BENG0004 Biochemistry and Molecular Biology

Protein Practical Lab Report

Purification and Concentration Measurement of Protein

Cheuk Ka, Chan

Group 10

Group members: Yihang, Zhang & Yukun, Yuan

Department of Biochemical Engineering, University College London

This series of experiments aims to measure the concentration and weights of proteins, use its peripheral principles to purify Tinsel Purple and measure enzyme activity. A standard curve of absorbance was generated by plotting the absorbances of a dilution series with known concentrations, which was later referenced to calculate the concentration of unknown protein samples. We also investigated the use of a spectrophotometer to measure the optical density of whole cell culture samples to generate samples with consistent cell concentration when given samples with varying cell proliferation. Ion exchange chromatography was used to isolate and extract Tinsel Purple protein from a culture extract via a series of elution steps with a final yield of 86.3%. Finally, we explored using the light absorbance properties at a specific wavelength of an enzyme product to measure the enzyme's activity.

1 Introduction

The Bradford Assay technique is often employed to measure a protein's concentration. First, a dilution series with known concentrations is created with a stock solution. The dilution series and the unknown sample are added onto a 96-well plate. Bradford dye reagent is then added to each sample. The dye in the reagent, Coomassie brilliant blue G-250, exhibits a reddish-brown colour but will hue-shift towards blue in the presence of protein when it transitions from a cationic state to an anionic state and forms a complex with the protein [1]. Hence, the hue of the sample colour can be used to determine protein concentration [2].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), a versatile and pivotal technique in protein separation, is often used to separate proteins between 5 and 250kDa based on their size [3]. This technique employs a charge-neutral semi-porous matrix to provide resistance while the proteins are drawn downwards towards the anode. The resistance aggregates the proteins by mass and size, offering a comprehensive view of the protein composition. The molecular weights of the proteins can be estimated if a protein ladder of known weights is electrophoresed alongside the sample. The weights of a protein can often be used to determine the proteins present approximately without having to sequence the peptide, making SDS-PAGE a powerful tool in protein analysis.

The spectrophotometer measures the samples' light absorption at specific wavelengths following the Beer-Lambert law, where the amount of light absorbed is proportional to the concentration of the light-absorbing species [4]. The absorbance wavelengths of many protein species are often similar because most proteins have similar fractions of tyrosine residues [4]. For E.coli and other bacterial cells, the optical density, which is a measure of turbidity, at 600nm OD_{600} is often used to estimate the number of cells in a sample [5]. If the protein or enzyme species to be measured has a known light absorption characteristic, the concentration or activity of the species can be easily measured, with the values weighted using the number of cells in the sample.

Ion exchange chromatography is a common protein purification technique. The modern technique was first described in 1975, where a stationary phase was used for separation while a second column was used to detect and monitor the eluted ions [6]. The column's stationary phase is a porous insoluble resin matrix with fixed cations or anions and mobile counter-ions of the opposite charge [7]. Due to the electrochemical properties of the side chains, a protein exhibits a negative charge when it is in a solution with a pH higher than its isoelectric point (pI), while it exhibits a positive charge when the solution has a lower pH than its pI [8]. This leads to the protein being electrostatically attracted to the fixed ions in the resin, given that the appropriate resin type is used, while the mobile counter-ions in the matrix dissolve into the solution to maintain charge neutrality; hence, this process is called an "ion exchange". This results in the protein being pulled out of the sample mixture, which is later eluted from the column and analysed [9].

2 Materials & Methods

2.1 Concentration Measurement of Unknown Protein Sample

Three dilution series of 80, 50, 30, 25, 20, 15, 10, 5, and 0μg/mL are prepared from a 100μg/mL standard BSA protein stock by diluting with various amounts of water. 5μL of each is added to a well plate in rows A, B, and C, each representing a dilution series. 5μL of an unknown protein is added to an empty well at the end of each row. 200μL of Bradford dye reagent is added to each well. (*See Figure 1 below*)

The absorbance of each sample at 595nm was measured using a plate reader and plotted in a graph to generate a standard curve for each dilution series (*See Figure 2 below*). The concentrations of the unknown protein samples were estimated by referencing the curves.

