Defence Life Science Journal, Vol. 8, No. 4, October 2023, pp. 321-325, DOI : 10.14429/dlsj.8.18616

A Method for Determination of Protein Concentration in a Given Unknown Sample Using Absorbance Difference Between 205 nm and 280 nm

Puspendu Karmakar and Jai Prakash Keshri*

Phycology Laboratory, Department of Botany,
The University of Burdwan, Burdwan–713 104, West Bengal, India
**E-mail: keshrijp@gmail.com*

ABSTRACT

Nowadays, determining the protein content of a sample is a common experiment that is conducted in laboratories. Although there are several ways to measure protein content, dye-based spectrophotometric methods are most frequently used in laboratories. In dye-based approaches, protein assays are mostly carried out at a certain wavelength. Protein concentration tests like Lowry's take a long time; whereas Bradford's is quick but requires expensive chemicals. In order to reduce the usage of time and money associated with protein assay, we first looked into and then proposed an easy, affordable, and more accurate technique of determining protein concentration that uses standard curves but doesn't use any dyes. The difference of two UV wavelength absorbance values at 205 nm and 280 nm was used to determine the protein concentration where one absorbance was recorded for the presence of peptide bonds and another for aromatic proteins. The proposed method has many advantages as it consumes minimum time and chemicals but the major setback is anionic detergents, which can shift the absorbance spectra abruptly.

Keywords: Spectrophotometric determination; UV absorbance; Beer's law; Protein quantification; Protein concentration

1. INTRODUCTION

Proteins are nitrogen-based compounds mainly formed by the polymerization of amino acids.[1] Peptide bonds between the carboxylic group of one amino acid and the α-amino group of the following amino acid are formed when amino acids polymerize themselves. Peptides present in protein quantitatively may be two to many.[2] In general, proteins are found in living organisms as necessary macromolecules that control important metabolic processes by assisting in the synthesis of enzymes, hormones, and various structural elements. In living beings, proteins can also be used as an alternative energy source. Moreover, proteins are involved in every cellular function in living beings. At the cellular level, particular DNA codes RNAs, and proteins are coded by specific RNAs.[1,3]

To determine the protein concentration in a particular biological organism is a common technique practiced before protein purification, protein isolation, or other protein assays. Several methods has been developed over the years to measure the protein concentration but UV and visible spectrophotometric assay is the easiest and most rapid.[4-5] However, determining accurate protein concentration is difficult as different assays have their own merits and demerits.[6] Colorimetric assay is performed extensively to measure protein concentration with the help of spectrophotometer readings. The measurement is based on particular dyes that bind with unknown proteins. A standard curve also needs to be prepared with the absorbance versus known concentration of known protein solution and the concentration of unknown protein mixture of the extract which is expressed relative to that known concentration of known protein already used in standard curve.[7]

The biuret test is suitable when the total protein concentration is high and low concentration prefers other assays like Lowry's assay, Bradford assay, etc.[8] The Bradford assay performs in an acidic environment and relies on the interaction of certain residues of amino acids (such as lysine, arginine, and histidine) with non-conjugated groups of the Coomassie brilliant blue G-250 whereas, the Lowry's assay depends on the reaction between protein and alkaline copper tartrate solution in presence of Folin's reagent.[9-10]

If the absorbance (A), molar extinction coefficient (∈), and cuvette path length (L) are known, the Beer-Lambert rule can be used to directly determine the protein concentration (C). For the purpose of calculating protein concentration, absorbance should be measured at 205 nm or 280 nm of wavelength. While aromatic amino acids are responsible for the protein's absorbance at 280 nm, peptide bonds are responsible for the absorbance at 205 nm. Despite the fact that the sensitivity is best observed at 205 nm, 280 nm is the most commonly employed wavelength.[11] The commonly used extinction coefficient

Received : 29 October 2022, Revised : 13 July 2023
Accepted : 21 July 2023, Online published : 21 December 2023

(∈) for protein concentration at a wavelength of 205 is 31 mL/mg cm.[12]

In the present investigation, two UV wavelengths, 205 nm and 280 nm have been used where one absorbance was recorded due to peptide bonds and another was recorded due to aromatic proteins.[11-12] Spotlight has especially been made to develop an easy, cost-effective, more accurate protein concentration determination method by using standard curve but without the involvement of any dyes.

2. METHODOLOGY

2.1 Preparation of Buffer Solution

0.1M Saline Phosphate Buffer (PBS) was prepared up to 1L of volume with double distilled H_2O according to the following recipe and pH of the buffer was maintained 7.4.

