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Spectrophotometric Protein Assays

Introduction

There are numerous methods for quantifying the amount of protein in a sample including well established spectrophotometric methods such as the Bradford[1], Lowry[2], Biuret[3] and Bicinchoninic acid[4] (BCA) assays. The Bradford assay relies on protein binding to Coomassie Brilliant Blue G dye in an acidic environment, whereas the other assays form protein-copper complexes in alkaline solution. In addition, due largely to amino acids with aromatic rings, proteins absorb strongly in the UV region and can be measured directly at 280nm. Each of these methods has its advantages and disadvantages as summarised in Table 1.

Assay	Advantages	Disadvantages	Range (mg/ml)
BCA	Compatible with detergents	Incompatible with reducing agents Requires heating	0.2-1.0
Biuret	Easy to prepare reagent Compatible with detergents	Low sensitivity Incompatible with ammonium salts	0.5-10
Bradford	Compatible with reducing agents Fast	Incompatible with detergent High protein to protein variation	0.2-1.4
Lowry	Compatible with detergents	Incompatible with reducing agents 2-step reaction	0.2-1.5
Direct UV	No standard required Sample not consumed	Variability between proteins	<0.1-3.0

Table 1: Summary of various spectrophotometric protein assay methods.

The Jenway Genova life science spectrophotometer is pre-programmed with parameters for each of these assays, allowing them to be performed quickly and easily. We demonstrate here determination of the protein concentrations of four unknown samples using each of the five Genova protein assay modes. We also demonstrate the effect of detergent and a reducing agent on each of the methods.

Methods

Protein assay kits were purchased where available from the suppliers described below. The Bio-Rad DC kit is based on the Lowry method.

- Bradford Reagent - Sigma-Aldrich (B6916).
- BCA Protein Assay Kit - Sigma-Aldrich (BCA1).
- Bio-Rad DC Protein Assay Kit - Bio-Rad (500-0111).

The Biuret reagent was prepared as described in Layne (1957)[3]. Briefly, 1.5g copper (II) sulphate pentahydrate and 6g sodium potassium tartrate were dissolved in 500ml water. To this, 300ml 10% (w/v) sodium hydroxide was added and the volume made to 1 litre with water. 1g potassium iodide was added to inhibit the reduction of copper.

For a protein standard, the γ-globulin supplied with the Bio-Rad kit was re-constituted to a concentration of 10mg/ml using molecular biology grade water. A working solution of 2mg/ml was prepared from this by a 1 in 5 dilution. The concentration was measured using the Direct UV mode on the Genova and adjusted as necessary to give a 2.0mg/ml solution.

The four unknown samples tested were as follows:

1. Prostatic acid phosphatase (PAP) at a nominal concentration of 10mg/ml (suspension). This was diluted 1 in 10 for all assays except the Biuret assay.
2. Egg white. This was diluted 1 in 100 for all assays except the Biuret assay where it was diluted 1 in 10.
3. Acetylated BSA, approximately 10mg/ml. Diluted 1 in 10 for all assays except the Biuret assay.
4. BSA standard solution, stated as 1mg/ml. Not diluted. Not used in the Biuret assay.

All primary dilutions were performed with molecular biology grade water.

The samples were then further diluted as follows:

a. 1:1 with water
b. 1:1 with 6mM dithiothreitol (DTT)
c. 1:1 with 2% TWEEN® 20

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This dilution was to investigate the effect of the reducing agent DTT and the non-ionic detergent TWEEN® 20 on each of the different assays. 100µl of each final sample dilution was measured using the BCA, Bradford and Bio-Rad assays. For the Biuret assay, 100µl of the primary sample was diluted with 150µl water and 250µl of either water, 6mM DTT or 2% TWEEN® 20 to give a total sample volume of 500µl.

Where kits were used, each assay was performed according to the manufacturer's instructions. For the BCA, Bradford and Bio-Rad assays a standard curve consisting of six standards ranging from 0.2 to 1.5mg/ml γ-globulin was prepared. For the Biuret assay the standard curve ranged from 0.5 to 3mg/ml. Tables 2 and 3 detail the preparation of the standard curves.

Final concentration (mg/ml)	Volume of a 2mg/ml standard (µl)	Volume of water or dilution buffer (µl)
0 (blank)	0	100
0.2	10	90
0.4	20	80
0.6	30	70
0.8	40	60
1.0	50	50
1.5	75	25

Table 2: Standard dilutions for the BCA, Bradford and Bio-Rad assays. The final volume is 100µl in each case.

