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Tannin Assays in Ecological Studies: Lack of Correlation Between Phenolics, Proanthocyanidins and Protein-Precipitating Constituents in Mature Foliage of Six Oak Species

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Summary. There is no correlation between protein-precipitating capacity and either total phenolic or proanthocyanidin content of extracts of mature foliage from six species of oaks: *Quercus alba* (white oak), *Q. bicolor* (swamp white oak), *Q. macrocarpa* (bur oak), *Q. palustris* (pin oak), *Q. rubra* (red oak), and *Q. velutina* (black oak). It is argued that studies which probe the role of tannins in the selection and utilization of food by herbivores should include a protein-precipitation assay, since such an assay provides a measure of the property of tannins which is presumed to contribute to their utility as defensive compounds. A convenient modification of the bovine serum albumin (BSA) precipitation assay, which measures the amount of protein precipitated when a plant extract is added to a BSA solution, is described. Advantages of this procedure recommend its routine adoption in studies of the role of tannins in plant-herbivore interactions.

Introduction

Tannins are naturally occurring, water-soluble phenolic compounds in the 500 to 3,000 molecular weight range, having the property of precipitating proteins from aqueous media (Ribéreau-Gayon 1972; Haslam 1979; Swain 1979a, b). They have been accorded an important role in protecting plant tissues from herbivore attack (Feeny 1976; Rhoades and Cates 1976). Commencing with Feeny's classic investigation of the inhibiting effects of oak leaf tannins on larval growth by the winter moth, *Operophthera brumata* (Feeny 1968, 1970), a series of papers (Fox and Macauley 1977; Chan et al. 1978; McKey et al. 1978; Rhoades 1979; Bernays et al. 1980, 1981; Moran and Hamilton 1980; Oates et al. 1980; Waterman et al. 1980; Lawson et al. 1982) has appeared shaping contemporary views concerning the significance of these substances in plant-herbivore interactions. Since tannins constitute a structurally heterogeneous class of compounds, they do not lend themselves to easy quantification, and investigations of the importance of tannins in food selection and utilization have been hampered by deficiencies in the methods used to assay for tannin content.

The procedures most frequently used in ecologically oriented studies have been the Folin-Denis assay (Swain and Hillis 1959), the proanthocyanidin assay (Hillis and Swain 1959; Govindarajan and Mathew 1965) and the vanillin/HCl assay (Burns 1971; Price et al. 1978). The appeal of these three methods is the ease with which they can be performed, but each has serious shortcomings as an assay for tannin content (Swain 1979a). In all three the extent of color development depends upon the molecular structures as well as the amounts of the tannins present. In addition, non-tannic constituents can contribute to color development. Furthermore, the proanthocyanidin and vanillin/HCl assays depend upon structural elements present only in the condensed tannins, and hence provide no measure of hydrolyzable tannins.

Since tannins form insoluble complexes with proteins (van Sumere et al. 1975), another approach to measuring the amount of tannin in an extract has been to determine its capacity to precipitate proteins from solution. Assays involving the precipitation of β-glucosidase (Goldstein and Swain 1965), hemoglobin (Bate-Smith 1973; Schultz et al. 1981), and bovine serum albumin (Hagerman and Butler 1978) have been described. Since it is the capacity of tannins to precipitate proteins, especially digestive enzymes or ingested food plant proteins, which is postulated to be responsible for the adverse effects which these substances have on many organisms, these assays would appear to be particularly appropriate ones in studies of the significance of tannins in herbivory. To date, however, such procedures have found only limited use by investigators of plant-herbivore interactions.

In this study we have performed Folin-Denis, proanthocyanidin, and two different protein-precipitation assays on extracts of the mature foliage of six species of oaks: white oak (*Quercus alba*), swamp white oak (*Q. bicolor*), bur oak (*Q. macrocarpa*), pin oak (*Q. palustris*), red oak (*Q. rubra*), and black oak (*Q. velutina*). While the results of the two protein-precipitation assays are highly correlated, the protein-precipitation assays, the Folin-Denis assay, and proanthocyanidin assay provide three entirely different pictures of the chemical defenses of these species. Bate-Smith (1977) and Swain (1979b) have pointed out the problems inherent in using chemical, functional group assays to evaluate tannin content. Our comparison of the foliage of six oak species underscores the cogency of their remarks, and argues strongly for the use of a protein-precipitation assay in studies designed to probe the significance of tannins in plant-herbivore interactions.

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Materials and Methods

Preparation of Extracts

Mature foliage (Lawson et al. 1982), frozen immediately after collection and subsequently lyophilized, was ground in a Wiley mill (40-mesh) after removal of the midrib. Ground leaf powder was stored in a desiccator until analysis. Leaf powder (60.0 mg) was extracted twice for 8 minutes with 4 ml of boiling 50% (v/v) aqueous methanol in a centrifuge tube (capped with a marble) placed in a heat block at 95° C. After centrifugation ($12{,}000 \times g$, 15 min, 5° C), the pellet was resuspended in a small volume of 50% methanol and centrifuged as before. The volume of the combined supernatants was adjusted to 10 ml, and dilutions appropriate to each assay were prepared from aliquots of this stock solution.

Assays

Folin-Denis Assay (Swain and Hillis 1959; Ribéreau-Gayon 1972). Aliquots of the stock extract derived from 0.06, 0.12, 0.18, and 0.24 mg (dry weight) of leaf powder were diluted to 2.8 ml by the addition of water, and 0.2 ml of the Folin-Denis reagent was added with vigorous vortexing. Three minutes later, 0.4 ml of a saturated solution of sodium carbonate and 0.6 ml of water were added. After 60 min at room temperature, absorbance at 725 nm (A_{725}) was measured, zeroing the spectrophotometer with a tube containing all of the reagents plus water in place of the extract. A calibration curve was constructed from commercial tannic acid, TA (Sigma Lot 73C-1480), found to contain 6.4% moisture: $A_{725} = 0.0199$ (μg TA in assay mixture) -0.0034, in the range 5–25 μg TA ($r = 0.998$, standard error of regression coefficient = 0.0004). This regression was used to calculate the tannic acid equivalents (μg TAE) present in the extracts.

