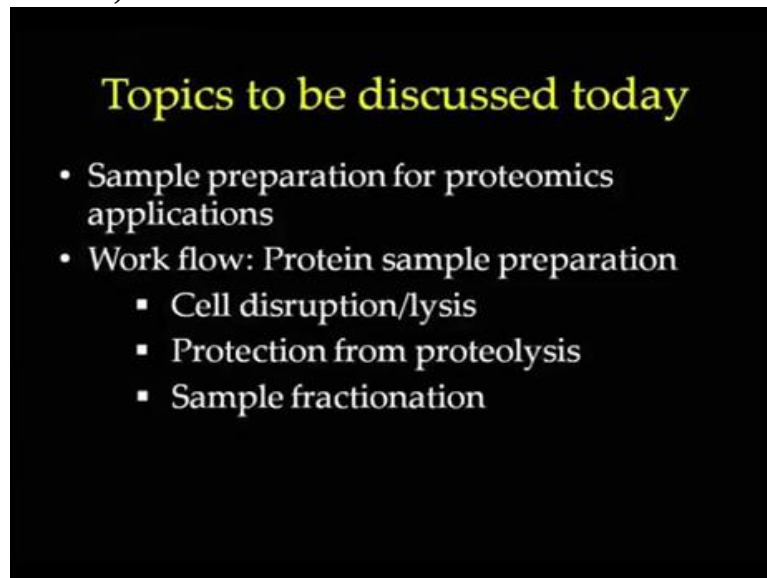
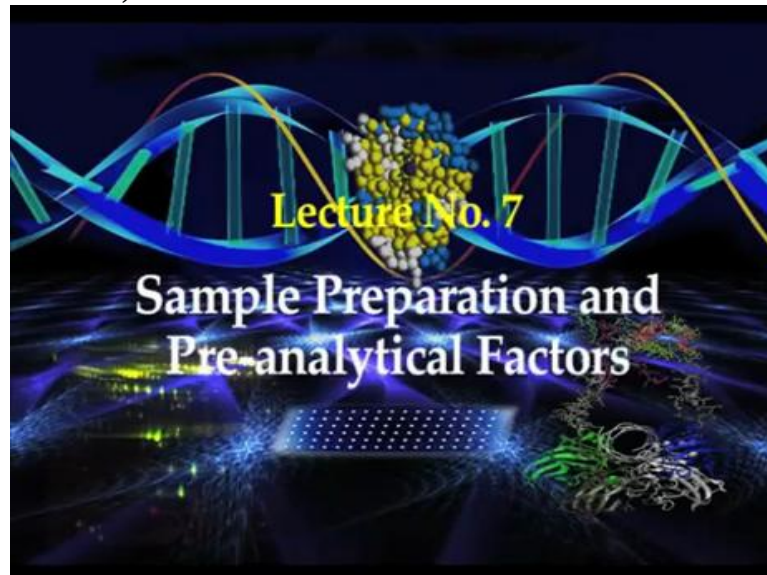


Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 03 Lecture Number 8

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Welcome to the Proteomics course. In today's lecture we will talk about sample preparation for proteomics applications.

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.

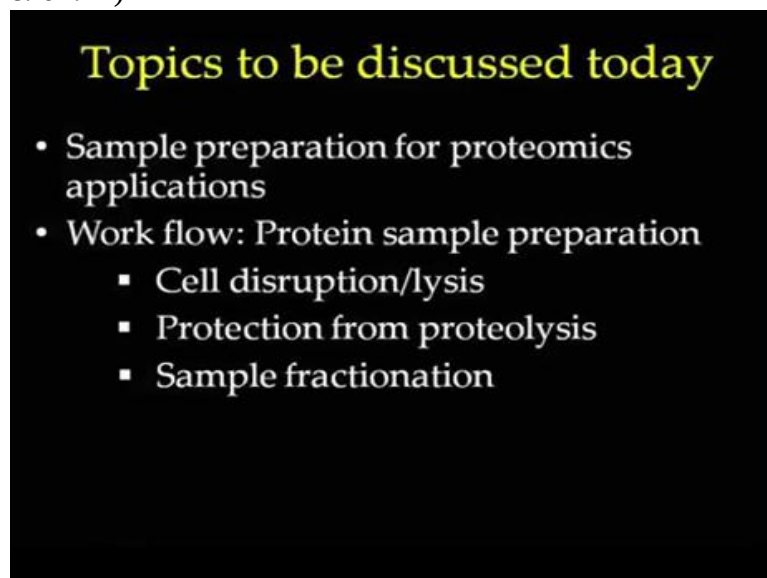
An ideal sample preparation step ensures that you have no contamination. You have very good protein yield and no interfering substances are present in your extract.

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 several 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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Topics to be discussed today

- Sample preparation for proteomics applications
- Work flow: Protein sample preparation
 - Cell disruption/lysis
 - Protection from proteolysis
 - Sample fractionation

In today's lecture we will talk about sample preparation for proteomics application. 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 the mass spectrometry and different types of label-free techniques you have to ensure that you are starting with a very good protein extract.

