LECTURE-11

SAMPLE PREPARATION FOR PROTEOMICS APPLICATIONS: BACTERIAL & PLANT PROTEOME, QUANTIFICATION

TRANSCRIPT

Welcome to the proteomics course. Today we will talk about sample preparations for proteomics applications with special emphasis on bacterial and plant proteome analysis and protein quantification. In the last two lectures we discussed about different methods which can be used for very good protein sample preparation for proteomics applications. We talked about different type of lysis method; we talked about how to prevent proteolysis during lysis, different type of protein pre-fractionation methods then we talked about protein precipitation, protein solubilization and how to remove various interfering components.

So we discussed about work flow for protein sample preparation, different type of precipitation methods, and removal of interfering substances and then in the last lecture I started talking about specific examples first, we discussed about how to analyze serum proteome, how to prepare sample for proteome analysis and then we move on to bacteria and we started talking about sample preparation strategies for bacterial proteome analysis.

Today we are going to continue from bacterial proteome analysis. In today's lecture we will first talk about different type of methods available for bacterial protein extraction. I will recommend one method and elaborate on that then we will talk about how to prepare good samples for plant proteome analysis. We will then talk about how to quantify the proteins accurately so that you are ready to perform proteomics experiments.

Let's start with the bacterial proteome analysis.

So if you remember in the last lecture I stopped on the slide where I showed you various steps which are involve in bacterial protein sample preparation. So now let's elaborate on this and continue-

Different type of sample preparation strategies which people apply for bacterial protein extraction, these are all reported methods available in literature. I have just compiled it for your brief overview. For details of each of the protocol you can refer to each of the journal. So 2% SDS and heat treatment has been applied for bacterial sample preparation similarly lysozyme and acetone precipitation method has been used. TCA (tri-chloro acetic acid) and acetone together have been effectively used for precipitation.

Direct protein extraction and solubilization in the solubilization buffer has also been used and then Trizol method has been used for bacterial sample preparation. I will elaborate on the Trizol method.

So why we want to elaborate on Trizol method because this method provides you an opportunity for recovering DNA, RNA and protein, all three major biomolecules from same sample in a sequential extraction method one can obtain DNA, RNA and protein all by using this method. Trizol having Guanidinium isothiocyanate is one of the inhibitor for RNAase and gives good quality RNA. This method also excludes any possibility of nucleic acid contamination because you are already removing DNA and RNA, salts are also getting rid of by using this method. There is no lipid contamination because chloroform is being used along with trizol which dissolves lipids so this method is able to getting rid of various type of interfering components as well as different type of contaminant and finally protein are easy to resolubilize after extraction from the trizol method. So this method is very useful for extracting bacterial and other proteome analysis.

Let me brief you about procedure this is not detailed for the protocol, just I am giving you few points for your reference one can modify these depending upon how much protein they want to extract and based on their sample. So you can start with 1 ml trizol reagent and then add that in bacterial sample then add 200 µl chloroform immediately to the mixture and vortex vigorously and incubate for 15 min at the room temperature. Centrifuge it at 12000 g for 15 minute.

After the centrifugation carefully remove upper layer containing RNA using micropipette and add 300 µl ethanol to bottom layer. Again you need to centrifuge at 5000 g for 5 minutes to remove DNA. In fact, this step can be used for keeping the material safe for further DNA extraction. Same applies to the previous step where you can collect the RNA which can be further used for RNA clean up and preparation. So once you have removed the supernatant, which contains the proteins then you can collect that in a fresh tube and in this supernatant you can add 4 volumes of chilled acetone and incubate this mixture for 4-6 hours at -20^{o} C.

Once incubation is done then start centrifugation step at 12000 g for 5 minutes, discard the supernatant and retain pellet. This pellet can be washed with 95% ethanol, 3-4 times then dry this pellet at the room temperature and this pellet can be reconstituted in a buffer suitable for your analysis. If you are going to perform 2-D electrophoresis you need to add the lysis buffer which contains urea, CHAPS, thiourea and different other components which we have discussed previously or if you want to analyze on SDS-

PAGE you can add the laemmli buffer. So depending upon your application you can reconstitute the dried pellet in the right buffer composition.

So let me show you the bacterial protein sample preparation in the interactive animation.