Table 1. Recipe of 0.1M Saline phosphate buffer solution for 1 litre. [13]

Components	Amount (g)
NaCl	80
KCl	2
Na_2HPO_4-$7H_2O$	21.7
KH_2PO_4	2.59

2.2 Preparation of Protein Solutions

Two types of protein solutions with two different concentrations were prepared where 10mg of Bovine Serum lbumin (BSA) was mixed with 10 mL of PBS buffer to make the solution concentration 1mg/mL. The other solution was prepared by mixing 5 mL of 1 mg/mL BSA solution and 45 mL of PBS buffer to make the concentration 100µg/mL.

2.3 Determination of Protein Concentration of Unknown Sample

In the present investigation, we have used Chara sp. to determine the unknown protein concentration. The protein extraction process of the sample was done by Barbarino and Lourenco[14] described method. 1g dried sample was immersed in distilled water and then grounded. The grounded sample was subjected to centrifuge at 4 °C with 15,000 g for 20 minutes where supernatants were collected for protein estimation and pellets re-extracted with 1mL. 0.1N NaOH with 0.5% β-marcaptoethanol (v/v). The mixture was then centrifuged at 21°C with 15,000g for another 20 minutes. The second supernatant was combined with the first one to make the volume 9 mL and the pellets were being discarded. Protein precipitation of *Chara sp.* was done by using chloroform and methanol. Estimation of protein was followed according to Bradford[15] described method and absorbance of particular dilution mixtures at 595nm were taken from test tubes and a standard curve was made to quantify the protein content in the sample.

2.4 Spectrophotometric Assessment

2.4.1 Preparation of Standard Curve

Ten different test tubes of ten different BSA concentrations from 0 µg/mL to 160 µg/mL were prepared from 1mg/mL BSA solution. The absorbance of different BSA solutions were taken at three particular wavelengths of 205 nm, 280 nm and 340 nm respectively. Absorbance value at 340 nm was subtracted from the absorbance value taken at 205 nm and 280 nm used to correct light scattering. Now, the subtracted absorbance value at 205 nm and 280 nm were used further. Two standard curves at 205 nm and 280 nm relative to BSA with PBS buffer solution were prepared.

2.4.2 Determination of Protein Concentration and Molar Extinction Coefficient

Total three different test tubes were prepared separately where two different test tubes with two different measured concentrations (15 µg/mL and 30 µg/mL) of BSA protein solutions were prepared and other one was protein solution of Chara sp. with unknown protein concentration. Absorbance values for three mentioned wavelengths were measured for separately prepared protein samples. Protein concentrations of each protein samples were measured separately from two previously prepared standard curves at 205 nm and 280 nm separately. Molar extinction coefficients (∈) of protein solutions were calculated by using Beer's law for two wavelengths where 'A' stands for absorbance and 'L' stands for path length.

2.4.3 Final Determination of Protein Concentration

Finally, the exact protein concentrations were measured by using the following formula in µg/mL:

$$Concentration\ (C) = \frac{(A_{205} - A_{280})}{(Difference\ of\ \in value)}$$

2.5 Preparation of Detergent Solution

Another two test tubes with two anionic detergents: 1% Triton 100 x and 1% SDS were prepared with PBS buffer to observe the absorbance shifting.

Each of the experiments was performed thrice and data presented here are mean values to observe the appropriate results.

3. RESULTS

3.1 Protein Concentrations in Samples

From Bradford's assay, it was calculated that the protein concentration of collected Chara sp. was 16 µg/mL where by taking two wavelength's absorbance value at 205 nm and 280 nm, the calculated protein concentration value was 10.35 µg/mL. The calculated protein concentration values of two measured BSA solutions (15 µg/mL and 30 µg/mL) were recorded 12.88 µg/mL and 26.26 µg/mL. Table 2 summarises calculated protein concentrations of three samples.

Table 2. Calculated protein concentrations of *Chara sp*. and two measured (15 µg/mL and 30 µg/mL) BSA solutions by taking 205 nm and 280 nm absorbance value.

Samples	Absorbance at 205 nm	Concentration at 205 nm (mg/mL)	Molar extinction co-efficient value at 205 nm	Absorbance at 280 nm	Concentration at 280 nm (mg/mL)	Molar extinction co-efficient value at 280 nm	Final Protein Concentration (µg/mL)
Chara sp.	0.196	10.2	0.019267	0.007	6.33	0.001166	10.35
Measured 15µg/mL BSA solution	0.244	13	0.018769	0.016	17	0.001063	12.88
Measured 30 µg/mL BSA solution	0.472	26.41	0.017872	0,028	29	0.000965	26.26

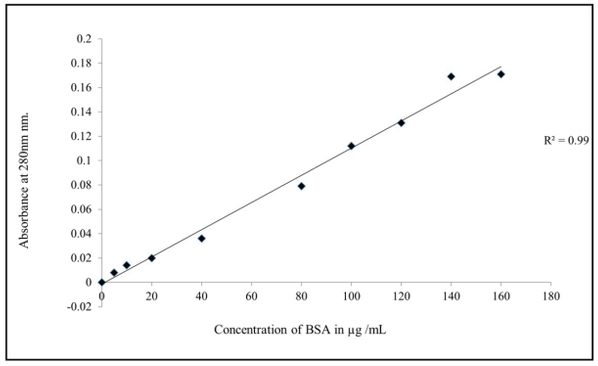

Figure 1. Standard curve of BSA at 205 nm.

