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USE OF DYE-LABELED PROTEIN AS SPECTROPHOTOMETRIC ASSAY FOR PROTEIN PRECIPITANTS SUCH AS TANNIN

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Abstract—Bovine serum albumin has been covalently labeled with Remazol brilliant blue R to provide a substrate for a convenient spectrophotometric assay for protein precipitants. The blue protein is especially useful for measuring protein precipitation by vegetable tannins because its absorption maximum is at a wavelength where plant pigments exhibit minimum absorption. Blue BSA has been used to determine, by competition experiments, the relative affinity of various proteins for tannins. A procedure for purifying condensed tannin from commercially available quebracho extract is described.

Key Words—Tannin assay, protein precipitation, sorghum tannin, quebracho tannin, condensed tannins

INTRODUCTION

The biological effects of the complex polyphenols known as tannins are considered to be the result of their binding and precipitating proteins (McManus et al., 1981). Assays of protein binding and precipitation are therefore of considerable importance in characterizing tannins.

Unfortunately, the assays presently available for measuring protein binding/precipitation are not satisfactory in all respects. The use of hemoglobin as a spectrophotometric marker for protein precipitation (Bate-Smith, 1973; Schultz et al., 1981) is absolutely dependent on freshly prepared hemoglobin (I. Baldwin, personal communication); commercial (lyophilized) preparations are unsatisfactory. Pigments such as anthocyanidins, often present in plant extracts containing tannins, absorb at similar wavelengths as hemoglobin, interfering with the precipitation assay and causing high blank values. The hemoglobin

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precipitation assay has given unsatisfactory results on high-tannin sorghum (Bullard et al., 1981).

Martin and Martin (1983) devised an indirect assay using the Bradford protein test (Bradford, 1976) to measure unprecipitated protein. This technique is versatile with respect to assay conditions and has recently been adapted to the analysis of multiple samples (Wilson, 1984). It is inherently less accurate because it does not measure the precipitated protein directly. Moreover, controls to eliminate the effect of interfering materials are laborious.

There have been several attempts to estimate tannin concentration by its inhibition of various enzymes (Davis and Hoseney, 1979; Becker and Martin, 1982), but the correlation between tannin concentration and degree of inhibition is unsatisfactory (Daiber, 1975; Gupta and Haslam, 1980; Earp et al., 1981; Bullard et al., 1981). This may be due to retention of variable activity in enzyme–tannin complexes (Armstrong, 1983; Butler et al., 1984).

In this laboratory we have directly measured protein binding and precipitation by utilizing standard proteins labeled with radioisotopes in order to facilitate their detection (Hagerman and Butler, 1980a; Asquith et al., 1983). This method is sensitive and reliable, but it depends upon the availability of radioisotope equipment, and preparation of labeled protein may be difficult.

We report here a direct spectrophotometric assay which obviates most of the difficulties mentioned above. The assay utilizes a standard soluble protein, bovine serum albumin (BSA), covalently labeled with a blue dye. The assay can be adapted to measurement of materials which do not precipitate proteins but which compete with precipitants for binding them.

METHODS AND MATERIALS

All chemicals were reagent grade and used without further purification. Bovine serum albumin (fraction V, fatty acid free), chicken egg ovalbumin, and fetuin were purchased from Sigma Chemical Corp. (St. Louis, Missouri). Calf skin gelatin was from Eastman Organics (Rochester, New York). Rat submaxillary gland glycoprotein GP_{66}-SMX (Mehansho and Carlson, 1983) was generously provided by Dr. Haile Mehansho. Remazol brilliant blue R was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Cyanidin was purchased from K & K Laboratories Inc. (Plainview, New York). Condensed tannin (Gupta and Haslam, 1980) was purified from *Sorghum bicolor* Moench, DeKalb BR 64, as described by Hagerman and Butler (1980b). Crude quebracho condensed tannin (Roux, 1957) was obtained from Trask Chem. Corp. (Marietta, Georgia).

