Mass Spectrometry Based Proteomics
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Mod 01 Lecture Number 02

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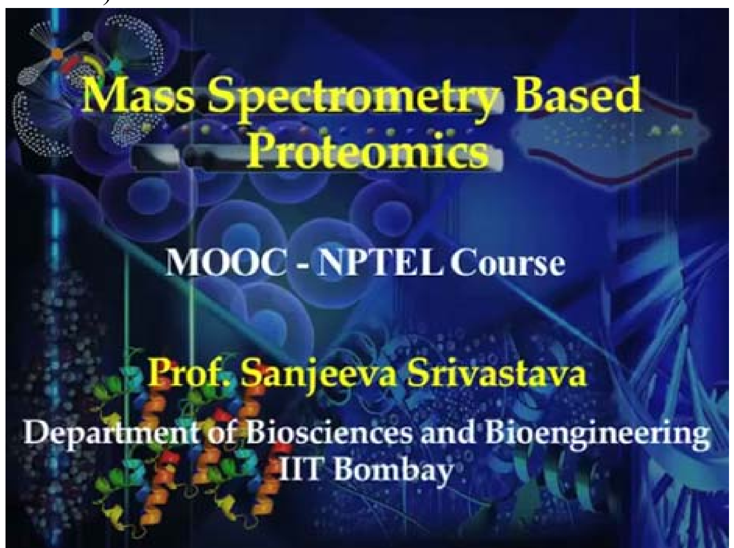

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Topics to be Discussed Today

Why proteomics
Sample preparation
Workflow for sample preparation
Proteomics techniques
Protein solubilization

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Welcome to the Proteomics course.

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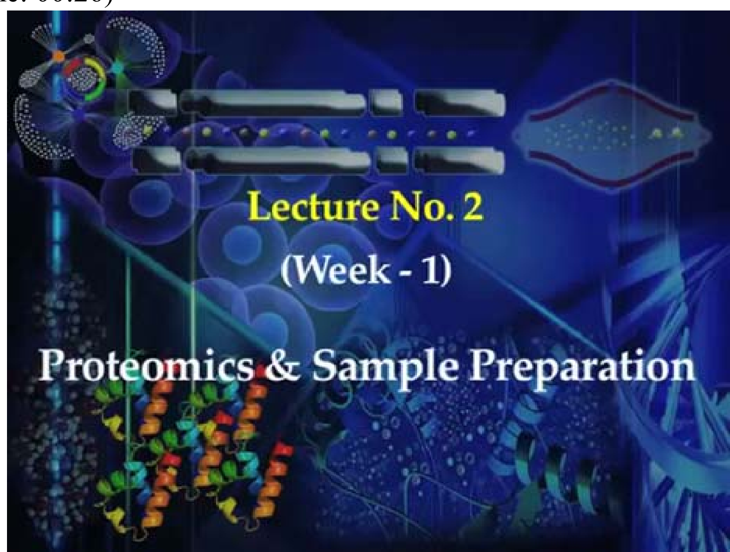

In today's lecture we will talk about sample preparation for proteomics applications.

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Section I
Why Proteomics

As you know, a very good sample is essential to perform any good experiment and especially when you want to characterize thousands of proteins in a given experiment, it is very essential that you start with a very good sample.

An ideal sample preparation step ensures that you have no contamination. You have very good protein yield and no interfering substances are present in your extract.

Often each type of biological sample poses its own challenges. For example, if you are working on bacterial cultures, working on plants, working on human samples, different body fluids such as serum, urine, saliva or cerebral spinal fluids, each sample type brings its own unique composition and its own challenges.

Now when you are performing sample preparation, you have to be very cautious that what sample you are processing. You cannot follow a generic protocol. You have to optimize conditions depending upon your sample.

An ideal sample preparation will ensure that you have all the protein present in your sample without different types of contaminants such as nucleic acids, salts and other interfering components.

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Section II
Sample Preparation

In today's lecture we will talk about sample preparation for proteomics application.

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- Sample preparation for proteomics applications
- Work-flow: protein sample preparation
    1. Cell disruption/ lysis
    2. Protection from proteolysis
    3. Sample fractionation
    4. Protein extraction and solubilization

I will give you a workflow for protein sample preparation, such as the first step, how to disrupt the cell, how to lys them, how to protect the cells during the lysis step so that there is no proteolysis occurring, how to fractionate the samples.

Often you need to simplify the proteome. You need to ensure that sample is simple enough to show the good protein throughout the proteome coverage. So to obtain comprehensive proteome coverage, often it is important that you should prefractionate your samples. Protein extraction and solubilization, these are another very essential components.

Now when we are talking about the sample preparation, this is a quite generic for different types of proteomic applications, whether one wants to use for gel-based approaches or gel-free approaches.

So whether you want to use two-dimensional electrophoresis, DIGE or different types of gel-based applications or you want to perform mass spectrometry and different types of label-free techniques you have to ensure that you are starting with a very good protein extract.

But sample solubilization and some other components are added more when you are performing the gel-based proteomics. So during this lecture I will talk to you about different types of components which are essential to make a good sample preparation for proteomic applications. I will give you step-wise work flow.

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Section III
Workflow for Sample Preparation

So the proteome is very complex. Whether you want to perform…

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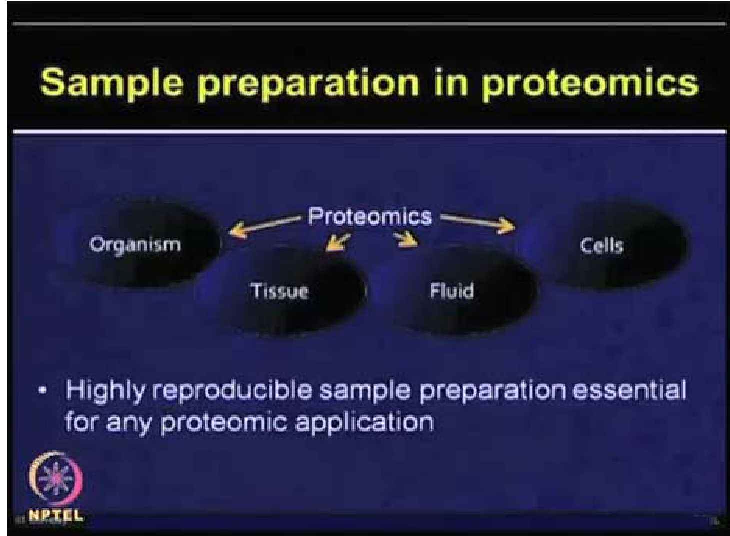

… the proteomic analysis for the whole organism, it means you want to know all the proteins present in a given organism or in a tissue or in body fluids or in different types of cells Proteomics can be global or it can be very targeted or expression based.

So, very highly reproducible sample is very important for performing comparative proteomic analysis. If you want to know the difference in your sample as compared to the controls, you need to ensure that your sample preparation is very reproducible. If you introduce some artifacts to begin with, then obviously you are not going to identify the reproducible biological changes.

