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Color reaction of hydrolyzable tannins with Bradford reagent, Coomassie brilliant blue

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Abstract

Bradford protein-binding dye, Coomassie brilliant blue G-250, formed intensively blue-colored complexes with hydrolyzable tannins. The tannin–dye aggregates displayed a broad absorption maximum around 700 nm, with a shoulder at 620 nm. Pronounced reactivities were observed with tetra- to nonagalloylglucoses. Gallic acid, β-glucogallin and digalloylglucose were inactive and trigalloylglucose gave only a weak reaction. Moderate color formation (65% relative to pentagalloylglucose) was observed for the ellagitannin, tellimagrandin II. Monomeric and dimeric proanthocyanidins gave only traces of color. These complexation characteristics parallel the binding of hydrolyzable tannins to proteins.

Keywords: Bradford reagent; Coomassie brilliant blue G-250; Galloylglucose esters; Hydrolyzable tannins

1. Introduction

Since its introduction more than 20 years ago, the protein determination method of Bradford (1976) with Coomassie brilliant blue G-250 (CBB, **1**) has gained increasing importance and popularity because of its rapidity and sensitivity, and particularly because of its supposed specificity for protein. This later property has been critically investigated by Compton, and Jones (1985) who concluded that compounds reacting with CBB "must have both a macromolecular form and an active basic or aromatic functional group in order to bind". This statement was supported by recent observations in the course of studies on the biosynthesis of gallotannins. Crude enzyme extracts from tannin-producing plants were found to give a pronounced color reaction with CBB that by far exceeded the values expected for the usually observed protein concentrations obtained from such sources. Closer analyses revealed that this effect was due to complexation of Bradford reagent with endogenous plant phenolics that contaminated the cell-free extracts. This unexpected finding prompted us to investigate the affinity of CBB towards hydrolyzable and condensed tannins. As reported below, it was found that the binding pattern of this dye closely resembled the well-known complexation of tannins with proteins or alkaloids like caffeine.

2. Results and discussion

General characteristics of CBB–tannin complexation were studied with 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose as tannin model substance under the conditions of Bradford's 'micro protein assay' (Bradford, 1976). The absorption spectrum obtained under these conditions revealed a broad curve, exhibiting a maximum at 700 nm, plus a flat shoulder at ca. 620 nm. Similar spectra were recorded for commercial tannic acid (0.002–0.02% concentration range) while the reverse situation was encountered at very low concentrations of tannic acid (0–0.002%), with a flat maximum at 620 nm and

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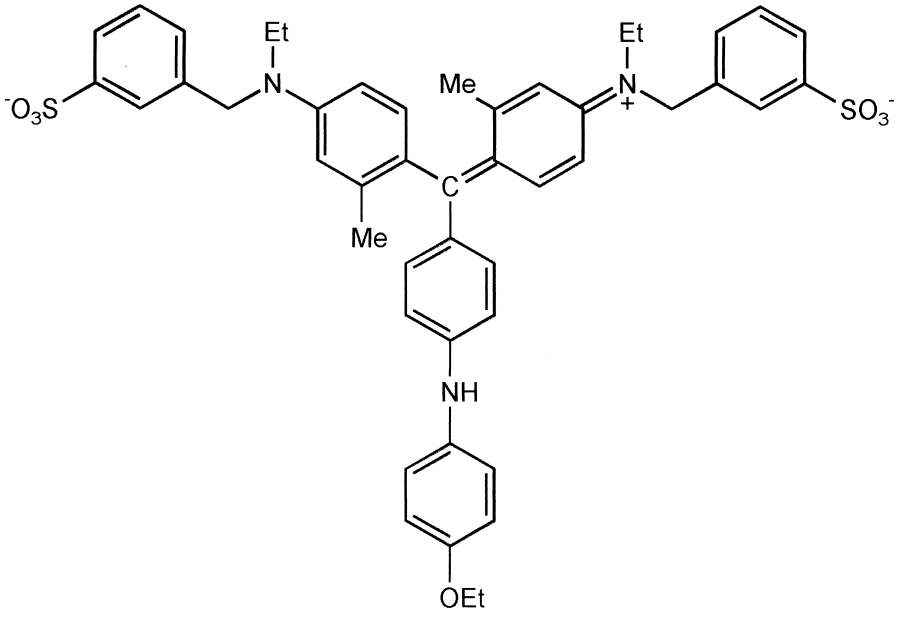

Coomassie Brilliant Blue G-250 (**1**)

a shoulder at 700 nm. For this reason, 660 nm was chosen as standard because this wavelength was found to be unaffected by different concentrations of reactant. This latter value is close to the absorption maximum of 650 nm reported for complexes of CBB with various flavonoids (Compton, & Jones, 1985). Analogous to Bradford's results (Bradford, 1976), maximal absorption was reached after ca. 2 min and was maintained with negligible decrease for at least 30 min. Increasing precipitation and sedimentation of the blue complex was observed after prolonged incubation, a result which is in accordance to previous observations on protein determinations with this reagent (Marshall, & Williams, 1992, 1993). Calibration curves with 0–10 nmol pentagalloylglucose per standard assay revealed slight nonlinearity of color yield versus tannin concentration (Fig. 1), a feature that was already apparent from the data of Bradford (1976) and that has also been recognized by other authors (Splittgerber, & Sohl, 1989).

