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Antioxidant Activity of Extracts, Condensed Tannin Fractions, and Pure Flavonoids from *Phaseolus vulgaris* L. Seed Coat Color Genotypes

CLIFFORD W. BENINGER* AND GEORGE L. HOSFIELD

Sugarbeet and Bean Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Plant and Soil Science Building, Michigan State University, East Lansing, Michigan 48824

Twelve different seed coat color genotypes of *Phaseolus vulgaris* L. were extracted and pure flavonoids isolated from 10 of these. The seed coat methanol extracts, tannin fractions, and pure flavonoids all displayed antioxidant activity in a fluorescence-based liposome assay. The relatively high activity of the condensed tannin (proanthocyanidin) fractions indicates that these may play an important role in the overall activity of the extracts. This activity also indicates that although these polyphenols cause problems in digestibility, they may be important dietary supplements with beneficial health effects. The pure anthocyanins delphinidin 3-*O*-glucoside (**1**), petunidin 3-*O*-glucoside (**2**), and malvidin 3-*O*-glucoside (**3**) and the flavonol quercetin 3-*O*-glucoside (**4**) isolated from seed coats also had significantly higher antioxidant activity than the Fe^{2+} control. The activity of kaempferol 3-*O*-glucoside (**5**) was not different from that of the Fe^{2+} control. These findings suggest that variously colored dry beans may be an important source of dietary antioxidants.

KEYWORDS: ***Phaseolus vulgaris*; antioxidants; extracts; condensed tannin fractions; anthocyanins; flavonols**

INTRODUCTION

Dry beans (*Phaseolus vulgaris* L.) are widely consumed throughout the world and are an important source of plant protein in many countries (*1*). In the United States, dry beans have an annual farm gate value in excess of $700 million (*2*). Considerable variability exists in common beans (*P. vulgaris* L.) for seed characteristics, and consumers have acquired specific preferences for various combinations of size, shape, and color of the dry seeds (*3*). In many countries these preferences distinguish the various commercial (market) classes, which must meet specific consumer expectations and industry standards. Seed coat color and seed size are the two main criteria that identify the numerous market classes recognized throughout the world.

Prakken (*4*, *5*) reviewed the literature for the genetics of seed coat color and showed that eight Mendelian loci contribute: *P*, *C*, *D*, *J*, *G*, *B*, *V*, and *Rk*. According to Prakken's interpretation, *C*, *D*, and *J* are the color genes; *G*, *B*, and *Rk* are modifying genes (have an intensifying effect or darkening influence upon pale colors formed by the action of the color genes); and *V* is called the violet factor. The dominant allele, V, causes bluish or violet to black colors to develop in the seed coat.

The seed coat color of dry beans is determined by the presence and amounts of flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins) (*6*). Recently, a variety of seed coat color genotypes have been obtained, and the phenolic compounds responsible for color were isolated and identified (*7*−*12*). To date, work on *P. vulgaris* has focused mainly on the antinutritional effects of seed coat polyphenolics such as condensed tannins (*13*−*15*), but nothing has been reported on what beneficial effects, if any, the wide array of phenolic compounds found in *P. vulgaris* seed coats may have. For example, polyphenols from dry beans may act as antioxidants to inhibit the formation of damaging free radicals that result from the natural degradation of foods (*16*). Flavonoids obtained commercially (*17*−*19*) and isolated from plant species (*20*) are known to be effective free radical scavengers. Recently, condensed and hydrolyzable tannins of relatively high molecular weight have also been shown to be effective antioxidants with greater activity than simple phenolics (*21*).

The purpose of the current study was to determine whether *P. vulgaris* L. methanolic seed coat extracts exhibited antioxidant activity by inhibiting lipid peroxidation in a fluorescence-based lipid bioassay (*22*). We also wanted to determine whether isolated condensed tannin fractions and pure flavonoid compounds were responsible, at least in part, for antioxidant activity that may be observed in the methanol fractions. Therefore, fractions containing condensed tannins and pure compounds isolated from the methanolic extract of a variety of color genotypes were also tested to determine their effectiveness as antioxidant compounds.