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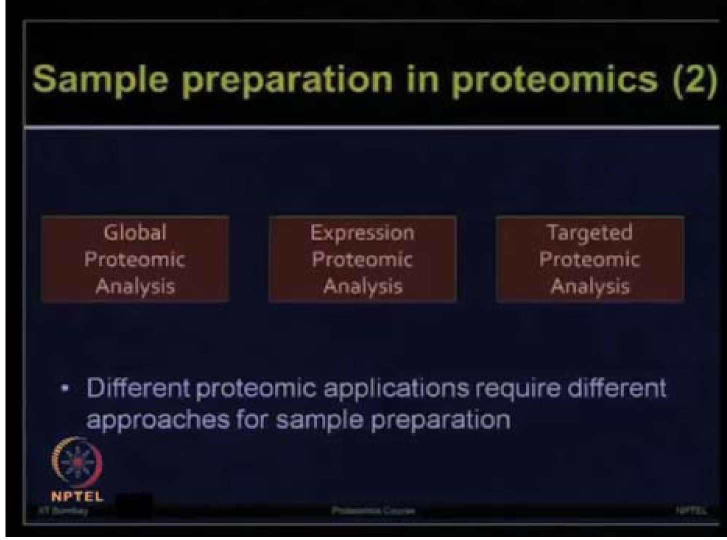

So let me give you three different terminologies here for proteomic analysis. One is global proteome analysis, expression proteome analysis, and targeted proteome analysis.

When I am talking about global proteome analysis, it means your aim is to characterize all your proteins present in the given sample.

Expression proteome analysis, it means you are mainly interested to look for those changes which are due to any chemical or your treatment those are induced either going up or down, the protein amount is changing. So the protein expression analysis that is most commonly used for various types of clinical and different studies

Targeted proteomic analysis, if you are very focused for a given organelle or a given sample type, often you would like to know what is happening in that particular proteome, for example mitochondrion proteome; so, one need to try different types of strategies when thinking about performing a sample preparation.

What is your objective? Whether you want to do global profiling or whether you want to do expression profiling? In either case, you need to extract all the proteins present in that particular target sample.

Now when you are looking at targeted proteomic analysis, you just want to prefractionate your sample in such a way that only that particular component is isolated and then all the proteins from that organelle or cell is being extracted; so different types of strategies need to be used to perform these types of proteomic analysis.

Now all of this sample processing involves solubilization, denaturation, reduction and treatment of sample proteins.

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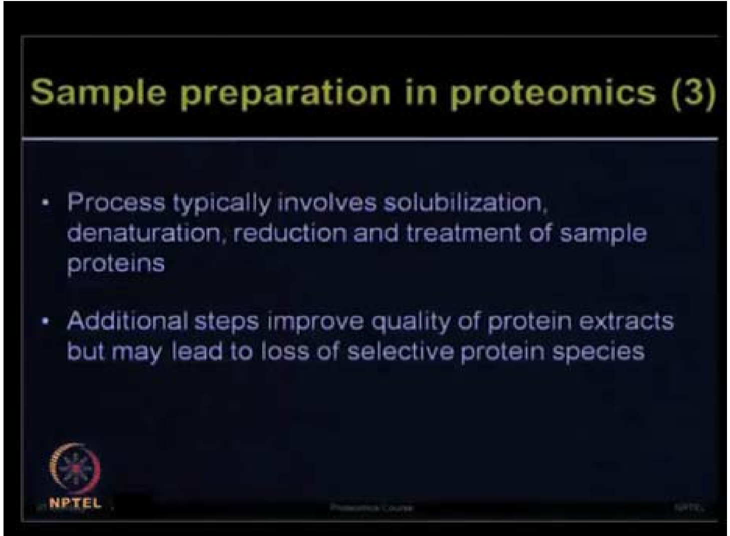

But you need to involve additional steps depending upon the type of samples and your type of objective so that the protein quality, the protein extract can be improved and while you are doing this, you have to be very cautious that when you are performing various steps and sequential type of extraction, you may also lose a small fraction of the proteins.

So, one has to be careful when adding various additional steps during the sample preparation. Now protein extraction protocols, they need to ensure that most, if not all the proteins in a cell or its organelle are extracted.

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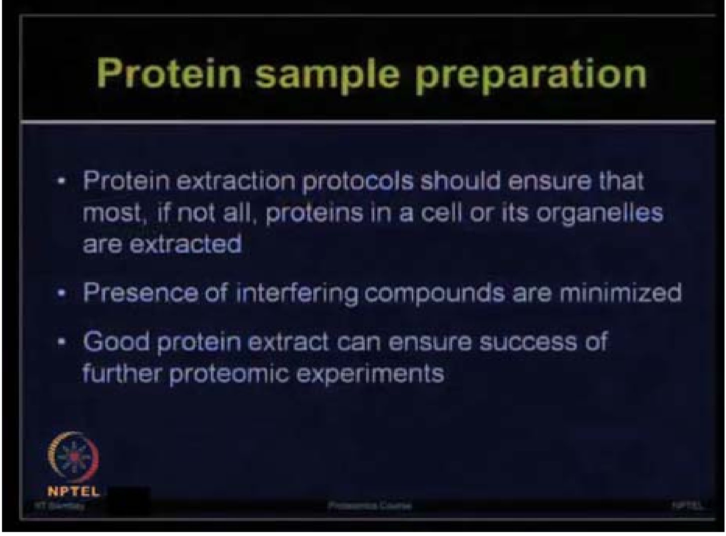

The presence of interfering compounds should be minimized. So you have optimized a very good protein extraction procedure that should ensure that you have a very wide proteome coverage and that is ultimately going to determine the success of your proteomic experiment.

So ideal protein preparation step involves solubilizing all the proteins present in the given mixture, preventing protein aggregation….

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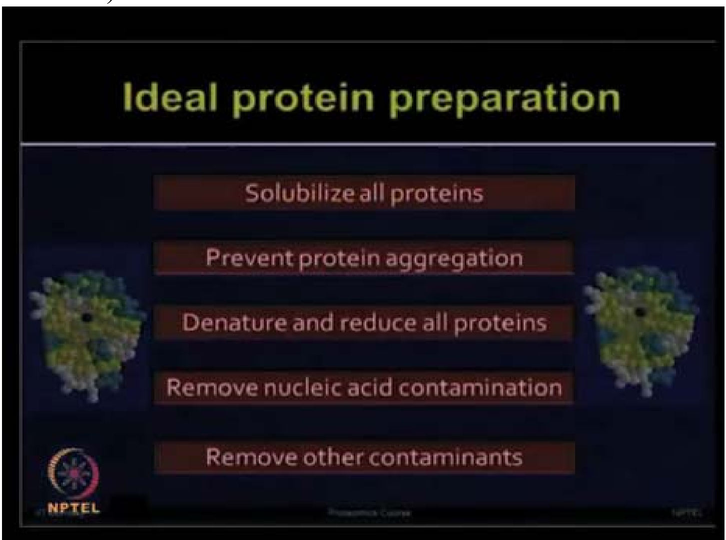

…denaturing and reducing all the proteins which are present in that mixture, removal of nucleic acids and other contaminants as well as removing salt and some other small interfering components

Again depending upon your sample type, you may have to think what all different types of interfering components could be present in that sample type? If you are talking about plant roots, you may have to get rid of phenolic components.

