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Bradford assay as a high-throughput bioanalytical screening method for conforming pathophysiological state of the animal

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ABSTRACT

Proteins are the essential components of the tissues that play a key role in the body. Its expression in the cell or tissue under a specified set of conditions and at a particular time regulates the different body conditions either as a normal body function or as a disease state. Protein is an important building block of muscles, skin, cartilage, bones and blood. Bradford assay is a reliable advanced and cost-effective protein estimation test for determining the exact concentration of protein in different tissues of the animal. In this study, we have taken a rat suffering from protein deficiency disorder and total protein concentration in the heart, brain, liver, blood and kidney was determined. It was found that the total protein concentration in different tissues of rat i.e., heart, brain, liver, plasma and kidney was found to be 8.39 ± 0.75, 10.46 ± 0.76, 6.74 ± 0.39, 8.12 ± 0.32 mg/g of tissue and 61.27 ± 0.95 mg/mL of plasma respectively (mean ± SEM). As compared to earlier published reports the total protein concentration in different tissues like hear, brain, liver and kidney found much lower to standard value as reported by Beyer, the reason behind obtaining this kind of results may be due to the presence of insufficient amount of the protein content in different tissue of animal as suffering from protein degeneration disorder. The rat was unable to digest and store the protein or catabolism was much faster than anabolism.

Keywords: Anabolism, Bradford assay, Catabolism, Protein estimation.

INTRODUCTION

Proteins are large, complex molecules that play many critical roles in the body. They do most of the work in cells and are required for the structure, function, and regulation of the body's tissues and organs. The cellular structure has many components that help to maintain different body function like DNA carries genetic information, RNA helps to synthesize various proteins and proteins make different enzymes, hormones and other body chemicals [1]. Protein is an important building block of muscles, skin, cartilage, bones and blood. Every protein has a specific role in the body. Its expression in the cell or tissue under a specified set of conditions and at a particular time regulate the different body conditions either as a normal body function or as a disease state [2]. Apart from that, proteins also induce the enzyme activity & transport of nutrients or biochemical compounds through the different biological membranes. It has an extensive role in the maintenance and growth of the human body and also provides energy as a nutrient [3]. Total protein content in tissues plays an important role to maintain the state of health in a living organism [4].

With the help of rapid advancements in molecular biology and genetic engineering, a rising number of biotherapeutics are being developed and marketed. The quality and safety requirements for this class of active ingredients has steadily increased over the decades since Eli Lilly put forth the first insulin manufactured using genetically modified organisms in 1982. This has led to dynamic developments in protein analysis and proteomics intended to meet a growing demand for new technologies and sophisticated analytical techniques to characterize therapeutic proteins [5]. To understand the correlation between protein concentrations with physiological activities, protein content profiling is needed. Protein content profiling includes identifying the protein content in a particular tissue under a specified set of conditions and at a particular time. Usually determination of protein content in tissue done as compared to a reference solution containing bovine serum albumin [2].

Although, estimation of the protein content of a variety of biological samples consisting of complex protein mixtures and a multitude of other biological macromolecules, has remains as challenging task for biochemists. Determination of absolute protein content can be best performed by measuring the amino acid content of the proteins in the studied samples. This procedure, however, requires specialized equipment, which is not readily available. Thus a number of other methods, which yield relative estimates of protein content, are being employed as an acceptable compromise depending on the researcher's needs [6].

