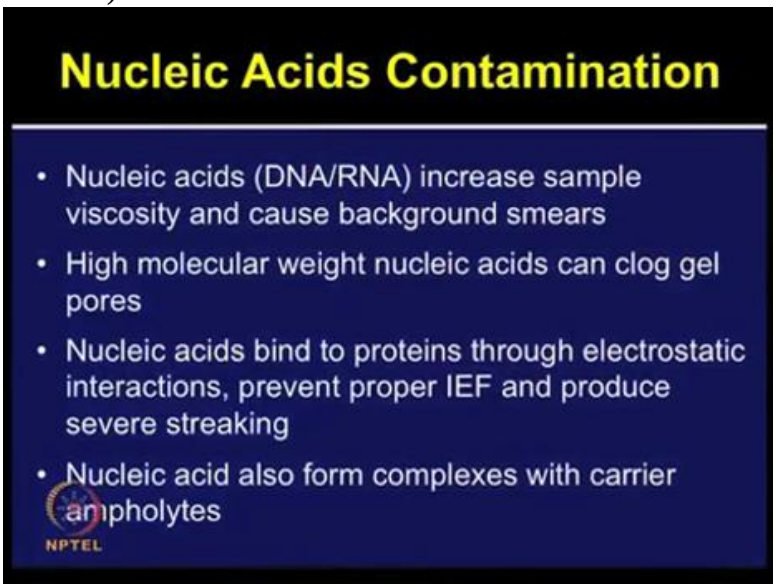
For example, if you are talking about biological fluid such as urine, plasma or serum, these samples are already very much rich in the salt content. Similarly there are different types of plant cells which are quite rich in the salt contents. So if you want to remove these salts, you have to follow different types of salt removal methods. These methods could be dialysis, spin dialysis, gel filtration method, precipitation and re-solubilization.

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 component. And in the water or 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 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 the amount in the protein extract, they can increase the sample viscosity. And later on, if you are separating the proteins on two-dimensional electrophoresis gels, it is going to show background smear or different type of streaking.

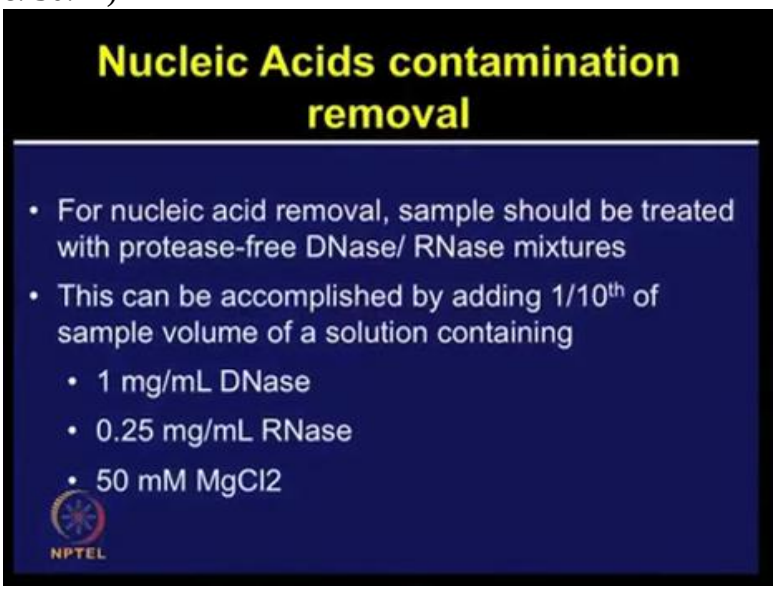
(Refer Slide Time: 29:44)



The high molecular weight nucleic acids 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.

So, how to remove the nucleic acid contamination removal? To remove the nucleic acid contamination your sample should be treated with protease-free DNase or RNase mixtures.