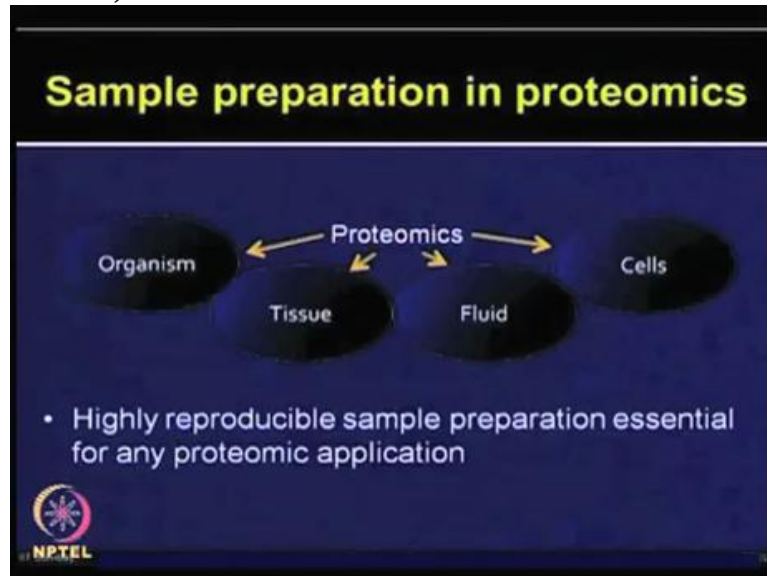
But sample solubilization and some other components are added more when you are performing the gel-based proteomics. So during this lecture I will talk to you about different types of components which are essential to make a good sample preparation for proteomic applications. I will give you step-wise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

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So let's start workflow for proteomic sample preparation. So the proteome is very complex.

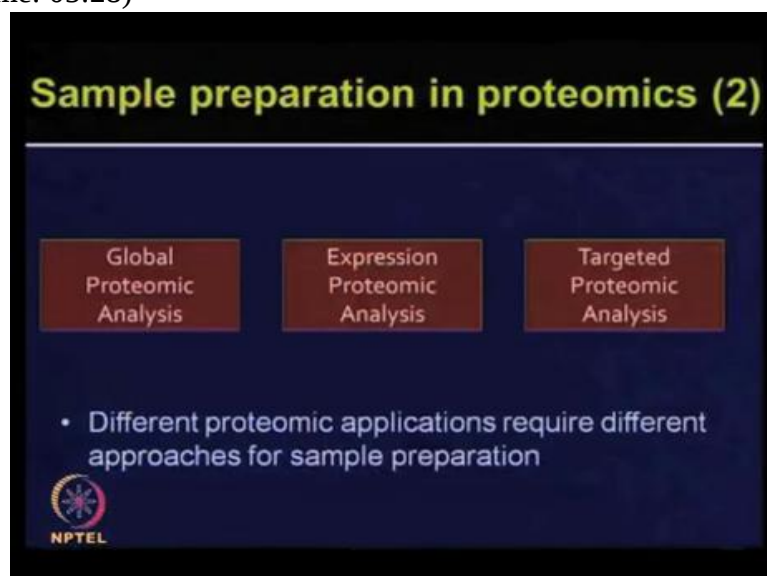
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Whether you want to perform your proteomic analysis for the whole organism, it means you want to know all the proteins present in a given organism or in a tissue or in body fluids or in different types of cells. Proteomics can be global or it can be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

So, very highly reproducible samples are 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.

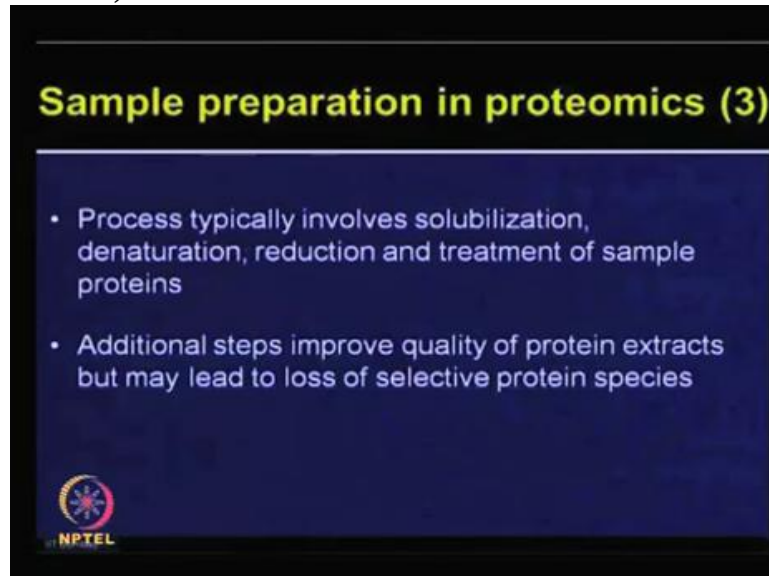
Expression proteome analysis, it means you are mainly interested to look for those changes which are due to any chemical or your treatment those are induced either going up or down, the protein amount is changing. So the protein expression analysis that is most commonly used for various types of clinical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

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Sample preparation in proteomics (3)

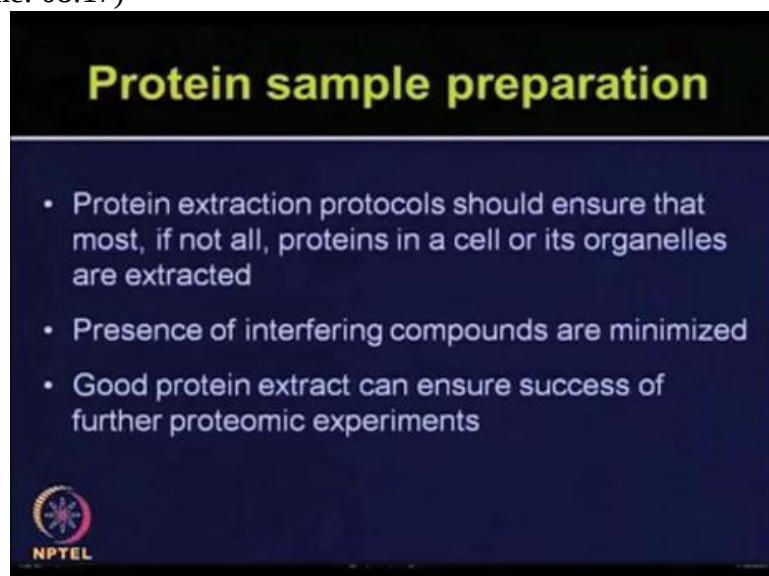
- Process typically involves solubilization, denaturation, reduction and treatment of sample proteins
- Additional steps improve quality of protein extracts but may lead to loss of selective protein species

NPTEL

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

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.