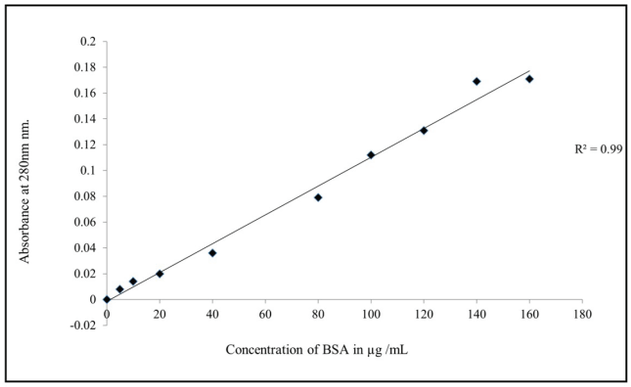

Figure 2. Standard curve of BSA at 280 nm.

3.2 Effect of Detergents

Two mostly used anionic detergents for preparation of protein lysis buffer; Triton X-100 and SDS were used here to detect the shifting of absorbance. Interestingly, the absorbance shifted broadly. Table 3 shows the shifting of absorbance after adding the detergents.

4. DISCUSSION

The measured protein concentrations: 15µg/mL BSA solution and 30µg/mL BSA solution were used as reference to observe the difference between calculated protein concentrations performed in this study. The results depict that calculated final protein concentration values by using 205 nm and 280 nm wavelengths differ from the measured protein concentration values because of the peptide bonds and possibly because of the protein structure. Recalling the fact; two UV wavelengths at 205 nm and at 280 nm exhibit absorbance due to aromatic proteins and peptide bonds respectively. So, the difference between the absorbance value at 205 nm and 280 nm can be used as the key tool to determine the protein concentration.

Table 3. Shifting of absorbance value at 205nm and 280nm in presence of anionic detergents.

Detergents	Absorbance at 205 nm.	Absorbance at 280 nm.
1% Triton X-100	2.491669	3.00333
1% SDS (Sodium dodecyle sulphate)	2.236667	1.62133

There are many advantages of the method: (a) Determination of unknown protein concentration without previous knowledge of molar extinction coefficient (∈); (b) Measurements of wavelengths are easily accessible to all UV spectrophotometer; (c) No dye is used. Therefore, the method is cost effective. Further, the advantages of the method are: (d) Independent of specific amino acid composition of the protein; (e) Time saver process; (f) No interference of scattering absorbance as it is corrected by taking 340 nm absorbance reading; (g) Correction of nucleic acid interference; (h) Interference of phenol groups are corrected by taking absorbance of two wavelengths. The proposed method is near about 86% sensitive of the usual Bradford's protein assay method.

The study exhibited that detergents have ability to shift the absorbance value drastically. So, the major setback of the method is the presence of detergents that can influence the result strongly. The absorbance changes of anionic detergents are demonstrated in (Fig. 3).

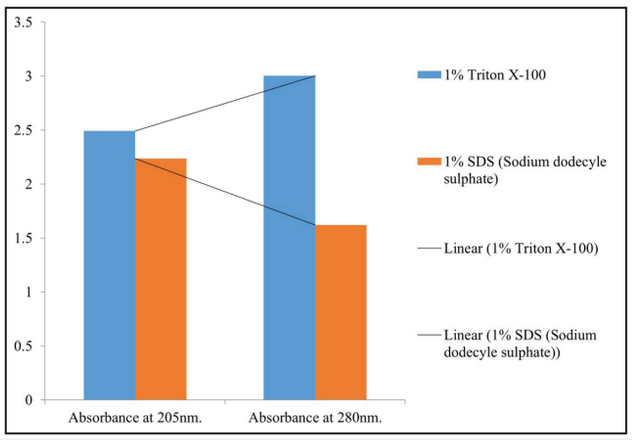

Figure 3. Shifting of absorbance value at 205 nm and 280 nm in presence of anionic detergents.

5. CONCLUSIONS

Huge efforts have been made over the year to develop methods for protein quantification but nowadays, spectrophotometric methods practiced popularity due to their easy dealings. Our proposed method is easy to deal with as only two standard curves are the key steps here. The determination of protein concentrations and molar extinction coefficients at each mentioned wavelengths are the calculation steps only. Moreover, the proposed method is less time consuming and obviously does not require much costly chemicals. Though, the proposed method is advantageous over many spectrophotometric protein quantification methods but the study revealed limitations to deal with anionic detergents.