For any bacterial proteome analysis, first you need to grow bacterial culture on LB media containing suitable antibiotic. Now the proper 37°C temperature is provided for overnight or 6-8 hours growth. The overnight cultures are diluted with fresh LB and grown at 37°C for 6-8 hours. Continue growing the culture till it reaches mid exponential phase. The bacterial cells can be harvested by centrifuging cells at 12000 rpm for 10 minutes at 4°C. Wash the bacterial pellet with phosphate buffer of pH 7.4 for 4 times to remove the media. Resuspend the pellet with protease inhibitors and lysozyme and cells can be further ruptured by sonication in ice to prevent foaming and heat. The sonication step helps to release the content of bacterial cells. Sonication can be performed by using a sonicator for set cycles of 5 seconds pulse with 30 seconds gap in between at 20% amplitude. Sonication involves the use of high energy sound waves those are capable of opening of outer membrane of cell. Cell debris and unbroken cells can be separated by centrifugation step.

To the supernatant add 1 ml of Trizol and 200 µl of chloroform and mix vigorously for 15 seconds by vortexing it at room temperature to allow the phase separation. After phase separation centrifuge the sample at 12000 rpm for 15 minutes at 4°C. As I mentioned earlier the different protocols one can use for bacterial proteome analysis, here we are demonstrating the Trizol method. In which the top pale yellow layer contains RNA, the middle white layer contains protein and lower phenol layer have both proteins and DNA. Remove the upper layer and use it for RNA isolation using Isopropanol if you want to use RNA for some other application. To the bottom layer add 300 µl of absolute alcohol per 1 ml of trizol and mix gently to suspend the white precipitate and keep at room temperature for 3 minutes. Centrifuge the mixture at 12000 rpm for 15 minutes at 4°C to precipitate the DNA. To the clear pink layer add 4 volumes of chilled acetone and keep it at -20°C for 20 minutes. Protein pellet can be washed with 95% ethanol for 3-4 times, during the washing step you have to also vortex so that the pellet is properly washed. You need to allow the pellet to dry at the room temperature and then add the lysis buffer containing 7M of urea, 2M of thio-urea, CHAPS, IPG buffer, DTT and bromophenol blue.

I hope this animation was informative for bacterial protein extraction by using trizol method and you must appreciate that in this method you can obtain DNA, RNA and protein all the biomolecules together.

So to give you further information about the detailed protocol as well as how to perform these experiments in the lab, I will show you a video for the laboratory demonstration of bacterial protein sample preparation for proteomics applications so let's watch this video.

Bacterial protein extraction-

This process involves culturing of bacteria, harvesting and sonication of the obtained culture followed by protein extraction.

Bacterial culturing-

Clean the laminar work space thoroughly with ethanol and keep the master plate containing bacterial culture ready, light the spirit lamp or Bunsen burner to maintain sterile or aseptic conditions throughout the process. Carefully remove a bacterial colony from the master plate and inoculate the autoclaved growth media. Incubate this inoculated sample at 37^{o}C for 6-8 hours with constant shaking to allow bacteria to grow. The turbidity of the culture which gradually increases indicates bacterial growth.

Culture harvesting-

Transfer the grown bacterial culture to a fresh tube under sterile condition. Centrifuge this tube at 12000 rpm for 10 minutes, maintain the temperature 4^{o}C. Transfer the pellet obtained containing intact bacterial cell in a fresh microfuge tube. Wash this pellet thoroughly with phosphate buffer to remove any unwanted debris.

Sonication

The resuspended pellet is sonicated on ice to enable the bacterial cells to break open so that it contains a released. Sonication involves the use of high energy sound waves that are capable of breaking open the outer membranes of cells. All cellular contents including proteins of interest leak out of this disrupted membrane. Carry out the sonication procedure for 30 seconds with a pulse of 1 second at 40% amplitude. Once it is complete, centrifuge the contents and collect the supernatant that is obtained.

Protein extraction- The trizol extraction protocol allows efficient separation of not just bacterial proteins but also their DNA and RNA. Add the trizol reagent containing Guanidinium isothiocyanate, phenol and chloroform to the supernatant obtained after sonication. Mix the content thoroughly by vortexing. Add chloroform to this solution, mix the contents and place the tube on ice for few minutes. Centrifuge the tube at 2000 rpm for 5 minutes. 3 distinct layers will be obtained. The uppermost layer is aqueous layer containing RNA, the centre is interphase containing proteins and bottom layer is organic

and consists of DNA. Discard the transparent top layer having the RNA then add absolute alcohol to the remaining layer and mix the solution well. Centrifuge the contents at 2000 rpm for 5 minutes. The DNA forms a white precipitate at the bottom of the tube while the proteins remain in the clear supernatant. Collect the supernatant in a fresh tube. Then add chilled acetone to this tube and mix well by vortexing. Store this solution at -20^{o}C for at least an hour before centrifuging it to obtain the protein pellet. Discard the supernatant and dry the pellet at room temperature. Reconstitute the dried pellet with rehydration buffer and store overnight at -20^{o}C before carrying out protein quantification.