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Protein sample preparation

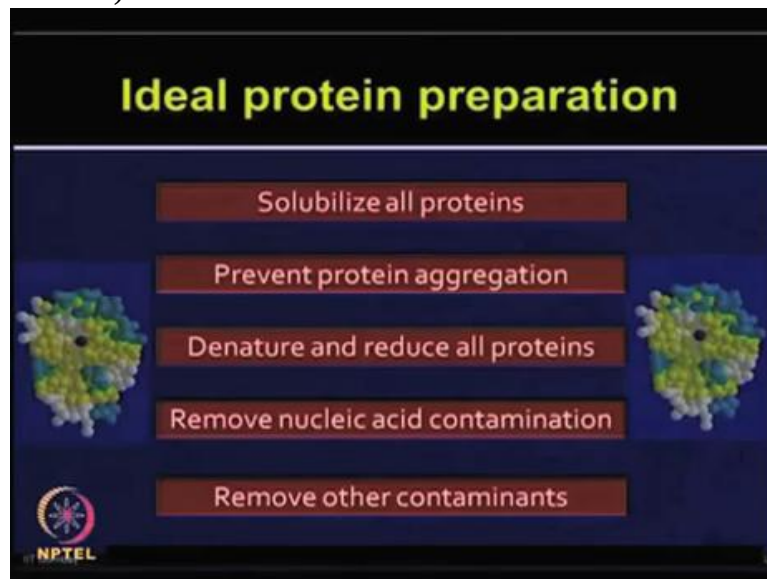
- Protein extraction protocols should ensure that most, if not all, proteins in a cell or its organelles are extracted
- Presence of interfering compounds are minimized
- Good protein extract can ensure success of further proteomic experiments

NPTEL

Now protein extraction protocols, they need to ensure that most, if not all the proteins in a cell or its organelle are extracted.

The presence of interfering compounds should be minimized. So if 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.

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

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Guidelines for sample preparation

- Starting point - literature search for good sample preparation strategy
- Remove nucleic acids, salts and particulates
- Prepare sample freshly and store at -20°C in aliquots
- Avoid repetitive freezing and thawing of samples

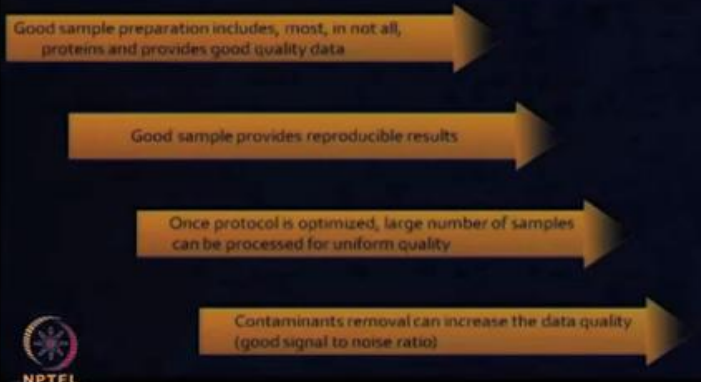


So I am giving you the guidelines for sample preparation. Starting point, one should start with finding a good reference from the literature as a starting point and then try to modify the protocol depending upon the objective of that experiment.

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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Importance of good protein sample preparation



- Good sample preparation includes, most, in not all, proteins and provides good quality data
- Good sample provides reproducible results
- Once protocol is optimized, large number of samples can be processed for uniform quality
- Contaminants removal can increase the data quality (good signal to noise ratio)



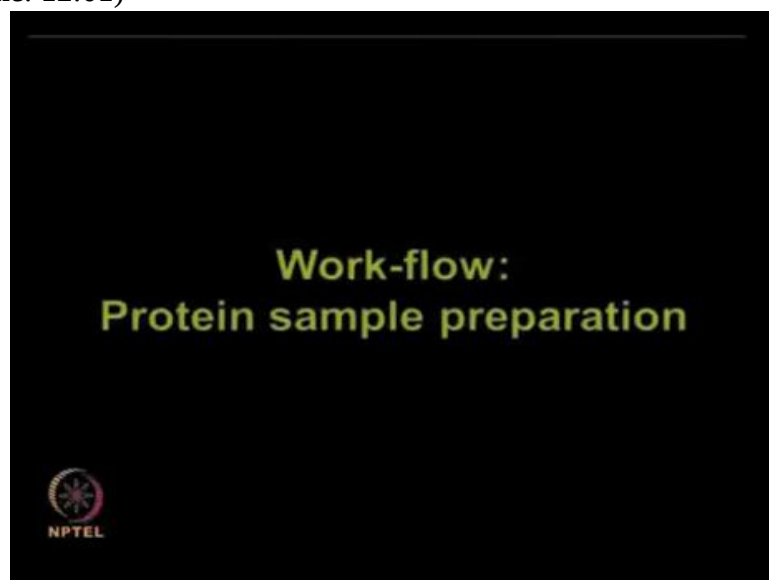
A good protein sample preparation includes all the proteins present in that mixture and it is going to provide you high quality data because there will be less interference from the artifacts.

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.