Preparation of Labeled BSA. The protein-labeling procedure was adapted from Rinderknecht et al. (1968). To 2 g of BSA dissolved in 40 ml of 1% (w/v) $NaHCO_3$, pH 8.2, was added 150 mg of Remazol brilliant blue R, and

the solution was stirred for 30 min at room temperature. The solution was dialyzed against 0.2 M acetate, pH 4.8 (solution A), at 4°C overnight to replace the reaction buffer with a buffer more suitable for protein-binding assays. Protein concentration was determined by the method of Lowry et al. (1951). When the labeled protein had been diluted to about 1 mg/ml, the dye did not interfere with the Lowry assay.

Protein Precipitation. To 4.0 ml of blue BSA (2.0 mg/ml) in solution A was added 1.0 ml of methanol containing 0.1–0.7 mg of tannin [either purified tannin or a crude methanol or acidic methanol extract of plant tissue (Asquith et al., 1983)]. Five minutes after vigorous mixing at room temperature, the tubes were centrifuged (bench top centrifuge). The supernatant layer was removed with a Pasteur pipet and discarded. Precipitates were dissolved in 3.5 ml of 1% (w/v) sodium dodecyl sulfate–5% (v/v) triethanolamine–20% (v/v) isopropanol (solution B), and the absorbance at 590 nm was measured spectrophotometrically. Complete precipitation gave A_{590} values of about 0.90. For blanks, methanol was substituted for the sample. Blank values are zero for properly prepared protein; no precipitate forms. Sorghum grain samples were extracted initially with hexane to remove lipids that otherwise precipitate out of the methanol extract in the aqueous assay. The assay was standardized with purified sorghum tannin. Assays of tannins from other sources should be standardized with purified tannin from that source.

Competition Assays. Conditions were adapted from Hagerman and Butler (1981). Varying amounts of competitor were mixed with 1 mg of blue BSA to give a total volume of 1.6 ml of solution A. To this mixture was added 0.4 ml of methanol containing enough tannin to precipitate 70–80% of the blue protein as determined in the absence of competitor. After vortexing and centrifuging as described above, the supernatant layer were removed. The pellet was dissolved in 3.5 ml of solution B and the absorbance was measured at 590 nm. For comparing the tannin-binding capacities of several proteins, it is useful to determine the concentration of competitor which inhibits the precipitation of labeled BSA in the standard assay by 50%. Relative affinity is defined as the weight of the blue BSA present divided by the weight of competitor which prevents 50% of the labeled BSA from precipitating.

Preparation of Quebracho Tannin. Crude quebracho tannin (20 g) was dissolved in 1 liter of 0.001 M acetic acid and extracted four times with equal volumes of ethyl acetate. Residual ethyl acetate was removed by rotary evaporation and the aqueous tannin solution mixed with a thick aqueous slurry (1 ml of resin/mg of tannin) of Sephadex LH-20 (Pharmacia). After stirring for 2 min, the slurry was transferred to a sintered glass funnel and washed successively with water and ethanol. Tannin was eluted with acetone–water (50:50, v/v). Acetone was removed from the tannin solution under reduced pressure, and the material was lyophilized.

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Lyophilized tannin was further purified by applying 50 mg of tannin, in 10 ml of water, to a 2 × 15-cm column of Sephadex LH-20 equilibrated with water. The loaded column was washed with four column volumes each of water, 5% acetone (v/v), and 30% acetone (v/v). Tannin was eluted with 60% acetone (v/v) and lyophilized after the acetone was removed by rotary evaporation.

Purified quebracho tannin contained 1.3% contaminating protein as determined by Kjeldahl analysis; comparable preparations of sorghum tannin contained similar amounts of protein (Hagerman and Butler, 1978). On HPLC most of the A_{280} absorbing material eluted at retention times characteristic of high-molecular-weight procyanidin polymers (Putman and Butler, unpublished data). Digestion of the tannin by HCl-*n*-butanol (Gupta and Haslam, 1980) and analysis by TLC using Analab silica gel plates and toluene-formic acid-acetone (60:30:10) (Armstrong, 1983) gave cyanidin and another compound with characteristics consistent with that of fisetinidin (Roux, 1957). Sorghum tannin yields only cyanidin when digested (Strumayer and Malin, 1975). The average chain length (Butler et al., 1982) of the purified quebracho tannin was about 70% of the chain length determined for purified sorghum tannin.

