Introduction to Proteomics
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Lecture - 06
Sample Preparation and Pre-Analytical Factors

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 1000s of protein 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 (()) (01:31) spinal fluid, each sample type brings its own unique composition and its own challenges.

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

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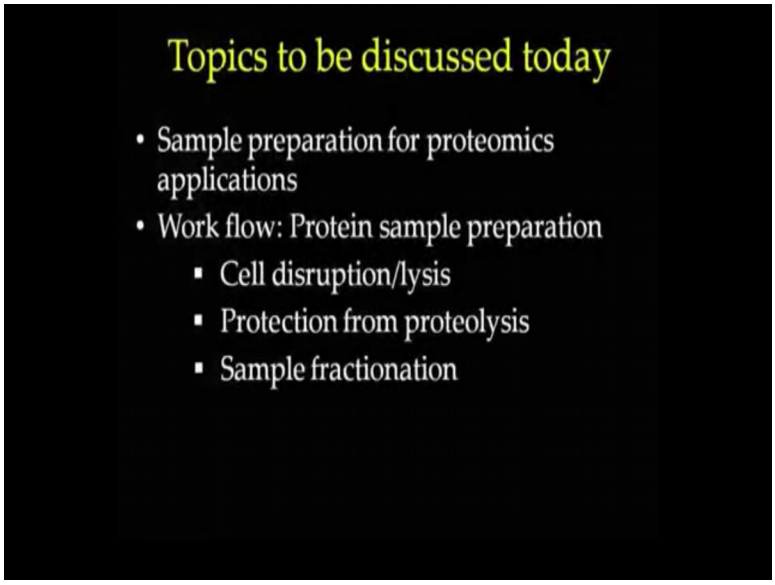

In today's lecture, we will talk about the sample preparation for proteomics application. I will give you a workflow for protein sample preparation such as the first step, how to disrupt the cell, how to lysis them, how to protect the cell during the lysis step so that there is no proteolysis occurring. How to fractionate the sample? Often you need to simplify the proteome.

You need to ensure that the sample is simple enough to show the good protein throughout the proteome coverage. So to obtain a comprehensive proteome coverage often it is important that you should pre fractionate your samples. Protein extraction and solubilization, these are another very essential component. Now when we are talking about the sample preparation, this is quite generic for different type of proteomic application whether one want to use for gel-based approaches or gel-free approaches.

So whether you want to use two dimensional electrophoresis, dyes or different types of gel-based applications or you want to perform mass spectrometry and different type 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 have added more when you are performing the gel-based proteomics.

So during this lecture I will talk to you about different type of components which are essential to make a good sample preparation for proteomic application. I will give you a stepwise workflow.

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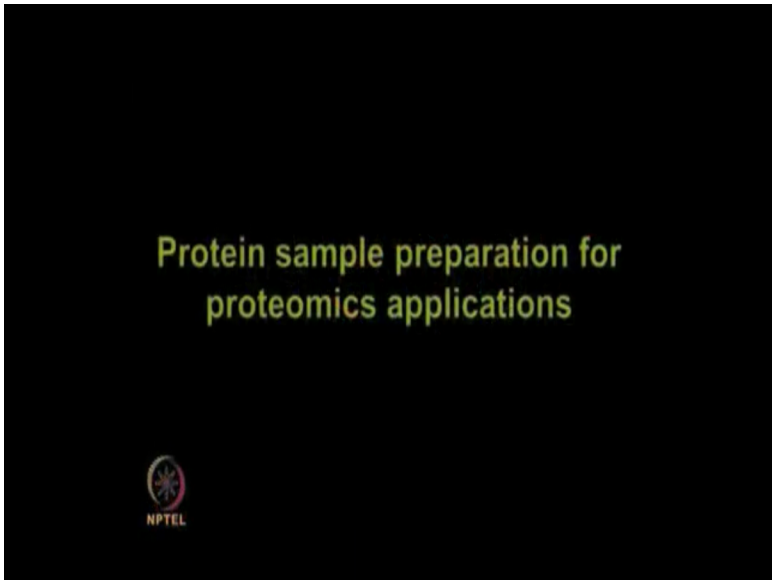

So let us start workflow for proteomic sample preparation.

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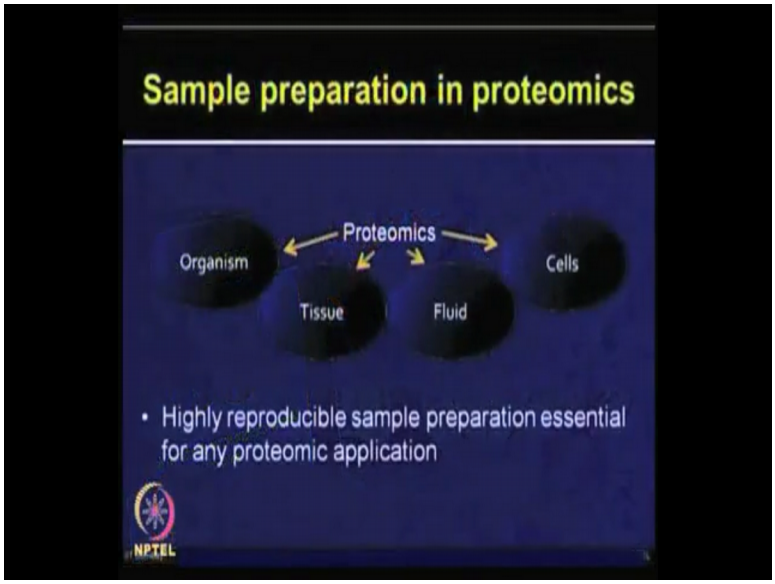

So the proteome is very complex, whether you want to perform your proteomic analysis for the whole organism, it means you want to know all the proteins present in a given organism or in a tissue or in body fluids or in different types of cells. Proteomics can be global or it can be very targeted or expression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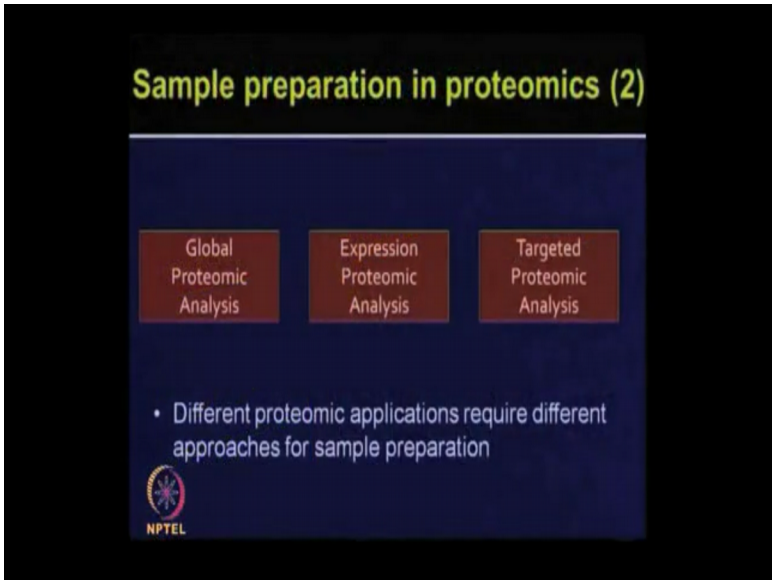