Various estimation assay and methods are utilized to determine protein concentration in different tissues (Table 1 and 2). The most common used methods for protein estimation are the Biuret method, Bradford assay and Lowry method. Biuret method indicated the lowest content of proteins or albumin which was probably due to the low sensitivity of this method. The Biuret assay is not much good for protein concentrations below 5 mg/ml. By using the Folin-Ciocalteu reagent to detect reduced copper makes the Lowry assay nearly 100 times more sensitive than Biuret reaction alone [7]. A rapid and accurate method for the estimation of protein concentration is essential in many fields of protein study. An assay originally described by Bradford has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and non-protein components of biological samples [8].
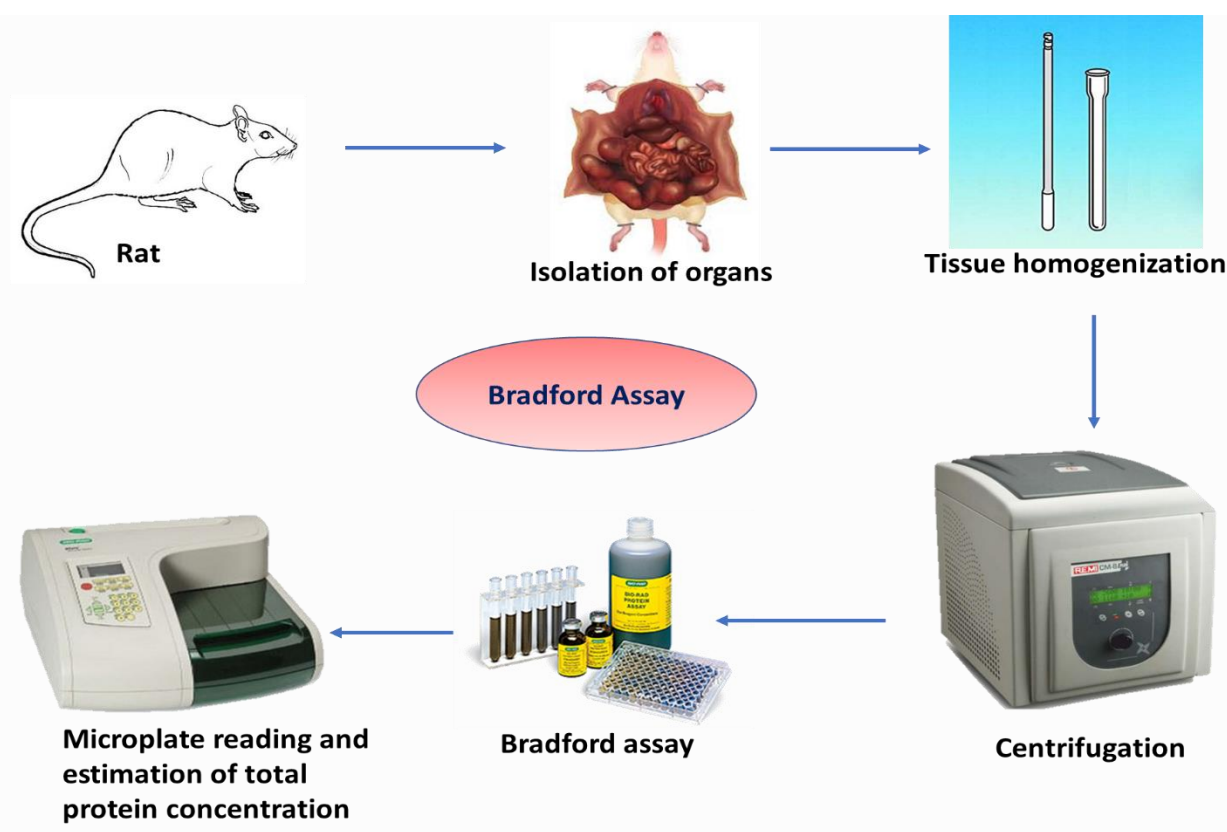

Fig.1: Schematic representation of sequential estimation of total protein content by Bradford assay

One of the main challenges in proteomics nowadays is absolute protein quantification, a must in order to get a deeper understanding of protein functions and their interaction networks. In particular, that type of quantitative knowledge turns out to be critical now to assess the potential of biomarkers in clinical applications [9]. The Coomassie brilliant blue protein assay, known as the Bradford assay, is widely used because of its ease of performance, rapidity, relative sensitivity, and specificity for proteins. The reagent used in this assay bind to the protein and gives a blue color that absorbs at 595nm in UV spectrophotometer [10].

The aim of this study was to estimate total protein content in different tissues of rat and correlating with earlier reported values in literature and thus conforming to the physiological state of the animal under investigation. Bradford test was selected for protein estimation analysis because it is less time consuming, sufficiently selective and sensitive, reagents used were available easily and comparatively fewer steps involved to perform a test.

