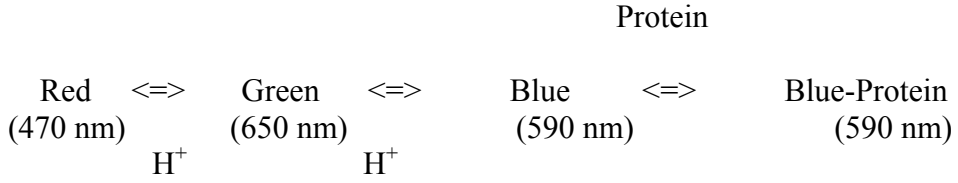
BT 510 Analytical Biotechnology Lab

Protein Extraction & Protein estimation by Bradford method

Theory/Principle: The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form.



The Bradford assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response, its response is prone to influence from non protein sources, particularly detergents, and becomes progressively more nonlinear at the high end of its useful protein concentration range. The response is also protein dependent, and varies with the composition of the protein.

a) Materials Required:

(i) **Equipments:** Spectrophotometer.

Glass or polystyrene cuvettes

(ii) **Chemicals/reagents:**

- Bradford reagent
- Bovine serum albumin (BSA)

(iii) **Glass wares and others:**

- Test-tubes
- Pipettes
- Micro centrifuge tubes

b) Reagent Preparation:

- **Protein extraction buffer:**

Tris HCl pH 8.1 ------10mM

EDTA pH 8.0----------10mM
β-Mercaptoethanol---- 5mM
PMSF ----------0.1 mg/ml

- **BSA stock solution**: 2.0mg/ml in extraction buffer

c) Procedures:

Protein Extraction

1. Weigh 1-1.5g leaves/seeds/roots/flowers harvested from test plants. Add 1ml of protein extraction buffer per gram of leaf in a cold mortar and pestle.

2. Grind the tissue in the presence of fine sand or liquid nitrogen until a thick paste is produced. Collect the paste and place them in a 1.5ml micro centrifuge tube and centrifuge for 20 minutes at 12000 rpm at $4^{o}C$.

3. Transfer the supernatant to another 1.5ml micro centrifuge tube.

4. Place about 100µl of the supernatant in a tube for quantification of the extracted protein by Bradford method.

Protein Estimation by Bradford method

1. Take 100µl of Protein extract containing approximately 10-100µg. As you do not know the protein content of the extract, you will be obliged to run a preliminary assay. Dilute two different concentrations of the extract i.e 20µl and 5µl make up the volume to 100µl with extraction buffer. Add 5ml of dye reagent and mix well. At the same time, prepare a set of standards containing 5, 10, 20, 30, 40, 50, and 100µl of Bovine Serum Albumin (BSA 2.0mg/ml stock in extraction buffer) in separate tubes. Add extraction buffer to each tube to bring the volume to 100 µl. To these tubes also add 5ml of dye reagent and mix well by vortexing. After 5 minutes and before one hour, read the absorbance at 595nm (OD_{595}) against a reagent blank (100 µl of extraction buffer with 1 ml of dye reagent)

2. Calculate the protein concentration in the extract by comparison with the standard curve for BSA. If the OD_{595} for the diluted extract is too high or too low, prepare a more suitable dilution.

3. Different proteins show considerable variation in their dye-binding capacities and so give different responses in the assay. In particular, bovine serum albumin gives a high OD_{595} value and so it is not totally representative of proteins. It is used here for convenience with total leaf extract. If you wish to measure the concentration of a specific protein, it is advised to use a purified form of the same protein as standard.

Table1:

Sl No	PROTEIN		Extraction buffer (µl)	Dye Reagent (ml)	Incubate at at Room Temperature for 30mins to 1Hr	A595 before one hour
	(µl)	(µg)				
1	-	-	100	1.0		
2.	5	10	95	5.0		
3.	10	20	90	5.0		
4	20	40	80	5.0		
5.	30	60	70	5.0		
6.	40	80	60	5.0		
7.	50	100	50	5.0		
8.	100	200	-	5.0		
9.	Unknown Sample (A)	-	-	0.9		
10.	Unknown Sample (B)	-	-	0.9		
11.	Unknown Sample (C)	-	-	0.9		
12.	Unknown Sample (d)					

Calculation: Prepare a standard curve of absorbance versus micrograms of protein and determine the slope y/x from the standard curve, which gives the A_{595} per unit of protein (µg).Hence determine the amount of protein in the unknown sample.

References:

1. Method of Bradford, Anal. Biochem.72:248 (1976); see also Anal. Biochem. 86: 142 (1978)

Sl No	PROTEIN		Extraction buffer (µl)	Dye Reagent (ml)		A595 before one hour
	(µl)	(µg)				
1	-	-	100	1.0		
2.	10	10	90	1.0		
	10	10	90	1.0 (Brad ford dye)		
3.	20	20	80	1.0		
4	40	40	60	1.0		
5.	60	60	40	1.0		
6.	80	80	20	1.0		
	80	80	20	1.0 (Brad ford dye)		
7.	100	100	Nil	1.0		
9.	Leaf extract - 10µl	-	-	1.0		
10.	Leaf extract 50µl			1.0		
11.	Root extract 10µl	-	-	1.0		
12.	Root extract 50µl			1.0		
13.	Seed extract 10µl	-	-	1.0		
14.	Seed extract 50µl			1.0		

Incubate at at Room Temperature for 30mins to 1Hr