2.2 SDS-PAGE of Total Cell Protein

Samples of a starter culture of cells were incubated for 50 minutes before IPTG was added. Three samples were obtained right before induction, 2 hours post-induction, and 4 hours post-induction.

To ensure equal loading of cell equivalents in each lane of the PAGE gel, we diluted the different samples to the same OD_{600} level. A desired OD_{600} level of 0.2 was chosen. The samples were first diluted 20 times before OD_{600} measurement using a spectrophotometer. A dilution is necessary since prior testing indicated that the undiluted OD_{600} level lies outside the spectrophotometer's linear range of 0-1 (*See Table 2 below*), which would introduce errors in future calculations.

An equation was used to determine the amount of cell sample needed to obtain the desired OD_{600} level (*See Equation 1 below*). The appropriate amount of cell cultures was extracted and centrifuged to remove the supernatants. 50μL of SDS sample buffer was added to each to resuspend the cell pellets before loading them on a 12% SDS-PAGE gel. After SDS-PAGE, the gel was stained with Coomassie Blue stain and destained to image the proteins.

2.3 Purification of Tinsel Purple

A 20mM Tris-Cl pH 8.5 buffer is used for the ion exchange chromatography. Since the isoelectric point of Tinsel Purple is 7.12, which is lower than the buffer pH, it exhibits an overall negative charge in the buffer [10]. A weak anion exchange resin such as diethylaminoethanol can be used [11]. A Tinsel Purple extract was obtained. Passing 25mL of 25mM NaCl, 350mM NaCl, and 1M NaCl with Tris-Cl buffer through the samples yielded three fractions: wash, elution, and high salt wash.

To measure the concentrations of the fractions, a 5μL sample of the original lysate and three 5μL samples of each fraction in 2×, 5×, and 10× dilutions are added to the wells along with another row of protein dilution series. The Bradford Assay is performed again as described above. The yields of the Tinsel Purple protein can then be calculated.

2.4 Enzyme Activity Measurement

Cell culture samples were obtained at 0, 2, and 4 hours of incubation. The sample absorbances at 600nm were measured with appropriate dilutions to stay within the spectrophotometer's linear range. 50μL of 100mM catechol was added to each sample and let sit for 5 minutes before centrifuging. The supernatants' absorbances at 380nm were measured. The concentration and amount of 2-hydroxymuconic semialdehyde, a product of catechol 2,3-dioxygenase given catechol as reagents, can infer enzyme activity.

3 Results & Discussion

3.1 Concentration Measurement of Unknown Protein Sample

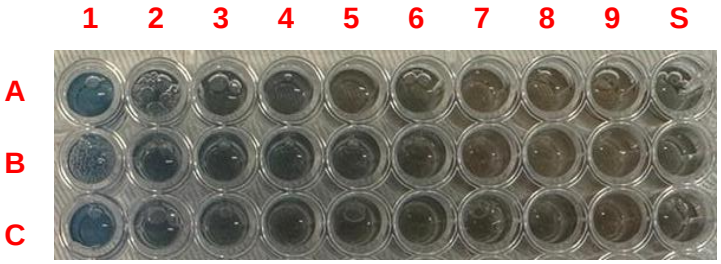

Figure 1 - Three dilution series with varying dilutions of BSA and an unknown sample with Bradford reagent.

Wells:
***Column 1** – 5µL of 80µL/mg BSA, **Column 2** – 5µL of 50µL/mg BSA, **Column 3** – 5µL of 30µL/mg BSA,*
***Column 4** – 5µL of 25µL/mg BSA, **Column 5** – 5µL of 20µL/mg BSA, **Column 6** – 5µL of 15µL/mg BSA,*
***Column 7** – 5µL of 10µL/mg BSA, **Column 8** – 5µL of 5µL/mg BSA, **Column 9**– 5µL of 0µL/mg BSA,*
***Column S** – 5µL of unknown protein sample.*
200µL of Bradford reagent was added to each well in addition to the samples.
The contents of each row are identical.

Table 1 - Protein sample absorbances at 595nm.