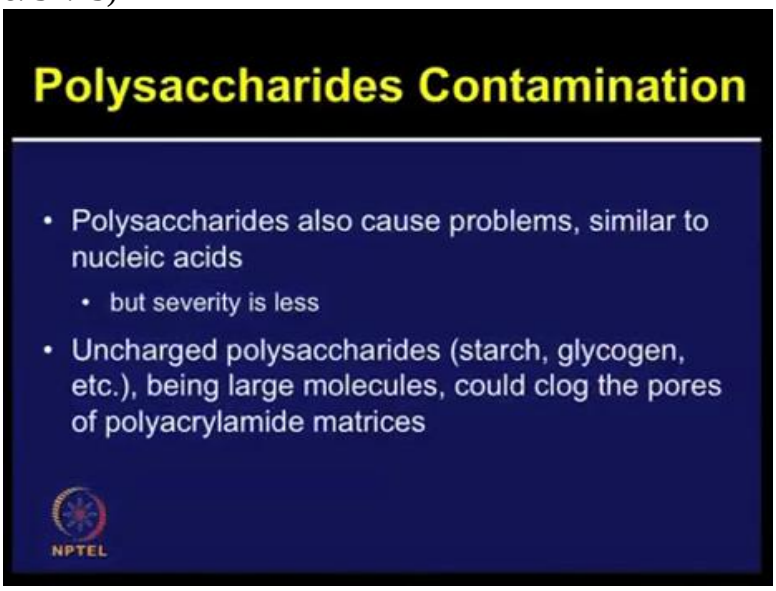
(Refer Slide Time: 30:47)



And you can accomplish this by addition of one-tenth of the sample volume of the solution containing 1 mg per ml of DNase, 0.25 milli gram per ml of RNase and 50 milli molar of magnesium chloride. Please perform these steps in the cold conditions. Try to keep this reaction iced so that you are effectively performing the nucleic acid contamination removal.

So now let's talk about polysaccharide contamination.

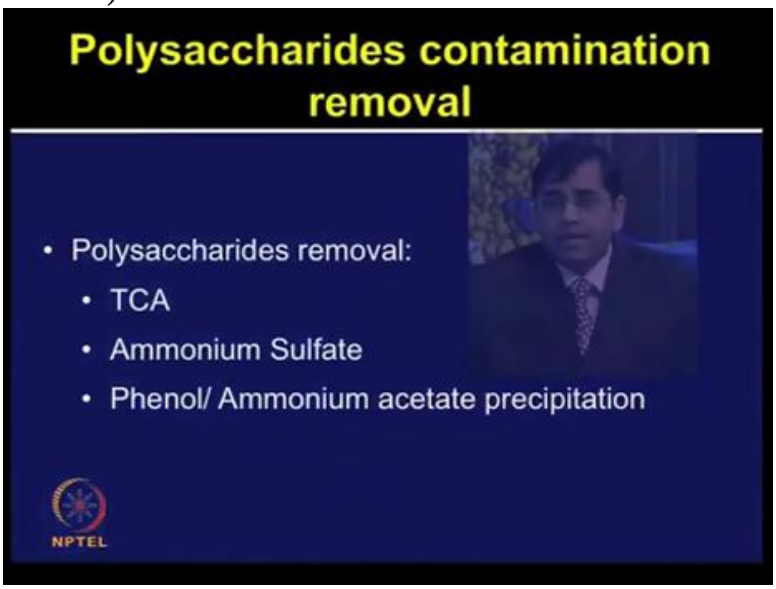
(Refer Slide Time: 31:23)



Similar to the nucleic acid, polysaccharides...they may also cause problem. However the severity will be less as compared to the nucleic acid contamination. There are different types 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 acids. So, how to remove these types of polysaccharides contamination?

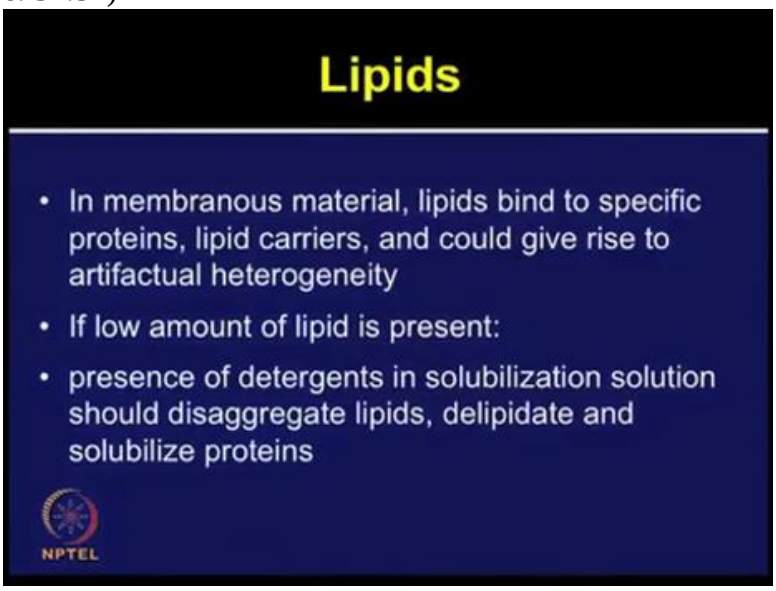
(Refer Slide Time: 32:02)



