

Precipitation of Phlorotannin by Protein

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Introduction

Because phlorotannins do not have the ortho phenolic groups typical of condensed or hydrolyzable tannins, the protein precipitable phenolics method cannot be used to determine phlorotannin in precipitates. This method was devised to take advantage of the reaction between phlorotannins and dimethoxybenzaldehyde. The phlorotannin and protein are reacted in a buffer solution to form a precipitate. The precipitate is dissolved in N,N-dimethylformamide and the amount of phlorotannin in the precipitate is measured using 2,4 DMBA.

The protein and tannin are reacted in a buffer containing a reducing agent (DTT) because of the sensitivity of phlorotannin-protein interactions to oxidation. Both the original method described in Stern, J. L.; Hagerman, A. E.; Steinberg, P. D.; Mason, P. K. Phlorotannin-protein interactions. *J. Chem. Ecol.* 22, 1877-1899 1996, and a microscale method are given here. The method can be coupled with the radiolabeled protein precipitation assay to allow determination of protein in the precipitates.

Reagents

- Buffer J: 0.1 M acetate, 0.43 M NaCl. Mix 5.7 mL glacial acetic acid with about 800 mL water and add 25.16 g NaCl. Adjust to pH 5.0 with conc NaOH. Bring volume to 1 L with water. Store refrigerated.
- 1 M dithiothreitol (DTT): Bring 1.54 g DTT up to 10.0 mL with water. Divide into 150 uL aliquots and store frozen.
- Buffer J with DTT: Because DTT is unstable, the DTT is added to buffer J on the day of the assay. Bring 150 uL of 1 M DTT up to 10.0 mL with buffer J (final DTT concentration is 15 mM).
- Saline: 6.5 g NaCl up to 1 L with water.
- Protein: Prepare the protein (standard is BSA, others can be used) at 10 mg/mL in saline. Keep refrigerated, prepare fresh frequently. (For Ribulose biphosphate carboxylate prepare 2.5 mg/mL and centrifuge to remove insoluble materials. Use 80 uL instead of 20 uL in assay and reduce the buffer to 120 uL).
- Tannin: 10 mg/mL in methanol

- 16% HCl in glacial acetic acid: 16 mL conc HCl made up to 100 mL with glacial acetic acid. Stable at room temperature.
- DMBA: 2.0 g of 2,4-dimethoxy benzaldehyde (Sigma D 3269) made up to 100 mL with glacial acetic acid. Prepare fresh daily. Prepare only the amount needed. Will require 1.25 mL DMBA per sample (original method) or 700 uL per sample (microscale method).

Original method

1. Bring water bath to 30 C.
2. Dispense buffer (180 uL) into 15 mL conical centrifuge tubes. Add tannin (10 uL), mix, add protein (20 uL). Mix. Incubate 30 min at room temp.
3. Need to prepare at the same time "no protein" blanks, each has 210 uL buffer and 10 uL tannin.
4. Centrifuge all tubes 15 min at 3200 rpm, then aspirate off supernatant. Wash ppt by gently adding 100 uL J+DTT to each tube, and centrifuging 5 min at 3200 rpm (maximum speed on a clinical centrifuge).
5. Aspirate off wash and immediately add 10 uL of DMF to each tube. Vortex.
6. Add 1.25 mL of 16% HCl to each sample.
7. Prepare the tubes for two types of controls by rinsing with 200 uL J+DTT. Total phlorotannin controls contain 10 uL phlorotannin, 10 uL DMF, 1.25 mL 16% HCl. Reagent controls contain 1.25 mL 16% HCl.
8. At timed 1 min intervals, add 1.25 mL of DMBA reagent to all tubes. Mix, cover tubes, and place in 30 C water bath.
9. After exactly 60 min, start timed reading of absorbance at 510 nm. Note any tubes that contain precipitate at time of reading.
10. Spec should be zeroed with 16% HCl.

Microscale method

The advantage of the microscale method is use of less reagent and snap cap microfuge tubes which contain the fumes very well.

Reagents are as for original method, but tannin is at 5 mg/mL and protein is at 5 mg/mL.

Buffer (180 uL), tannin (up to 10 uL) and methanol (up to 10 uL, to make final volume after protein addition up to 210 uL) are vortexed in a 1.5 mL microfuge tube.

Protein (20 uL) is added with immediate vortexing.

Steps 2-4 from the original method (above) are followed, except that samples can be centrifuged in a microfuge at about 4000 rpm. High speed centrifugation is not recommended, since it may make the precipitate difficult to dissolve.

Steps 5-7 are followed as in original procedure, except that 700 uL of the HCl reagent and 700 uL of the DMBA reagent are used.