So let me give you 3 different terminology here for proteomic analysis. One is global proteome analysis, expression proteomic analysis and targeted proteome analysis. When I am talking about global proteomic analysis it means your aim is to characterize all the proteins present in the given sample.

Expression proteome analysis it means you are mainly interested to look for those changes which are big 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 type of clinical and different studies. Targeted proteomic analysis, if you are very focused for a given organelle or given sample type, often you would like to know what is happening in that particular proteome.

For example, mitochondria proteome. So one need to try different type of strategies, one, thinking about performing a sample preparation, what is your objective? Whether you want to do global profiling or 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 proteome analysis you just want to pre fractionate your sample in such a way that only that particular component is isolated and then all the proteins from that organelle or cell is extracted. So different type of strategies need to be used to perform these type of proteomic analysis. Now all of the sample processing involves solubilization, denaturation, reduction and treatment of sample proteins.

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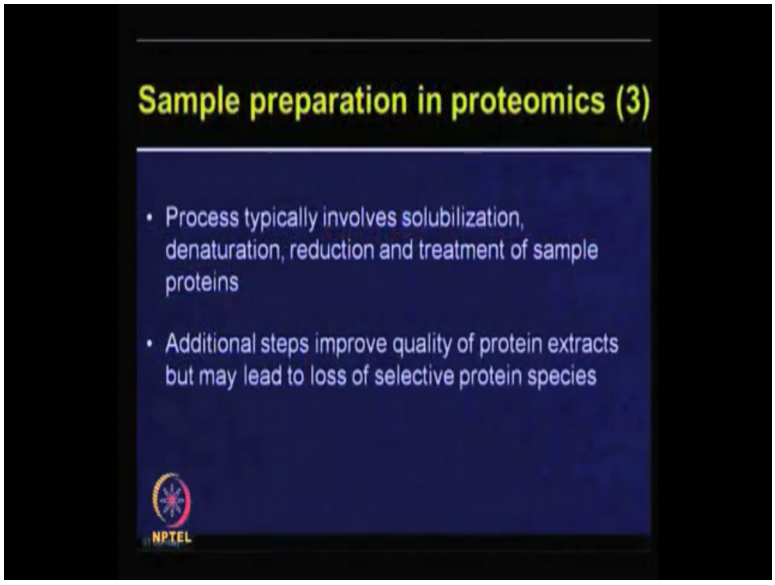

But you need to involve additional steps depending upon the type of samples and your type of objectives so that the protein quality, the protein extract can be improved and while you are doing this you have to be very cautious that when you are performing various steps and sequential type of extraction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

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

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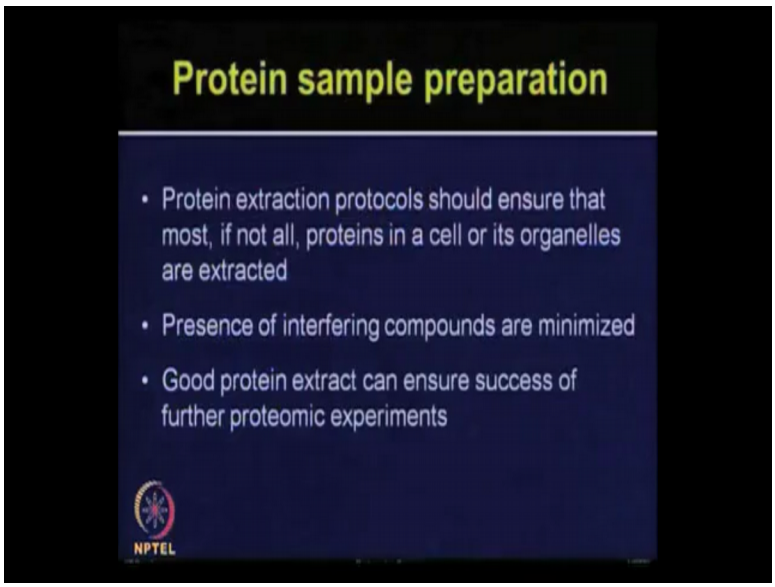

The presence of interfering compounds should be minimized. So if you have optimized a very good protein extraction procedure that should ensure that you have a very wide

proteome coverage and that is ultimately going to determine the success of your proteomic experiment.

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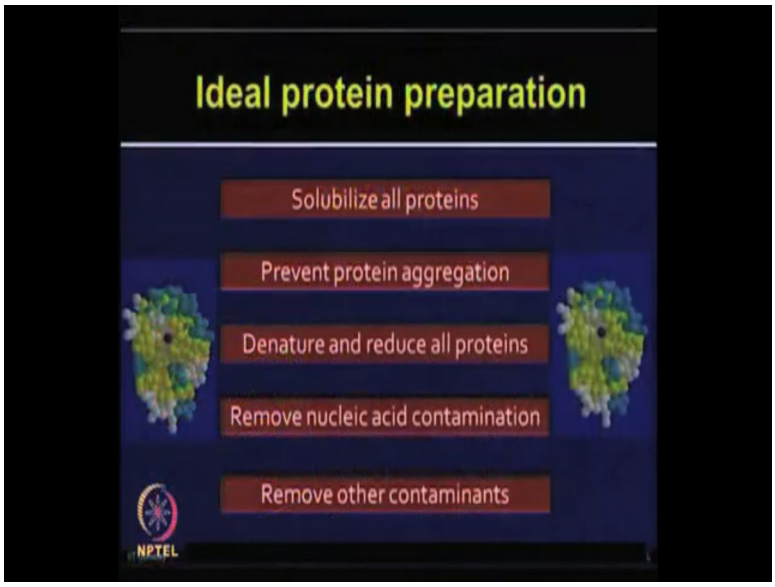

Ideal protein preparation is involved solubilizing all the proteins present in a given mixture preventing protein aggregation, denaturing and reducing all the proteins which are present in that mixture, removal of nucleic acid and other contaminants as well as removing salt and some other small interfering component.

