

Blue BSA Method for Determining Protein Precipitated by Tannin

Ann E. Hagerman © 2002

Contents

Introduction	1
Making the blue BSA	1
Standardizing the blue BSA	2
Reagents.....	2
Lowry Assay (determining concentration of blue BSA)	2
Converting blue color to mg protein.....	2
Blue BSA precipitation by plant extracts or other tannin preparations	3
Isopropanol/SDS/TEA Reagent	3
Precipitation.....	3

Introduction

A standard protein, bovine serum albumin, is labeled with a blue dye so that it can be selectively measured in tannin-protein precipitates (Asquith and Butler, *J. Chem. Ecol.* 11, 1535-1544, 1985). The method is simple but less sensitive than the radiolabeled BSA precipitation method. Extracts must not contain acetone, which inhibits protein precipitation by tannins. The method involves: making the blue BSA; standardizing the blue BSA (allows conversion of blue dye color to mg protein); using the blue BSA in precipitation assays

Making the blue BSA

1. Prepare 100 mL of 1 % NaHCO₃ by dissolving 1.0 g of sodium bicarbonate in 100 mL water.
2. Dissolve 2.0 g bovine serum albumin (Sigma A 6003) in 40 mL of the NaHCO₃.
3. Add 150 mg of Remazol brilliant blue dye (Sigma R 8001) to the protein solution.
4. Let it stir gently for 30 min at room temperature.
5. Prepare 3 L of acetate buffer by diluting 34.2 mL of glacial acetic acid with about 1800 mL of water, and then adjusting the pH to 4.8 by adding 2 N NaOH (80 g NaOH dissolved in 1 L of water) drop by drop and monitoring the pH continuously at a pH meter. After the pH has been adjusted, add water to make the final volume 3 L. Store this in the cold.
6. Put the BSA-dye mixture into a dialysis bag made with 12-14,000 MW cutoff dialysis tubing, and dialyze against 1 L of the acetate buffer overnight in the cold (4 °C). Discard the acetate buffer

and dialyze again overnight with fresh buffer. Discard the buffer again. Dilute the 40 mL of dialyzed blue BSA to 1 L with acetate buffer. Store this diluted sample in the cold.

Standardizing the blue BSA

(using the Lowry assay as modified by Peterson, *Meth Enz* 91, 95-119).

Reagents

- CTC Dissolve in 100 ml water:
 - 0.1 g copper sulfate ($\text{CuSO}_4 \times 5 \text{H}_2\text{O}$)
 - 0.1 g sodium potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \times 4 \text{H}_2\text{O}$)
 - 10 g sodium carbonate (Na_2CO_3)
- SDS Dissolve in 100 mL water:
 - 5 g sodium dodecyl sulfate (sodium lauryl sulfate, SDS)
- NaOH Dissolve in 100 mL water: 3.2 g NaOH
- Known protein solution for standardizing the method: Prepare approximately 1 mg/ml BSA (Sigma) by dissolving 10 mg BSA in 10 mL acetate buffer. Determine the exact concentration of this standard spectrophotometrically by placing a sample in a cuvette, pathlength 1 cm, and determining the absorbance at 280 nm. Calculate the concentration from the known extinction coefficient of BSA (extinction coefficient, 280 nm, 1% (w/v) solution = 6.6). $A = (\text{extinction coeff}) * (\text{path length}) * (\text{concentration})$ So for an absorbance of 0.599 the calculated concentration of the standard would be 0.91 mg/mL. (You can also purchase premade protein standards, at 1 mg/mL)
- Just before running the assay, prepare Reagent A
 - 1 part CTC 2 parts SDS 1 part NaOH
- Just before running the assay, prepare Reagent B
 - 1 part commercial Folin's reagent (stored at 4 °C) 5 parts water

Lowry Assay (determining concentration of blue BSA)

Run the Lowry assay on replicate samples of the standard protein and the unknown (blue BSA).

1. Use 10- 100 uL of the standard, making each sample to 1 mL with water.
2. Use 10-50 uL of the blue BSA, making each sample to 1 mL with water.
3. To 1 mL of sample, add 1 mL of reagent A. Mix.
4. About 10 min later add 0.5 mL of reagent B. Mix.
5. About 30 min later read absorbance at 750 nm in a 1 cm cell.

Converting blue color to mg protein

To calculate amounts of protein precipitated by tannins, the blue color in the precipitate must be converted into ug blue BSA precipitated, and a standard curve is needed for that conversion. The absorbance properties of the dye are dependent on the solvent composition, so this calibration is done

in the isopropanol/SDS/TEA solution that is used to dissolve the precipitated protein in the assay (see below). Aliquots of the blue BSA solution are brought to a final volume of 3 mL with the isopropanol/SDS/TEA solution, and the absorbance at 590 nm is determined. The absorbance of the blue solution is plotted as a function of the protein concentration (determined from the Lowry assay, above) to yield a calibration curve to convert absorbance at 590 to mg protein.

Blue BSA precipitation by plant extracts or other tannin preparations

Blue BSA is precipitated by tannin, and the precipitate is redissolved and color determined.

Isopropanol/SDS/TEA Reagent

Add to a one liter graduated cylinder:

50 mL triethanolamine (2,2',2''-nitrilotriethanol)

200 mL isopropanol

10 g SDS (sodium dodecyl sulfate, also called sodium lauryl sulfate)

Bring to 1 L with water.

Precipitation

1. Put a volume of blue BSA solution equivalent to 4 mg of blue BSA plus enough acetate buffer to make the volume of protein 4.0 mL into a screw cap glass test tube.
2. Add tannin containing sample (1 mL extract, or extract diluted to 1 mL).
3. Vortex. Allow the mixture to incubate 2 h in the cold (4 C).
4. Centrifuge 15 min at 3000 x g (high speed in a clinical or table top centrifuge).
5. Carefully pour off the supernatant, without disturbing the blue precipitate.
6. Add 3.0 mL of isopropanol/SDS/TEA to the precipitate, and vortex vigorously to completely redissolve the precipitate.
7. Read the absorbance at 590 nm, and calculate the amount of protein precipitated from the calibration curve.
8. Sometimes plant extracts have pigments which interfere with the blue color. If the color of the redissolved precipitate is different than the color of just blue BSA, set up a sample-only blank containing the sample plus isopropanol/SDS/TEA and measure its absorbance. Subtract this value, and note the unusual presence of these interfering pigments.