Final concentration (mg/ml)	Volume of a 10mg/ml standard (µl)	Volume of water or dilution buffer (µl)
0 (blank)	0	500
0.5	25	475
1.0	50	450
1.5	75	425
2.0	100	400
2.5	125	375
3.0	150	350

Table 3: Standard dilutions for the Biuret assay. The final volume is 500µl in each case.

BCA assay
2.0ml of BCA working reagent was added to each sample, the tubes vortexed and heated at 60ºC for 15 minutes. After cooling, the samples were transferred to plastic cuvettes.

Bradford assay
3.0ml of the Bradford reagent was added to each sample, the tubes vortexed and incubated at room temperature for 30 minutes. The samples were then transferred to plastic cuvettes.

Bio-Rad DC assay
0.5ml of reagent A' was added to each sample and the tubes vortexed. 4.0ml of reagent B was then added and the tubes vortexed again immediately. The samples were incubated at room temperature for 15 minutes then transferred to plastic cuvettes.

Biuret assay
2.5ml of the Biuret reagent was added to each sample, the tubes vortexed and incubated at room temperature for 30 minutes. The samples were then transferred to plastic cuvettes.

Measurement
For each colorimetric assay, the relevant mode was selected on the Genova and the standard curve constructed following the instructions displayed on the screen. The sample concentrations were read directly following standard curve generation. For the Bradford assay, the wavelength was adjusted to 595nm.

Direct UV
100µl of each sample was measured in a quartz microcuvette using the direct UV mode, blanking with water.

Results
The standard curves from each of the assays are shown in Figure 1.

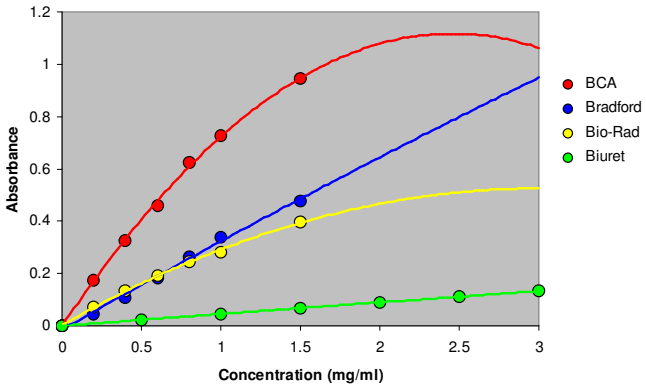

Figure 1: Standard curves for each of the colorimetric assays.

The Genova uses a polynomial curve fit for each of the assays; however it can be seen that for the BCA, Bradford and Bio-Rad assays, the curves are approximately linear up to at least 1mg/ml protein. The Biuret assay, although less sensitive, is linear up to the 3mg/ml protein tested. Absorbance varies quite significantly between assays with the BCA assay giving the highest absorbance signal above background suggesting that it is the most sensitive of the colorimetric assays tested.

Based upon the individual standard curves, the concentrations of each of the protein samples were determined by each assay. These were calculated back to those of the undiluted samples so that all the assays could be compared directly. The results are shown in Table 4.

	UV	BCA	Bradford	Bio-Rad	Biuret
PAP	13.98	5.36	7.46	2.18	8.97
Egg	129.2	131.8	106.0	49.00	123.0
AcBSA	7.40	11.20	25.18	3.82	10.90
BSA std	0.60	1.07	2.66	0.36	-

Table 4: Original sample concentrations (mg/ml) as calculated from the protein assay standard curves and sample dilution factors.

The Bio-Rad assay consistently gave lower values for all the samples than any of the other assays. The BCA assay gave values closest to those expected for the acetylated BSA and BSA standard samples. However the Bradford reagent gave values for BSA samples approximately two-fold higher expected; this is a phenomenon which has been previously noted[5] and for this reason it is recommended that BSA is not used as a standard with the Bradford assay.

Each of the colorimetric assays is reported to be affected to various extents by reagents which may be present in the protein extract. This often limits the choice of assay which can be performed. Common interfering substances include detergents, which are used to help extract and solubilise proteins and reducing agents, added to protect the proteins during extraction. Other reagents which change the pH or chelate metal ions will also interfere.