* Address correspondence to this author at the Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1 [telephone (519) 824-4120, ext. 54846; fax (519) 837-0442; e-mail cbeninge@uoguelph.ca].

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Table 1. Flavonoid Compounds Present in Seed Coats and Names, Seed Coat Colors, and Abbreviations of the 12 Genetic Stocks Used for Antioxidant Activity Analysis

seed coat color, common name, and (abbreviation)	color genotype	compounds present in seed coat
white		
V0059	*p*^{gri}[*C r*] *d J G b v Rk*	condensed tannins*
gray white (GW)	*p*^{gri}[*C r*] *D J G B V Rk Asp*	UNK
brown		
buffy citrine (BC)	*P* [*Cr*] *D J g B v Rk Asp*	K3G, condensed tannins
mineral brown (MB)	*P* [*C r*] *D J G B v Rk Asp*	K3G, condensed tannins
matte mineral brown (MMB)	*P* [*C r*] *D J G B v Rk asp*	K3G, condensed tannins
yellow		
manteca (MT)	*P* [*C r*] *d j G b v*^{lae} *Rk*	K3GX, K3G
yellow brown (YB)	*P* [*C r*] *D J G b v Rk Asp*	K3G, condensed tannins
blue to black		
5-593	*P* [*C r*] *D J G B V Rk Asp*	D3G, P3G, M3G, condensed tannins
matte black (MTB)	*P* [*C r*] *D J G B V Rk asp*	D3G, P3G, M3G, condensed tannins
dark brown violet (DBV)	*P* [*C r*] *D J G b V Rk Asp*	D3G, P3G, M3G, condensed tannins
red		
dark red kidney (DRK)	*P* [*c*^{u}] *D J g B v rk*^{d}	Q3GX, Q3G, K3G, condensed tannins
light red kidney (LRK)	*P* [*c*^{u}] *D J g B v rk*	Q3G, condensed tannins

MATERIALS AND METHODS

Plant Material. The genetic stocks used for this investigation were developed by M. J. Bassett by backcrossing selected recessive genes for seed coat color into a recurrent parent Florida breeding line 5-593. Line 5-593 has dominant genes for seed coat color in all eight seed coat color loci (*P*, [*C r*], *D*, *J*, *G*, *B*, *V*, and *Rk*), except for the dominant red locus *R*, which is tightly linked to the *C* locus (*23*). The genetic stocks are in the third backcross to the recurrent parent 5-593 (*24*) and are not isogenic with respect to the recurrent parent 5-593, but an isogenic condition is not needed for our experimental purpose. For each seed coat line, the genotype and the compounds it contains are given in **Table 1**.

Genetic stocks were increased in the summer of 1996 in a nursery at the Saginaw Valley Bean and Sugarbeet Research Farm, Saginaw, MI. The soil type on the farm is a Mistequay silty clay [fine, illitic (calcareous), frigid typic Haplaquolls]. After the beans were harvested in the autumn, seeds were frozen at −20 °C. Over several months beans were removed from the freezer and soaked in distilled water, and the seed coats were removed. Seed coats were then frozen in a −80 °C freezer, freeze-dried, and ground to a fine powder.

Extraction. One hundred grams of dried (lyophilized), ground seed coats from each genetic stock was loaded into glass columns and extracted sequentially with hexane, ethyl acetate, methanol, and methanol/water (1:1). Extracts were dried under reduced pressure and heat (40 °C) in a rotary evaporator and stored at −20 °C prior to use.

Isolation of Condensed Tannin (Proanthocyanidin) Fractions. The crude methanol extract (3 × 30 mg) from each genetic stock was dissolved in 1.0 mL of methanol and then loaded directly onto the surface of a column (14.0 × 1.0 cm i.d.) packed with Sephadex LH-20 gel, which had been equilibrated overnight with 100% methanol. Flavonoids, simple phenols, and hydrolyzable tannins were then eluted from the column using methanol/water (8:2) as the eluting solvent. Condensed tannins were selectively retained on the Sephadex gel (*25*), and these fractions were then eluted using 50% aqueous acetone as a solvent, evaporated to dryness with a rotary evaporator, weighed, and then diluted for use in the antioxidant assay.