Table 1: Comparative protein estimation test part 1

Protein estimation tests	Principle	Sensitivity	Selectivity	Time	Easiness	Cost
Biuret test	A violet-purplish color is produced when cupric ions (Cu2+) interact with peptide bonds under alkaline conditions.	**1mg/ml** less sensitive to protein type due to availability of peptide bond to all protein [11]. **	Peptide bonds detection at λ 540nm, ***	20-30min	Easy ****	Rs 16,990 INR for 500ml ****
Folin-Lowry test	It complexes copper with the nitrogen gives blue-green color which absorbs at 650–750 nm [12].	**1µg/ml** Endpoint assay with a stable result, i.e comparing it with a previous standard curve. ***	Aromatic amino acids, as tryptophan, tyrosine, cysteine ***	2hrs	Incompatibility with EDTA, Tris, reducing agents [13]. **	500 ml (Folin-Ciocalteu phenol) cost is 12,358 INR **
Bradford test	Negatively-charged Coomassie brilliant blue dye binds to positively-charged proteins.	**1µg/ml** [14] ****	Aromatic amino acids arginine, tryptophan and proline at λ 465-595nm ****	15 min	The Bradford reaction is fast, easy, and stable for up to an hour [15]. ***	500 ml cost is 6,868 INR ****
Bicinchoninic acid assay	Colorimetric, 1st complex the protein with copper ions. secondly, this chelates BCA to give an intense purple color [16].	**0.5µg/ml** Doesn't have a set endpoint, need to be compared with standard [17]. ***	**Aromatic amino acids λ 562 nm.** interference with surfactant if present more than 5% **	35 min	Not easy **	1 L BCA is 33,211 INR (Very Costly) **

*- Very less useful, ** - Less useful, ***- Useful, **** Highly useful

Table 2: Comparative protein estimation test part 2

Protein estimation tests	Principle	Sensitivity	Selectivity	Time	Easiness	Cost
Direct UV analysis	Estimates the amount of protein by measuring the characteristic absorption of tyrosine and tryptophan at 280 nm [18].	**50 ng/ml** Nonspecific ****	Absorbance at 280nm, Simple but often unreliable **	5 min	Simple, easier ****	cheaper ****
Kjeldahl	Nitrogen sin a protein sample after it's been converted to ammonia	**1g sample** Tedious and time-consuming **	A series of terrifying steps involving heated sulfuric acid, steam distillation, and back-titration with sodium hydroxide. **	60 min	Difficult *	Costly and tedious due to multi-step, highly impractical [19]. *
Fluorometric method	Attaching fluorophore to protein and recording fluorescence [20]	Less *	Primary amines in proteins **	90 min	 **	Average ***
Turbimetric method	Protein molecules which are normally soluble in the solution can be made to precipitate by the addition of certain chemicals, e.g., trichloroacetic acid.	Less sensitive compared to Bradford **	Protein precipitation causes the solution to become turbid. Thus the concentration of protein can be determined by measuring the degree of turbidity [21]. **	10 min	Easy ***	Rs 12,869 for 500 gm ****

*- Very less useful, ** - Less useful, ***- Useful, **** Highly useful

MATERIALS AND METHODS

Materials

Bradford reagent, distill water, bovine serum albumin (BSA), sodium chloride, potassium chloride, disodium phosphate, potassium dihydrogen phosphate was purchased from Sigma Aldrich Bangalore India. Microplate, absorbance reader (BIO-RAD), Volumetric flask (500 L), Pipettes (200 μL, 10μL, 1 mL), Weighing balance, Centrifuge, Glass tube homogenizer, Beaker, Centrifuge tubes. Glassware was of borosilicate and other chemicals were of analytical grades.

Methods

Phosphate buffer saline (pH 7.4)

Phosphate buffer saline (PBS) was prepared by using 8g of sodium chloride (NaCl), 0.2g of potassium chloride (KCl), 1.44g of disodium phosphate (Na_2HPO_4), and 0.24g of potassium dihydrogen phosphate (KH_2PO_4) and dissolved in some amount of distilled water. Further, more amount of distilled water was added to make up to the total volume of 1000ml. The pH of the obtained buffer was adjusted to 7.4 with dil. Hydrochloric acid [22].

Preparation of bovine serum albumin (BSA) stock solution

Accurately weighed 10 mg of BSA was dissolved in 10 mL of phosphate buffer saline (pH 7.4) to produce a solution of 1 mg/mL concentration.