RESULTS

Characteristics of Blue BSA. Blue BSA, prepared as described, can be stored for several months at 6°C, at concentrations up to 50 mg/ml, with no loss of precipitating activity. It is soluble in solution B, and the color formed is stable indefinitely. Blue BSA dissolved in solution B has an A_{max} at 590 nm and a second peak at 620 nm (Figure 1). When subjected to SDS gel electrophoresis (Laemeli, 1970), the labeled protein yielded a single major blue band with an R_f slightly greater than that of unlabeled BSA. Only a trace of unlabeled BSA was detectable by silver staining of these gels (BioRad, 1982).

After lyophilization, blue BSA is less soluble. Increasing the pH of the labeling reaction increases the amount of dye bound (Laytner and Finnemore, 1973), but the more heavily dyed protein is less soluble. In both cases, addition of methanol without tannin results in precipitation of blue BSA. The conditions described here, 1 mg of dye to 13 mg of BSA, represent a satisfactory compromise between color intensity and solubility properties similar to those of the native protein. Although batch to batch variation was small, each batch should be restandardized with purified tannin (see below).

Direct Precipitation Assay. When excess blue BSA is present, the amount of protein precipitated is proportional to the amount of added tannin (Figure 2), with sorghum tannin precipitating about twice as much blue BSA as does the same amount of quebracho tannin. Extracts from oak leaves were also assayed satisfactorily using the procedure (data not shown). The assay is sensitive to the nature of the sample solvent. Both of the purified tannins tested precipitate more

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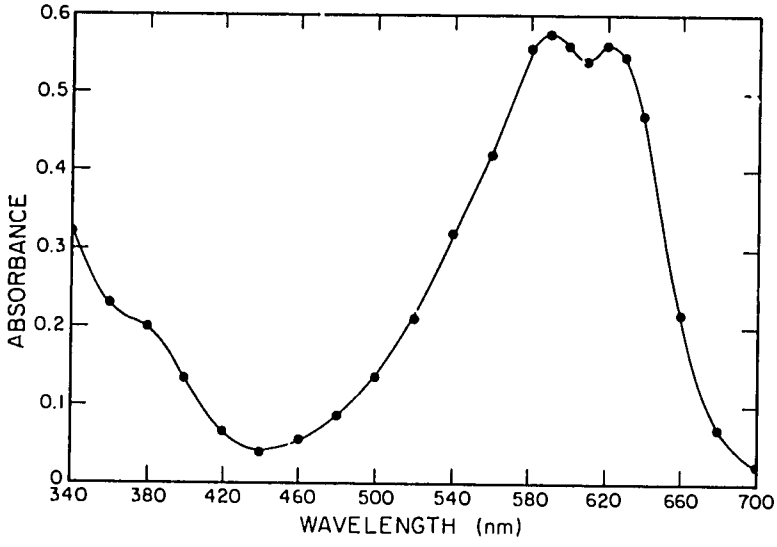

Fig. 1. Spectrum of blue BSA dissolved in solution B (0.6 mg/ml).