I hope this video was informative and now you are able to appreciate the complexity involved in this procedure as well as how useful this method can be for different type of biomolecular extraction including DNA and RNA and how protein can get rid of various type contaminants using trizol method

So now let's move on to the plant proteome analysis, the third specific example. So in the flow of sample preparation strategies, I tried to give you a feel of different type of samples available and the challenges which are associated with each sample type. We talked about human serum; we talked about bacteria and now let's talk about plant leaf and how to analyze the plant proteome.

So as you are aware plants are very crucial because they provide food source for human and animals. To understand the detail of molecular events happening inside the plant cells, one need to study its genome, transcriptome and proteome to understand events in systems approach. The plant proteome analysis is very crucial because it can reveal various molecular mechanisms, which are underlying plant growth, development and its interaction with other biomolecules as well as with the environment.

The analysis of plant proteome can provide information for various properties such as protein abundance, protein modification, where the proteins are localized the sub-cellular localization, the 3-D structure of the proteins, its interaction with the proteins as well as other biomolecules. So similar to other proteome analysis plant proteome analysis is very crucial.

So let me explain you a protocol, a method you how to perform leaf proteome analysis. Let's start with the leaf protein extraction. So if you want to analyze the leaf proteome, you first need to extract all the proteins present in the leaf. So you can take 300 mg of leaves (you can adjust the weight depending upon your experimental requirement) and homogenize using a mortar pestle or homogenizer or different type of lysis methods can be used so homogenize this 300 mg leaf in the liquid nitrogen by using mortar pestles.

Add 1.5 µl of TCA in acetone, one can use 10% TCA in acetone with minor addition of DDT, 0.07% percent of DDT and add this to the ground tissue again grind it so that it becomes very fine and then incubate this homogeneous solution at -20°C for 1 hour. Then centrifuge this mixture at 14000 rpm for 30 minutes at 4°C. Remove supernatant layer and wash pellet 3-4 times with chilled acetone containing 0.07% DTT.

So in the leaf protein extraction then you need to dry the pellet at room temperature, reconstitute the pellet in lysis buffer or a buffer which is suitable for your proteomic sample, centrifuge the contents at 14000 rpm for 15 minutes at 4°C and then collect the supernatants for further proteomic applications.

So here in the pictorial form, I am giving you an overview of steps involved in the plant protein sample preparation starting from the leaf collection, weighing the leafs, transfer into the mortar, grinding by using liquid nitrogen with a pestle, homogenization in the presence of TCA and acetone, after centrifugation removal of the supernatant, how the protein pellet is formed then need to be washed with acetone couple of times and then the pellet can be reconstituted with lysis buffer.

So let me give a laboratory demonstration; a video for the plant protein sample preparation.

Plant protein extraction- This process involves homogenization of the plant leaf sample followed by acetone precipitation for protein extraction.

Leaf homogenization- Select the plant leaf sample of interest and weigh around 300 mg of the leaf on an aluminum foil. Transfer these leaves to a chilled mortar and carefully add liquid nitrogen to it which helps in drying up leaves instantaneously. Grind the leaves well using a pestle to obtain a fine powder. To this powder add around 0.5 ml of lysis buffer containing TCA, acetone and DTT and grind it well until a fine paste is obtained. The lysis buffer causes the plant cells to swell and finally break open thereby disrupting the membrane and releasing all its intracellular contents. Add another 1 ml of lysis buffer to the paste and then transfer the solution to a fresh tube after grinding thoroughly to obtain a uniform mixture. Centrifuge the sample at 10000 rpm for 5 minutes and maintain a temperature of 4°C during the process to ensure that there is no denaturation of the proteins. Discard the supernatant and retain the pellet containing plant proteins along with various other intracellular components. Incubate the pellet at -20°C for 1 hour.

