PLANT CONSTITUENTS INTERFERING WITH THE LOWRY METHOD OF PROTEIN DETERMINATION

Jeffrey L. Smith

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019

Twenty-four compounds were tested for interference with the Lowry method of protein determination. Twenty-three were found to cause significantly high values at one or more of the concentrations tested per compound. Hippuric acid was the only test compound that did not interfere. Interference is believed to be caused by the reduction of the Folin phenol reagent resulting in increased color formation. Many of the test compounds are plant secondary metabolites and caution is suggested when determining plant protein content by the Lowry method.

INTRODUCTION

The method of protein determination commonly known as the Lowry method is widely used in determining the protein content of biological samples. The method of Lowry et al. (1) is based on the Folin phenol reagent of Folin and Denis (2), the active constituent of which is a phosphomolybdic-tungstic mixed acid. Proteins effect a reduction of the mixed acid by a loss of one, two, or three oxygen atoms from tungstate and/or molybdate, producing one or more of several possible reduced species which have a characteristic blue color (750 nm). Lowry et al. (1) stated that "Few substances encountered in biological work cause serious interference." Folin and Denis (2), however, reported 26 substances which have a color reaction with their reagent, several of which (salicylic acid, tannic acid, and vanillin) are frequently encountered in plant material. Since the work of Lowry et al., there have been several reports in the literature of interfering substances (3-6). Reports on interference by plant products are generally limited to: 1) synthetic polysucrose and other carbohydrates (7), 2) hexosamines (8-9), 3) sulfhydryl compounds (10-11), and 4) phenolics (12). In many instances the interfering compound was identified, but the relationship of quantity present to amount of interference was not investigated. The purpose of this study was to examine 24 compounds, many of which are common plant phenolics or their derivatives, for interference with the Lowry method and relate the significance of the interference to the quantity present.

MATERIALS AND METHODS

Three concentrations of each compound were tested for interference. A 100-μM solution was made of each compound (except saponin), and diluted to give additional concentrations of 10 μM and 1 μM. Saponin is a mixture of compounds and was therefore made as 1.0%, 0.1%, and 0.01% solutions. Kaempferol and quercetin are only slightly water soluble and were dissolved in a 1:1 mixture of water and 0.1 N NaOH.

Bovine serum albumin was used as the protein for all standards and tests. Tests for interference were made by adding 0.5 m*l* of a test solution to a 1-m*l* sample containing 100 μg protein. The reagents for color development were then added and the optical density was read at 750 nm on a Bausch and Lomb Spectronic 20 colorimeter. Standards were made for each group of four to five compounds tested and all standards and tests were run in triplicate.

A standard curve for each group of test compounds was generated by fitting a second-degree regression line to the observed optical densities of protein standards. The equation of the form $y = ax^2 + bx + c$ for each standard curve was obtained. Use of the quadratic equation made it possible to solve for the apparent protein concentration given any observed optical density. In cases where the observed optical density was too high to allow direct computation of apparent protein concentration (because of zero or negative values under the square root in the quadratic equation) a maximum protein value was calculated and the

value was recorded as greater than (>) this maximum value. Significant differences between the observed protein value in presence of a test compound and the 100 μg protein/m*l* standard of that group were detected by a single classification analysis of variance (ANOVA).

RESULTS

All but one of the compounds tested gave a significantly higher μg protein/m*l* value than the standard at one or more of the concentrations used (Table 1). Out of 22 compounds, one was significantly higher at the 1 μM concentration, 11 were significantly higher at the 10 μM concentration, and all 22 were significantly higher at the 100 μM concentration. Saponin showed significantly higher values at all of its concentrations. Hippuric acid was the only compound tested that did not show any significant interference.

DISCUSSION

The review by Peterson (5) suggests that there are several methods by which substances can interfere with the Lowry method: 1) compounds may react with the standard protein of the blank, resulting in a lowered standard color, 2) compounds may absorb at the same wavelength as the standard, causing an increase in apparent

TABLE 1. *Apparent protein levels (μg/ml) observed in the presence of compounds tested for interference in the Lowry method.*