ACKNOWLEDGEMENT

The authors are grateful to the Head of the Department (Department of Botany, The University of Burdwan) for providing laboratory facilities. This work was supported by the Department of Science and Technology and Biotechnology (DST and BT), Government of West Bengal, India.

REFERENCES

1. Hoffman, J.R. & Falvo, M.J. Protein – which is best?. *J. Sports Sci. Med.*, 2004, **3**, 118-130.
2. Guzmán, F.; Barberis, S. & Illanes, A. Peptide synthesis: chemical or enzymatic. *Electron. J. Biotechnol.*, 2007, **10**, 279-314. doi: 10.2225/vol10-issue2-fulltext-13.
3. Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K. & Walter, P. Molecular Biology of the Cell. Ed. 5, Garland Science, New York, 2007. doi: 10.1201/9780203833445.
4. Noble, J.E. & Bailey, M.J.A. Quantitation of Protein, In: Burgess, R.R. & Deutscher, M.P. (Eds.), Methods in Enzymology, Academic Press, Cambridge, 2009, pp. 73-95. doi: 10.1016/S0076-6879(09)63008-1.
5. Selzle, K.R.; Tchipilov, T.; Backes, A.T.; Tscheuschner, G.; Tang, K.; Ziegler, K.; Lucus, K.; Pöschl, U.; Nowoisky, J.F. & Weller, M.G. Determination of the protein content of complex samples by aromatic amino acid analysis, liquid chromatography-UV absorbance, and colorimetry, *Anal. Bioanal. Chem.*, 414, 4457-4470. doi: 10.1007/s00216-022-03910-1.
6. Olson, B.J.S.C. & Markwell, J. Assays for determination of protein concentration, *Curr. Protoc. Pharmacol.*, 2007, **38**, A.3A.1–A.3A.29. doi: 10.1002/0471141755.pha03as38.
7. Noble, J.E.; Knight, A.E.; Reason, A.J.; Matola, A. & Bailey, M.J.A. A comparison of protein quantitation assays for biopharmaceutical applications, *Mol. Biotechnol.*, 2007, **37**, 99-111. doi: 10.1007/s12033-007-0038-9.
8. Krohn, R.I. The colorimetric detection and quantitation of total protein *Curr. Protoc. Food Anal. Chem.*, 2001, **00(1)**, B1.1.B1.1.28. doi: 10.1002/0471142913.fab0101s00.
9. Lu, T.S.; Yiao, S.Y.; Lim, K.; Jensen, R.V. & Hsiao, L.L. Interpretation of biological and mechanical variations between the Lowry versus Bradford method for protein quantification. *N. Am. J. Med. Sci.*, 2010, **2(7)**, 325-328. doi: 10.4297/najms.2010.2325.
10. Redmile-Gordon, M.A.; Armenise, E.; White, R.P.; Hirsch, P.R. & Goulding, K.W. A comparison of two colorimetric assays, based upon Lowry and Bradford techniques, to estimate total protein in soil extracts. *Soil. Biol. Biochem.*, 2013, **67**(100), 166-173. doi: 10.1016/j.soilbio.2013.08.017.
11. Simonian, M.H. Spectrophotometric determination of protein concentration. *Curr. Protoc. Food Anal. Chem.*, 2002, **4**(1), B1.3.1–B1.3.7. doi: 10.1002/0471142913.fab0103s04.
12. Anthis, N.J. & Clore, G.M. Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm., *Protein Sci.*, 2013, 22(6), 851–858. doi: 10.1002/pro.2253.
13. Cold Spring Harbor Protocols, Phosphate-Buffered Saline (PBS; 0.1M, pH 7.4). http://cshprotocols.cshlp.org/content/2014/12/pdb.rec085126, 2014 (accessed 12 November 2021).
14. Barbarino, E. & Lourenco, S.O. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae, *J. Appl. Phycol.,* 2005, **17**, 447-460. doi: 10.1007/s10811-005-1641-4.
15. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976, **72**, 248-254.

CONTRIBUTORS

Mr Puspendu Karmakar completed his MSc degree from Sambalpur University, Odisha, India in Life Sciences (Botany Stream). He is presently working as a Ph.D. student in the Department of Botany, The University of Burdwan, West Bengal, India. His research work is based on the protein profiling, lipid profiling of algal biomasses and their commercial potentials. In the present study, he has performed all the experiments, analyzed the data and prepared the final manuscript.

Prof Jai Prakash Keshri received his PhD degree from The University of Burdwan, West Bengal, India. He is currently working as professor in the Department of Botany, The University of Burdwan, West Bengal, India. His area of interest in research includes algal taxonomy and systematics, ecology, algal physiology,

For the present study, he conceptualized and outlined the research work. He also guided to carrying out the total research work and helped in preparing final draft.