応用化学実験第一

片山研究室

提出日：2023 年　1 月　18 日（水）

担当教員：新居　輝樹

題目：　BCA 錯体の呈色反応を利用するタンパク質の定量

実験日：2023 年　1 月　11 日

学籍番号：　1TE21306Y
氏名：　　シフエンテス　ゴンザロ
実験班：　　IV 班

共同実験者：　安武大地

Contents

1 Introduction

Protein quantitation is a fundamental experimental technique in biochemical research. Since the amount of protein that can be handled is generally very small, weighing is difficult, and concentration is determined by absorption spectrophotometry. Among the amino acids that make up proteins, tryptophan and tyrosine, which have heterocyclic rings, are amino acids that absorb in a characteristic wavelength range (280 mm). Therefore, if the amino acid composition (tryptophan and tyrosine content) of the protein is known, it is possible to calculate the molar absorption coefficient.

However, since the molar absorption coefficients of these amino acids are small, a reasonable amount of protein is required for quantification from absorbance at 280 nm. In addition, protein solutions often contain additives to maintain quality, so there are not many cases where concentration determination using the molar absorption coefficient is possible. Therefore, protein concentrations are generally determined by weight concentration as a relative value to a reference protein. In such relative determination, it is required that the protein sequence does not affect the determination.

In this experiment, protein quantification by the BCA method is studied. In protein quantification, the reduction of Cu^{2+} to Cu^{+} by peptide bonding, called the Biuret test, is used. The BCA method uses the purple color (λ_{max}=562 nm) of the Cu(I)-BCA complex produced by complexing Cu^{+} with bicinchoninic acid (BCA) for quantification. Since this color reaction uses peptide bonding, it has the advantage of being insensitive to protein sequence effects. In this experiment, the concentration of bovine serum albumin (BSA) (69 kDa, 607 amino acids) is determined. BSA is a protein often used in biochemical experiments because of its advantages such as high stability, low cost, and biological inactivity.

2 Materials

2.1 Reagents

- 0, 100, 200, 300, 400, 500 ug/mL BSA solutions (standard sample)
- X ug/mL BSA solution (unknown concentration sample)
- Solution A: 1%BCA-Na_2 solution (containing 0.4% NaOH/ 2% Na_2CO_3/ 0.95% $NaHCO_3$/ 0.16% sodium tartrate)
- Solution B: 4% $CuSO_4$

2.2 Glassware

- Eppendorf microtubes
- 1 UV cuvette ($l = 1.0\ cm$)

2.3 Equipment

- UV-Visible spectrophotometer
- One 20-200 ul adjustable micropipette
- One 100-1000 ul adjustable micropipette

3 Methodology

1) A 1.0 mg/mL aqueous solution of BSA was prepared by weighing 12.3 mg of BSA and dissolving it in deionized water.
2) Six standard samples (200 uL each) were prepared from this BSA solution.
3) Working reagent (WR) was prepared by mixing solution A and B at 50:1 (8mL:160uL).
4) 50 uL of standard and unknown samples were each taken in 1.5-mL microtubes. 1mL of WR was added and mixed well by using a vortex (the pH of the solution was about 11.25).
5) The microtubes were left at 37°C for 30 minutes to promote color reaction (standard and unknown samples were run simultaneously).
6) After the color reaction, the tubes were brought to room temperature and the absorbance of each sample at 562 nm was recorded within the next 10 minutes.
7) The results of the standard samples were plotted on a graph (Fig. 3) to create a calibration curve of absorbance versus concentration. From this calibration curve, the concentration of the unknown sample was determined.

4 Results

4.1 Quantitative results

The UV-vis absorption spectra values obtained for the standard samples are shown in Fig. 1. For the purpose of this experiment, the graph shows only wavelengths from 450 nm to 750 nm.