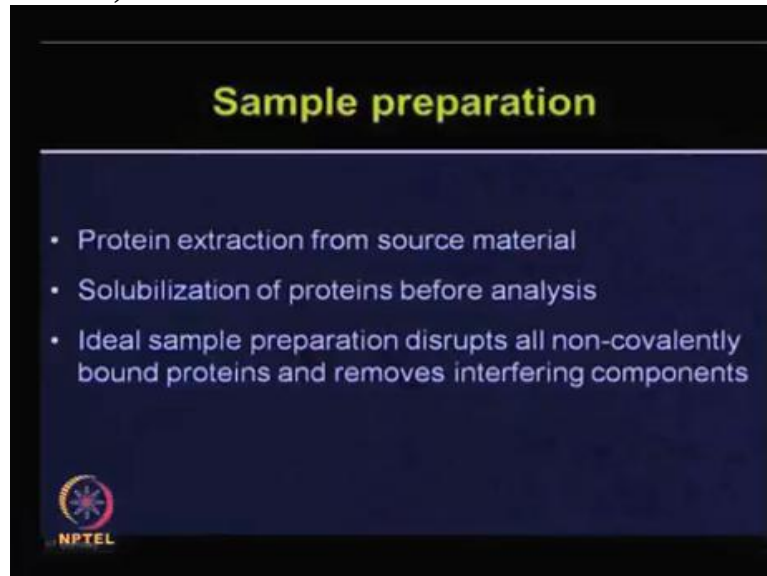
Now if you are able to remove lots of contaminants or artifacts present in your sample, then your signal to noise ratio will improve. You have to literally see your good signal and don't have to worry too much of the background and the noise.

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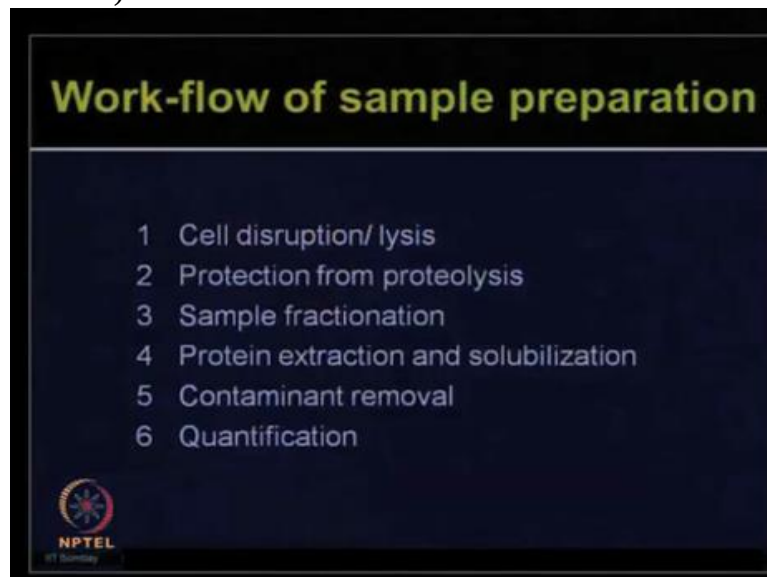
So I am giving you the workflow of protein sample preparation. Let's go step by step

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

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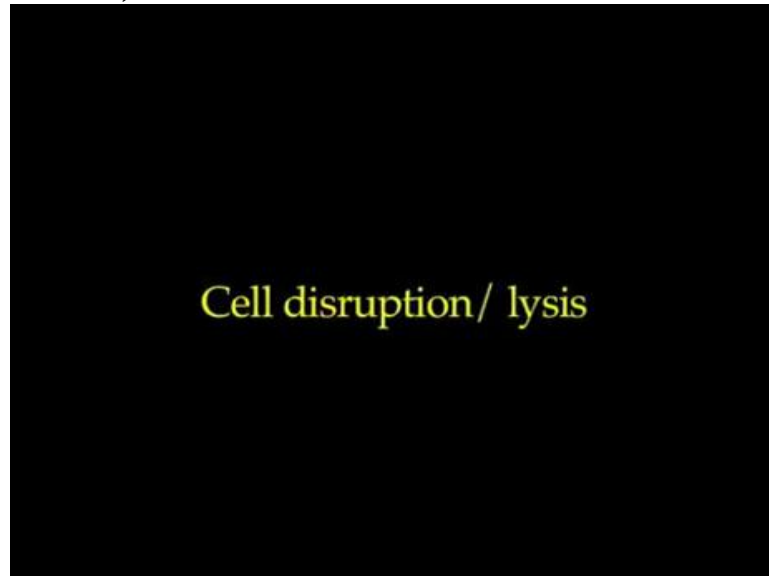


So the workflow of sample preparation would involve first, cell disruption or lysis, second protection from the proteolysis, third sample fractionation, fourth protein extraction and solubilization, fifth removal of contaminants and sixth quantification.

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.

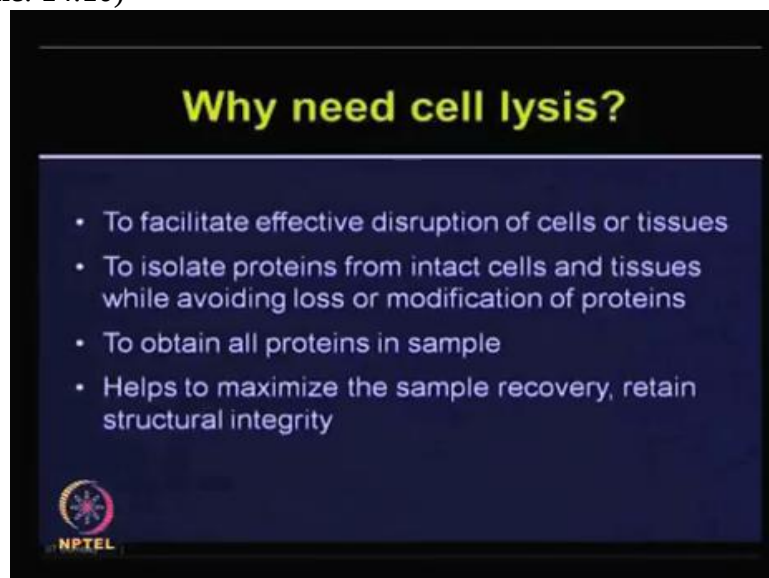
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So cell disruption or lysis... The lysis is very important step, because first of all, you would like to break open the cells and remove all the cell components outside so that you can get good protein yield. So cell disruption or lysis is very important.