Similarly you have to think specific sample types and what could be the major contaminants present in that sample; for example, serum. That is rich in lot of salt components. You need to get rid of those. So an ideal protein preparation should involve all of the steps as I mentioned previously.

So I am giving you the guidelines for sample preparation. Starting point…

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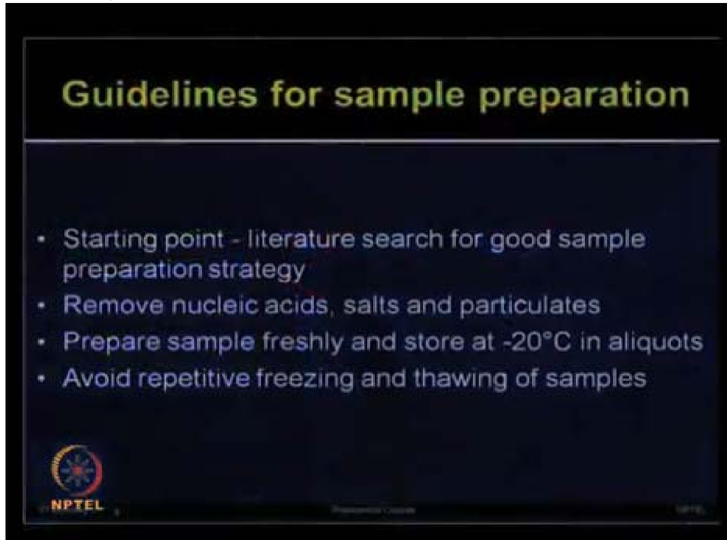

… one should start with finding a good reference from the literature as a starting point and then try to modify the protocol depending upon the objective of that experiment

You need to ensure that you remove nucleic acids, salts and different particulates. Prepare the samples as freshly as possible and store it in -20 degrees in small aliquots. You should avoid repetitive freezing and thawing strips of sample. So what a good protein sample preparation is important?

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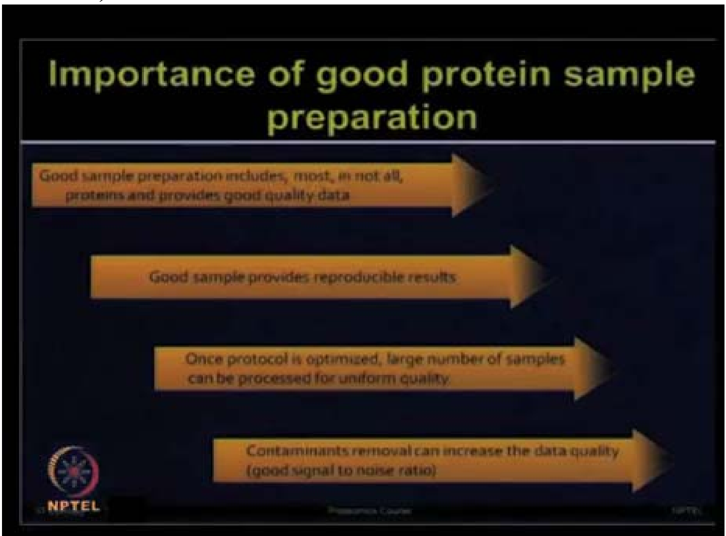

A good protein sample preparation includes all the proteins present in that mixture and it is going to provide you high quality data because there will be less interference from the artifacts.

A good sample will provide reproducible results. You have to perform biological replicates and technical replicates of a given experiment. So a good sample will provide very reproducible results. Once you have optimized a protein extraction protocol, then you can apply the same protocol for the large studies.

For example if you are performing a clinical trial study on 200 patients. So once you have optimized the protocol with a small population then the same protocol can be applied for large number of samples. So that is going to ensure the success of the clinical studies.

Now if you are able to remove lots of contaminants or artifacts present in your sample, then your signal to noise ratio will improve. You have to literally see your good signal and don't have to worry too much of the background and the noise.

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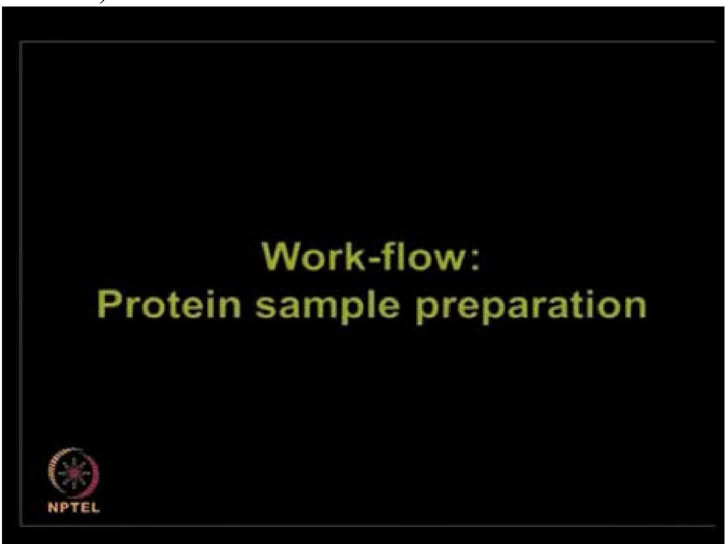

So I am giving you the workflow of protein sample preparation. Let’s go step by step.

Sample preparation: The protein extraction should be performed from source material…

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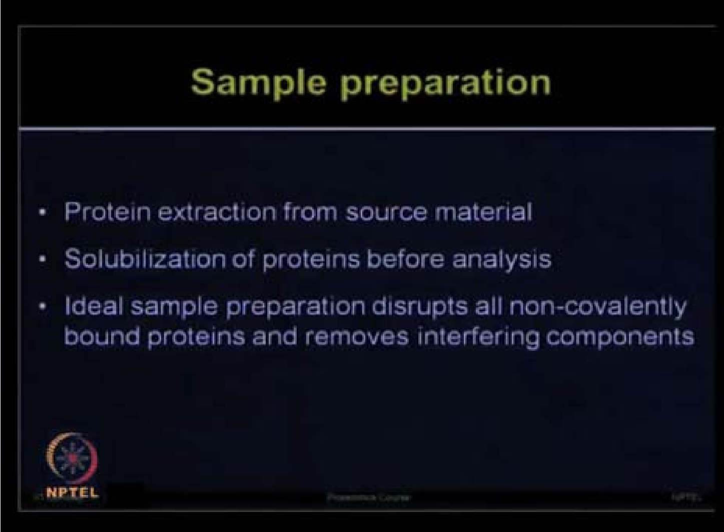

… and then you need to solubilize the proteins before starting the analysis The ideal sample will disrupt all the non-covalent bonds present with the proteins and it will remove the other interfering compounds.

