Summer project

On

Protein estimation methods and its application in bio-pharmaceutics

Under the supervision of

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Submitted by

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Project duration:

16th May 2016 – 11th July 2016

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A SANOFI COMPANY

11th July 2016

TO WHOMSOEVER IT MAY CONCERN

This is to certify that **Mr. Akhilesh Kesavan** pursuing Master of Science in Biological Sciences from **IIT, MADRAS** has completed an Industry Oriented internship on **"PROTEIN ESTIMATION METHODS AND ITS APPLICATION IN BIOPHARMACEUTICALS"** at our facility from **16.05.2016 to 11.07.2016.**

He is sincere, hardworking and punctual and has taken keen interest while completing the project.

We wish him good luck for his future endeavors.

For **Shantha Biotechnics Private Ltd.**

DVB KISHORE
Assistant Manager –Human Resources

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Abbreviations

Abbreviation	Full form
SDS	Sodium dodecyl sulphate
PAGE	Poly acrylamide gel electrophoresis
CBB	Coomassie brilliant blue
BCA	Bicinchoninic acid
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
NaCl	Sodium chloride
FAV	Final assay volume
SILAC	Stable isotope labelling by amino acids in cell culture
ICAT	Isotope coded affinity tagging
iTRAQ	Isobaric tags for relative and absolute quantitation
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline

Introduction

Pharmaceutics is the science of preparing and dispensing drugs. Drugs, specially vaccines and antibiotics contain proteins in the form of enzymes or antibodies as the active component. In most cases these foreign proteins can be dangerous or harmful in larger concentrations for the body. Therefore it is crucial for the accurate and precise estimation of the protein concentration in the final product. This estimation of proteins is not only important for the final product but it also helps in the optimization of the production. The quantification of proteins at each step of a new procedure helps to identify

1. The degree of purification in each step
2. The functional step that is critically dependant on the protein concentration
3. The step where the most unwanted substances are removed

Depending on the different steps involved in the production, extraction and purification of the proteins and the nature of the protein itself, different protein estimation methods are used. These methods can involve

1. The assay of the activity of the enzyme
2. The absorbance of the protein with or without treatment with a reagent
3. The estimation of the mass itself
4. Tagging the protein to an assayable substrate

In most cases not all of the methods are convenient however and have to be selected according to the conditions. The criteria for the selection of an appropriate method of determination are:

1. Sensitivity
2. Interfering compounds
3. Available sample
4. Need to reuse the sample

Methods

1. Estimation by UV-VIS spectrometry

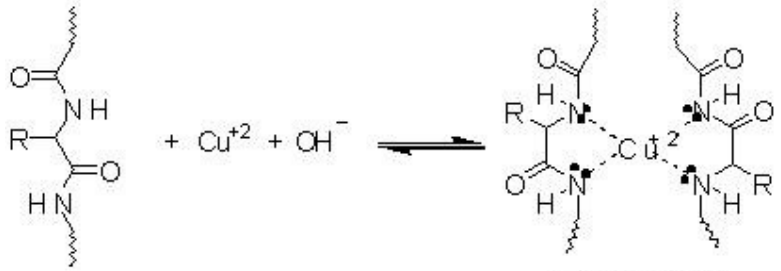
1.1. ESTIMATION OF PROTEIN BY BIURET METHOD

PRINCIPLE:

Biuret assay is one of the basic assays used for protein concentration estimation. The biuret reagent consists of sodium hydroxide, hydrated copper (II) sulphate, together with potassium sodium tartrate. The tartrate is used to stabilize the cupric ion by complex formation.

During the reaction, the cupric ions react with the nitrogen atoms in the peptide bond in an alkaline solution to form a violet coloured complex, with absorption maxima at 565 nm wavelength.

The intensity of the colour formed is proportional to the amount of protein in the sample. Thus using Beer – Lambert law, the concentration of protein is estimated by measuring the absorbance of standard concentrations, plotting the standard curve using a least square error fit and plotting the unknown concentration on the curve.



The reagent is added to the sample and incubated for 30 minute in room temperature. The absorbance is measured at 450 nm, and the concentration is determined by the use of beer-lambert law. This reaction is the first step in other spectrometric assays like Lowry's, BCA, etc.