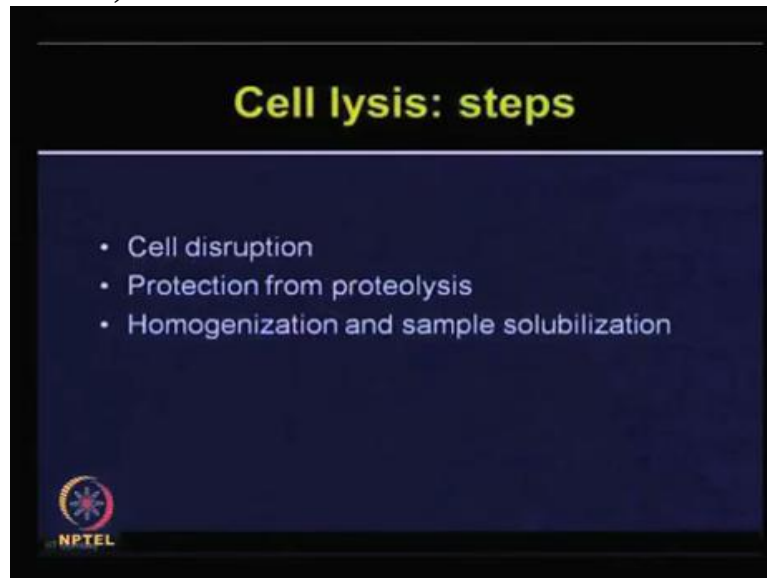
And it is often challenging because all the sample types, you cannot lys with the same type of method. So the lysis strategy has to be modified depending upon the sample type.

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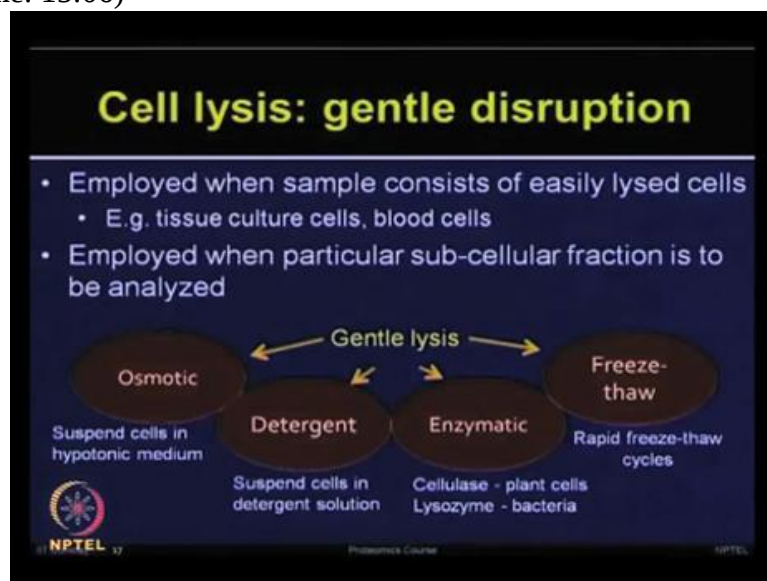
So why need cell lysis? To facilitate the effective disruption of cell or tissue, to isolate the proteins from intact cells and tissue while avoiding the loss or modification of proteins, to obtain all the proteins which are present in a given sample and to help to maximize the sample recovery and retain 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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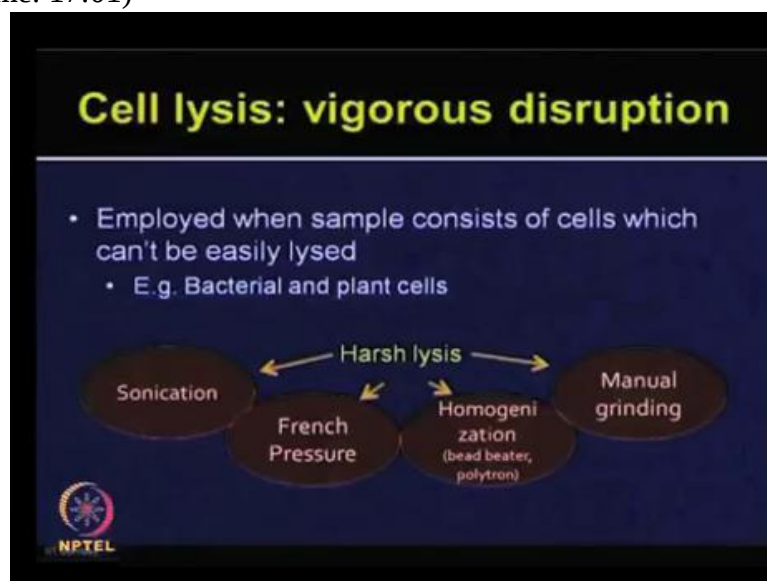
So cell lysis can be performed with the gentle way or in harsh condition depending upon the type of cells you want to disrupt. When you are employing gentle disruption method, you need to think that you have to break open all the cellular components.

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.