So the workflow of sample preparation would involve first, cell disruption or lysis, second protection from the proteolysis…

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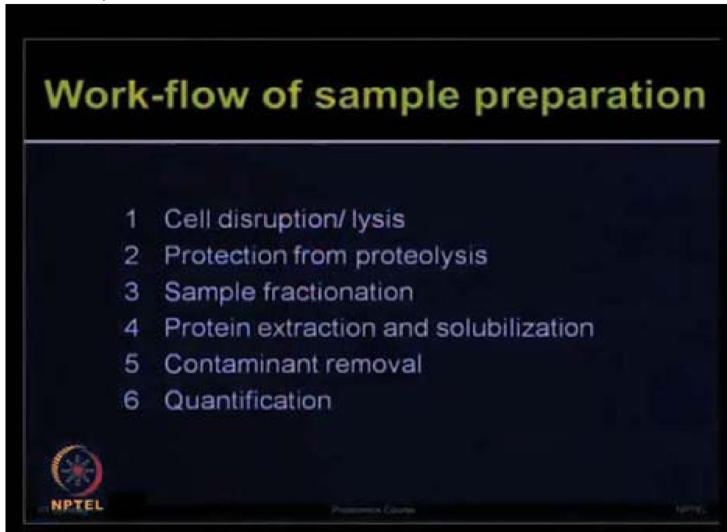

…. third sample fractionation, fourth protein extraction and solubilization, fifth removal of contaminants and sixth quantification.

Obviously this workflow can be modified depending upon your sample type and few steps can be moved in that sequence. So let's follow this workflow and during this workflow, I will

give you some examples of different types of samples, how to extract proteins from those and what types of challenges these samples are going to impose.

So cell disruption or lysis, the lysis is very important step….

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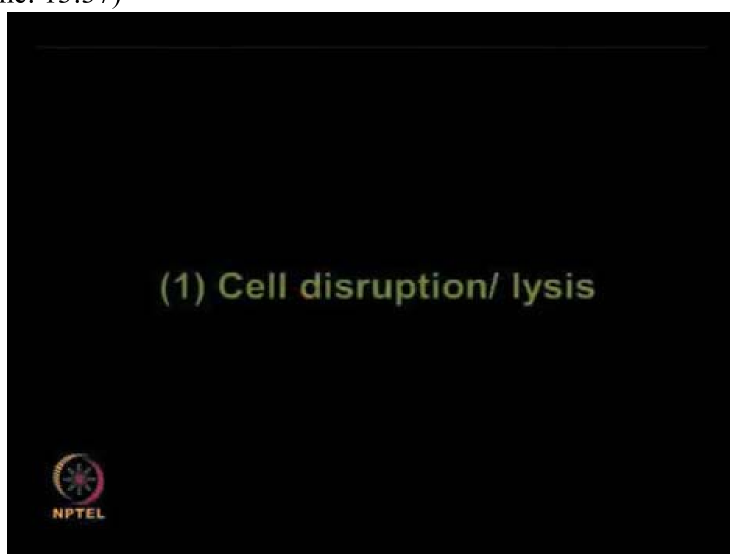

…because first of all, you would like to break open the cells and remove all the cell components outside so that you can get good protein yield So cell disruption or lysis is very important.

And it is often challenging because all the sample types, you cannot lys with the same type of method. So the lysis strategy has to be modified depending upon the sample type.

So why need cell lysis?

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To facilitate the effective disruption of cell or tissue, to isolate the proteins from intact cells and tissue while avoiding the loss or modification of proteins, to obtain all the proteins which are present in a given sample and to help to maximize the sample recovery and retain the structural integrity. So cell lysis is very important due to all of these factors.

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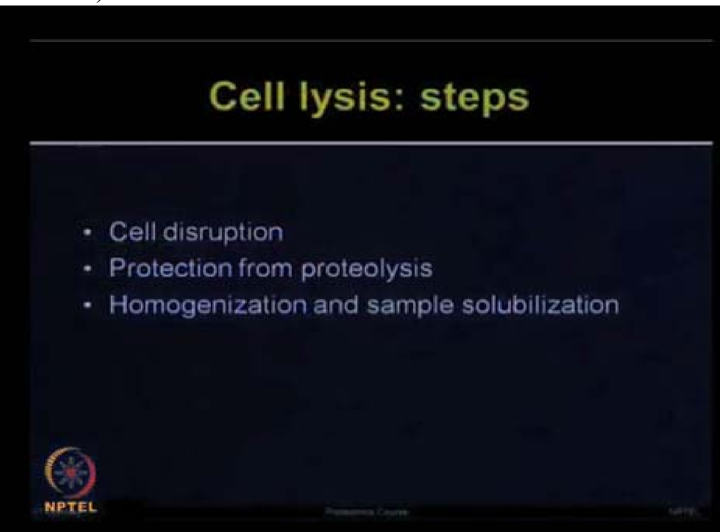

The different steps are involved in cell lysis. You need to disrupt the cell, protect from the proteolysis during the lysis step, homogenize and solubilize your sample. I will describe all of this in more detail.

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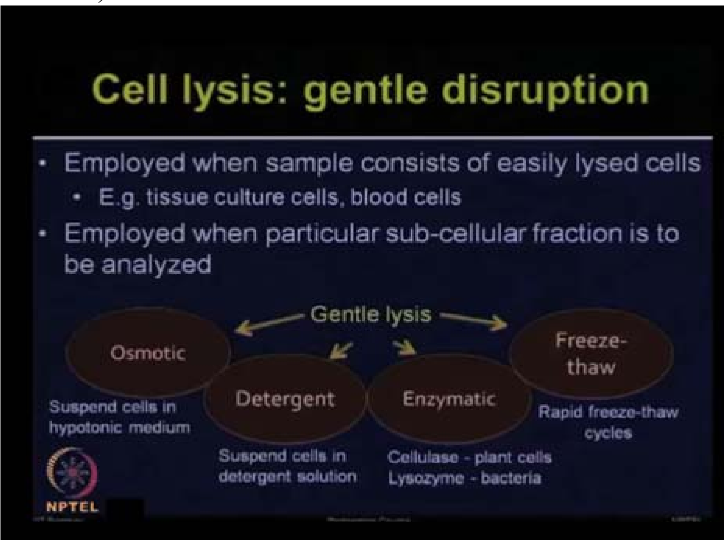

So cell lysis can be performed with the gentle way or in harsh condition depending upon the type of cells you want to disrupt. When you are employing gentle disruption method, you need to think that you have to break open all the cellular components.

So your gentle disruption should be efficient enough to disrupt the cell. So if you are looking at those cell types which can be easily lysed such as blood cells or culture cells, then you can involve the gentle disruption methods.

There are different types of lysis methods available such as osmotic lysis, in which one can suspend the cell in hypotonic medium; detergent lysis, you need to suspend the cells in detergent solution; enzymatic lysis, if you are using plant cell for example, one can use cellulase enzyme, if you are using bacterial sample one can use lysozyme. There are different types of enzymes present which are used for enzymatic lysis.

Freeze-thaw: That is one of the very commonly used methods for gentle disruption which involves rapid freezing and thawing cycle. You need to cool your sample in a very, very cold condition, for example liquid nitrogen and then immediately put it in the boiling water. While doing this rapid freezing thawing, the cells will break open.

Now there are different types of challenges being imposed by different types of cells.