Materials Required:

Sodium hydroxide, hydrated copper sulphate, potassium sodium tartrate, known protein standards or stock solution, UV/VIS Spectrophotometer, pipettes, cuvette.

Preparation of Biuret reagent:

For 1 L final volume: Dissolve 1.5 g hydrated copper (II) sulphate and 6.0 g of sodium potassium tartrate in 500 mL water. Add 300 mL 10% sodium hydroxide and make to 1 L with distilled water. For long term storage add 1 g potassium iodide. Note: discard if black precipitate is formed.

Procedure:

1. Prepare the standard solutions from the stock along with a blank. Also prepare the Biuret reagent.
2. Add 9 mL reagent to all the samples and standards and incubate for 20 minutes.
3. Measure the absorbance spectra around 565nm for each standard and sample in a spectrophotometer.
4. Plot the standard curve and determine the unknown concentration

Advantages:

1. The variation between different proteins is less
2. Not affected by common detergents used in extraction of the proteins
3. Simple and can be done in room temperature

Disadvantages:

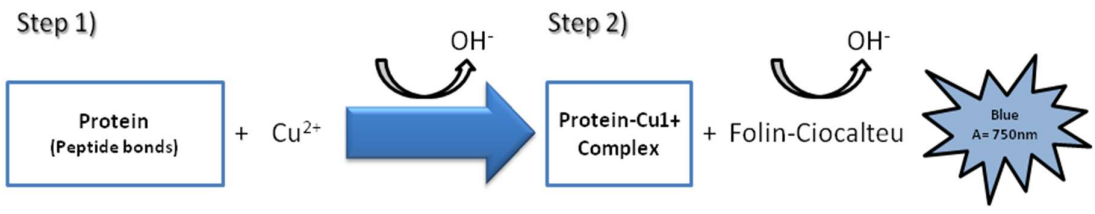
1. Sample not reusable.
2. Takes 30 – 60 minute for accurate estimation

1.2. ESTIMATION OF PROTEIN BY LOWRY'S METHOD

PRINCIPLE:

Similar to biuret assay, Lowry's assay is one of the most cited procedures for the estimation of proteins. In addition to the copper (II) complex, the Lowry's reagent contains a mixture of phosphomolybdate and phosphotungstate called Folin's reagent.

During the reaction, the cupric ions react with the nitrogen atoms in the peptide bond in an alkaline solution to form a violet - blue coloured complex. The blue colour is further intensified by the reaction of the Folin's reagent with the phenolic groups. The final product has an absorption maximum around 640nm.



The intensity of the colour formed is proportional to the amount of protein in the sample. Thus using Beer – Lambert law, the concentration of protein is estimated by measuring the absorbance of standard concentrations, plotting the standard curve using a least square error fit and plotting the unknown concentration on the curve.

Materials Required

Sodium hydroxide, sodium carbonate, hydrated copper sulphate, potassium sodium tartrate, Folin's reagent, known protein standards or stock solution, UV/VIS Spectrophotometer, pipettes, cuvette.

Preparation of Lowry's reagent

Lowry's reagent has 4 parts: A, B, C, D.

Lowry's Reagent A: 0.5 g hydrated copper sulphate + 1 g sodium citrate in 100 mL water. Citrate is more stable compared to tartrate.

Lowry's Reagent B: 20 g sodium carbonate + 4 g sodium hydroxide in 1 L water.

Lowry's Reagent C: 50 mL of B + 1 mL of A.

Lowry's Reagent D: 1:1 Dilution of Folin's Reagent.

Procedure

1. Prepare the standard solutions from the stock along with a blank. Also prepare the Lowrys Reagent.
2. Add 4.5 mL reagent to all the samples and standards and incubate in room temperature for 10 minute.
3. Add 0. 5 mL of Folin's Reagent to all the samples and standards and incubate in room temperature for 20 minute.
4. Measure the absorbance spectra around 640nm for each standard and sample in a spectrophotometer.
5. Plot the standard curve and determine the unknown concentration

Advantages:

1. The variation between different proteins is relatively less
2. Widely used and accepted method
3. Simple and easy to perform

Disadvantages:

1. Sample not reusable.
2. Takes 30 – 60 minute for accurate estimation

3. Hindered by many reagents

1.3. Estimation of Proteins by Measuring Absorbance at 280nm and 205nm

Principle:

All atoms, bonds and molecules absorb and radiate light at specific wavelengths. The absorption of light by an atom or bond is measured by absorbance. Therefore this intrinsic property is used to find the concentration of the proteins by measuring the absorbance at 280nm and 205nm.