Again depending upon your sample type you may have to think what a different type of interfering components could be present in that sample type if we 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 soluble 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 guidelines for sample preparation.

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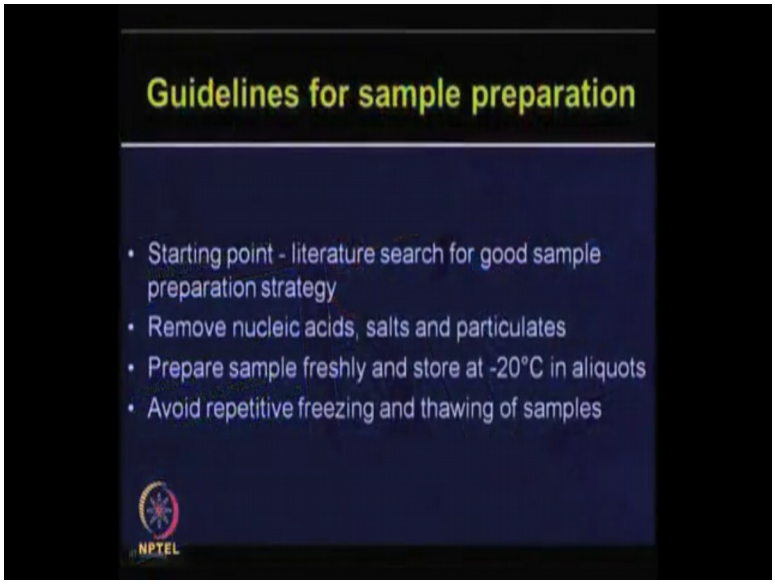

Starting point, one should start with finding a good reference from the literature as a starting point and then try to modify the protocol depending upon the objective of that experiment. You need to ensure that you remove nucleic acids, salts and different particles, prepare the samples as freshly as possible and store it in minus 20 degrees in a small (()) (10:22). You should avoid repetitive freezing and thawing of sample.

So why a good protein sample preparation is important.

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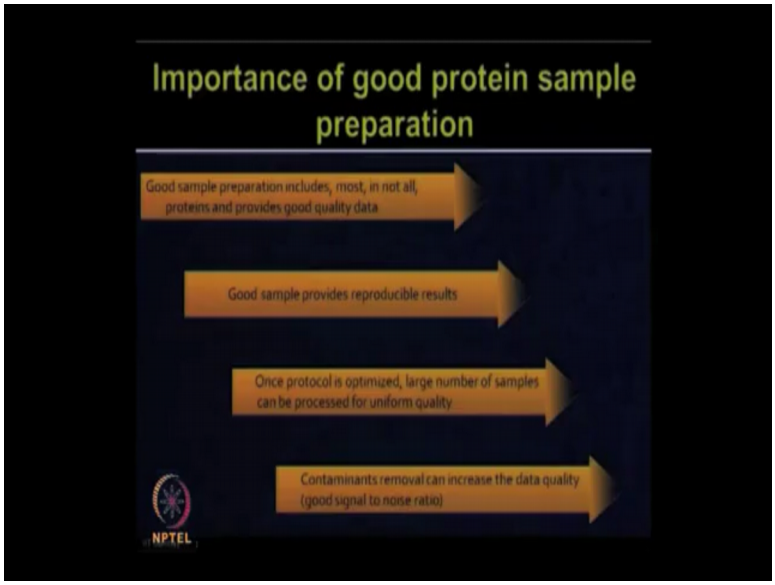

A good protein sample preparation includes all the proteins present in that mixture and it is going to provide you high quality data because there will be less interference from the artifacts. A good sample will provide reproducible results. You have to perform by the skill

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 a protocol with a small population then the same protocol can be applied for the large number of samples. So that is going to ensure the success of the clinical study.

Now if you are able to remove lot 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 how to remove the background and the noise.

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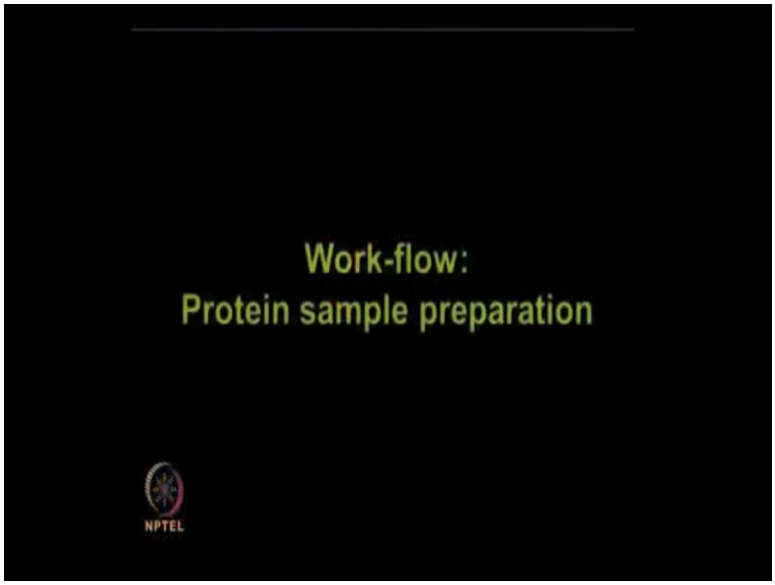

So I am giving you the workflow of protein sample preparation. Let us go step by step. Sample preparation.

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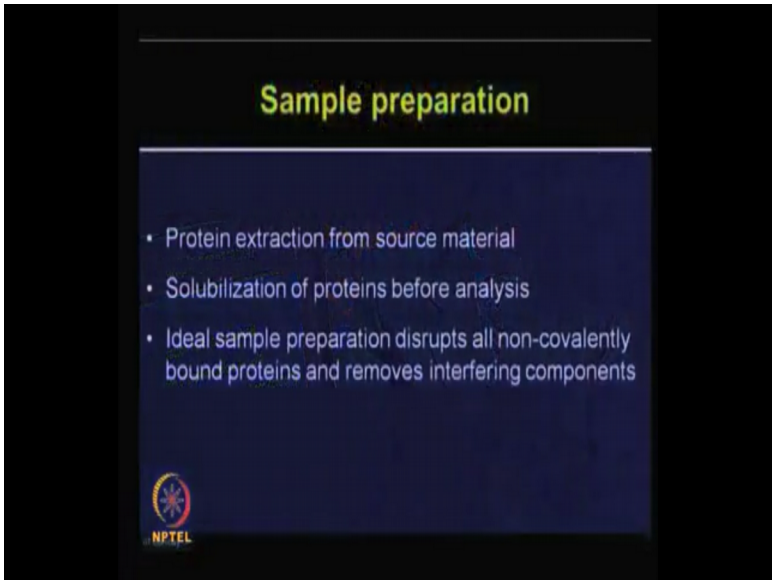

The protein extraction should be performed from source material and then you need to solubilize the proteins before starting analysis. The ideal sample will disrupt all the non-covalent bonds present with the proteins and it will remove the other interfering compound.