The reactivity of a variety of hydrolyzable tannins and related compounds was tested under standard assay conditions, revealing that significant color formation only occurred with tetra- to nonagalloylated galloylglucoses (higher substituted analogs were not available). Quantitative determinations showed that the increasing molecular weight of these galloylglucoses was paralleled by a (nonlinear) increase of color formation (Fig. 2), while gallic acid, β-glucogallin (1-*O*-galloyl-β-D-glucose) and 1,6-digalloylglucose were inactive. 1,2,6-Trigalloylglucose displayed only 11% reactivity as compared to pentagalloylglucose. Commercial crude tannic acid (Sigma; main components tetra- to octagalloylglucoses) gave an absorbance of ca. 0.2 (77% relative to pentagallyolglucose) at these conditions. The monomeric ellagitannin, tellimagrandin II, showed a reactivity of 65% relative to the standard, pentagalloylglucose. Negligible activities were observed in experiments with monomeric and dimeric proanthocyanidins (epicatechin, fisetinidol-(4α-8)-catechin, fisetinidol-(4β-8)-catechin, catechin-(4α-8)-epicatechin (procyanidin B4)). Undefined tannin extracts from wattle and quebracho, known to be characterized by significant amounts of 'angular' trifla-

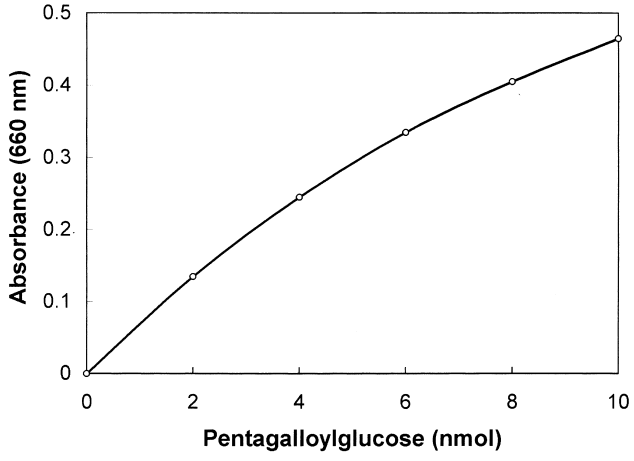

Fig. 1. Calibration curve for the reaction of Coomassie brilliant blue G-250 with 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose under standard assay conditions (cf. Section 3).

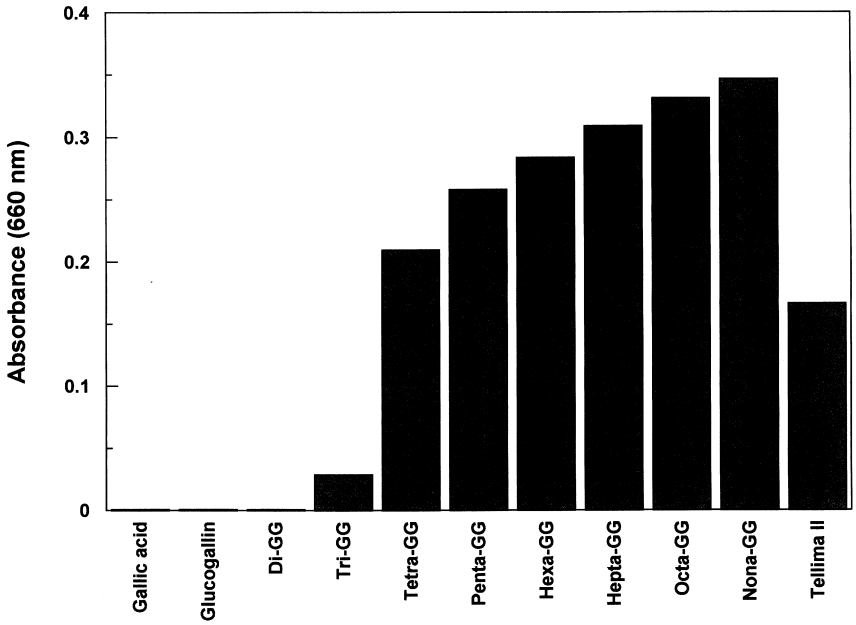

Fig. 2. Coomassie brilliant blue G-250-binding responses of hydrolyzable tannins. Absorbance values refer to 5 nmol tannin per standard assay. GG, galloylglucose; tellima II, tellimagrandin II.

vonoids (Haslam, 1989), displayed under standard assay conditions A_{660} values of ca. 0.05 and 0.13, respectively (19 and 50% corresponding to pentagalloylglucose). Ascorbic acid, which has been reported to cause underestimations in the Bradford assay (Compton, & Jones, 1985), was inactive. It should be noted that also common organic solvents, like methanol, ethanol or acetone, interfered with the color reactions which made it mandatory to dissolve all reactants in water to avoid elevated background absorbance.