Near UV spectrum i.e. around 280nm for the estimation of proteins is based on the absorbance of the different aromatic side chains present in amino acids namely Phenylalanine (257nm), Tyrosine (274nm) and Tryptophan (280nm). Since this estimation is dependent on the peptide sequence it accurate if the standards are the same of the unknown protein. Also the standard plot can vary drastically between different proteins.

Far UV spectrum i.e. around 205nm for the estimation of proteins is based on the absorbance of the peptide bonds between the amino acids rather than the side chains. The N-C peptide bond has an absorption maximum around 192nm. However, around this wavelength the spectrum can be hindered by the absorption due to oxygen. Therefore the spectrum around 205nm is taken where the effect of oxygen is negligible and the absorptivity is still significant.

These two spectra are simple, don't destroy the sample (so it can be recovered) and is instantaneous. Also the only interference here is due to materials that absorb in the UV region. This assay doesn't require the addition of any reagent.

Materials Required

Known protein standards or stock solution, UV/VIS Spectrophotometer, pipettes, cuvette.

Procedure

1. Prepare the standard solutions from the stock along with a blank.
2. Measure the absorbance spectra around 205nm and 280nm for each standard and sample in a spectrophotometer.
3. Plot the standard curve and determine the unknown concentration

Advantages:

1. Quick and simple estimation

2. Sample is reusable
3. Less protein is required for 205nm

Disadvantages:

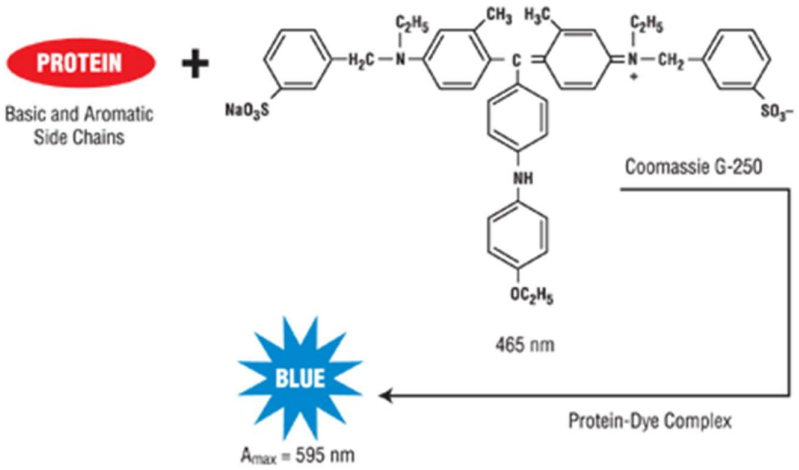
1. Variations between different protein standards are huge for 280nm

1.4. Estimation of Proteins by Bradford's Method

Principle:

The Bradford's assay is a dye based assay for the estimation of proteins in a solution. This assay requires the use of the dye Coomassie Brilliant blue G250 (CBB G250).

In the Bradford's reagent, the dye is protonated by a strong acid and is reddish brown in colour. When proteins are present, the dye binds to the peptide and a shift in the pKa restores its blue colour.



The intensity of the colour formed is proportional to the amount of protein in the sample. Thus using Beer – Lambert law, the concentration of protein is estimated by measuring the absorbance of standard concentrations, plotting the standard curve using a least square error fit and plotting the unknown concentration on the curve.

Materials Required

Coomassie Brilliant Blue G250, known protein standards or stock solution, UV/VIS Spectrophotometer, pipettes, cuvette.