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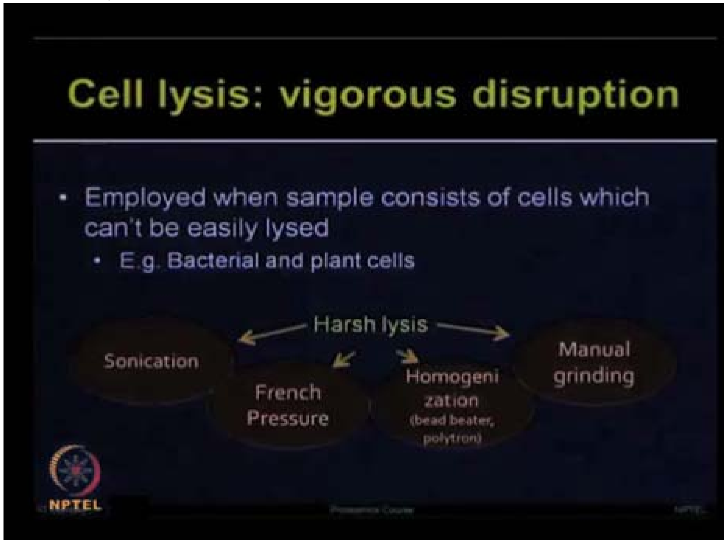

If the cells are very difficult to lys, very difficult to break open, then you need to involve vigorous disruption methods such as sonication, French pressure, homogenization or manual grinding.

We will talk of different types of cell lysis methods and some of its principles involved as we go along with more specific types of samples. But in all the samples preparation, mostly people use manual grinding or homogenization by using bead beaters or Polytron homogenizers. So these are very commonly used homogenization methods.

The manual grinding is performed by using mortar pestle. Often it is very efficient but if you have very less sample and you want to avoid any contamination or you have some samples which are going to pose challenges with mortar pestle, then you need to use the electronic homogenizers.

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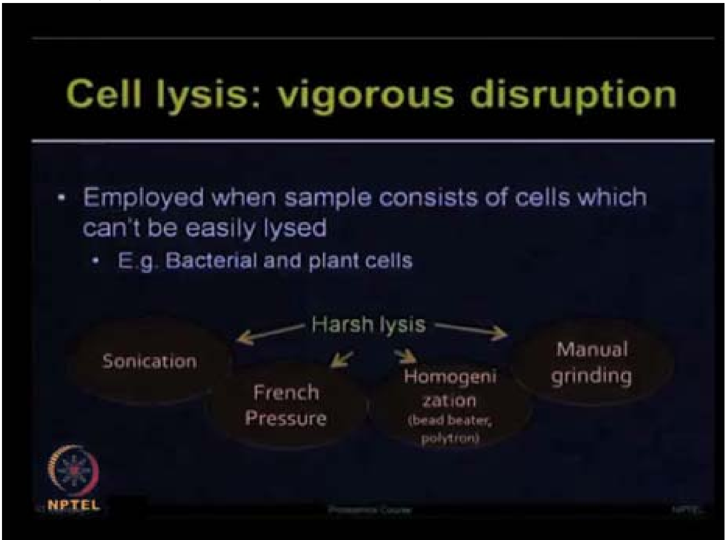

Then sonication and French pressures are more used when you are applying bacterial and yeast or different type of those cells which are difficult to open. We will talk about these in more detail in the more specific example when we talk about how to extract the proteins from bacterial sample.

So now I will give you…

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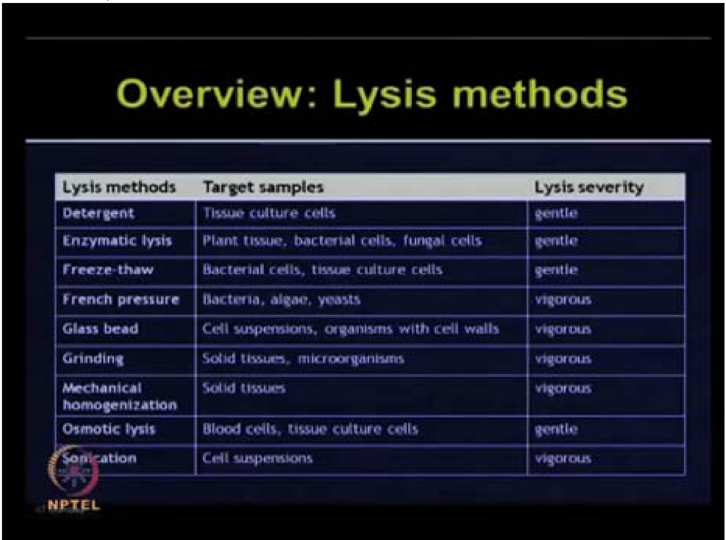

Lysis methods	Target samples	Lysis severity
Detergent	Tissue culture cells	gentle
Enzymatic lysis	Plant tissue, bacterial cells, fungal cells	gentle
Freeze-thaw	Bacterial cells, tissue culture cells	gentle
French pressure	Bacteria, algae, yeasts	vigorous
Glass bead	Cell suspensions, organisms with cell walls	vigorous
Grinding	Solid tissues, microorganisms	vigorous
Mechanical homogenization	Solid tissues	vigorous
Osmotic lysis	Blood cells, tissue culture cells	gentle
Sonication	Cell suspensions	vigorous

…the overview of lysis methods; we have talked about all of these methods briefly. I am giving you an overview now.

One can use detergents if your target sample is tissue culture cells; this is going to provide gentle lysis method. Enzymatic lysis can be used for plant tissues, bacterial cells, fungal cells etc. This is again a gentle lysis method. Freeze thawing can be used for bacterial cells or cultured cells and this is again a gentle lysis method.

French pressure is often applied on bacteria, algae, yeast. This is a vigorous lysis method. Glass beads are used with cell suspensions or organism with cell wall; this is again a vigorous lysis method. Grinding of solid tissues and micro-organisms is another vigorous lysis method. The mechanical homogenization of solid tissues is another vigorous lysis method.

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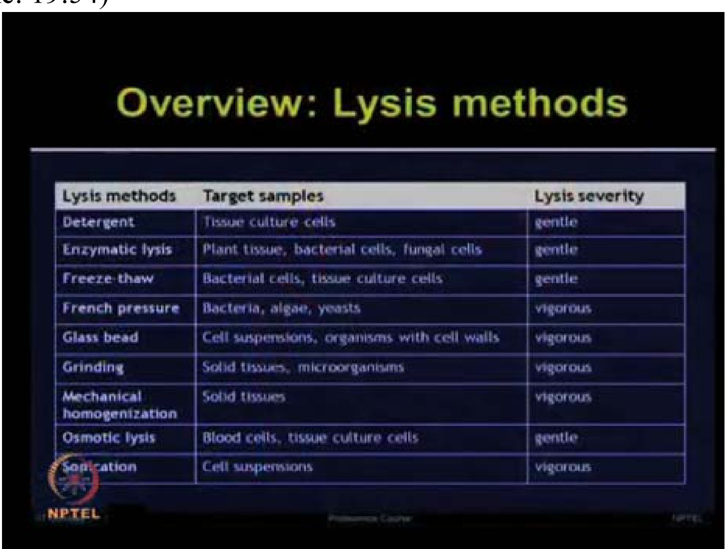

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Mechanical homogenization	Solid tissues	vigorous
Osmotic lysis	Blood cells, tissue culture cells	gentle
Sonication	Cell suspensions	vigorous

Osmotic lysis can be used for blood samples and tissue culture cells which is a gentle lysis method. Sonication can be used for cell suspension and other bacterial samples which is another vigorous lysis method. I hope these methods give you options to lys your cells effectively.