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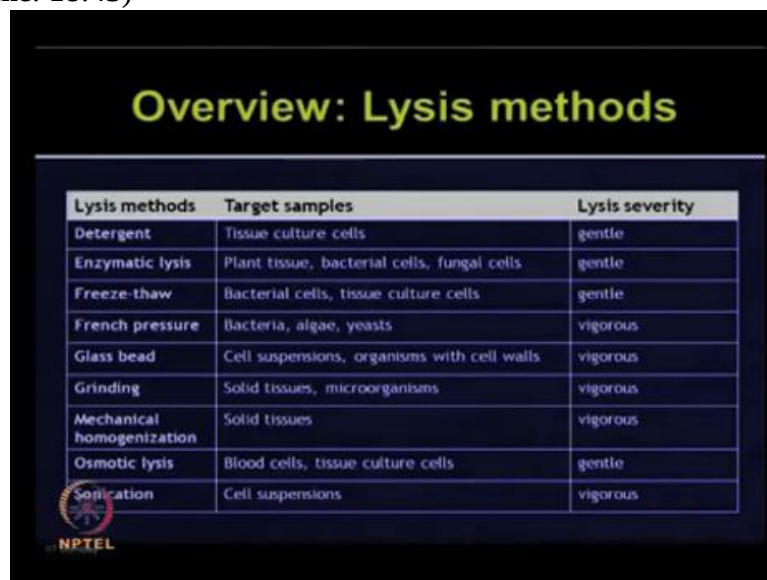
Now there are different types of challenges being imposed by different types of cells. If the cells are very difficult to lys, very difficult to break open, then you need to involve vigorous disruption methods such as sonication, French pressure, homogenization or manual grinding.

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.

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.

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Overview: Lysis methods

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

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So now I will give you the overview of lysis methods. We have talked about all of these methods briefly. I am giving you an overview now.

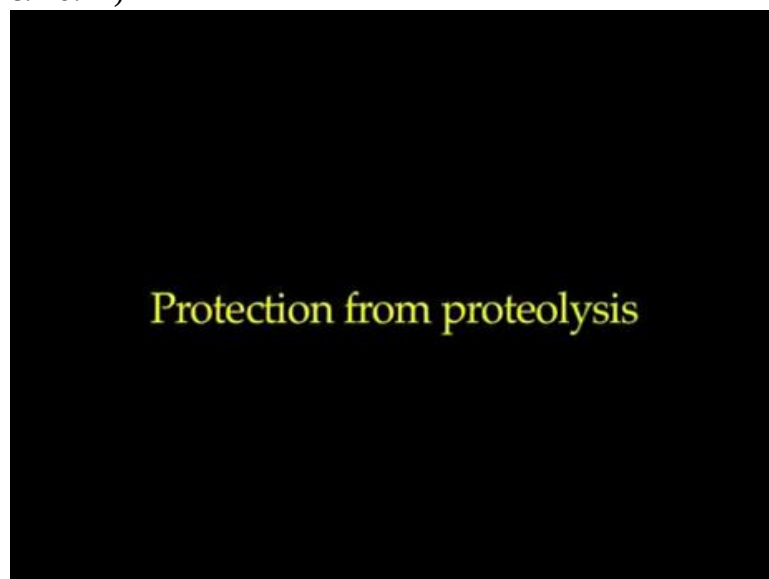
One can use detergents if your target sample is tissue culture cells; this is going to provide gentle lysis method. Enzymatic lysis can be used for plant tissues, bacterial cells, fungal cells

etc. This is again a gentle lysis method. Freeze thawing can be used for bacterial cells or culture 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.

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.

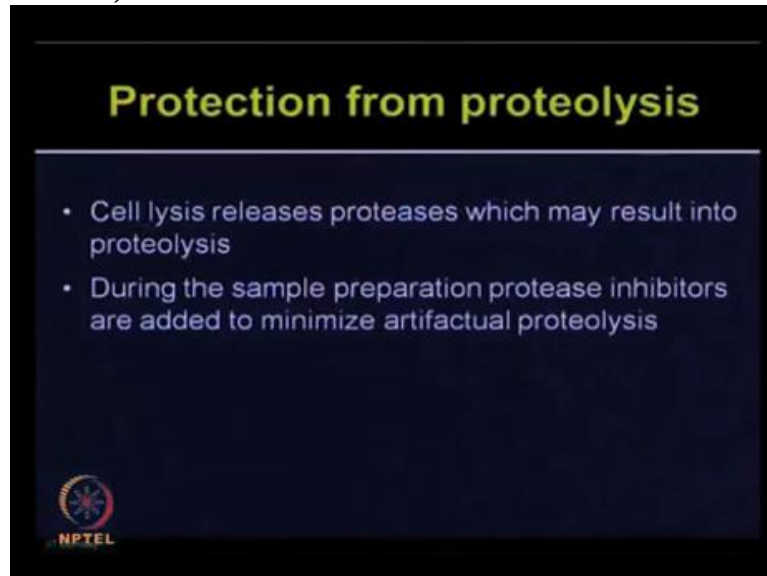
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Now you have tried to break open your cells and release all the protein components and other cellular components present inside the cell. But often during this process of grinding and lysis, you may degrade some of the protein and you need to ensure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