Procedure

1. Prepare the standard solutions from the stock along with a blank.
2. Add 5 mL of dye and incubate for 15 minute in room temperature.
3. Measure the absorbance spectra around 590nm for each standard and sample in a spectrophotometer.
4. Plot the standard curve and determine the unknown concentration

Advantages:

1. Fast and accurate estimation of proteins

Disadvantages:

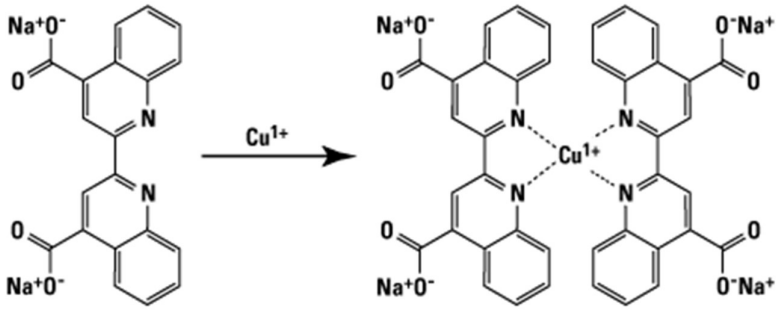
1. Dye can be adsorbed on to the surface of the cuvette leading to in accurate results

1.5. Estimation of Proteins by BCA Method

Principle:

A modified version of the Lowry's assay, the BCA assay is used for more sensitive and detergent compatible estimation of the proteins. The BCA Reagent contains the same copper tartrate complex along with Bicinchoninic acid.

During the reaction, the cupric ions react with the nitrogen atoms in the peptide bond in an alkaline solution to form a violet coloured complex. During this reaction the cupric ions are reduced to cuprous ions. These cuprous ions are then estimated by the Bicinchoninic acid by the formation of a violet coloured complex with absorption maxima at 562nm.



The intensity of the colour formed is proportional to the amount of protein in the sample. Thus using Beer – Lambert law, the concentration of protein is estimated by measuring the absorbance of standard concentrations, plotting the standard curve using a least square error fit and plotting the unknown concentration on the curve.

Materials Required

Sodium hydroxide, sodium carbonate, hydrated copper sulphate, potassium sodium tartrate, Bicinchoninic acid, known protein standards or stock solution, UV/VIS Spectrophotometer, pipettes, cuvette.

Procedure

Prepare working solution:

1. Mix reagent A (in general the blue bottle in a BCA kit) and reagent B with ratio of A: B=1:20 for enough volume of using.
2. Pipette working solution into microplate wells (100 µL/well)
3. Add gradient volume of standard protein (2 mg/ml) to each well (5 or more) followed by adding of sample protein (5 µL/well)
4. Mix wells thoroughly with pipet.
5. Cover the plate and incubate at 37°C for 30 minutes.
6. Cool down the plate to RT.
7. Measure the absorbance at or near 562 nm in a plate reader.
8. Make a standard curve using data of standard protein. Calculate concentration of sample protein from the standard curve.

Advantages:

1. Widely used and accepted method
2. Simple and easy to perform
3. Not hindered by as many reagents as Lowry's

Disadvantages:

1. Sample not reusable.
2. Takes 30 – 60 minute for accurate estimation

2. Estimation with separation of proteins in a sample

2.1. Determination Proteins by SDS-PAGE

Principle:

Poly-Acrylamide Gel Electrophoresis or PAGE is a technique used for the separation of proteins based on their charge or molecular weight. The concentration of the protein separated can be estimated by staining the gel with an appropriate reagent and comparing the intensity with those of standard concentrations.

The Poly-Acrylamide Gel or PAG is a cross linked polymer of Acrylamide and N, N'-Methylenebisacrylamide. The Acrylamide forms straight chains and the bisacrylamide forms the cross-links. The cross linking gives the gel a porous nature as there are a lot of gap between the bisacrylamides. The size of the pore is determined by the concentration of bisacrylamide. During electrophoresis in the gel, smaller molecules move through the gel faster compared to the larger molecules. The concentration of the gel is selected such that a proper resolution is observed. The polymerization of the gel is initiated by ammonium persulphate (APS). APS provides free radicals for the polymerization to occur. These free radicals are stabilized by the addition of N, N, N', N'-Tetramethylethylenediamine (TEMED).