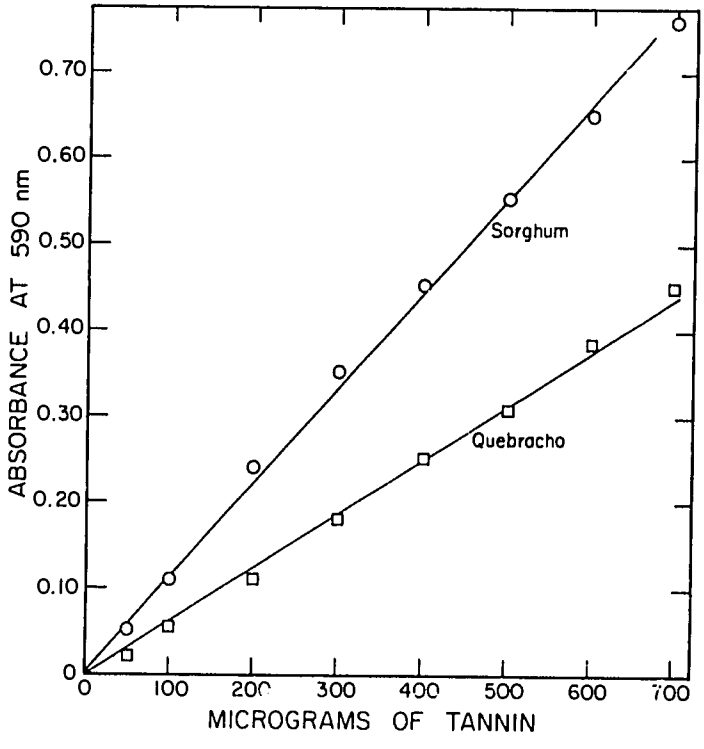

Fig. 2. Precipitation of blue BSA by sorghum or quebracho tannin under the standard assay conditions outlined in Methods and Materials. Values presented are average of duplicates.

protein when the solvent contains 20% (v/v) alcohol than in 100% water; methanol gives more precipitation than ethanol. A similar effect was observed with ^{125}I-labeled BSA (Hagerman and Butler, 1980a). Calderon et al. (1968) noted that ethanol increased the amount of gelatin precipitated by quebracho tannin, possibly by decreasing the solubility of the tannin/protein complex.

Increasing the ionic strength decreases the amount of blue BSA precipitated (data not shown). Below pH 4 and above pH 5 very little blue BSA precipitates, in accordance with the observations of Hagerman and Butler (1978) on unlabeled BSA.

When acidic methanol is used as an extractant for tannins (Asquith et al., 1983), a few low-tannin sorghums yield a hydrophobic protein, mol wt 16,000 on SDS-PAGE (Laemeli, 1970), which coprecipitates with blue BSA in the assay to give a cloudy white haze with A_{500} up to 0.4. The hazy material interferes with making accurate spectrophotometric measurements and gives a false-positive test for tannins by precipitation of blue BSA. Precipitates formed from extracts of these lines tested negative for protein precipitable phenols (Hagerman and Butler, 1978).

Competitive Binding Assay. A technique similar to that of competitive antibody binding assays has been utilized by Hagerman and Butler (1981) to measure the relative affinity of various proteins and other ligands for tannin. A standard labeled protein is mixed with enough tannin to precipitate 70–80% of the labeled protein. Relative affinities are established by mixing an unlabeled competitor with the labeled protein before tannin is added. Less labeled protein is precipitated in the presence of competitor than is precipitated in the absence of competitor. The effect of the competitor is a function of its concentration, its affinity for tannin, and its capacity for binding tannin. The competitor need not precipitate the tannin, but only bind it and thus prevent it from precipitating the labeled protein.

Using this assay, the set of proteins tested were found to vary by as much as three orders of magnitude in their relative affinities for the tannin (Figure 3). Results are presented as semilog plots in order to accommodate the wide range of competitor concentrations. The relative affinities of sorghum tannin for gelatin, fetuin, and unlabeled BSA were almost identical to the relative affinities of quebracho tannin for these proteins (Table 1). Compared on the basis of amount of competitor required to inhibit 50% of the precipitation due to tannin, fetuin is 10 times more effective than unlabeled BSA at binding tannin, and gelatin is about three times more effective than fetuin. Unlike other proteins, GP_{66}-SMX differs in its affinity for the two tannins. Soluble polyvinylpyrrolidone (PVP), a nonprotein synthetic polymer used for binding tannins in plant extracts (Gray, 1978), bound tannin in this assay with an affinity similar to that of gelatin (data not shown).