Isolation and Identification of Individual Flavonoid Compounds. A variety of thin-layer, medium- and high-pressure liquid chromatography techniques were used to isolate the pure flavonoid compounds. Isolated compounds were then identified by ^{1}H and ^{13}C NMR spectroscopy. These methods and identification of these compounds are described in a series of papers (*7*−*12*).

With the exception of quercetin and kaempferol 3-*O*-diglycoside, all of the pure flavonoids isolated from bean seed coat genotypes were tested in the antioxidant assay. Each flavonoid was purified three times to give three repetitions for the antioxidant assay.

Antioxidant Assay. Preparation of liposomes and the fluorescence spectrographic assay were modified from those of Wang et al. (*26*) and Arori and Strasburg (*22*). A mixture of 157.5 μL of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 86.7 μL of the fluorescence probe 3-[4-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (Molecular Probes, Inc., Eugene, OR), diluted 20-fold, was dried under vacuum. The resulting film was suspended in 500 μL of buffer (NaCl, 0.15 M; EDTA, 0.1 mM; MOPS, 10 mM) and then subjected to 10 freeze−thaw cycles in an EtOH−dry ice bath. The suspension was then passed 29 times through a polycarbonate membrane with a pore size of 100 nm using a LiposoFast extruder (Avestin, Inc., Ottawa, ON, Canada). Lipid solution was kept out of light during preparation. Prepared liposomes were stored at 4 °C for a maximum of 4 days prior to use.

Fluorescence Assay. Liposome solution was kept out of light during the assay. The blank and iron ($FeCl_2 \cdot 4H_2O$, 0.1 M) control solutions were a mixture of 100 μL of the buffers (HEPES, 50 mM; Tris, 50 mM). Buffers were stored in Chelex 100 to remove metal ions. In the experiments the control solution had 100 μL of buffer, 200 μL of 1 M NaCl, 1.66 mL of N_2-sparged Millipore water, 20 μL of DMSO, and 20 μL of liposome solution added. Twenty microliters of iron solution ($FeCl_2 \cdot 4H_2O$, 0.1 M) was then added to the control solution. The test sample solution was 100 μL of buffer, 200 μL of 1 M NaCl, 1.66 mL of N_2-sparged Millipore water, 20 μL of sample solution (1 mg of sample/1 mL of DMSO diluted 10-fold), 20 μL of liposome solution, and 20 μL of iron solution. The final concentration of the samples tested was, therefore, approximately 1.0 μg/mL or 1.0 ppm. Fluorescent intensity of the lipid suspensions was monitored for 21 min using a Turner (Dubuque, IA) model 450 fluorometer.

Statistical Analysis. Data were analyzed using the SAS Institute (*27*) statistical package. Analysis of variance utilized a general linear models procedure (GLM) with a Student−Newman−Keuls comparison of means to test for significant differences between means.

RESULTS AND DISCUSSION

Antioxidant Activity of Extracts, Tannin Fractions, and Pure Compounds. Significant differences between the various methanol extracts are given in **Table 2** for the readings taken at 15 min into the assay. Red kidney, light red kidney, and the yellow brown extracts were the most active and did not differ from each other significantly. The next most active extracts were those of matte mineral brown, mineral brown, V0059, and buffy citrine, which did not differ significantly in their activity. These genotypes (except for V0059) are varying shades of brown. The next most active extracts were the blue or black series represented by Florida dry bean breeding line 5-593, matte black, and dark brown violet. The least active extracts were those of gray white and manteca.