SDS PAGE is a denaturing gel electrophoresis where Sodium Dodecyl Sulphate or SDS, an anionic detergent, is added to the gel. SDS is a strong anionic detergent that is capable of denaturing the proteins and gives them a uniform negative charge based on their size i.e. molecular weight. Therefore during electrophoresis, since the charge/ unit mass ratio is same for all proteins, the smaller proteins move faster towards the anode and are found lower on the gel.

Typically SDS Page is made of two gels that vary in the bisacrylamide concentration stacked one over the other. The lower one is called Resolving gel. In this gel, the bisacrylamide concentration is higher and has a pH of 8.8. It is here that the resolving of proteins happens. The upper gel is called the Stacking gel. Here the pH is about 6.8 and the pore size is larger. The purpose of this gel is to make sure that all the samples that will be run start from the same line.

There are two types of buffer used in SDS PAGE apart from the Tris buffers used to make the gels: the Running buffer and the sample loading buffer. The Running buffer the buffer poured in the electrophoresis tank is a source of counter ions for the balance of the intrinsic charge of the gel and proteins. Normally glycine is used as a counter ion as it has a high pKa and its mobility can be

controlled. The running buffer also contains SDS. The sample loading buffer is mixed with the sample before it is loaded in the gel. It contains of a tracking dye like (Bromophenol Blue), 2-beta Mercaptoethanol (Reducing agent, helps denature the protein), SDS, pH 6.8 Tris buffer and Glycerol (To pull down the proteins).

Once the gel is run the gel is stained (using dyes like Coomassie Brilliant Blue R-250 or Silver Staining for higher sensitivities) and then de-stained to remove the excess stain. During the staining process, the proteins take up the stain proportional to their concentration in each band. Then when the gel is imaged and processed, the concentrations of the proteins can be found by comparing the band intensities with known standard concentrations. Alternately the proteins can be separated from the gel and used in a chemical assay for the quantification.

Materials Required:

1. **For a 5 mL stacking gel:**

H_2O	2.975 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL
10% (w/v) SDS	0.05 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 mL
10% (w/v) ammonium persulfate (AP)	0.05 mL
TEMED	0.005 mL

2. **For a 10 mL separating gel:**

Acrylamide percentage	**6%**	**8%**	**10%**	**12%**	**15%**
H_2O	5.2 mL	4.6 mL	3.8 mL	3.2 mL	2.2 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2 mL	2.6 mL	3.4 mL	4 mL	5 mL
1.5M Tris(pH=8.8)	2.6 mL	2.6 mL	2.6 mL	2.6 mL	2.6 mL
10% (w/v)SDS	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL

10% (w/v) ammonium persulfate (AP)	100 µL	100 µL	100 µL	100 µL	100 µL
TEMED	10 µL	10 µL	10 µL	10 µL	10 µL

Note: AP and TEMED must be added right before each use.

3. **5X Sample buffer (loading buffer):**

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

Make sure your target protein dissolved in the liquid phase, and no inappropriate ingredients present (e.g. guanidine hydrochloride can interact with SDS and cause precipitation) Generally, to treat your unprepared sample, you can use sonicator, lysis buffer or both to sufficiently make your target protein released, and centrifuge to make supernatant and pellet separated.

4. **1x Running Buffer:**

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

(Approximately vol. of less than 1 litre is needed depending on the type of your electrophoresis system.)

5. **Coomassie blue (R-250) staining:**

Sensitivity: 0.2~0.5 µg/band

5.1. Staining solution:

5.1.1. 0.3 % Coomassie Brilliant Blue R-250 (w/v)

5.1.2. 45 % Methanol (v/v)

5.1.3. 10 % Glacial acetic acid (v/v)

5.1.4. 45 % distilled water

5.2. De-staining solution:

5.2.1. 20%Methanol (v/v)

5.2.2. 10% Glacial acetic acid (v/v)

5.2.3. 70 % distilled water

Procedure:

1. Make the **separating gel**:

Set the casting frames (clamp two glass plates in the casting frames) on the casting stands. Prepare the gel solution (as described above) in a separate small beaker. Swirl the solution gently but thoroughly. Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates. To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until overflow. Wait for 20-30 minutes to let it gelate.

2. Make the **stacking gel**:

Discard the water and you can see separating gel left. Pipet in stacking gel until overflow. Insert the well-forming comb without trapping air under the teeth. Wait for 20-30 minutes to let it gelate.