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Now you have tried to break open your cells and release all the protein components and other cellular components present inside the cell. But often during this process of grinding and lysis, you may degrade some of the protein and you need to ensure that you have to protect your protein from the proteolytic activities of various enzymes.

So protection of your protein components from the proteolytic step is very important during the protein sample preparation. So cell lysis will release various proteases…

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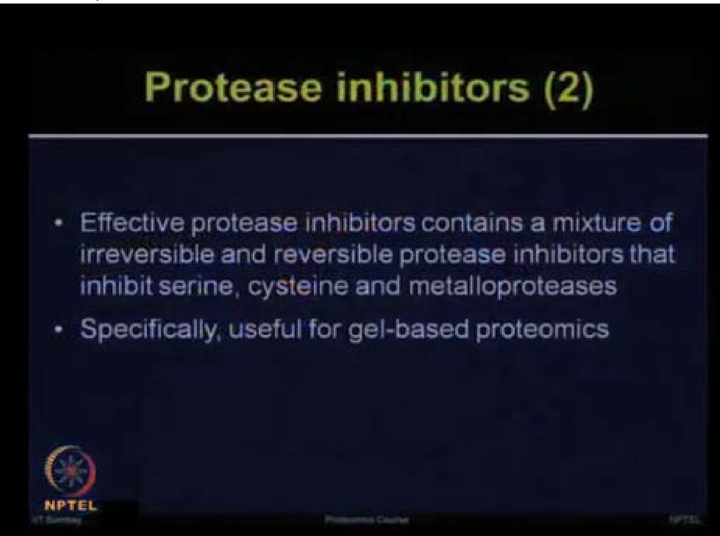

… which may result into proteolysis During the sample preparation, one can use different types of protease inhibitors which can minimize the artifactual proteolysis.

The effective protease inhibitors contain a mixture of different types of protease inhibitors, irreversible and reversible, which inhibits serine, cysteine and different metalloproteases. This step is more important when you are preparing your sample for gel-based proteomic applications.

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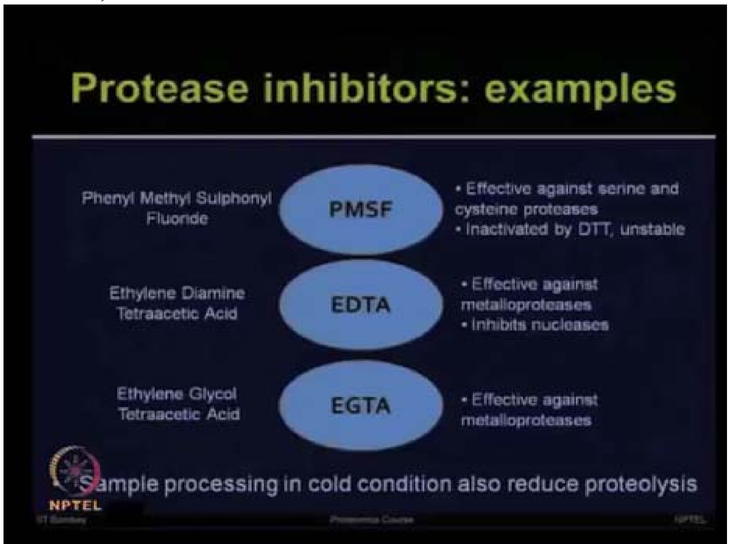

Now I will give you few specific examples of protease inhibitors such as Phenyl Methyl Sulfonyl Fluoride PMSF which is very effective against serine and cysteine proteases. It can be inactivated by DTT and it is unstable when you are preparing the sample for gel-based applications.

EDTA, Ethylene Diamine Tetraacetic Acid, it is effective against specifically metalloproteases. It inhibits nucleases as well. So EDTA can serve both purpose of protecting from proteolysis and also partial inhibition of nuclease activity.

EGTA or Ethylene Glycol Tetraacetic Acid, it is also effective against metalloproteases. Now when you are making your sample preparation for proteomic applications, it is very important that you process the samples in the cold conditions to reduce any proteolysis step.

Now these proteolysis artifacts can be minimized by using cold conditions during the grinding as well as during the centrifugation step.

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Section IV
Proteomic Techniques

When you are applying your protein samples on 2D gels or other type of gel-based proteomic techniques this will provide you less clouded protein map. So again fractionation can serve a good deed in both gel-based and gel-free proteomic applications.

So let’s talk about different types of fractionation methods available. Simplest for looking for the organelle or specific type of proteome people use different ways of centrifugation ultra-centrifugation

When you are looking for sub-cellular fractionation of organelle such as mitochondria or chloroplast or cell compartment such as plasma membrane

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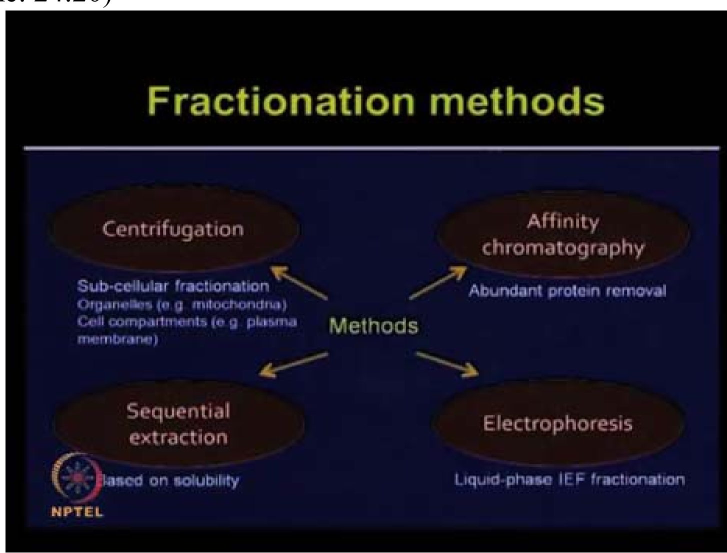

…different types of chromatography methods can be used for fractionation.

If you are interested in looking at all the serum proteins but there are certain proteins which are highly abundant in the serum such as serum albumin protein, so by using affinity chromatography methods one can remove those highly abundant proteins so that all the proteins in that given mixture can be well resolved.

Now affinity chromatography methods can be used for fractionation in different context. Sequential extraction which is again going to simplify your proteome, it is based on solubility and different types of chemicals are used so that in sequential way, one can extract the proteins.

Few proteins may be more soluble in one particular composition of reagents and other proteins are more soluble in different solubilization buffer. So by applying different type of recipe of chemicals one can obtain the bigger coverage of whole proteome in doing the sequential extraction.

Electrophoresis can be used for fractionation. For example, if we are using gel-free method, directly you want to extract the protein and want to analyze that using mass spectrometry, so rather than applying the whole sample directly with the liquid chromatography, one can first simplify the proteome by using isoelectic focusing and doing the IEF process in the liquid phase itself.