Each protein sample was determined in the presence of either 3mM DTT or 1% TWEEN® 20 to investigate the extent of interference. The results are shown in Figure 2 and clearly demonstrate the incompatibility of the protein-copper assays with the reducing agent DTT, shown in red. The non-ionic detergent TWEEN® 20 also shows a small effect on these assays, which is why it is always advisable to prepare the standards in the same buffer as the unknown samples. The Bradford reagent is very slightly affected by DTT but exhibits a very large absorbance in the presence of TWEEN® 20.

The presence of both DTT and TWEEN® 20 also resulted in increased absorbance values using the direct UV method. This is presumably an effect on protein folding which changes the absorption coefficient of the protein.

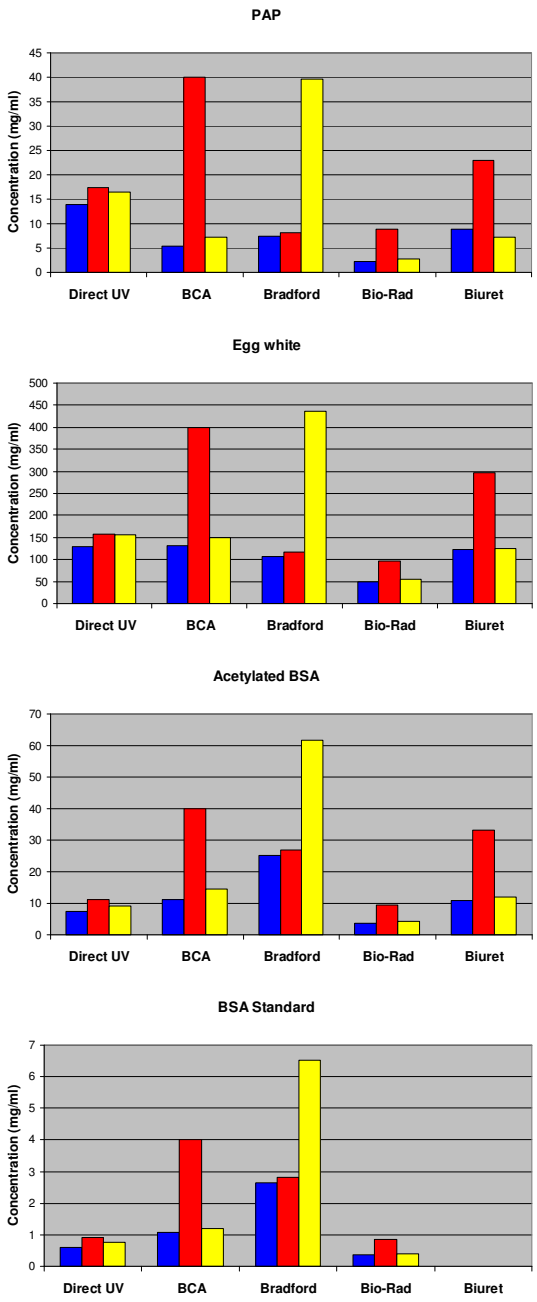

Figure 2: Protein determination of the four unknown samples with no added reagent (blue), in the presence of 3mM DTT (red) or 1% TWEEN® 20 (yellow).

Conclusions

Protein quantitation is often required after sample extraction and prior to further downstream analysis. It is also often used as quick check to identify column chromatography fractions containing proteins after separation. The Jenway Genova is pre-programmed for five of the most common methods currently used in

protein determination. These include both direct absorbance measurements in the UV region and colorimetric assays.

It is important to remember that the components of the sample will dictate which assay is most suitable. Points to consider are:

1. Protein concentration.
2. Presence of detergents.
3. Presence of reducing agents.
4. Presence of metal ion chelating compounds (e.g. EDTA and EGTA).
5. Presence of ammonium salts.

It is always advisable to prepare the standard protein dilutions in the same buffer as the sample to minimise any interference effects.

With colorimetric assays, each protein will respond in a different way. These differences are related to differences in amino acid composition, sequence, isoelectric point and secondary structure. These factors all play a role in how the protein interacts with the dye or forms a complex with copper ions[6]. Therefore wherever possible the standard used should ideally be a purified sample of the protein to be determined. As this is often not possible, it is important to accept that the assays represent an estimation rather than an exact quantitation of the amount of protein present and that it is not possible to compare data obtained with different assays.

References

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