3. Make sure a complete gelation of the stacking gel

Take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

4. Prepare the samples:

Mix your samples with sample buffer (loading buffer). Heat them in boiling water for 5-10 minute.

5. Load prepared samples into wells and make sure not to overflow.

Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.

6. Set an appropriate volt and run the electrophoresis when everything's done.
7. As for the total running time, stop SDS-PAGE running when the down most sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel possessing higher percentage of acrylamide, the time will be longer.
8. Immerse the gel with staining solution, and slowly shake it on horizontal rotator for about 20-30minute.
9. Immerse the gel in de-staining solution and put it on the same shaker for about 20-30minute. Change the de-staining solution for 3-5 times until you can see clear bands with almost no blue background.

Note: Various factors affect the properties of the resulting gel.

1. Higher concentration of ammonium persulfate and TEMED will lead to a faster gelation, on the other hand, a lower stability and elasticity.
2. The optical temperature for gel gelation is 23°C-25°C. Low temperature will lead to turbid, porous and inelastic gels.
3. The pH is better to be neutral and the gelation time should be limited in 20-30 minute.

Advantages:

1. Can be used to estimate multiple proteins in a mixture of proteins
2. If the given sample is a mixture of proteins, the proteins are also separated for further use, for example the separated proteins can then be used for mass spectroscopic studies
3. Versatile and can be used for proteins of different lengths and concentrations

Disadvantages:

1. Length process and needs to be done carefully

2. The casted gels cannot be stored for long

2.2. Estimation by High Performance Liquid Chromatography

Principle:

High performance liquid chromatography is an efficient technique for the separation, identification and quantification of proteins in a mixture. This technique separates different components of a mixture based on their affinity to a certain adsorbing material. It relies on a pump that passes pressurised solvent containing the mixture through a column filled with a solid adsorbing material. Since the affinity of different components vary and are characteristic, the components are separated and identified by the difference in flow rates.

During separation the components are seen as bands and can be eluted out to purify the component. In the last stage of the process a detector is placed which can detect the different band and separate accordingly. This detector is usually a photodetector and identifies a band by reading the absorbance at 280nm for proteins. The output of the HPLC detector gives an intensity vs time plot, with the peaks representing bands.

After the peaks have been integrated and identified, the next step in the analysis is quantification. Quantification uses peak areas or heights to determine the concentration of a compound in the sample.

A quantitative analysis involves many steps that are briefly summarised as follows:

1. Know the compound you are analysing
2. Establish a method for analysing samples containing this compound
3. Analyse a sample or samples (the Standard) containing a known concentration or concentrations of the compound to obtain the response due to that concentration (called 'Calibration')
4. You may analyse a number of these samples with different concentrations of the compounds of interest if your detector has a non-linear response (referred to as 'multi-level calibration'), or if a wide concentration range is to be measured in the samples
5. Analyse the sample containing an unknown concentration of the compound to obtain the response due to the unknown concentration
6. Compare the response of the unknown concentration to the response of the known (standard) concentration to determine how much of the compound is present

Advantages:

1. Can be used to estimate multiple proteins in a mixture of proteins
2. If the given sample is a mixture of proteins, the proteins are also separated for further use, for example the separated proteins can then be used for mass spectroscopic studies

Disadvantages:

1. Length process and needs to be done carefully
2. To obtain a valid comparison for the unknown sample response to that of the known standard, the data must be acquired and processed under identical conditions**.**

3. Determination by weight

3.1. Determination by dry weight

A simple but lengthy method for the estimation of the protein, determination by dry weight gives a very accurate amount of the protein in a given sample. Drying a protein-containing sample in a 104^{o}C to 106^{o}C oven for a few hours removes water and volatile materials. The sample is then weighed on a balance to measure the aggregate weight of protein plus whatever non-volatile material remains, such as salts and many buffers.