	1	2	3	4	5	6	7	8	9	10
A	0.8016	1.1464	0.5851	0.5139	0.3944	0.4992	0.3476	0.3525	0.4377	0.4541
B	1.3378	0.6376	0.5956	0.5409	0.4672	0.4390	0.2950	0.3257	0.3038	0.4043
C	0.8326	0.7327	0.5850	0.5046	0.5061	0.4159	0.4271	0.3665	0.3135	0.4269
Avg.	0.9907	0.8389	0.5886	0.5198	0.4559	0.4514	0.3566	0.3482	0.3517	0.4284

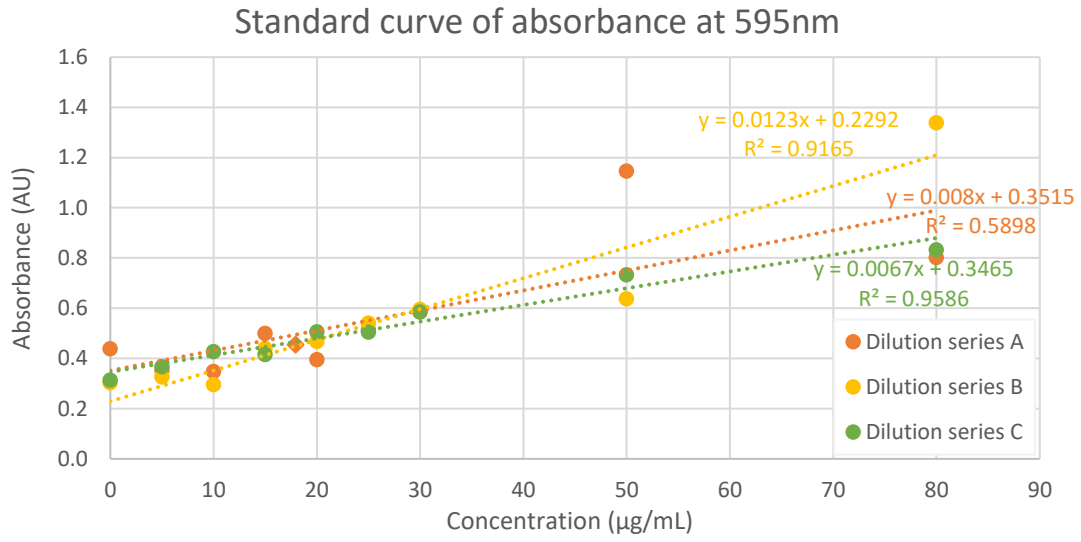

Figure 2 - Standard curve generated with a scatter plot of protein sample absorbances at 595nm with the data obtained in Table 1 above.

The concentration of the unknown samples can be calculated using the equations obtained in Figure 2 above by substituting the measured absorbances into y. We got $x_A = 17.965$, $x_B = 15.276$, $x_C = 12.651$, and $\bar{x} = 15.297$.

3.2 SDS-PAGE of Total Cell Protein

Using Equation 1 below, the appropriate volume of cell culture necessary for the SDS-PAGE can be calculated.

Equation 1 - Equation for determining the volume necessary to obtain the desired OD_{600} level.

$$\text{Volume } (\mu\text{L}) = \frac{0.2}{\text{OD}_{600} \text{ of the original}} \times 1000 = \frac{0.2}{\text{OD}_{600} \text{ of the 20 times dilution} \times 20} \times 1000$$

Table 2 – OD_{600} of the cell samples and the following calculations using Equation 1 above.

Samples	0 hours post-induction	2 hours post-induction	4 hours post-induction
20× dilution OD_{600}	0.036	0.114	0.123
Original OD_{600}	0.72	2.28	2.46
Necessary amount of cell sample (µL)	277.7	87.7	81.3

From Table 2 above, we can observe that the OD_{600} of the cell samples did increase over time following induction and incubation; therefore, the necessary amount of cell samples to reach the same final OD_{600} decreases with increasing incubation time.

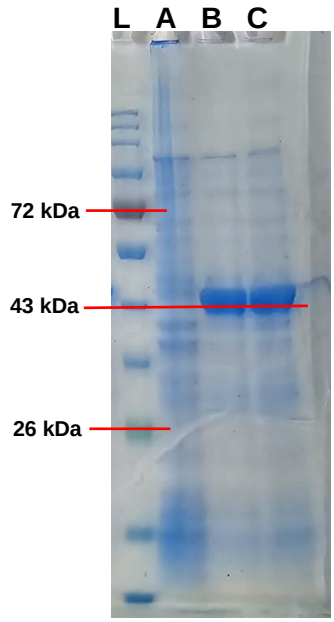

Figure 3 - SDS-PAGE gel showing the protein extracted from the cell culture.