During the precipitation step itself, lots of polysaccharides, they get removed. So TCA, TriChloro Acetic acid, ammonium sulfate or phenol or ammonium acetate precipitation, they are efficient ways of removing the polysaccharide contamination.

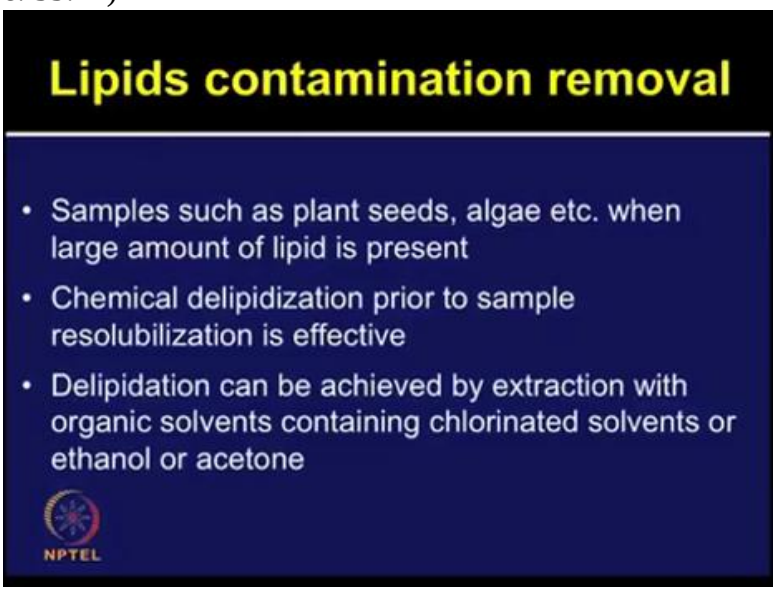
Lipids, they are very important. They are used for various types of biological problems to probe but if your context is to study about the proteins, you would like to get rid of any lipids or any other nucleic acid or any other interfering components because you just want to analyze only proteins. Since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

(Refer Slide Time: 32:57)



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 solubilizable solution should dis-aggregate the lipids, delipidate and solubilize the proteins.

(Refer Slide Time: 33:44)



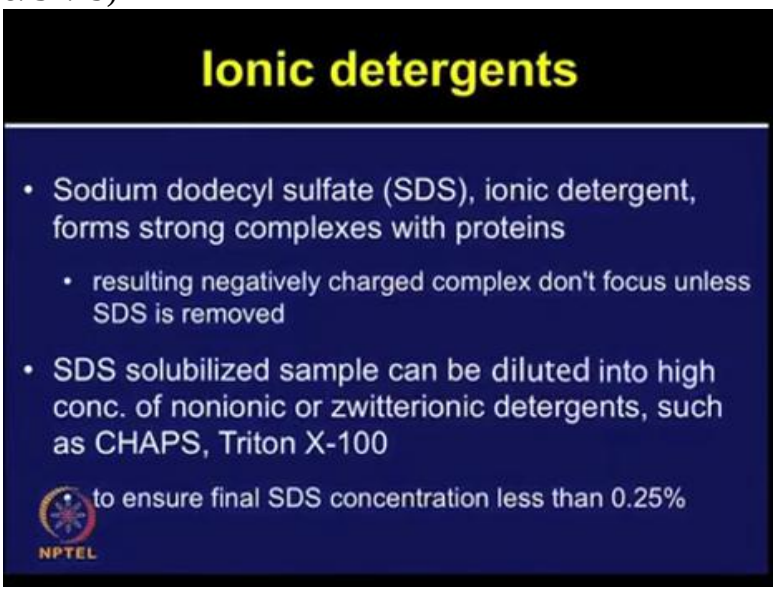
But if your samples are very much rich in lipid contamination, few samples which are intrinsically rich in the lipids such as plant seeds or algae, so you need to treat the samples by using chemical delipidization prior to the sample resolubilization.