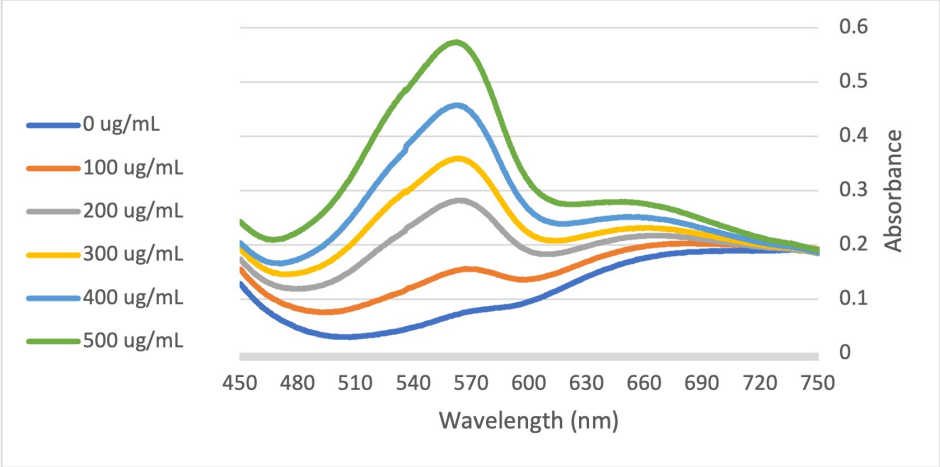

Figure 1: Absorbance vs wavelength graph

Subsequently, the table in Fig. 2 summarizes the absorbance values at 562 nm of the standard samples as well as the unknown sample.

BSA concentration (ug/mL)	0	100	200	300	400	500	x
Absorbance at 562 nm	0.0715	0.1526	0.281	0.3587	0.4571	0.5733	0.3816

Figure 2: Absorbance at 562 nm

From the data shown in the above table, the Absorbance vs Concentration was plotted. The graph as well as its linear regression are displayed in Fig. 3.

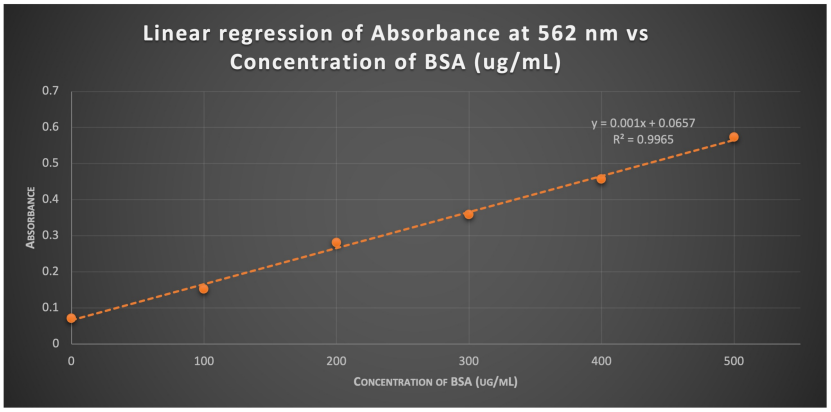

Figure 3: Absorbance vs Concentration of BSA graph

From the linear regression obtained in Fig. 3, the concentration of the unknown sample can be determined. The following equation is the linear regression of the calibration curve, where A represents Absorbance at 562 nm, and C represents concentration of BSA (ug/mL)

$$A = 0.001[C] + 0.0657 \tag{1}$$

Therefore, the concentration of the unknown sample is determined from Eq. 1 by using the absorbance at 562 nm shown in Fig. 2.

$$\begin{aligned} 0.3816 &= 0.001[C] + 0.0657 \\ C &= 315.9\,\mu g\,mL^{-1} \end{aligned} \tag{2}$$

4.2 Qualitative results

The following picture shows the color of the microtubes of the standard samples (different concentrations) as well as the sample with the unknown concentration after the color reaction.

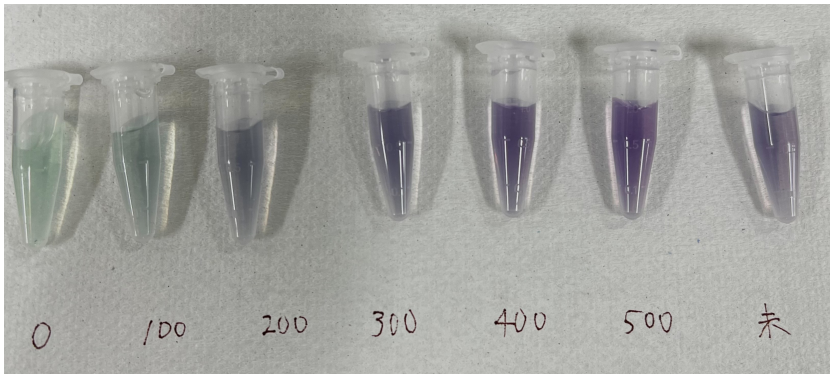

Figure 4: Color of standard samples and unknown concentration BSA solution

5 Discussion

The BSA sample with the unknown concentration had a crystal-powder-like texture and a light yellow color.

The results obtained in Fig. 1 are according to expected, the peak was generated near 562 nm, and the arrange according to concentration was accurate. Moreover, the linear regression showed that the coefficient of determination ($R^2 = 0.9965$) is close enough to 1. Therefore, the data fits well the regression model.