The activity of the crude methanol extracts is due to the presence of flavonoid monomers and polymers (condensed

Table 2. Antioxidant Activity of Methanol Extracts and Condensed Tannin Fractions of Seed Coats of 12 Genetic Stocks Differing in Seed Coat Color

	mean relative fluorescence[a] (1.0 μg/mL)											
	V59	GW	BC	MB	MMB	YB	MT	593	MTB	DBV	DRK	LRK
methanol extracts	0.58b ± 0.02	0.30d ± 0.04	0.56bc ± 0.01	0.60b ± 0.01	0.60b ± 0.01	0.74a ± 0.01	0.17e ± 0.02	0.46c ± 0.01	0.53bc ± 0.01	0.50bc ± 0.02	0.77a ± 0.03	0.70a ± 0.05
condensed tannin fractions	0.83a ± 0.01	N/A	0.67b ± 0.02	0.72b ± 0.03	0.44d ± 0.02	0.69b ± 0.01	N/A	0.10e ± 0.01	0.46d ± 0.01	0.53c ± 0.02	0.73b ± 0.01	0.81a ± 0.01

[a] V59, V0059; GW, gray white; BC, buffy citrine; MB, mineral brown; MMB, matte mineral brown; YB, yellow brown; MT, manteca; 593, Florida breeding line 5-593; MTB, matte black; DBV, dark brown violet; RK, dark red kidney; LRK, light red kidney. Means followed by a different letter within a row are significantly different at $P \leq 0.05$.

tannins), hydrolyzable tannins, and phenolics. Recently, polyphenolic compounds from plants such as condensed and hydrolyzable tannins have been shown to be powerful antioxidants. For example, tannins from *Sorghum bicolor* Moench were found to be 15−30 times more effective than simple phenols at quenching peroxide radicals (*21*). Similarly, tannins isolated from adzuki bean (*Vigna angularis* Ohwi et Ohashi) were also found to have antioxidant properties (*28*). Finally, tannin-containing extracts from a variety of dry beans (*P. vulgaris*) have been shown to inhibit iron-catalyzed oxidation of soybean (*Glycine max* [L.] Merr.) oil (*29*).

To determine the role the condensed tannin fractions from our seed coat extracts play as antioxidants, condensed tannin fractions were obtained by fractionation of the methanol extracts on Sephadex LH-20 gel as described. When these fractions were tested in the same antioxidant assay used to test the methanol extracts, the condensed tannin fractions were generally found to be as active or slightly more active than the methanol extracts (**Table 2**). This suggests that a significant amount of the antioxidant activity found in the methanol extracts may be due to the condensed tannins present. However, for matte black, matte mineral brown, and 5-593, the tannin fraction had less activity than methanol extracts. No condensed tannins were detected in the yellow manteca genotype, and its methanol extract (no condensed tannin fraction was obtained) also had the lowest activity of all the extracts tested. This also points to the importance of condensed tannins as responsible for some of the antioxidant activity from methanol extracts.

Because individual flavonoids have also been shown to exhibit antioxidant activity (*20*), these compounds were isolated and identified. Two of the main flavonol glycosides and three anthocyanins were tested in the antioxidant assay. Their structures are given in **Figure 1**, and their distribution among the different seed coat color genotypes is given in **Table 1**. Of the beans with white seed coats, V0059 tested positive for the presence of condensed tannins as detemined in the vanillin/HCl assay. Gray white seed coats did not give a positive response in this assay, and their phenolic composition is not known. Brown and yellow seed coat genotypes had predominantly kaempferol glycosides and tannins with the exception of the manteca type, which did not contain tannins but had an additional kaempferol diglycoside. The blue and black seed coat genotypes all had condensed tannins and contained the same three anthocyanins, but their concentrations differed (*12*). Finally, dark red and light red kidney had predominantly quercetin glycosides and condensed tannins.