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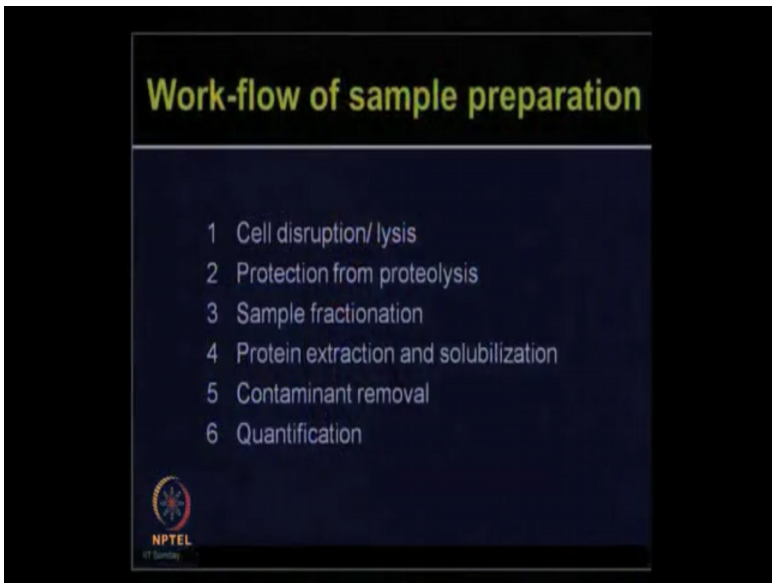

So the workflow of sample preparation would involve first some disruption or lysis second protection from the proteolysis, third sample fractionation, fourth protein extraction and solubilization, fifth removal of contaminants and sixth quantification. Obviously this workflow can be modified depending upon your sample size and few steps can be moved in that sequence.

So let us follow this workflow and during this workflow I will give you some examples of different type of samples, how to extract protein from those and what type of challenges these samples are going to impose.

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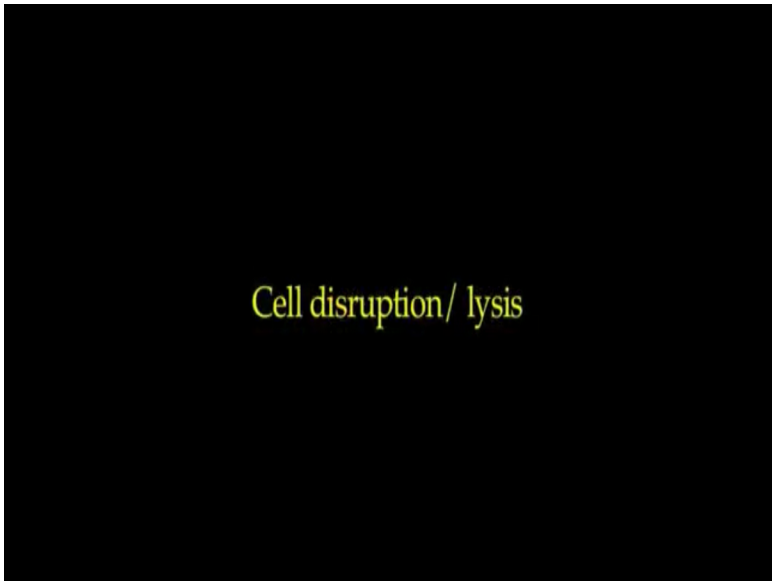

So cell disruption or lysis. The lysis is very important step because first of all you would like to break open the cells and remove all the cell components outside so that you can get good protein yield, so cell disruption or lysis is very important and it is quite challenging because all the sample type you cannot lysis with the same type of method. So lysis strategy have to be modified depending upon the sample size.

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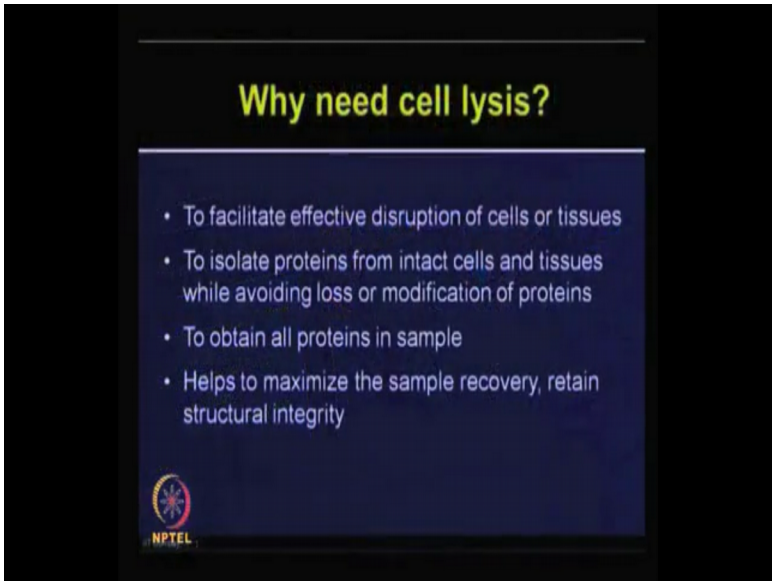