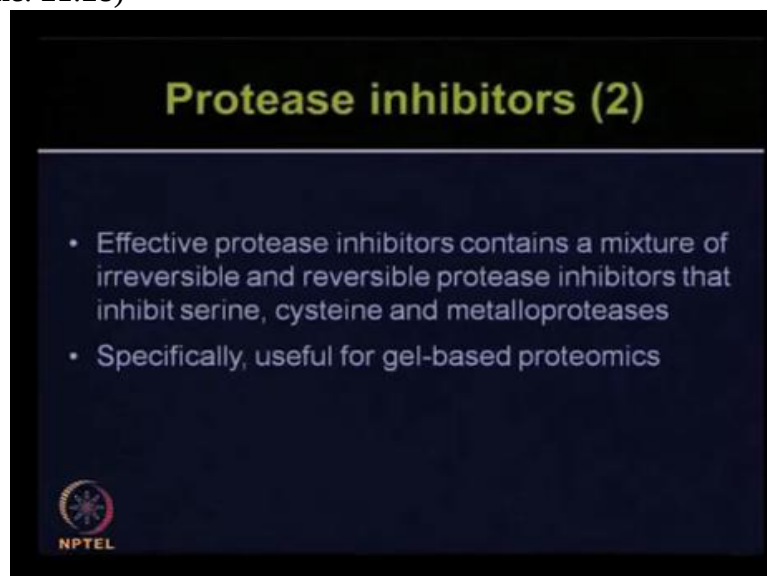
So protection of your protein components from the proteolytic step is very important during the protein sample preparation.

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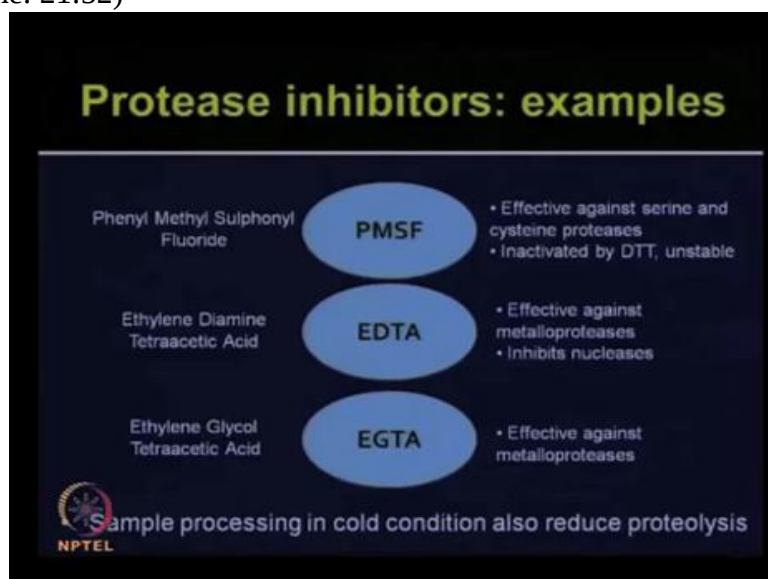
So cell lysis will release various proteases which may result into proteolysis. During the sample preparation, one can use different types of protease inhibitors which can minimize the artifactual proteolysis.

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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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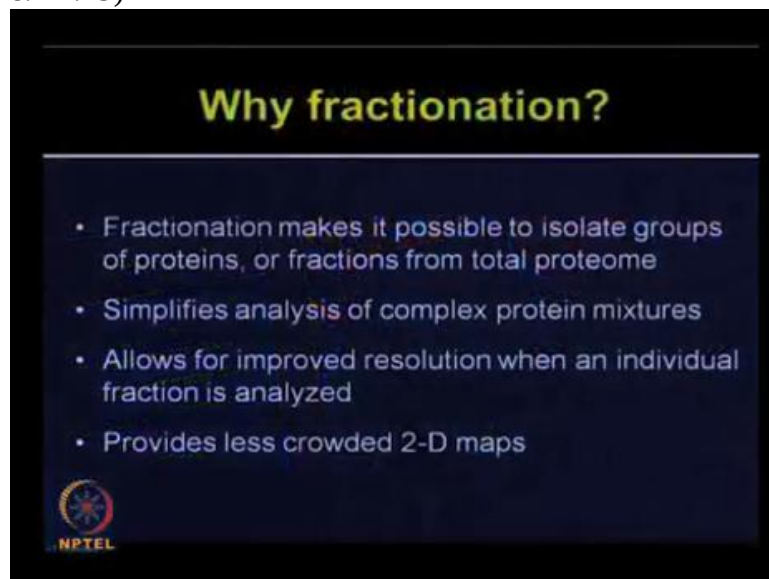


Now let's talk about sample fractionation. As I mentioned in the beginning, depending upon your applications you need to decide strategy, effective way of obtaining all the proteins so that you can study your biological problem in an effective way.

For example, if you are looking at a particular organelle, a proteome analysis for a given particular organelle, then you need to fractionate your sample.

Or if you are looking for wide proteome coverage but the proteome is posing lot of challenge to provide all the proteins mixture separation simultaneously, then one need to do the fractionation to reduce the complexity of sample.

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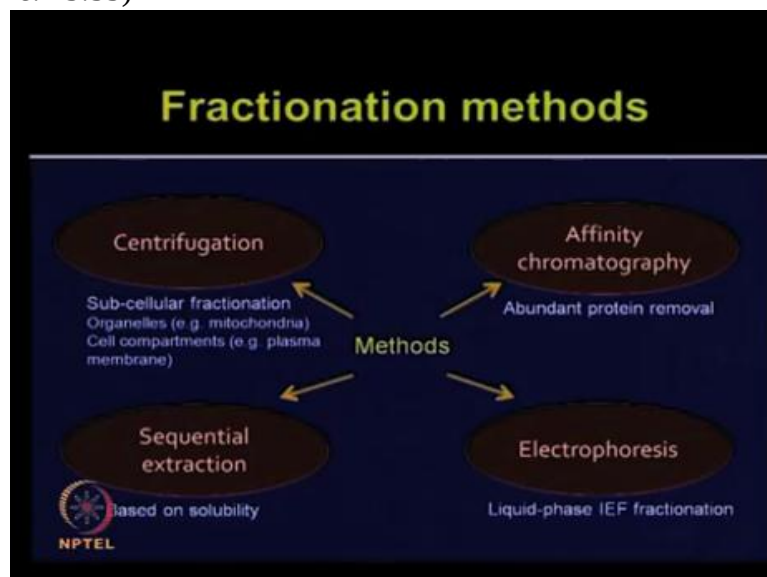
So why to perform fractionation? The sample fractionation makes it possible to isolate the group of proteins or fractions from the given total proteome. By doing fractionation, you can simplify the analysis of complex protein mixture.