This lipid, this process of delipidization 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 samples, those biological samples which are very rich in lipid components.

Now let's talk about ionic detergents. SDS, Sodium Dodecyl Sulfate, this is one of the anionic 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.

(Refer Slide Time: 34:49)

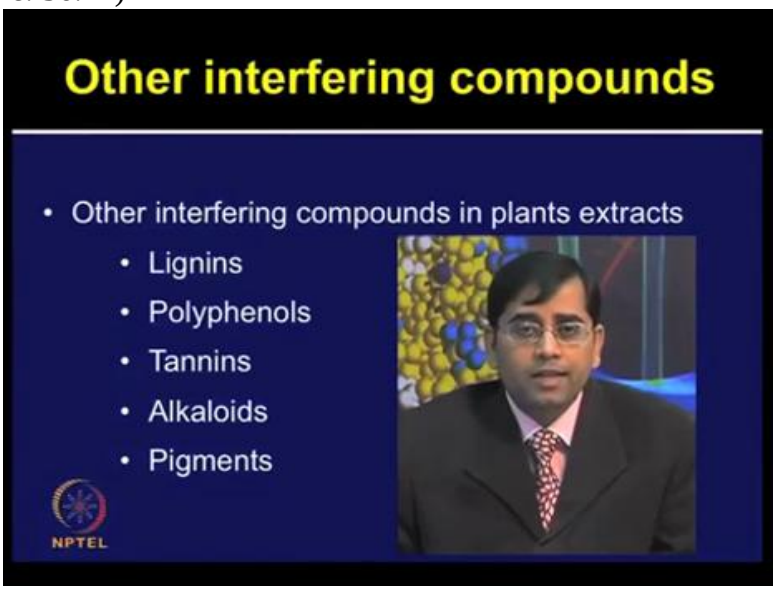


But in this context, when you are talking about protein preparation, SDS is one of the very efficient compounds. However, if later on, your aim is to perform protein separation by isoelectric focusing or other gel-based methods, it is going to create some problems because it will result into the..SDS 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 non-ionic or zwitterionic detergents which are CHAPS, Triton X-100 and we have talked of all different types of detergents in the last lecture.

So you can try different types of non-ionic 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 this SDS molecule.

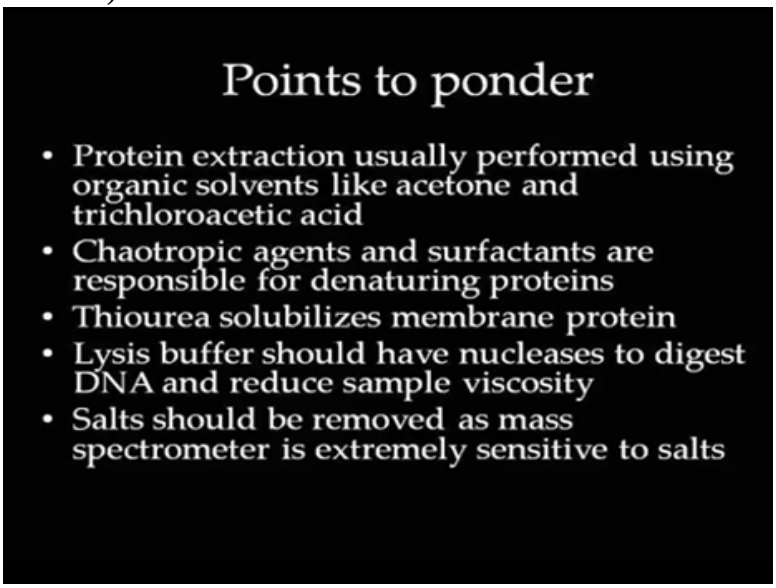
(Refer Slide Time: 36:14)



So we have talked about different types of interfering components. In the last bit, I will just say 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 interference 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.

(Refer Slide Time: 37:21)



(Refer Slide Time: 37:28)