So why need cell lysis? To facilitate the effective disruption of cell or tissue. To isolate the proteins from intact cells and tissues while avoiding a loss of modification of proteins to

obtain all the proteins which are present in a given sample and to help 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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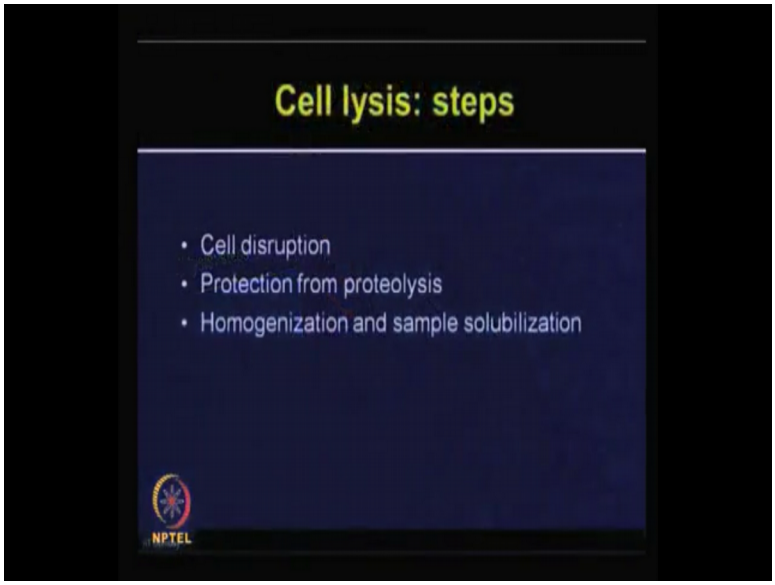

The different steps are involved in cell lysis, you need to disrupt the cell, protect from the proteolysis during the lysis is struck, homogenize and solubilize your sample. I will describe all of this in more detail.

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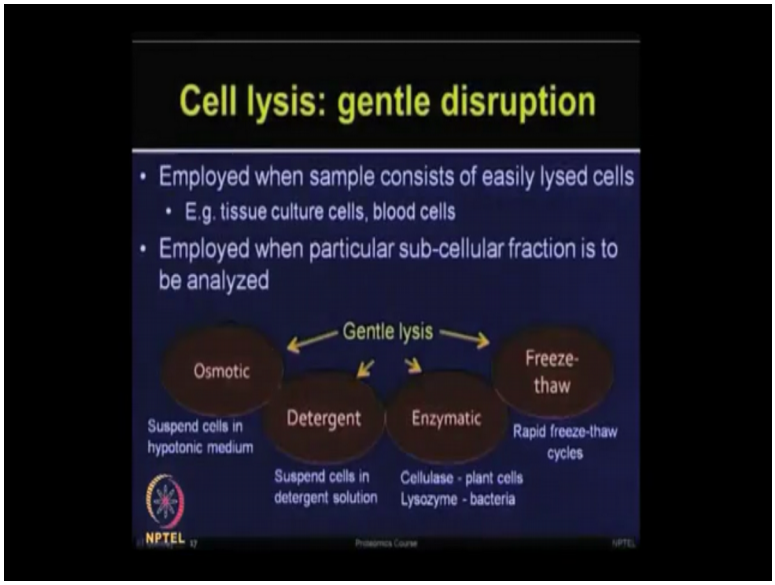

So cell lysis can be performed with a gentle way or in harsh condition depending upon, the type of cell you want to disrupt. When you are employing a gentle disruption method you need to think that you had to break open all the cellular components so your gentle disruption should be efficient enough to disrupt the cell. So if you are looking at those cell type which

can be easily lysed such as blood cells or culture cell, then you can involve the gentle disruption method.

There is different type of lysis methods available such as osmotic lysis in which one can suspend the cell in hypertonic medium, detergent lysis you need to suspend the cells in detergent solutions, isometric lysis if you are using plant cell for example. One can use cellulose enzyme. If you are using bacterial sample one can use lysozyme. There is different type of enzymes present which are used for isometric lysis. Freeze-thaw.

That is one of the very commonly used method for gentle disruption which involves rapid freezing and thawing cycle. You need to cool your sample in a very cold condition for example liquid nitrogen and then immediately putting a boiling water while doing this rapid freezing thawing the cells will break open.

Now there are different type of challenges being imposed by different type of cells. If the cells are very difficult to lyse, 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 about different type of cell lysis methods and some of its principle involved as we go along with more specific type of samples.

But in all the samples preparations mostly people use manual grinding or homogenization by using bead beater or polytrone homogenizes. So these are very commonly used homogenization method. The manual grinding is performed by using (()) (17:53). 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 post challenges with (()) (18:10) then you need to use the electronic homogenizers.

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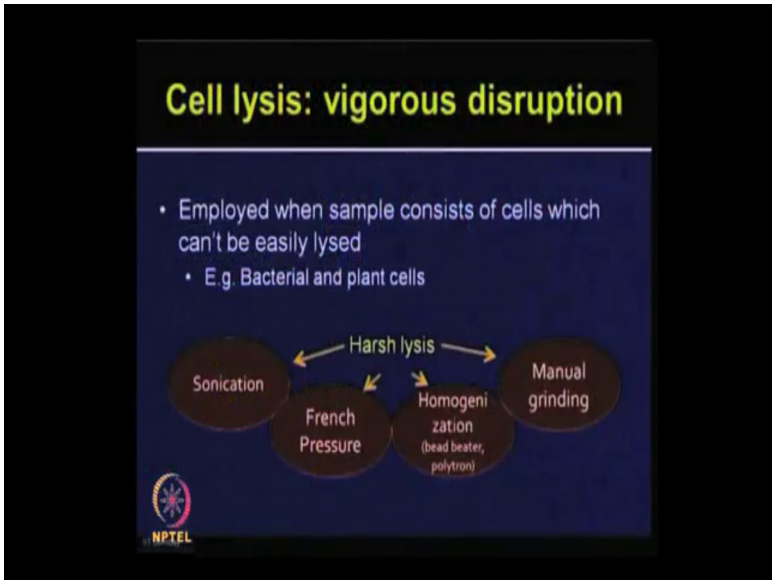

Then sonication and French pressures are more used when you are applying bacterial and yeast or different type of those cells which are difficult to open. We will talk about these in more detail in the more specific example when we talk about how to extract the proteins from bacterial sample. So now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