Proanthocyanidin Assay (Hillis and Swain 1959; Govindarajan and Mathew 1965). Aliquots of extract derived from 0.6, 0.9, 1.2, 1.5 and 1.8 mg leaf powder, diluted to 0.3 ml, were vortexed with 3.0 ml of a solution of 80% butanol-HCl (preheated to 97° C) containing 15.4% (w/v) ferrous sulfate. Heating at this temperature was continued for 15 min, during which time the assay tube was capped by a marble to allow refluxing of solvent. Absorbance at 550 nm (A_{550}) was compared immediately to an unheated control, zeroing against distilled water. A calibration curve was constructed using commercial bisulfited quebracho, BQ (Pilar River Plate Corp., Newark, New Jersey) found to contain 18% moisture: $\Delta A_{550} = 0.0020$ (μg BQ in the assay mixture) $+ 0.0418$, in the range 25–250 μg BQ ($r = 0.997$, standard error of regression coefficient = 0.00009). This regression was used to calculate the bisulfited quebracho equivalents (μg BQE) present in the extracts.

β-Glucosidase Precipitation Assay (Goldstein and Swain 1965). The aqueous methanolic solvent from 5.0 ml of the stock extract was removed at room temperature and reduced pressure using a rotary concentrator, and the residue was redissolved in 3.75 ml of acetate buffer (0.1 M, pH 4.8) by rotary agitation for 30 min. Aliquots of this solution, containing dissolved extractives from 0.24 to 1.68 mg of leaf powder, diluted to 0.3 ml with acetate buffer (0.1 M, pH 4.8), were vortexed with 0.3 ml of a solution of β-glucosidase (Sigma G-8625, Lot 40 F-4017) (0.5 mg/ml) in phosphate buffer (0.1 M, pH 7.0), and then allowed to stand at room temperature for 15 min. After centrifugation ($12{,}000 \times g$, 15 min, 5° C), the activity of the enzyme in the supernatant was determined in triplicate by combining a 50 μl aliquot with 3.0 ml of acetate buffer (0.1 M, pH 4.8) containing esculin (0.5 mM) and aluminum chloride (3.75 mM) at 25° C, and following the change in absorbance at 385 nm (ΔA_{385}) for 3.5 min on a self-zeroing Zeiss PMQ-II spectrophotometer zeroed against water. The change in A_{385} was demonstrated to be linear for at least 9 minutes. From a determination of the activity in the original enzyme solution (1.2–1.6 units/ml), the number of units of activity precipitated by the addition of the extract was calculated. One unit is the amount of enzyme which liberates 1 micromole of product per minute under the conditions of the assay. Assuming that the esculetin-aluminum chloride complex generated in this procedure has the same extinction coefficient as the one generated in the original procedure of Goldstein and Swain (1965), a ΔA_{385} of 1 A unit/minute corresponds to 0.45 units of enzyme activity. A plot of units of activity precipitated vs. mg leaf powder suggests a possible sigmoidal dependence, but the middle portion of the curve closely approximates linearity. Linear regressions were fitted to the nearly linear regions of these curves.

BSA Precipitation Assay: Determination of Protein Precipitated. Aliquots of the stock extract derived from 1.38, 2.07, 2.76, 3.45, or 4.14 mg of leaf powder, diluted to 0.69 ml with 50% aqueous methanol, were vortexed with 1.39 ml of a solution (1 mg/ml) of crystallized, lyophilized BSA (Sigma, Lot 70F-9350) in acetate buffer (0.2 M, pH 5.0) containing 0.17 M sodium chloride, and allowed to stand at room temperature for 15 minutes. After centrifugation ($12{,}000 \times g$, 15 min, 5° C), the pellet was rinsed gently with 0.42 ml of the same buffer, centrifuged as before, and the supernatants combined to give a final volume of 2.5 ml, which was applied to a 1.7×5.0 cm column of Sephadex G-25 (Pharmacia Fine Chemicals, PD-10 columns), which had been equilibrated with an acetate buffer (0.2 M, pH 5.0) containing 0.17 M sodium chloride and 13.8% methanol. Proteins were eluted completely in 3.5 ml of the same solvent mixture. This step removes all materials from the supernatant which absorb at 595 nm. The amount of protein in the eluent was determined by vortexing a 50 μl aliquot with 2.5 ml of Coomassie Brilliant Blue G-250 dye reagent (Bio Rad Protein Dye Reagent), and determining A_{595} after 6 minutes (Bradford 1976) using a blank consisting of 50 μl of buffer plus 2.5 ml of the dye reagent. The absorbance at 595 nm was transformed into mg of BSA by the use of a calibration curve constructed on the same day as the assay. From a determination of the amount of BSA in the original solution, the amount precipitated by the addition of the extract could be calculated. The amount of protein in the BSA-tannin precipitate cannot be measured directly, since the SDS required to redisolve the precipitate interferes with the protein assay.

BSA Precipitation: Determination of Phenols Precipitated (Hagerman and Butler 1978). Aliquots of the stock solution derived from 1.0, 1.5, 2.0, 2.5 or 3.0 mg of leaf powder diluted to 0.5 ml were vortexed with 1.0 ml of the same BSA solution described above. Following the rinsing proce-