Lanes:
***L** – Color Prestained Protein Standard, Broad Range (10-250 kDa),*
***A** – 0 hours post-induction sample, **B** – 2 hours post-induction sample, **C** – 4 hours post-induction sample.*

In Figure 3 above, we can observe that a high concentration of protein at ~43kDa can be seen in the samples extracted 2 and 4 hours post-induction, while a range of other protein sizes not of ~43kDa can be seen in the sample 0 hours post-induction.

3.3 Purification of Tinsel Purple

Using the same Bradford Assay technique as in 3.1 above, we generated the standard curve.

Table 3 – Tinsel Purple fraction absorbances at 595nm.
The values highlighted in yellow are dropped in future calculations.

µg/mL	80	50	30	25	20	15	10	5	0
Dilution series	0.8016	1.1464	0.5851	0.5139	0.3944	0.4992	0.3476	0.3525	0.3194
Dilutions	2×	5×	10×						
Clarified lysate	1.3981	1.0802	0.7683						
Wash	0.3348	0.3203	0.3229						
Elution	0.6505	0.4663	0.3918						
High salt wash	0.3844	0.3482	0.3438						

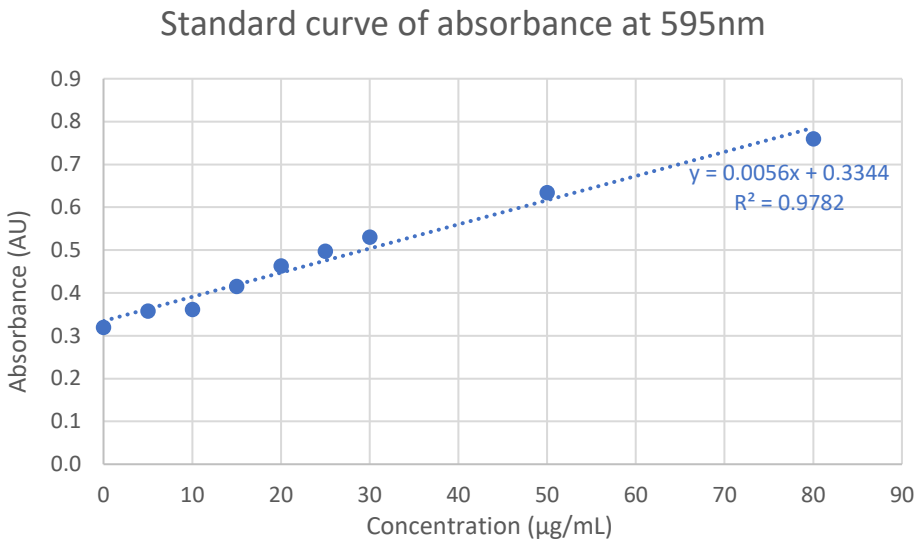

Figure 4 - Standard curve generated with a scatter plot of protein sample absorbances at 595nm with the data obtained in Table 3 above.

Using the same substitution technique as in 3.1 above, we calculated the concentrations of Tinsel Purple in each fraction.

Table 4 – Tinsel Purple fraction concentrations, amounts, and yields.
The values highlighted in yellow are dropped in the average calculation.

	Concentrations (µg/mL)				Sample volume (mL)	Protein amount (µg)	Yield
	2× dil.	5× dil.	10× dil.	Average			
Clarified lysate	185.05	129.92	75.824	758.24	0.2	151.65	
Wash	0.63918	-1.8756	-1.4247	1.2784	25	31.959	0.840%
Elution	55.393	23.446	10.525	111.09	25	2777.2	73.3%
High salt wash	9.2416	2.9632	2.2001	18.433	25	460.84	12.2%
Total extracted					75	3270.0	86.3%
Theoretical				758.24	5	3791.2	100%

As seen in Table 4 above, most of the yield came from the elution fraction, followed by the high salt wash fraction, whereas the wash fraction barely contributed anything. The total extracted yield was 86.3%. Note that some values, highlighted in yellow, were outside the

standard curve range (*See Table 3*) or did not yield a positive concentration estimation (*See Table 4*) and were dropped in future calculation steps