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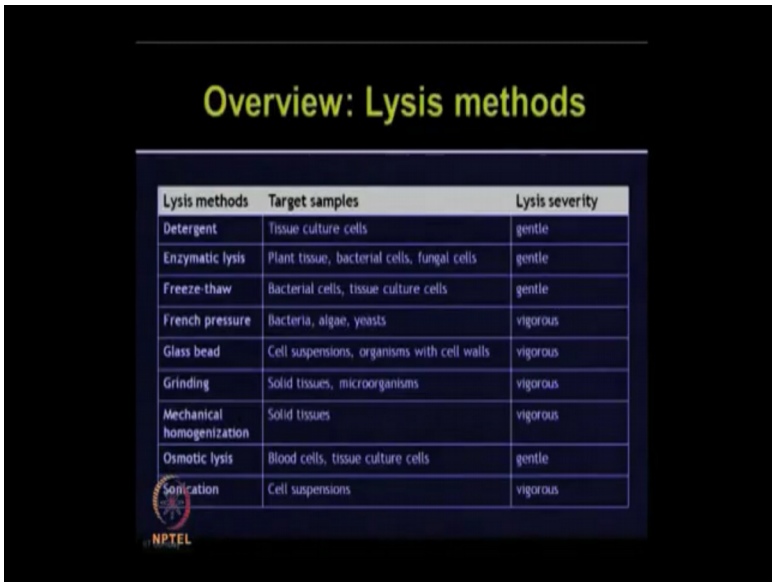

We have talked about all of these methods briefly. I am giving you an overview now. One can use detergents if your target sample is tissue culture cells, this is going to provide gentle lysis method. Enzymatic lysis can be used for plant issues, bacterial cells, fungal cells etc. This is again a gentle 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 bacterial, algae, yeast, this is 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 organism 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 suspensions and other bacterial samples which is another vigorous lysis method. I hope these methods give you some options to lyse yourself effectively.

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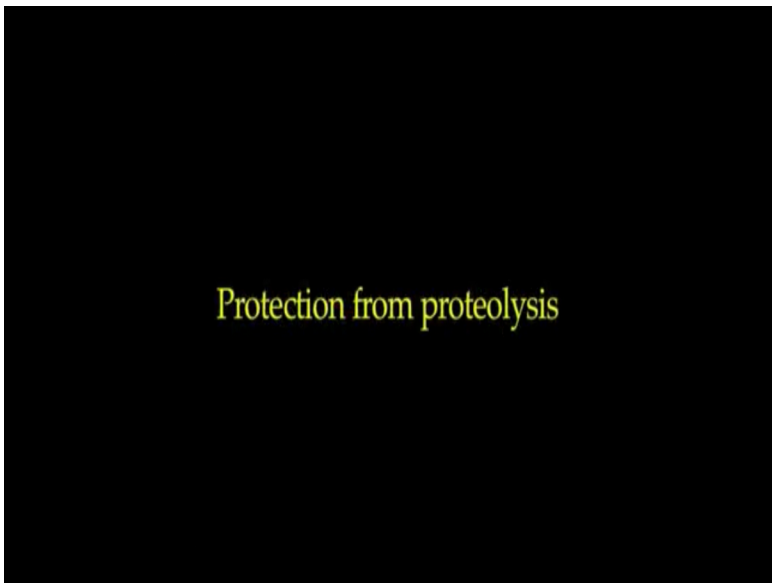

Now you have tried to break open your cells and release all the protein components and other cellular components present inside the cell. But often during this process of grinding a lysis you may degrade some of the proteins and you need to ensure that you have to protect the protein from the proteolytic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

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

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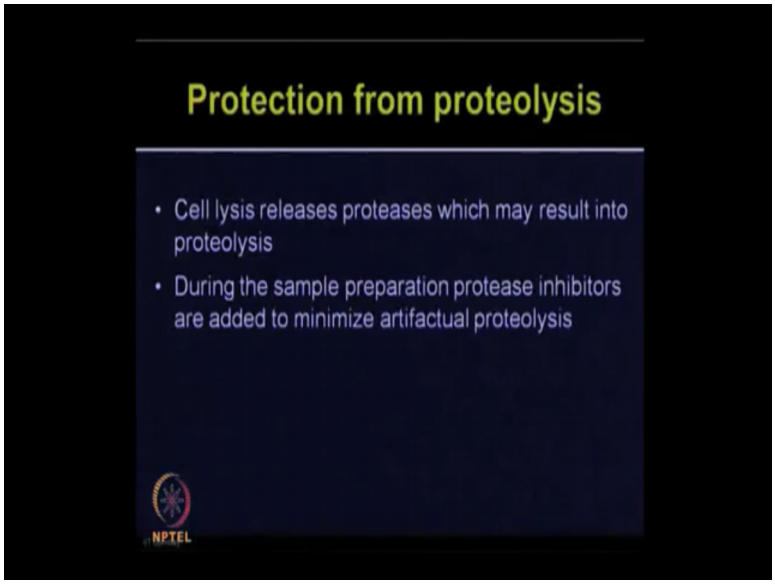

So lysis will release various proteases which may result into proteolysis. During the sample preparation, one can use different type of protease inhibitors which can minimize the artifactual proteolysis.

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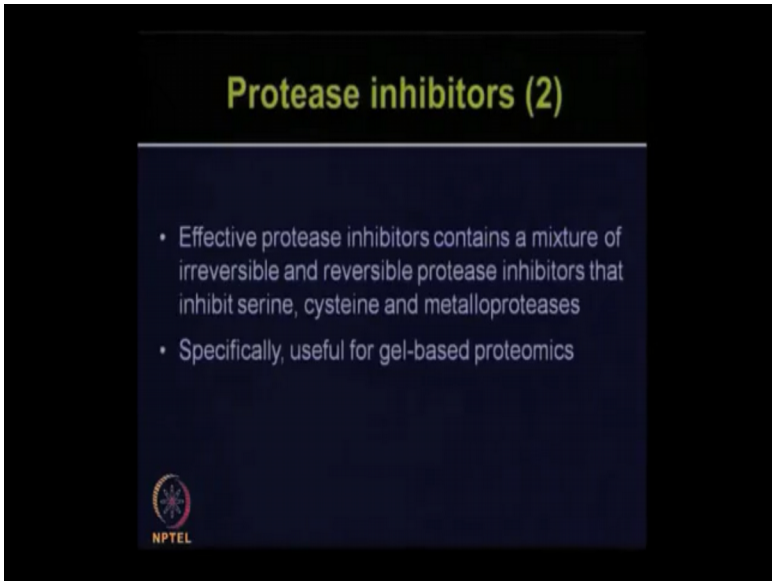

The effective protease inhibitors contain a mixture of different type of protease inhibitors, irreversible and reversible which inhibits serine, cysteine and different metalloproteases.