It is evident from these experiments that CBB was able to form colored aggregates only with those hydrolyzable tannins that exceeded a M_r of about 750 (tetragalloylglucose has M_r 790). This apparent dependence on a threshold molecular weight also explains the lacking color reaction with monomeric and dimeric proanthocyanidins which have M_r values of ca. 290–560, while plant extracts containing trimeric proanthocyanidins exceeding that threshold gave moderate to significant color reactions. The observed reactivities of hydrolyzable tannins with Bradford reagent exactly match earlier reports on the tanning potential of variously substituted galloylglucoses, as expressed by precipitation of peptides (e.g. bradykinin (Haslam, 1996)) and proteins (e.g. hemoglobin (Ozawa, Lilley, & Haslam, 1987) or bovine serum albumine (Kawamoto, Nakatsubo, & Murakami, 1995; Kawamoto, Nakatsubo, & Murakami, 1996)), inhibition of β-glucosidase activity (Ozawa et al., 1987) and complexation with caffeine (Spencer et al., 1988; Haslam, Lilley, Cai, Martin, & Magnolato, 1989). Also the evidently comparatively low reactivity of tellimagrandin II, a monomeric ellagitannin whose M_r of 939 is equal to that of pentagalloylglucose, fits into this picture. This behavior has been explained by reduced spatial flexibility of galloyl residues after biphenyl linkage that generates the characteristic hexahydroxydiphenoyl residues of ellagitannins and which obviously results in a significantly lowered complexing ability in comparison to pentagalloylglucose (Spencer et al., 1988; Haslam, 1989; Haslam et al., 1989).

Our results with CBB show striking similarities to an earlier report on the precipitation of another common dye, methylene blue, by a multitude of plant tannins and related compounds. Again, reaction rates were found to increase in relation to molecular weight (particularly in response to increasing galloylation degrees), while lower-molecular weight polyphenols lacking tanning properties were inactive in this investigation (Okuda, Mori, & Hatano, 1985). As quite recently discussed (Haslam, 1998), positively charged (i.e. electron deficient) aromatic compounds, like methylene blue, anthocyanins or the alkaloid berberine, appear to have strong tendencies of complexing with the electron rich aromatic residues of (poly)phenols to preferentially form apolar 'face to face' π–π stacks. Our results with CBB would nicely fit into this picture.

In summary, our observations suggest that formation of colored CBB–tannin aggregates depends on the tanning properties of the polyphenolic component and supports the above mentioned conclusions of Compton, and Jones (1985) which emphasized the requirement of a macromolecule that bears aromatic

residues as linkage sites. The color reaction described here is principally suitable for both qualitative and quantitative determinations of hydrolyzable tannins, as far as these possess tanning properties; this latter feature, however, applies to the majority of naturally occurring galloylglucoses. Eventually, this new method has the potential of contributing to solve the technical problems encountered in the definition and measurement of plant tannins (cf. Mole, & Waterman, 1987a, 1987b). As another consequence of our results, it must be recognized that Bradford assays with proteins from tannin producing plants bear a pronounced risk of overestimation and particular attention has to be paid on the depletion of polyphenols from such samples prior to their analysis.

3. Experimental

3.1. Chemicals and assay procedures

Mono- to nonagalloylglucoses, obtained by chemical synthesis or isolation from plant material (cf. Gross, 1993), were from the laboratory collection; except for 1-*O*-galloylglucose (β-glucogallin), 1,6-di-*O*-galloylglucose and 1,2,3,4,6-penta-*O*-galloylglucose, mixtures of isomers of the indicated galloylation degree were used. Crude tannic acid was obtained from Sigma. Tellimagrandin II and various proanthocyanidins were generous gifts of Dr. A. Scalbert (Avignon) and Dr. R.W. Hemingway (Pineville, LA).

Assays were done according to the 'microprotein assay' of Bradford (1976) ('standard assay'). Aq. solns (vol. 0.1 ml) with 2–10 nmol of tannin were mixed with 1 ml Bradford reagent in glass test tubes; absorbance of CBB–tannin complexes was measured after 10 min at 660 nm in micro-cuvettes.

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