Test compounds	Standard	Concentration of test compound 1 μM	10 μM	100 μM
Benzoic acids				
Gallic acid	96.9	98.4	98.4	133.7***[a]
2, 5-Dihydroxybenzoic acid	101.0	103.9	105.9	129.9***
p-Hydroxybenzoic acid	101.9	97.5	102.4	230.0***
Salicylic acid	94.8	89.8	97.9	> 200***[b]
Syringic acid	98.7	97.4	155.6***	> 365***
Vanillic acid	94.8	92.0	129.6***	> 200***
Cinnamic acids				
Caffeic acid	98.7	97.1	98.7	139.9***
Cinnamic acid	96.9	100.2	137.7*	> 290***
p-Coumaric acid	101.0	112.0**	126.9***	> 400***
Ferulic acid	96.9	100.2	134.8***	> 290***
Coumarins				
Coumarin	101.9	95.4	125.7**	> 337***
7-Hydroxycoumarin	101.9	100.1	118.0*	> 337***
Esculin	98.7	97.8	102.8	197.2***
Flavonols and Flavones				
Kaempferol	102.0	93.2	110.1	> 337***
Naringenin	102.0	92.0	101.0	215.2***
Quercetin	94.8	94.6	108.8*	> 200***
Miscellaneous Phenolics				
Catechol	101.0	104.4	107.7	147.5***
Chlorogenic acid	101.0	99.8	111.6*	176.1***
Tannic acid	94.8	100.9	145.3**	> 200***
Sulfonic acids				
1-Amino-2-naphthol-4-sulfonic acid	94.8	93.9	93.5	168.7**
6-Amino-1-naphthol-3-sulfonic acid	96.9	98.9	107.8*	> 290***
Miscellaneous Compounds				
Saponin[c]	101.0	119.4*	400***	> 400***
Hippuric acid	96.9	97.2	92.9	93.0
2-Methyl-1,4-naphthoquinone	98.7	96.0	93.0	117.7***

a. Values are significantly different from the standard at: * = .05 level, ** = .01 level, and *** = .001 level.
b. See text for explanation of values given as greater than (>).
c. Concentrations used were 1.0%, 0.1%, and 0.01%.

protein value, 3) compounds such as $HgCl_2$ and NaCl may decrease the protein ability to form the color complex, which decreases the apparent protein value, 4) compounds may form a precipitate with the reagents, causing an increase in turbidity and apparent protein value, 5) compounds may react with the Folin phenol reagent, preventing color formation and decreasing the apparent protein value, and 6) compounds such as detergents may reduce the Folin phenol reagent, causing an apparent increase in protein value. Peterson suggested that this last method of interference is not very common. He also did not present any evidence that naturally occurring compounds may cause this type of interference. All the compounds tested in this study, except hippuric acid, caused an increase in the apparent protein content of the samples. All appeared to do so by reducing the Folin phenol reagent, as none of them formed a precipitate with the reagents and the increase in color was very dramatic.

Peterson also warned that "Caution should be exercised in cases where a particular substance interferes only at high concentrations as impurities in the test compound may be the responsible agent." All the compounds tested in this study, other than saponin, had earlier been found to be free of impurities when examined by paper chromatography. It is feasible that some degradation may have since taken place, but the observation of interference by all but one of the test compounds makes it unlikely that the interference was caused by impurities.

The potential interference of these compounds in the Lowry method is certainly important in biological work. Many of the test compounds studied, particularly the benzoic and cinnamic acid derivatives, are common in many plant species. Although qualitative data may be available on their occurrence in plants, quantitative plant data are generally lacking. This study has shown that the amount of interference produced by the test compounds is related to their quantity. Without information on the quantity of these compounds in a biological sample, it would be difficult to predict *a priori* the amount of interference to be expected.

There are several alternatives available to investigators concerned about interference in protein determination. Many of the interfering compounds are secondary metabolites and it is possible that the pools of these compounds in a plant are not sufficiently high to cause interference. If the protein is isolated, the method used could be important. If the protein isolation technique can separate the protein from these compounds, their interference should be minimal. Techniques for enzyme isolation, for example, that use polystyrene-based anion exchange resins have been shown not only to improve the enzyme isolation, but also to free the sample from phenolics (13). These techniques can also be very important in studying the enzymes involved with the synthesis of secondary metabolites, as attempts at isolation of enzymes or enzyme complexes may fail without them (14). Other methods of protein determination are less subject to chemical interference than the Lowry method and may be desirable when interference is suspected. The Bio-Rad method is claimed to be subject to slight chemical interference, while the biuret, Kjeldahl, and absorbance (280 nm) methods are rated as moderate (15).

In summary, there appears to be a potential for many naturally occurring plant compounds to interfere with the Lowry method of protein determination. This may result in inaccurate high protein values. The types and concentrations of these compounds and the protein isolation technique used can affect the significance of the interference. It is suggested that investigators examine their study system for the presence of interfering compounds and use techniques to remove them before using the Lowry method or use methods that are less subject to chemical interference.

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