Protein precipitation - Remove the pellet from -20°C and add chilled acetone to it. Mix the sample well by vortexing to obtain a uniform solution. Centrifuge the contents at 4°C for 5 minutes at speed of 10000 rpm, discard the supernatant and repeat the acetone

washing at least three times to remove plant pigments to obtain a protein pellet at room temperature. Reconstitute the pellet with rehydration buffer. Vortex the sample to obtain a uniform solution, centrifuge the contents following morning at 10000 rpm for 5 minutes. Collect the supernatant containing proteins in a fresh tube and store at -20°C until protein quantification is performed.

By watching this video, now you must be familiar with TCA acetone precipitation method how it can be effectively used for plant protein sample preparation.

So now let's move on to the next topic which is protein quantification. So by now you have prepared your protein sample by using different type of methods and by keeping a careful eye on different interfering substances and contaminants and tried to eliminate all those components. Now you want to know how much protein quantity you have in your protein sample whether you can perform your experiment with that protein or not so one need to know protein quantity present in the sample. So there are different protein quantification methods available, we will talk about protein quantification in detail now.

The protein concentration determination by the UV absorption, it is one of the very commonly used method in fact oldest method to determine the protein concentration by the absorbance at 280 nm. This method is based on the absorbance of UV light by aromatic amino acids such as tryptophan and tyrosine residues which are present in the protein solutions. Phenylalanine is also aromatic amino acid but up to a lesser extent it is responsible for this absorbance.

So to determine the protein concentration at A_{280} method requires that your protein contains tryptophan and tyrosine aromatic residues. Since protein samples will have variability in aromatic amino acid content so the absorptivity at 280 nm will be variable also if you need very high protein concentration for your protein preparation for proteomic applications then this method is again limiting factor. So higher protein concentration is not always possible for you to obtain from your protein sample so you need to quantify protein concentration even if you have lower protein concentration so this method has some limitation- one that aromatic amino acid has to be there to obtain absorption at 280 nm and protein concentration should be in very high amount. To overcome these limitations there are various reagents and assays have emerged and these reagents can be used to determine the concentration of proteins in a given solution. These methods include Lowry assay, BCA assay and Bradford assay. There are several other assays but we will talk about these three more commonly used methods.

Let's talk about different type of colorimetric methods to determine protein concentration-

Let's first talk about Lowry assay - This is one of the very common methods, which have been used for quantification of soluble proteins. There are two main steps which are involved in performing Lowry assay- first- the alkaline cupric tartrate forms a complex with peptide bond of protein, second- after that a reduction step with Folin and Ciocalteu's reagent. This reaction yields purple color and absorption can be measured between 500-800 nm.

This method is very simple; it is very precise and sensitive. These are various advantages of using Lowry assay and that is why when people have abundant proteins in their samples they use conventionally Lowry assay. But if you are using a clinical sample or if you are using samples with very less yield probably this may not be the best method to use and that is why there are different other methods are also available.

So one of the problems of using this assay that it is unsuitable for those proteins which do not contain aromatic residues such as tyrosine, this assay depends on the reaction of tyrosine residues with the reagent so if you do not have tyrosine residues in you protein sample, then it would not be suitable. The sensitivity to the interfering reagents such as Tris, EDTA that is another limitation of this method but it can also be overcome if you can add precipitant such as TCA. By addition of TCA one can overcome this limitation.

Now let's move on to next assay which is BCA assay. In the BCA assay the proteins form complex with Cu^{2+} ion in alkaline solution. Now these are reduced to Cu^{+} ions in a Biuret reaction. It forms a violet color complex with BCA and then the amount of this reduction is proportional to protein present. So by using this chemistry one can know how much protein is present in the sample.

There are different advantages of using BCA assay. It is more sensitive than Lowry method or Biuret. This color complex is quite stable; it is less susceptible to the detergents. This method is also used for those samples which are rich in detergents as well as for the tedious samples which contain membrane proteins.

So this method definitely has lot of advantages as compared to the Lowry method but it still has some problems. This reagent can be disrupted by high concentrations of complex-forming reagents EDTA, ammonium sulphate; reducing material such as DTT. So people have tried different type of assays for protein quantification because there are many interfering components which could give you the false reading and if you are unable to accurately determine the protein concentration then your whole analysis is

going to be depending on that and it will not lead to right results, right comparison. Many times you want to compare the controls and the treatments, your 50 controls and 50 diseased samples. Now in all those if you are starting with equal amount of protein 100 µg for example but your quantification has some artifacts because you have some components which are interfering and giving the false reading so your quantification will not be accurate and rather than measuring only 100 µg for the controls and the treatments you are actually measuring 90 versus 100 or 100 versus 110 so there will be some artifacts if you do not start with the exact protein amount.