Materials Required

Protein sample Small weighing bottle or beaker 104" to 106'C oven

Procedure

1. Dry a small weighing bottle or small beaker by heating it 10 minute in a 104" to 106°C oven
2. Cool the weighing container in a desiccator 10 minute
3. Weigh the container on a balance to the nearest 0.1 mg (tare weight)
4. Add a 0.5- to 3-mL sample of protein solution to the container. Buffer salts, polysaccharides, the salt forms of amino acids, and high molecular weight pigments and sugars may not be driven off in a 4- to 6-hr drying cycle. They should be removed from the sample by dialysis, ion-exchange chromatography, or precipitation before it is dried
5. Dry the sample and container 4 to 6 hr (or overnight) at 104" to 106°C. Usually it is not necessary to carry out more than one cycle of drying, cooling, and weighing to approach a constant weight. Overnight drying is required for samples to 5 to I0 mL

6. Cool the container and reweigh it (dried weight)
7. Calculate the dry weight of the sample as: dry sample weight = dried weight of container and protein -tare weight. The first dry weights measured after 6 hr usually remain constant, but if volatile salts such as ammonium chloride and ammonium formate are present, the sample may have to be dried longer or for more cycles to completely drive them off Dry weight is reliable to within -3% for net weights of 2 to 4 mg protein and to within 1% to 2% for 3 mg protein.

Advantages:

1. Accurate and gives exact usable amount
2. The sample is reusable
3. Can be used to standardise the dry protein

Disadvantages:

1. Can only be used for pure samples
2. Length and laborious method

3.2. Determination by Mass Spectroscopy

Principle:

Mass spectroscopy is an analytical technique that is used to measure the mass of a substance by ionising the chemical species and sorting them based on their mass by charge ratio. The mass spectrometer attains this by first ionising the sample, with the help of chemicals, bombarding with other ions or by using a laser (MALDI), and then passing the ions through an electric and magnetic field to deflect it towards a detector. The strengths of the electric and magnetic field are varied to focus ions with a specific mass by charge ratio. The output, i.e. the mass spectrum, is a plot of the ion signal vs the charge by mass ratio. This plot is characteristic to proteins and can be used for the identification and quantification.

Recent methods allow for the quantification of the peptides on the basis of their mass spectrum by isotopically labelling either the standard or the sample with heavier isotopes of carbon or nitrogen. In this method, the heavier isotopes are incorporated into the standard or the sample. Then the standard and the sample are mixed before the analysis. The peptide from the standard and the sample can then be differentiated on the mass spectrum due their mass difference. The ratio of the peaks corresponds to the relative abundance ratio of the standard and the sample.

The most popular methods for isotope labelling are SILAC (stable isotope labelling by amino acids in cell culture), trypsin-catalysed ^{18}O labelling, ICAT (isotope coded affinity tagging), iTRAQ (isobaric tags for relative and absolute quantitation)

A ‘Semi-quantitative’ mass spectrometry can be performed without labelling of samples. Typically, this is done with MALDI analysis (in linear mode). The peak intensity, or the peak area, from individual molecules (typically proteins) is here correlated to the amount of protein in the sample. However, the individual signal depends on the primary structure of the protein, on the complexity of the sample, and on the settings of the instrument. Other types of "label-free" quantitative mass spectrometry, uses the spectral counts (or peptide counts) of digested proteins as a means for determining relative protein amounts. It is called ‘Semi-quantitative’ since the accuracy is less than that of the labelled method.

Advantages:

1. Accurate and gives exact usable amount
2. The sample is reusable
3. Can be used on samples from any source as long the components are known. To avoid interference from other components, it is combined with liquid chromatography and done in tandem repeats

Disadvantages:

1. The capabilities of this technique are not yet fully understood
2. Lengthy and laborious method

4. Determination by Enzyme Linked Immunosorbant Assay (ELISA)

Principle:

ELISA is a modern high sensitivity technique for protein estimation. It involves protein – protein interaction for the estimation of concentration. It is highly sensitive to both protein and the quantity of the protein.

Every protein has an antigen that it specifically binds to. The ELISA plate is coated with several such antigens that bind specifically only to certain proteins. When treated with an unknown sample, the antigens bind to the protein. Then all unbound sites are blocked and a secondary antibody is added that is conjugated with an easily assayable enzyme. Since the secondary antibody binding is directly dependent on the binding of the protein, the assay for the enzyme becomes the assay for the protein. This technique is mostly used for the detection and quantification of antibodies and antigens and when the sample is impure and/ or low concentration.