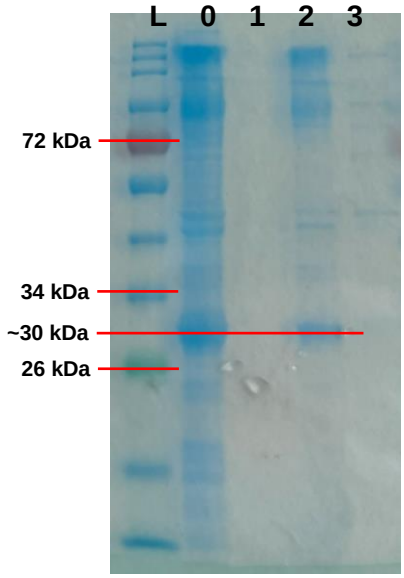

Figure 5 - SDS-PAGE gel showing the purified Tinsel Purple from different fractions.

Lanes:
***L** – Color Prestained Protein Standard, Broad Range (10-250 kDa),*
***0** – Clarified lysate, **1** – Wash fraction, **2** – Elution fraction, **3** – High salt wash fraction.*

In Figure 5 above, we can observe that a high concentration of protein at ~30kDa can be seen in the elution fraction, while virtually no protein can be observed in the other fractions. This result corroborates our findings in the calculations in Table 4 above. In literature, Tinsel Purple has a molecular weight of 25.55kDa, but only a band at ~30kDa can be observed. This could be due to the protein not being fully denatured or possessing a non-linear conformation, leading to a slower migration and an inflated apparent size estimate. Moreover, we can also observe that some unrelated proteins below 72kDa were extracted alongside Tinsel Purple. Hence, we can propose that the actual Tinsel Purple concentration should be lower than calculated.

3.4 Enzyme Activity Measurement

Table 5 - A_{600} of the whole cell samples at various times of incubation.

Sample	Dilution	A_{600} of the diluted sample	A_{600} of the original sample
0 hours of incubation	2×	0.312	0.624
2 hours of incubation	2×	0.722	1.444
4 hours of incubation	10×	0.278	2.780

In Table 5 above, we can observe that the cell absorbance increases with incubation time. Note the difference in dilution factors.

Equation 2 – Whole cell enzyme activity equation derived from the Beer-Lambert equation.

$$\text{Whole cell enzyme activity } \left(\mu\text{mol } {A_{600}}^{-1}\ \text{min}^{-1}\right) = \frac{(A_{380})(0.0005)}{(5)(A_{600})(4.4 \times 10^4)(3.8 \times 10^{-5})} \times 1000$$

Table 6 – Enzyme activity calculations from A_{380} of the whole cell samples with added catechol using Equation 2 above.

Sample	Dilution	A_{380} of the original sample	Whole cell enzyme activity ($\mu mol\ A_{600}{}^{-1}\ min^{-1}$)
0 hours of incubation	10×	0.260	0.0249
2 hours of incubation	10×	3.350	0.139
4 hours of incubation	10×	3.830	0.0824

In Table 6 above, we can observe that while the enzyme activity increased between the first two samples, it seemingly decreased between the second and third samples. A plausible explanation for this anomaly could be experimental pipette errors. Since we were working with a very diluted sample, any pipette error would be amplified greatly, affecting the final calculations significantly. It can be theorised that the enzyme saturation plateau effect acted to compound the impact of the error further, as suggested by the fact that the increase of A_{380} mismatched that of A_{600} by a non-trivial degree.

3.5 Conclusion

We successfully employed a range of protein analysis techniques to measure and purify proteins. For the Tinsel Purple purification, our final extracted yield was 86.3%; however, we observed that other unwanted proteins were mixed within the solution and that more work would be needed to purify the product further. We discovered that pipette errors can lead to a drastic calculation oddity when working with very diluted samples in the enzyme activity measurement. A similar issue also led to the linear regression returning a negative concentration value when constructing the standard absorbance curve with a dilution series. A note was made that the dilution factor should be minimised as much as possible, given that the measurement result is within the linear range of the spectrophotometer.

4 References

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