So there is another popular method available, which is Bradford assay.

This assay is based on complex formation between Coomassie brilliant blue G-250 dye and protein. Due to the binding absorption max the color shifts from 465 nm to 595 nm. This increase of absorption at 595 nm is used to measure protein concentration.

The Bradford assay has various advantages as compared to the Lowry or BCA method because it is compatible with reducing agents and thiols unlike Lowry and BCA method. This method is also very quick and compatible for microwell plate assays.But there are different problems with the Bradford assay as well such the dye binds most readily to arginyl and lysyl residues of proteins and this specificity may lead to the variations. Now there are various detergents such Triton-X100, SDS, and CHAPS they also interfere with the Bradford assay. So researchers have come up with various types of modified Bradford assays for specific applications again you have to keep an eye on what is the component of your buffer and you need to ensure that it is compatible with the assay conditions or not.

So let us talk about Bradford assay in little bit more about so that one can use that to determine protein concentration. So if you want to perform this assay what are the requirements so first of all you need one standard let's say BSA, you need some salt solution, coomassie brilliant blue solution and cuvette. So for standard preparation you can take BSA and add different concentration of this 5, 10, 15, 20, 25 µl or you can even go above than dilute this sample with 0.15 M NaCl and total volume make to 100 µl. One sample where there is no protein that can be used as the blank, it can be used to autozero spectrophotometer. For the unknown samples for which you want to determine the protein concentration you can take 10 or 15 µl of sample and dilute with the NaCl then same treatment can be performed for whole experiment. Now if your unknown is giving you higher absorbance than the standards prepared so it is better idea that you can try different dilutions of your unknown sample because it has to fall within the standard curve of the standard dilutions what you have taken.

Now you can add 1 ml of coomassie brilliant blue solution and vortex it, after that one can incubate it for 2 minutes so that color can be developed and then it can be measured for absorbance at 595 nm. Then you can use standard curve to determine protein concentration of unknown protein sample but often once you plot your unknown protein sample you may realize your values for the protein sample are either too high or too low to be plotted in the standard curve so then you may need to adjust your unknown sample. You may have to make dilution of it and use less volume or you may need to increase the volume of your sample so that it can fall within the range of the standard curve.

How to perform the quantification, so let me show you one video elaborated demonstration of how to perform protein quantification.

Quantification of proteins - After the protein sample has been extracted from its source it must be quantified to determine the protein content before any further processing. Label the tubes suitably for standard and test samples. Thaw the protein sample to be quantified by gently rubbing it between the palms. Prepare the sample buffer required for diluting samples during the assay and mix it well. Add an increasing concentration of standard protein sample to each of the designated tube. BSA is often used as a standard. Then add the unknown protein sample whose concentration is to be determined to the appropriately labeled tube. Dilute all the samples uniformly using the sample buffer then add the Bradford color reagent to each tube and mix well. An electron transfer reaction takes place between the red form of the coomassie dye of the reagent and the native protein. This disrupts the protein structure and establishes several non-covalent interactions between the dye and the protein. The transfer of electrons converts the dye into its blue form thereby giving the solution a blue color. Set the wavelength of the UV spectrophotometer to 595 nm. Adjust the reading to zero using a blank solution containing only the dye solution and no protein then measure the absorbance of all the standard protein sample of known concentrations followed by the unknown sample. Protein concentration of the unknown can be determined from the standard curve based on the absorbance values obtained. I hope it was useful for you to watch video of protein quantification and you got a feel how to perform this experiment in the laboratory.

So in the summary in the last three lectures we talked about strategies for sample preparation. I gave you a workflow where we talked about different type of lysis methods. We talked about how to pre-fractionate the samples. And then how to precipitate the samples, how to remove the interfering substances and then we moved

on to specific examples, we discussed in more detail different type of criteria being used for analysis of clinical sample and then we talked about Human serum sample. Then we discussed about bacterial protein extraction and how one can analyze bacterial proteome. Then we talked about plant protein extraction for the plant proteome analysis. Finally we talked about protein quantification, how to determine the protein quantity present in given sample. So we will continue our discussion on proteomics and now since you have prepared a good sample. It can be applied either for using the applications on gel based proteomics approaches such as 2-D gel electrophoresis or it can be used for gel free proteomic approaches such as mass spectrometry. So we will continue our lecture on gel based proteomics and demonstrate you how you can use your prepared sample further for analysis of complex proteome such as serum proteome, bacterial proteome and plant proteome. Thank you.