The anthocyanins delphinidin 3-*O*-glucoside and petunidin 3-*O*-glucoside and the flavonol quercetin 3-*O*-glucoside were the most active of the pure compounds tested (**Figure 2**). They differed significantly from the antioxidant activity of butylated hydroxytoluene (BHT) but still inhibited lipid destruction by >50% relative to the control. The third anthocyanin, malvidin 3-*O*-glucoside, was also active but had significantly less activity than BHT, delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and quercetin 3-*O*-glucoside. Finally, kaempferol 3-*O*-glucoside had the least antioxidant activity, inhibiting liposome breakdown by <20% relative to the control, and was not significantly different from the iron control (**Figure 2**).

Structure—Activity Relationships. The relatively high activity of the two anthocyanins and quercetin glucoside may be explained by their structures and the mechanism of free radical generation in the body. In the body O_2^- is formed by leakage of electrons from the mitochondria that reduce O_2 (*30*). Enzymatic or nonenzymatic conversion of O_2^- to H_2O_2 then

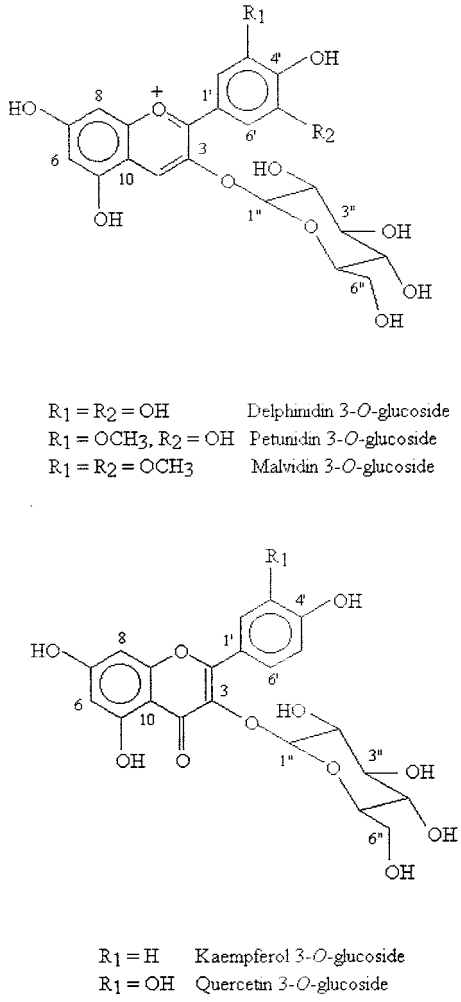

Figure 1. Anthocyanins (top) and flavonol glycosides (bottom) from *P. vulgaris* seed coats tested for antioxidant activity.

occurs, and in the presence of copper or iron atoms the peroxide forms the highly damaging hydroxyl radical (OH) (*30*). Therefore, the ability of flavonoids to complex with metals plays a part in their role as antioxidants. As a general rule, the greater the number of hydroxy groups on the flavonoid nucleus, the higher the antioxidant activity (*31*). For example, the aglycon flavonol quercetin has free hydroxy groups at the 3-, 3′-, 4′-, 5-, and 7-positions. Hydroxylation at the 3- and 5-positions of quercetin allows for ligand formation with iron at either of these sites (*32*). In our study the quercetins found in dry bean seed coats were all glycosylated at the 3-position, and, therefore, ligands could only form due to the presence of a 5-hydroxyl. The absence of a 3-hydroxyl in quercetin and kaempferol glycosides greatly reduces their metal complexing ability because chelation of the 3-hydroxy-4-keto group is the strongest metal complexing group (*33*).

Recent work has shown that the most important structural feature of flavonoids for antioxidant activity is the B-ring ortho 3′,4′-dihydroxy orientation (*34*−*36*). The most active flavonoids in our study were (1) delphinidin 3-*O*-glucoside and (2) quercetin 3-*O*-glucoside, which have a 3′,4′-dihydroxy group, and petunidin 3-*O*-glucoside, which has a 4′,5′-dihydroxy group. However, malvidin 3-*O*-glucoside, with both the 3′- and 5′-hydroxy groups methylated, had significantly lower activity than the above compounds, all of which have an ortho dihydroxy substitution on the B-ring. Wang et al. (*26*) tested three anthocyanins found in tart cherries, all of which had a B-ring 3′,4′ substitution, and these were all found to have good