From the graph in Fig. 3, the concentration of the unknown sample was determined to be $315.9\,\mu g\,mL^{-1}$ (Eq. 2). This value corresponds with the color obtained in 4, where it is suggested that the concentration value is somewhere between 300 and $400\,\mu g\,mL^{-1}$. In the color reaction, the intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. Therefore, it can be said that the experiment was succesfully conducted.

6 Conclusion

The purpose of the present experiment was to study protein quantification by the BCA method, and to determine the unknown concentration of a BSA solution. The results were according to expected for all the standard samples (known concentration BSA solutions), and the linear regression plot was accurate. Moreover, the unknown concentration of the sample was determined to be $315.9\,\mu g\,mL^{-1}$, which goes according witht the color obtained after the color reaction. In this way, the experiment was conducted succesfully, and the anticipated results were obtained.

7 Assignment

1. Explain why the color reaction is performed in a basic buffer (pH-11).

When the solution is neutral or acidic, the N electrons in the peptide bond (-NH-CO-) are attracted towards the C side by the highly electronegative oxygen molecule, and N is in a state of electron deficiency. Therefore, it is difficult to form coordination bonds with metal ions or H^+. On the other hand, when the solution is basic, H^+ is easily ionized from N in the peptide bond, and -NH-CO- and -N–CO- are in equilibrium. Then, the non-covalent electron pair of N forms a coordination bond with Cu^{2+} to form a stable complex (Fig. 5).

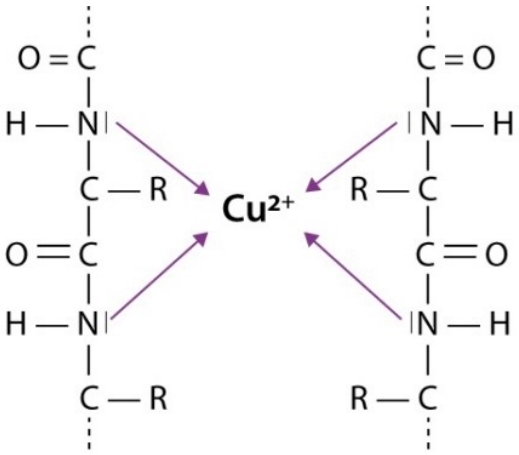

Figure 5: Complex formation of cupric ions with proteins

Therefore, the BCA method uses the biuret reaction, but it requires basic conditions for the divalent copper ion (Cu^{2+}) to be reduced to monovalent by the peptide bond in the biuret reaction.

2. As mentioned above, the molar absorption coefficient of a protein can be calculated as the sum of the molar absorption coefficients given for each amino acid (tryptophan and tyrosine). However, this value deviates somewhat from the molar absorption coefficient actually measured by weighing the protein. Explain the physicochemical reason for this.

The molar absorption coefficients given for each amino acid (tryptophan and tyrosine) in the protein are not accurate because they change depending on the environment of the amino acid molecules. For example, the absorption coefficient may change due to the dissociation of the OH group of tyrosine depending on the value of pH. Therefore, the sum of the molar absorption coefficients of tryptophan and tyrosine, even taking into account the error due to experimental manipulation, deviates from the value of all amino acids constituting the protein measured in the experiment.

3. Human serum albumin (HSA) has almost the same amino acid sequence as BSA and is found in human serum in concentrations as high as 4% Explain what the role of HSA is.

Human serum albumin (HSA) is the most abundant protein in blood and has many important biological roles: HSA is responsible for the transport of small and low molecular weight proteins, regulates colloid osmotic pressure, and is responsible for most of the tropic function in vivo. In medicine, they are widely used to treat shock, burns, and severe blood loss. HSA binds to small molecules easily due to its intramolecular polarity, easily changeable structure, and scattered hydrophobic regions. In biotechnology, they are used to improve drug delivery and maintain cell cultures.

4. What was difficult to understand in this experiment and what would you like to see improved?

The time that passed after the color reaction was finished and the UV-Vis spectrophotometry was performed was a bit longer than what was written in th laboratory guide. Besides from that, the experiment was performed smoothly.

References

[1] Sponsored by JAYHAWK Thermoset AdditivesMar 18 2020. (2022, June 29). What is polymer crosslinking?.

[2] Bianchi-Bosisio, A. (2005). Proteins | physiological samples. Encyclopedia of Analytical Science, 357-375. https://doi.org/10.1016/b0-12-369397-7/00494-5

[3] He, F. (2011). BCA (bicinchoninic acid) protein assay. BIO-PROTOCOL, 1(5). https://doi.org/10.21769/bioprotoc.44

[4] Lee, P.; Wu, X. (2015). Review: Modifications of human serum albumin and their binding effect. Current Pharmaceutical Design, 21(14), 1862-1865. https://doi.org/10.2174/1381612821666150302115025