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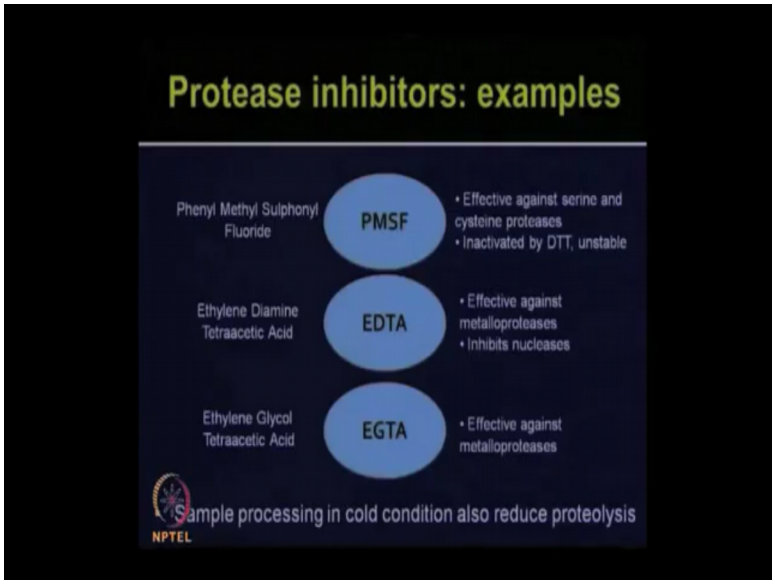

This step is more important when you are preparing your sample for gel based proteomic application. I will give you few specific examples of protease inhibitors such as Phenyl Methyl Sulphonyl Fluoride, PMSF which is very effective against serine and cysteine proteases. It can be inactivated by DTT and it is unstable when you are preparing the sample for gel-based application EDTA, Ethylene Diamine Tetra acetic acid.

It is effective against specifically metalloproteases. It inhibits nucleuses as well. So EDTA can serve both purpose of protecting from proteolysis and also partial inhibit of nucleus activity, EGTA or ethylene glycol tetra acetic acid. It is also effective against metalloproteases. Now when you are making your sample preparation for proteomic application it is very important that you process the samples in the core condition to reduce any proteolysis step.

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

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Now let us talk about sample fractionation. As I mentioned in the beginning, depending upon your application you need to decide a 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. So why to perform fractionation?

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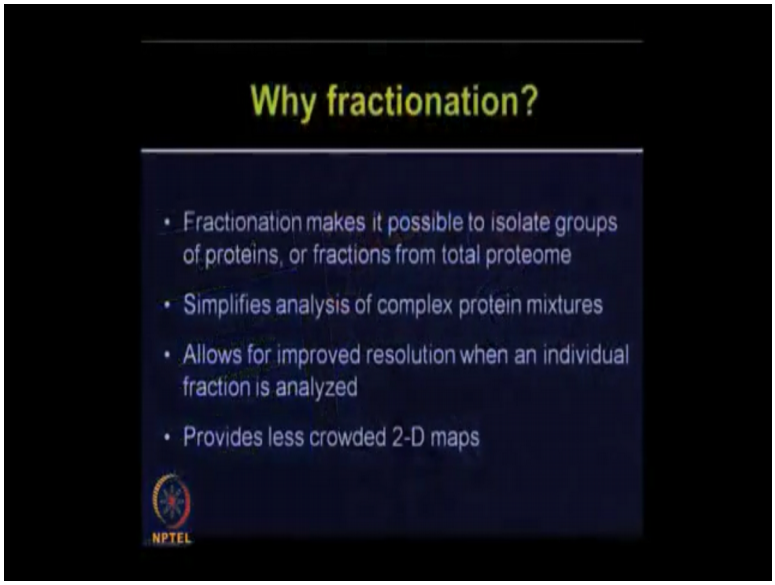

The sample fractionation makes it possible to isolate the group of proteins or fractions from a given total proteome. By doing fractionation you can simplify the analysis of complex protein

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.

Then you are applying your protein samples on to the gel or the other type of gel based proteomic techniques, this will provide you less clouded protein app. So again fractionation can serve a good need in both gel based and gel free proteomic applications. So let us talk about different type 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 compartments such as plasma membrane.

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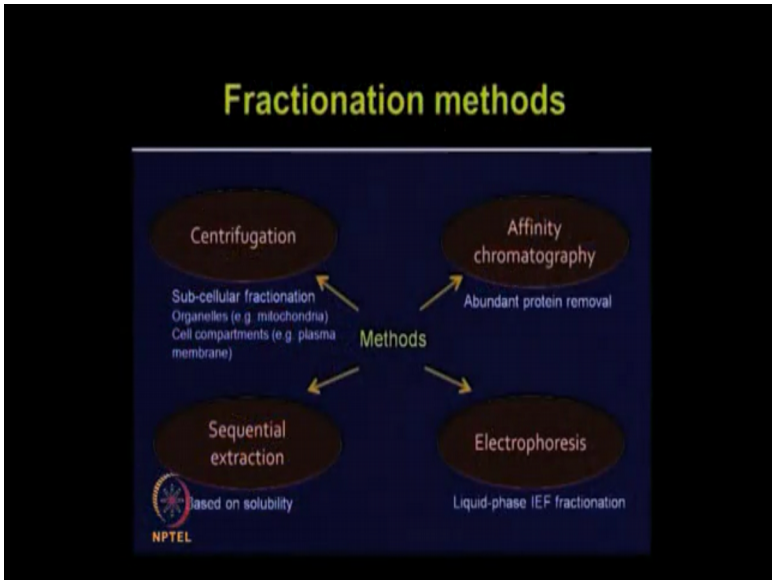

Different type of chromatography methods can be used for fractionation. If you are interested in looking at how the serum protein but there are certain proteins which are highly abundant in the serum such as serum albumin protein, so by using affinity chromatography method one can remove those highly 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 type of chemicals are used so that in a sequential way one can extract the

proteins, few proteins maybe more soluble in one particular composition of regions 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 start the protein and want to analyze that using mass spectrometry. So rather than applying the whole sample directly with electric chromatography one can first simply the proteome by using isoelectric focusing and doing the IEF process in the liquid phase itself.

The liquid phase IEF fractionation can simply the proteome based on the isoelectric point. In the next lectures when I will talk about a specific type of gel based and gel free techniques, I will describe you about a new method being used is (()) (28:00) fractionation which is simplifying such type of isolating proteins, separating proteins based on their isoelectric point in liquid phase.

So one can collect the fractions of different isoelectric point PI range and then further perform the proteomic analysis. So we have talked about different type of fractionation strategy.