Preparation of 10 % (w/v) tissue homogenate

The rat was sacrificed in CO_2 chamber and different organs were isolated and processed in the presence of ice packs in order to prevent degradation of proteins in the tissues. The tissue of the brain, liver, heart and blood plasma of the three rats were extracted as stated. The weighed amount of tissues was homogenized in PBS in a proportion of 1:10 (w/v) ratio by using a glass tissue homogenizer on ice bath. Then, obtained homogenates were collected in tubes and centrifuged (REMI cooling centrifuge) at 12000 RPM for 45 minutes at 4 °C [23].

Bradford Assay

The standard calibration curve of BSA was prepared by using, 6.25, 12.5, 25, 50, 100 and 200 μL of 1mg/mL BSA stock solution in an Eppendorf tube with a further addition of PBS up to the volume 1000 μL. The addition was done by using a micropipette. Each sample of the tissue homogenate (100 times diluted) and a standard solution of the calibration curve were taken in the microwell plate in a triplicate manner (10 μL). For the detection of protein content, 250 μL of Bradford's reagent was added to each microwell. The microwell plate was incubated at room temperature for 10 minutes in a dark place. Finally, the produced color was analyzed by using a microplate reader at 595 nm wavelength [24].

Preparation of calibration curve

The obtained absorbance of the different standard concentrations of BSA solution was calculated by subtracting

the mean absorbance of blank (PBS) from each absorbance value of a standard solution. Then the mean of triplicate value for each concentration was calculated. The standard curve was plotted between the BSA concentration on X-axis and the mean absorbance on Y-axis (Fig.2).
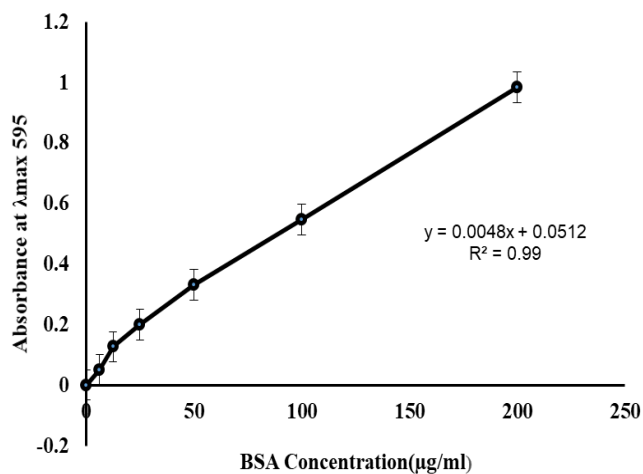

Fig. 2: Calibration curve of BSA in PBS at 595 nm. The obtained linear equation, $y = 0.0048x + 0.0512$ with $R^2 = 0.99$, where y is absorbance and x represent respective BSA concentration.

Statistical analysis

Protein concentration in the different samples was determined by using the GraphPad Prism software. Values are given as mean ± S.D. The significance of differences was analyzed by one-way analysis of variance and subsequent Tukey's multiple comparisons test. Differences among means were considered significant when $P < 0.05$.

RESULTS AND DISCUSSIONS

In this study, it was found that the total protein concentration in different tissues of rat i.e., heart, brain, liver, plasma, and kidney was found to be 8.39 ± 0.75, 10.46 ± 0.76, 6.74 ± 0.39, 8.12 ± 0.32 mg/g of tissue and 61.27 ± 0.95 mg/mL of plasma respectively (mean ± SEM). As compared to earlier published reports as mention as in table 3, the total protein concentration in different tissues like hear, brain, liver and kidney found to not up to standard value as reported by Beyer 1983, the reason behind obtaining this kind of results may be due to the presence of insufficient amount of the protein content in different tissue of animal as suffering from protein degeneration disorder. The rat was unable to digest and store the protein or catabolism was much faster than anabolism