This method can further allow the improved resolution of proteomic data and then, when you are obtaining the data from individual fractions and pooling it together, it is going to be more informative.

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 albumen 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.

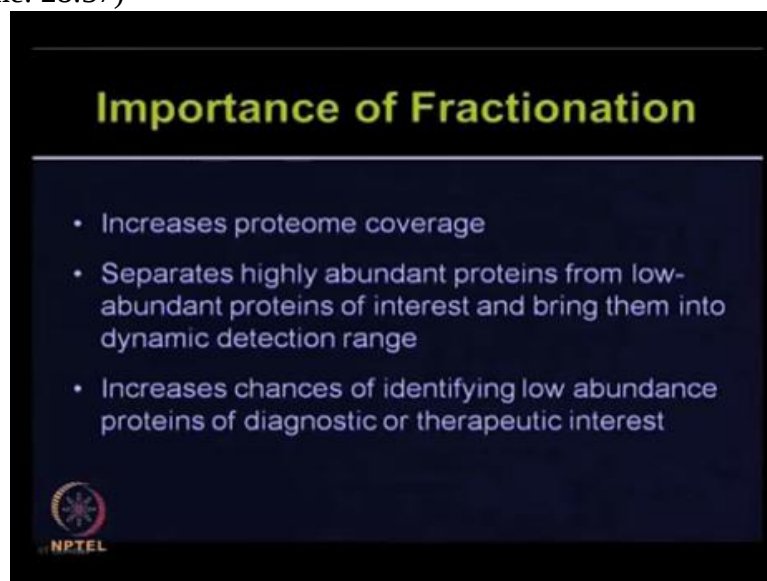
Electrophoresis can be used for fractionation. For example, if you are using gel-free method, directly you want to extract the protein and want to analyze that using mass spectrometry, so rather than applying the whole sample directly with the liquid chromatography, one can first simplify the proteome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

In the next lectures when I will talk about specific type of gel-based and gel-free techniques

I will describe you about new method being used is off-gel fractionation which is simplifying the step of isolating proteins, separating proteins based on their isoelectric points in liquid phase. So one can collect the fractions in different isoelectric points pI range and then further perform the proteomic analysis.

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So we have talked about different types of fractionation strategies. Now what is the significance of fractionation methods? The first most important thing, you would like to increase the proteome coverage. You would like to analyze as many proteins as possible from that given sample.

An effective fractionation method separates highly abundant proteins from the low abundant proteins of interest and brings them in the dynamic detection range. Often the low abundant proteins could be the proteins of interest which are being masked by the highly abundant proteins.

If you are able to separate the high abundant proteins, then probably you are able to increase the dynamic detection range and bring everything in the same range. Fractionation can also

increase the chances of identifying low abundant proteins of diagnostic or therapeutic interest.

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Points to ponder

- Sample preparation involves solubilization, denaturation, reduction and treatment of sample proteins
- Sample preparation starts with cell disruption and removal of contaminants
- Gentle lysis of cells can be performed by osmotic stress, detergents, enzymes and rapid freeze and thawing
- Plant and algal cells are subjected to sonication or liquid nitrogen crushing

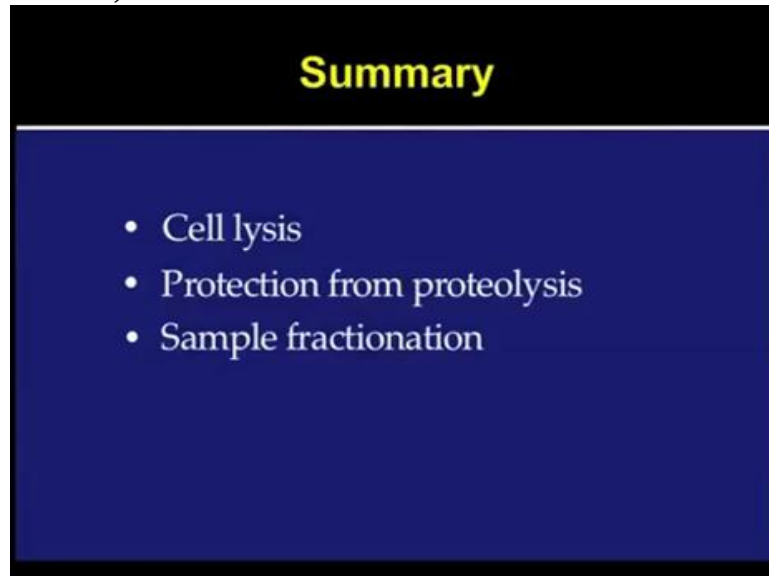
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Points to ponder

- Proteases fragment proteins in small pieces
- Proteases fall under 3 broad categories: serine proteases, cysteine proteases and metalloproteases
- Protease inhibitor cocktail usually consists of chemicals that target these key residues of proteases

So we will continue our discussion on how to perform the protein extraction and proteome analysis of serum and different types of biological samples in the next lecture.

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In summary today you have learnt about how to perform cell lysis, how to protect the cell from the proteins from the proteolysis by adding various types of protease inhibitors and sample fractionation methods. We will continue our lecture in the next class on serum proteome analysis followed by bacterial and plant protein proteome analysis. Thank you.