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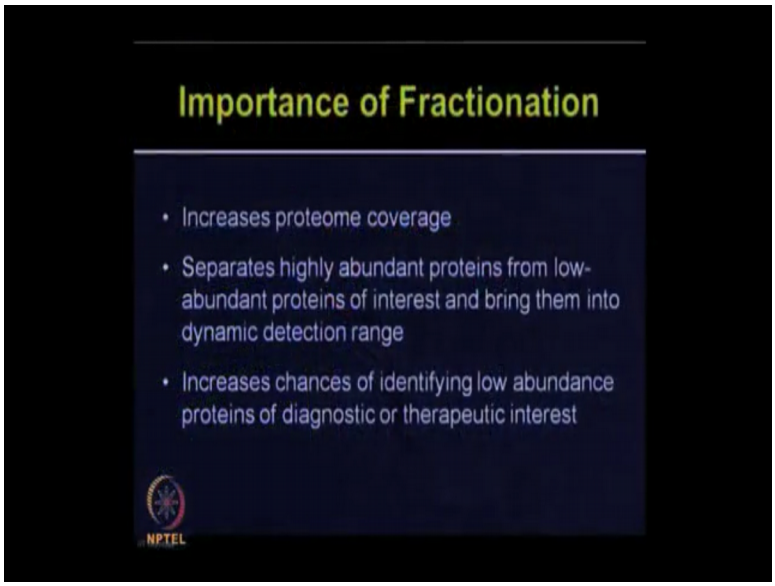

Now what is the significance of fractionation methods. The first most important thing we would like to increase is proteome coverage, you would like to analyze as many proteins as possible from that given sample. An effective fractionation method separates highly abundant

proteins from the low abundant proteins of interest and bring them into the dynamic deduction range.

Often the low abundant proteins could be the proteins of interest are being masked by the highly abundant protein if you are able to separate the high abundant proteins, then probably you are able to increase the dynamic deduction range and then everything in the same range. Fractionation can also increase the chances of identifying low abundance proteins of diagnostic or therapeutic interest.

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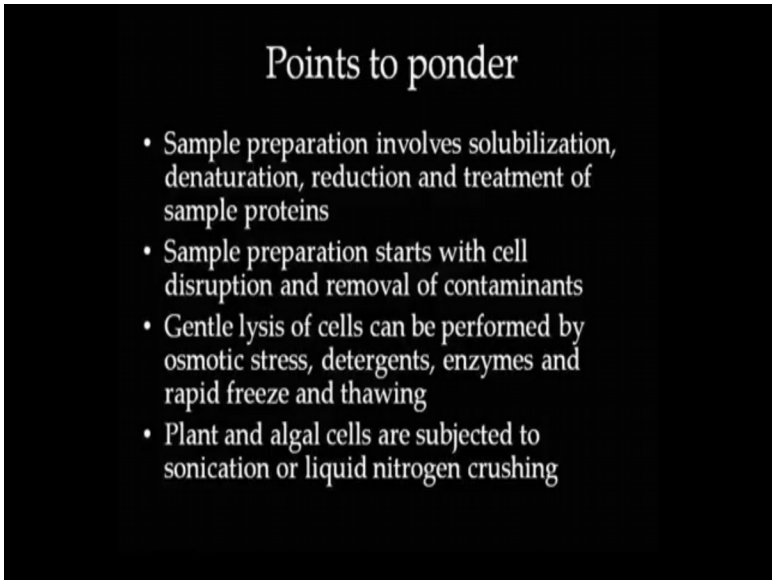

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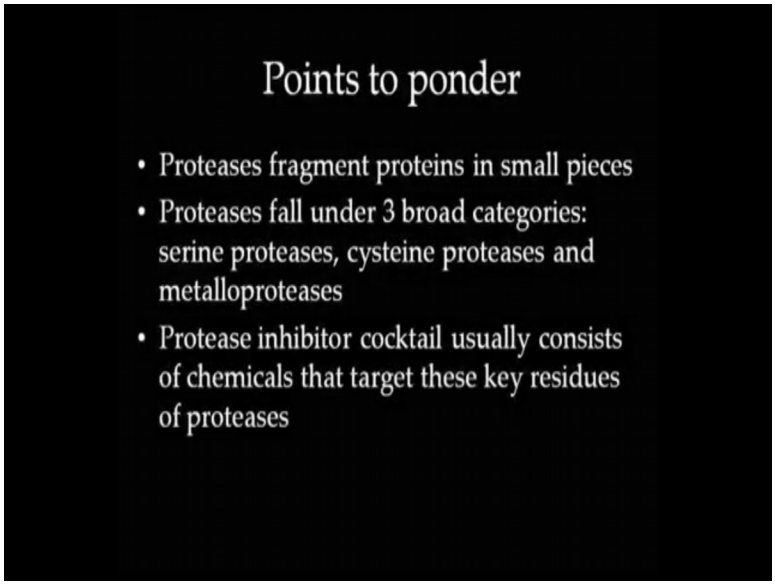

So we will continue our discussion on how to perform the protein extraction and proteome analysis of serum and different type of biological samples in the next lecture.

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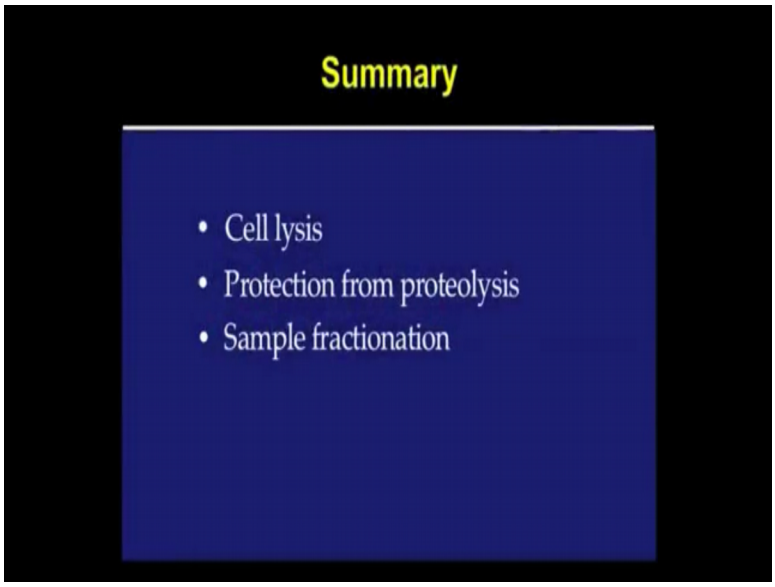

But the summary, today you have learned about how to perform the cell lysis; how to protect the cell from the proteins from the proteolysis by adding various type of protease inhibitors and sample fractionation methods will continue our lecture in the next class on serum proteome analysis followed by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