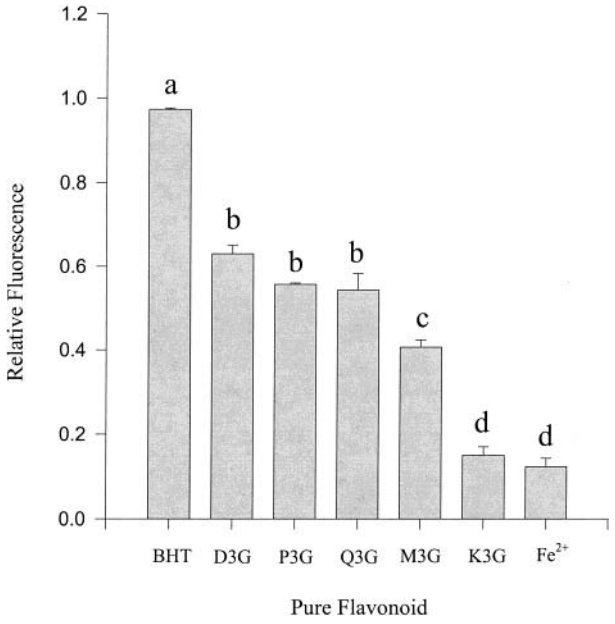

Figure 2. Antioxidant activity (1.0 μg/mL) after 15 min relative to the control of pure flavonoids isolated and identified from *P. vulgaris* seed coats. BHT, butylated hydroxytoluene; D3G, delphinidin 3-*O*-glucoside; P3G, petunidin 3-*O*-glucoside; Q3G, quercetin 3-*O*-glucoside; M3G, malvidin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside; Fe^{2+}, iron control. Letters above bars indicate significant differences ($P \leq 0.05$).

antioxidant activity. Finally, kaempferol 3-*O*-glucoside, which has only a single B-ring 4′-hydroxyl substitution, had no activity compared to the iron control. This finding is consistent with the results of the antioxidant assay of the methanol extract from the manteca type bean in which no significant activity was observed. This genotype had no detectable tannins and only two keampferol compounds present. In contrast, Gamez et al. (*20*) tested kaempferol 3-*O*-neohesperidoside in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay and also found this to have moderate antioxidant activity. However, the results from the fluoresence-based liposome assay used in our study may not be comparable to those of Gamez et al. (*20*).

Conclusions. There is increasing evidence that consumption of a variety of phenolic compounds present in natural foods may lower the risk of serious health disorders because of the antioxidant activity of these compounds (*37*, *38*). Dry beans are an integral part of diets in a significant portion of the world population, but the potential benefits of consuming beans from a "health benefits" point of view have largely been overlooked. In the current study we showed that pure flavonoid compounds such as anthocyanins, quercetin glycosides, and proanthocyanidins (condensed tannins) that are present in the seed coats of common bean had significant antioxidant activity relative to BHT, a commercial antioxidant in foods. Further resolution of the genes controlling flavonoid and tannin formation, along with knowledge of the antioxidant activity of these compounds, may enable plant breeders to select bean varieties that have a range of antioxidant activities and also, perhaps, balance the positive effects of antioxidant activity in diets with antinutritional effects.

ABBREVIATIONS USED

UNK, unknown; K3G, kaempferol 3-*O*-glucoside; K3GX, kaempferol 3-*O*-glucose-xylose; D3G, delphinidin 3-*O*-glucoside; P3G, petunidin 3-*O*-glucoside; M3G, malvidin 3-*O*-glucoside; Q3G, quercetin 3-*O*-glucoside; Q3GX, quercetin 3-*O*-glucose-xylose; *, presence or absence of condensed tannins

determined by TLC with vanillin/HCl as spray reagent (see ref *32*); for identification of all other flavonoid and anthocyanin compounds, see refs *7*−*12*.

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