The liquid phase IEF fractionation can simplify the proteome based on the isoelectric point.

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Section V
Protein Solubilization

So far we talked about how to perform the cell lysis then how to protect protein from the proteolysis. We have then looked at different types of strategies people involve to fractionate the proteome.

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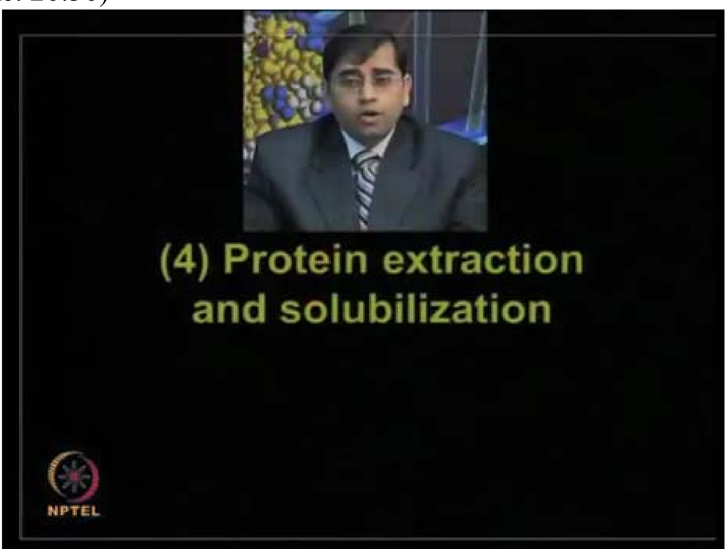

And now let's talk about protein extraction and solubilization Now this step will be more towards talking about gel-based proteomics where solubilization will be more important

The protein extraction after performing the sub-cellular fractionation…

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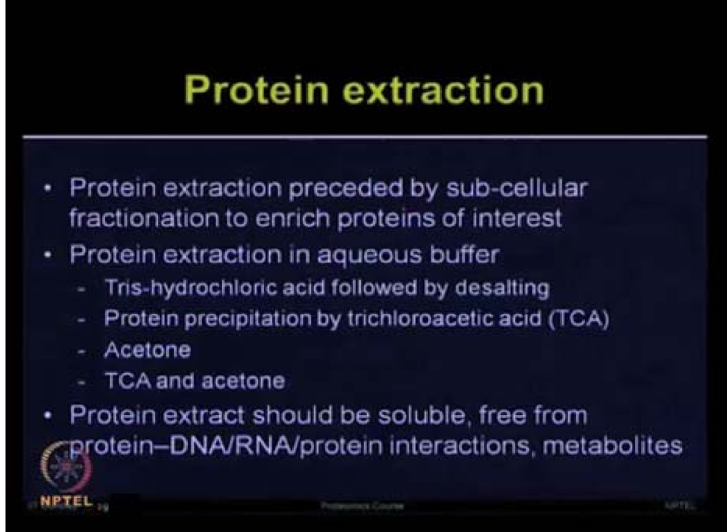

….so that the proteins can be enriched which is you are going to be analyzing in your experiment The protein extraction in the aqueous buffer one can follow different types of procedure.

Either you use TRIS hydrochloric acid followed by the desalting method, protein precipitation by TriChloro Acetic acid or TCA or acetone alone 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

Sample solubilization is important because …

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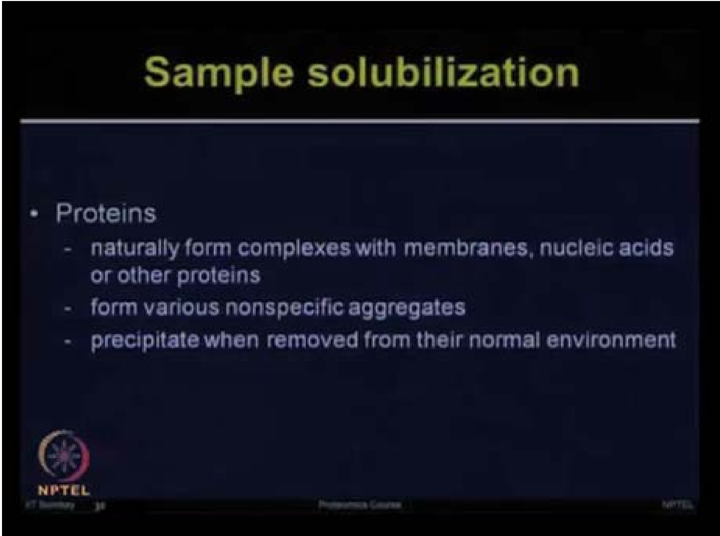

… proteins naturally form complexes with membranes, nucleic acids as well as other proteins. So to avoid all of these issues sample solubilization is very important.

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

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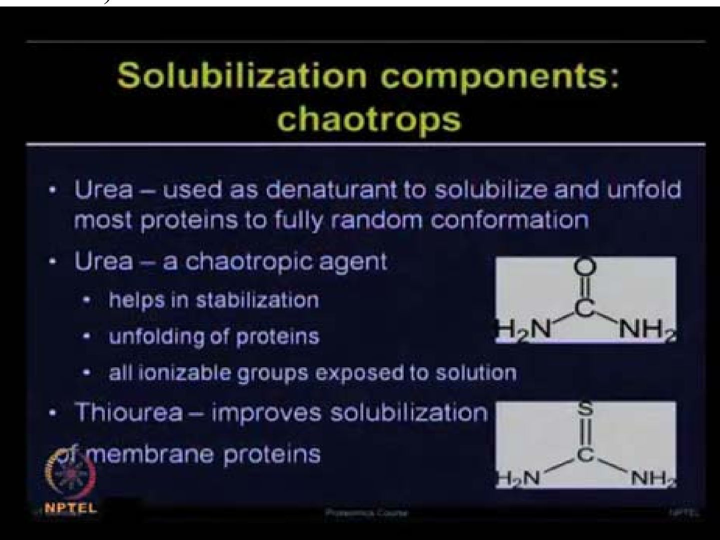

First of all let's talk about chaotrops, urea and thiourea. Urea is used as denaturant which can solubilize and unfold most of the proteins to fully random conformations. Urea is a chaotropic agent which helps in stabilization of the proteins unfolding proteins so that all the ionizable groups are exposed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

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

There are different types of detergents which are also used in solubilization such as SDS or

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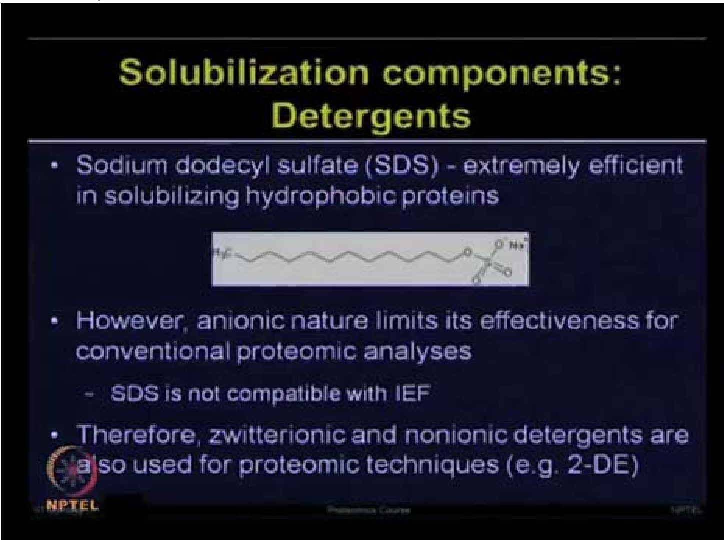

Sodium Dodecyl Sulfate which is very efficient in solubilizing hydrophobic proteins; if you want to solubilize hydrophobic proteins SDS can be very effectively used. But due to its anionic nature it limits its effectiveness for the conventional proteomic analysis. The SDS, the anionic detergent is not compatible for Isoelectric focusing.