Both direct precipitation and competitive binding assays have been done

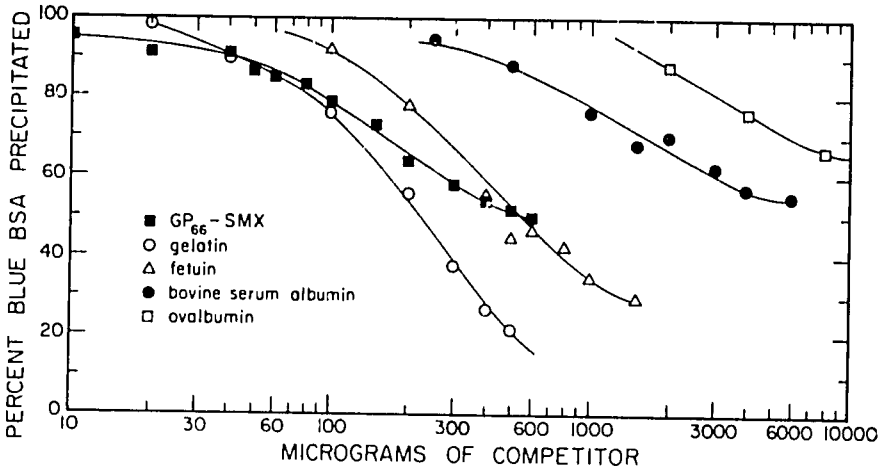

Fig. 3. Competition assays between blue BSA and standard proteins for sorghum tannin. The assays were performed as described in Methods and Materials, using 0.2 mg of purified tannin dissolved in 0.5 ml of methanol. Values presented are average of duplicates.

with tannic acid, with results similar to those obtained for the condensed tannins.

DISCUSSION

We chose Remazol brilliant blue R as a convenient spectrophotometric label for a protein to be precipitated by tannins. It reacts irreversibly with BSA

TABLE 1. RELATIVE AFFINITY OF PROTEINS FOR TANNINS

Protein	Relative affinity	
	Sorghum tannin[a]	Quebracho tannin[b]
Ovalbumin	0.05	0.003
BSA	0.17	0.17
Fetuin	1.8	1.8
Gelatin	4.5	5.8
GP_{66}-SMX	1.7	12.

[a]0.2 mg of sorghum tannin was used.
[b]0.3 mg of quebracho tannin was used.

under mild conditions without apparent denaturation. Its maximum absorption is at longer wavelengths than the anthocyanidins and other plant pigments often associated with tannins, thus minimizing their interference with the assay. Introduction of the hydrophobic dye reduces the solubility of the BSA and makes it precipitate more readily than unlabeled BSA.

This assay should be applicable to determination of tannin in a wide variety of plants. It must be noted, however, that direct precipitation assays, including Bate-Smith's hemanalysis (1973) and this assay with blue BSA, detect all protein precipitants. Formation of a precipitate should not be interpreted as indicative of the presence of tannin unless corroborated by assays for protein-precipitable phenols (Hagerman and Butler, 1978). It is possible that blue BSA will be useful in measuring other protein precipitants in addition to tannins.

Competition assays do not measure the concentration of tannin or any other protein precipitant. They measure the relative affinity of various materials, usually proteins, for the precipitating agent (in this case a tannin). Materials which bind tannin without precipitation are measured by this assay. The relative affinity of proteins for tannins varies widely, as previously noted (Hagerman and Butler, 1981).

The affinity of a protein for tannin may indicate functional significance (Mehansho et al., 1983). The high relative affinity for tannins of salivary proline-rich proteins such as GP_{66}-SMX, and their rapid induction in response to dietary tannins (Mehansho et al., 1983), suggests that these salivary tannin-binding proteins constitute a chemical defense system which binds and inactivates dietary tannins immediately on entering the digestive tract (Mehansho et al., 1983). Hamsters do not respond to dietary tannin in this manner and are relatively vulnerable to the effects of ingested tannins (Mehansho et al., 1985).

Possibly because of its higher degree of polymerization, sorghum tannin precipitates about twice as much blue BSA as an equal weight of quebracho tannin. Porter and Woodruff (1984) reported that procyanidin chain length was the determining characteristic in tannin astringency.

Tannins with different protein-precipitating capacities (Figure 2) cannot be directly compared in competition assays at the same tannin concentrations. We are preparing tannins labeled with ^{125}I for use in competition assays in order to compare relative affinities of sorghum, quebracho, and other tannins for standard proteins. Use of direct assays for measuring protein-precipitating capacity, and competition assays for measuring relative affinity, independent of precipitation, should increase our understanding of the specificity and mechanism of tannin binding by proteins.

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