Table 3: Comparison of the experiment results with respect to the earlier data

Tissue	Total protein concentration (mean ± SEM)	
	Present study	Earlier data
Heart (mg/g)	8.39 ± 0.75	83.64 ± 0.52 [25]
Brain (mg/g)	10.46 ± 0.76	44.45 ± 1.04 [25]
Liver (mg/g)	6.74 ± 0.39	102.92 ± 1.14 [25]
Blood (mg/mL)	61.27 ± 0.95	60.53 ± 2.65 [26]
Kidney (mg/g)	8.12 ± 0.32	51.38 ± 0.48 [25]

The protein content of Blood or plasma was found to be satisfactory and it is similar to the reported values in literature. The reason behind obtaining satisfactory results

in case of blood samples was due to the fact that animal can absorb the proteins but from the blood, it was unable to utilize for anabolic activities and also blood samples processing step does not involve any homogenization process so chances of degradation of protein during the process are very rare.

There may be several factors that may have affected the outcomes of the experiment i.e., large result deviations. Small volume measurement may be one of the limiting factors in the analysis. Temperature is another important factor because during the experiment it has been observed that temperature could affect Bradford reagent stability [27]. Thoroughly mixing of the protein sample and Bradford reagent may also affect the binding of the dye to the protein and thus the absorbance. When the total protein concentration was statistically compared within the different tissues, it was found that there is a statistically significant difference between the total protein concentration in the liver when compared to plasma and in the heart and brain when compared to the liver.
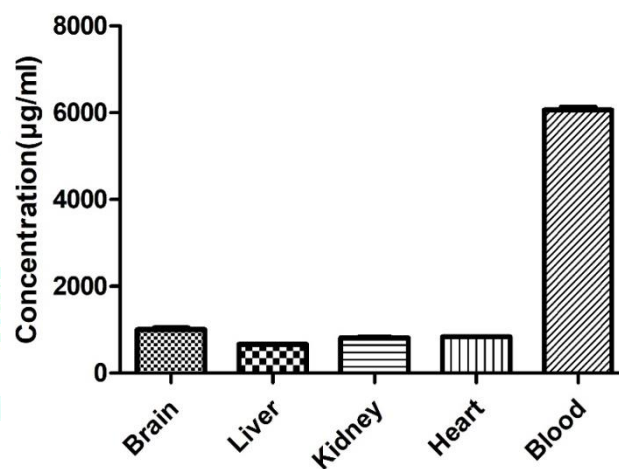

Fig. 3: Histogram of total protein concentration in different tissues of rat. Mean ± S.D.; $P < 0.05$, versus plasma, $P < 0.05$, versus liver.

Generally, the organs like heart, brain, and liver are highly perfused with the blood, and the total protein concentration in the liver is found to be highest as compared to other tissues. It may be due to the reason that the liver consists of a large number of metabolizing enzymes as well as protein synthesis takes place in the liver.

It was demonstrated that normalization using total protein analysis (TPA) with stains such as Coomassie and Instant blue provides a more robust baseline for performing quantitative Western blotting (QWB) experiments. The Western blotting is widely used for determining the presence or absence of a protein within a cellular homogenate. Total protein analysis is an accurate measure of protein load. The use of total protein analysis provides a measure of protein load that circumvents many of the problems associated with the use of single loading control proteins. [28]. Total protein analysis is an alternative simple technique in quantitative Western blotting to accurately determine if equivalent protein loading has been achieved within a gel [29]. While proteomics remains the tool of choice for discovery research, new innovations in proteomic technology now offer the potential for proteomic profiling to become standard practice in the clinical laboratory. Indeed, protein profiles can serve as powerful diagnostic markers and can predict treatment outcome in many diseases, in particular, cancer [30].

CONCLUSION

In this experiment, accurate and comparable measurement of the total protein concentration in different tissues of rats (heart, brain, liver, and plasma) was determined by using the Bradford assay. The assay was carried out in a 96-well plate and allowing less utilization of the ingredients. That gives the cost-effectiveness of the method with high-throughput screening. The results were statistically compared and it was found that total protein concentration in blood was significantly different as compared to other organs and the same was true for heart and brain when compared to blood plasma. The obtained results suggest that the rat was suffering from tissue protein degeneration disease. The estimation of total protein is helpful in experimental research as it provides a measure of protein load that avoids many problems associated with the use of single loading control proteins. Therefore, it is can be expanded to realize accurate and comparable biological measurements according to other reference methods.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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