So if you are 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

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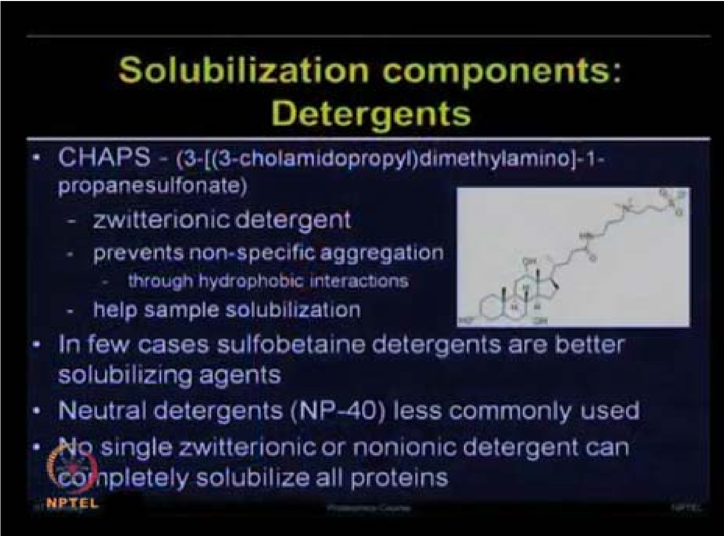

CHAPS one of the zwitterionic detergents is most commonly used detergent used in protein solubilization when your objective is to perform two-dimensional electrophoresis experiments. It prevents non-specific aggregations through hydrophobic interactions and it helps in sample solubilization.

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.

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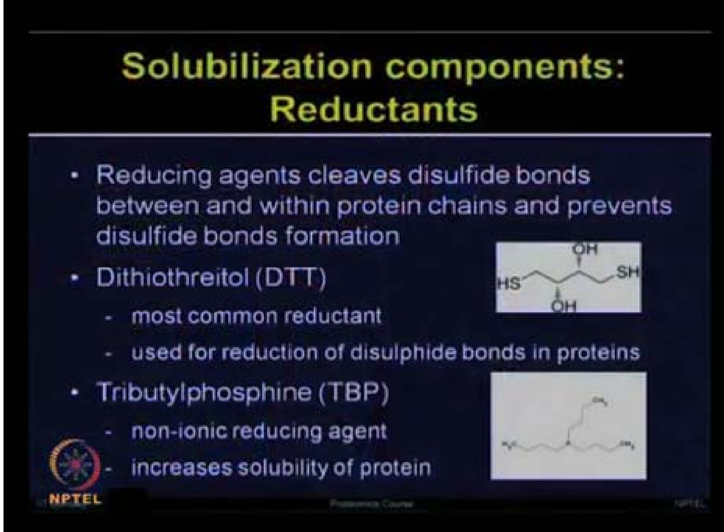

Most commonly used reductants are DiThoThreotal DTT or beta Mercaptoethanol. These are used for reduction of disulfide bonds which are present in the proteins.

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

If your aim is to perform Isoelectric focusing from your samples the solubilizing agent should include carrier ampholytes …

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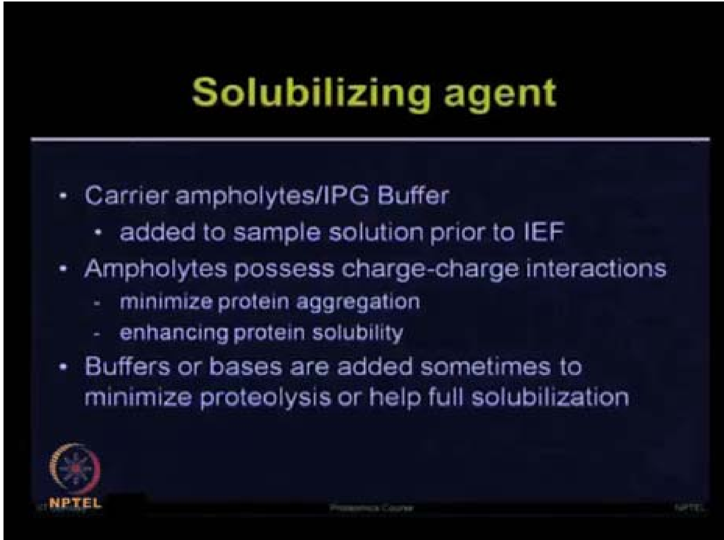

…or immobilized pH gradient buffers These are added in the sample solutions prior to the Isoelectric focusing step.

Different buffers or bases are added which sometimes minimize proteolysis and help in the complete solubilization of proteins. So we will continue our discussion on how to perform the protein extraction and proteome analysis on different types of biological samples in the next lecture.

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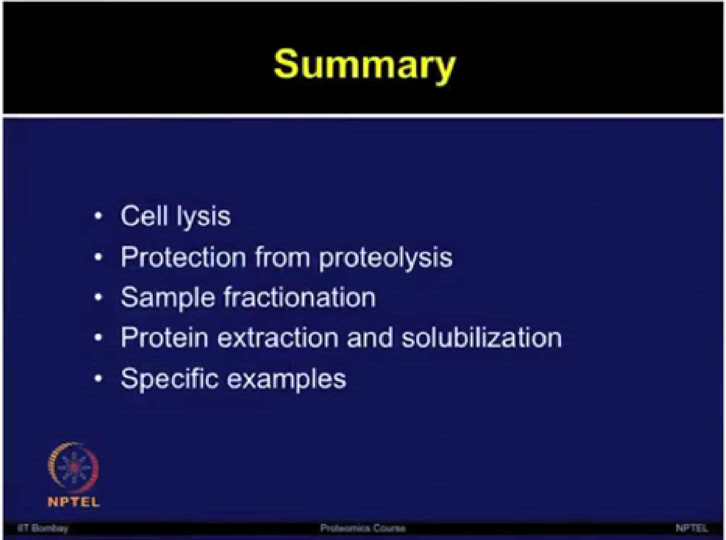

For the summary today you have learnt about how to perform cell lysis, how to protect the cell the proteins from the proteolysis by adding various types of protease inhibitors, sample fractionation methods, how to use protein extraction and solubilization for effective protein solubilization. And now we are talking about specific examples.

We will continue our lecture in the next class Thank you

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