The assay for the enzyme can be of three types:

1. Chromogenic i.e. generates coloured product. In this assay the concentration is found by Beer – Lambert Law.
2. Chemifluoresent i.e. generates a fluorescent product. In this assay the concentration is found measuring the extent of fluorescence. This assay is more sensitive than chromogenic.
3. Chemiluminescent i.e. generates light energy. In this assay the concentration is found by measuring the intensity of the light emitted which is proportional to the concentration. This assay is ultra-sensitive.

Materials Required:

96-Well Microtiter Plates, Eppendorf Tubes, Twelve-Channel Pipette, 1mL Adjustable Pipette, Humid Chamber, Wash Bottle or ELISA Plate Washer, ELISA Plate Reader, 3,3',5,5'-Tetramethylbenzidine, PBS (pH 7.3), Coating Solution: 0.1 M Sodium Carbonate (pH9.6) with 0.02% Sodium Azide, Wash Solution: 0.9% NaCl-0.05% Tween 20 (NaCl/T), PBS-TA: PBS-Tween 20 (0.05%) -

sodium azide (0.02%) pH 7.3, AP Substrate Buffer: 0.05 M sodium carbonate-0.001 M MgCl2 (pH 9.3), HRP Substrate: Mix equal parts TMB peroxidase substrate and TMB peroxidase solution B.

Notes: 1. Sodium Azide cannot be used in conjunction with HRP conjugates.
2. Wash cycles are critical steps to eliminate background.

Procedure:

1. Antigen (5-20 µg/mL) in coating buffer is added to plastic tubes or microtiter plates. Incubate for 4 hours at 37°C and then store at 4°C until use (if less than two weeks). For the vinyl plates, wash three times with washing solution, dispel liquid by slapping on paper towels and then cover and store at -70°C. These plates are good for at least 6 months.
2. Wash tubes or plates with washing solution three times, waiting 10 minutes between washes. Make sure all wells are filled with wash solution during each wash cycle.
3. Block tubes or plates with 0.5% BSA for 1 hr by completely filling tubes or all wells.
4. Wash tubes or plates with washing solution four times, waiting 10-15 minutes.
5. Add 100µl of antisera diluted in PBS-TA to each well, Incubate for 5 hours at RT or overnight in refrigerator. Normally a 1:100 dilution is sufficient. Monoclonal antibodies should be used undiluted.
6. Wash the plate as before and then add conjugate diluted with PBS-T (A). Incubate for 4 hours at RT for AP conjugates; 1.5 hours at RT or 1 hr at 37 C for HRP conjugates. Normally a 1:1000 dilution is sufficient.
7. Wash four times as before and then add 100 µl substrate to each well.

For AP conjugates: Substrate solution is p - nitro phenyl phosphate (Sigma) at 1 mg/mL in substrate buffer. Allow the colour to develop for 100 minutes and stop reaction by adding 10 µl of 1 N NaOH. Read absorbance at 405nm.

For HRP conjugates: Mix equal parts of the TMB system, place in wells, allow to develop until desired intensity (app. 5 minute) and stop reaction by adding 100 µl of 1M Phosphoric Acid. Read at 450 nm.

Conjugate Preparation

1. Centrifuge 0.3mL enzyme (alkaline phosphatase, Sigma type VII, sp act. 1,140 U/mL) at 8,000 x g for 2 minutes.
2. Add to the pellet 0.1 mL specific antibody affinity purified, resulting in a 3:1 ratio of enzyme to antibody.

3. Dialyze overnight against PBS.
4. Add 10 µl of glutaraldehyde to a final concentration of 0.2%.
5. Incubate at RT for 2 H, dilute to 1mL, and dialyze overnight against PBS.
6. Dilute to 10mL with 0.05 M Tris-5% BSA-1mM MgCl2-0.02% NaN3.
7. Store at 4°C.

Advantages:

1. Results not hindered by the presence of other proteins as long as the antibodies are chosen with care
2. Works with high specificity towards targeted protein
3. Gives accurate results

Disadvantages:

1. Length process and needs to be done carefully
2. To obtain a valid comparison for the unknown sample response to that of the known standard, the data must be acquired and processed under identical conditions**.**

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